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Research Article
In vitro Plant Regeneration from Mature Embryo Explants of *Jatropha curcas* L. (A Biodiesel Plant) on Two Standard Basal Nutrient Media

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Abstract

Background and Objective: The regeneration protocol for *Jatropha curcas* through mature zygotic embryo culture from healthy seeds were studied for large-scale production. This study was specifically undertaken to compare the two kinds modified basal media using zygotic embryos as explants and supplementing both basal media with two growth regulators-an auxin (α-naphthalene acetic acid) and a cytokinin (benzyl adenine) for studying morphogenesis of embryo explants. **Materials and Methods:** Zygotic embryos from mature seeds of *Jatropha curcas* were removed and inoculated on modified basal media under aseptic conditions. An auxin (α-naphthalene acetic acid) was used to supplement these media, in addition to a cytokinin (benzyl adenine, BA) either alone or in combination at concentrations between 0 and 10.0 mg L^{-1}. **Results:** The obtained results revealed that the two basal media used favoured complete *in vitro* plantlet growth from mature zygotic embryo explants of *J. curcas*. B5 medium differ notably from MS medium ($p = 0.05$) in all the growth parameters studied. However, spontaneous plantlet regeneration was observed in the absence of growth regulators after a prolonged period (up to 4 weeks) in culture. Also, in the presence of growth regulators, the simultaneous inclusion of both α-NAA and BA at 0.1 mg L^{-1} concentrations gave the best response for studying morphogenesis with the embryo explants while the least response was elicited by BA alone at 1.0 mg L^{-1} concentration on both media. **Conclusion:** The protocol reported here can be applied for maximum scale propagation of the recalcitrant *Jatropha* plants for enhanced seed production and the development of a viable conservation programme.

Key words: *Jatropha curcas* L., conservation, plantlet regeneration, zygotic embryo, recalcitrant seed

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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

The recent efforts in searching for renewable and alternative energy sources resulting from the imminent depletion of fossil fuels, coupled with the need to curb environmental hazards posed by these (fossil) fuels, have led to the intensification of efforts at mass propagation of bio-fuel plants which include *Jatropha curcas* (a bio-diesel plant)[1]. *Jatropha* is a non-food bio-diesel crop which can serve as an alternative to fossil fuel[2]. The oil-producing plant from the taxonomic family (Euphorbiaceae), with a life expectancy of about 50 years, is a small tree between 5-7 m in height[2-3].

Jatropha is also known as Physic nut contains about 170 known taxonomic species. This plant has numerous local names, namely, "Oboboloti" (Igbo); "Gadem" (Hausa) and "Lapalapa" (Yoruba) "Mapuluka" (Angola); "Kpoti" (Togo)[4].

Jatropha seeds and stems are used for conventional macropropagation, though it has been observed that propagation of *Jatropha curcas* by stem cuttings leads to low seed yields and poor root development[5]. Also, plants that are produced show low longevity and are less disease-resistant[4-6]. On the other hand, propagation by seed increases seed yield and provides good root formation, poor seed viability and low germination rate due to the recalcitrant nature of the seeds are encountered[7]. Also, seed propagation is relies solely on the amount of some climatic and cultural factors usually adequate rainfall, moisture and time and depth of sowing respectively[8]. It is believed that there will be a problem in the establishment of this plant, either by seeds or stem cuttings especially when these conditions are not met. To bridge this gap and proffer adequate and necessary solutions to such possible problems associated with seed-based propagation, plant micropropagation through embryo culture is encouraged.

A research on the influence of various plant growth regulatory substances on plantlet shoot regeneration from young cotyledons of *Jatropha curcas* using was carried out by Khemkladngoen *et al.*[9]. MS was fortified with 0.1 mg L^{-1} Indole Butyric Acid (IBA) and 3 mg L^{-1} Benzyl Adenine (BA) had the best regeneration frequency when compared to other treatments tested. Plantlets regenerated from this study had no tap root but did not differ phenotypically as the wild type plants raised from seeds. The authors concluded that the study would be necessary for improving on *Jatropha curcas* genetic lines.

Mohan *et al.*[10] studied the *in vitro* propagation of *Jatropha curcas* L. using immature embryo obtained from seeds. Murashige and Skoog basal medium (as the only medium used) with (0.1 mg L^{-1}) 6 benzyl-aminopurine (BAP) was used for initiation of embryos of *Jatropha curcas*. The basal medium was supplemented with 3% (w/v) sucrose and 0.8% agar. Datta *et al.*[5], Pan and Xu[3] and Khemkladngoen *et al.*[9] have successfully carried out investigations on the influence of several plant growth regulators on the plantlet regeneration of *Jatropha curcas* L. using MS as the sole medium but none used embryo (immature or mature) as explants.

In the callus induction of castor plant from both cotyledon and epicotyl tissues, Sujatha *et al.*[6] reported that Gamborg's B5 basal medium proved to be superior to MS basal medium. Warakagoda and Subasinghe[2] have reported that B5 medium was found best for seed germination of *Jatropha curcas* among the three media (MS, B5 and woody plant medium) used in their study. In contrast however, the work of Thangjam and Maibam[11] in a comparison between MS and B5 media showed MS to be better in the callus induction and formation from cotyledon explants of *Parkia biglobosa*. However, there is no report on the comparative effect of these basal media (B5 and MS) supplemented with plant growth regulators especially a cytokinin and an auxin using mature embryo as the explants.

Therefore, this study was specifically undertaken to compare the two kinds modified basal media, namely; Murashige and Skoog[12] and Gamborg *et al.*[13] using zygotic embryos as explants and supplementing both basal media with two growth regulators-an auxin (α-naphthalene acetic acid) and a cytokinin (benzyl adenine) for studying morphogenesis of embryo explants.

MATERIALS AND METHODS

Explant source and collection: *Jatropha curcas* fruits were taken from populations maintained over the years at Edem-ani, in Nsukka, Enugu State, Nigeria between September and October, from 2013-2017. The fruits were sun-dried for six days before being stored in plastic vials containing silica gel from which they were withdrawn as needed and dehusked. Seeds (Plate 1) were soaked in water for 24 h and seeds were cut open and zygotic embryos (Plate 2) that served as the explants for the experiment were aseptically removed. A dissecting microscope was used to excise the embryos which measured between 1.5 and 2 cm. The experiment was carried out from between June to September every year since 2013 till 2017. Every year similar experiments were repeated to ensure reproducibility.

Plate 1: Seeds of *J. curcas*

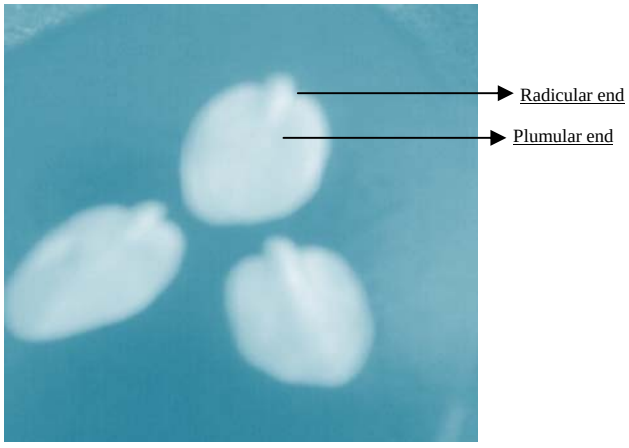

Plate 2: Embryos of *J. curcas* (explants)

Preparation of inorganic salts, vitamin and growth regulators stock solution: All chemicals used for this experiment were of analytical grade and were obtained from Sigma-Aldrich (USA), British Drug House (BDH) (England), Harkin and Williams (England), Qualikems (India), Fluka (Germany), May and Baker (England). Reagents used for all the experiments were commercial kits and products of Randox (USA) and Teco (TC) (USA) in this study, series of stock solutions of the media were made, from which appropriate strengths and concentrations were taken. The media were composed of macro salts, micro salts, iron compounds and organics (which included: Myo-inositol, thiamine-HCL, nicotinic acid, pyridoxine and glycine). Two major plant growth regulators used as supplements in this study were α-naphthalene acetic acid (NAA) which is an auxin and benzyladenine (BA), a cytokinin. These growth regulators were applied singly and in combinations at four concentrations between 0 and 10.0 mg L^{-1} giving rise to sixteen different growth regulatory treatments. These stock solutions of growth regulators were stored at 4°C.

Culture media: Culture media employed were modified basal media of Murashige and Skoog (MS)[12] and Smith (B5)[14] each was supplemented with concentrations ranging from 0-10.0 mg L^{-1} of NAA and BA, respectively. Cultures with no growth regulatory supplements were maintained as controls for the study.

Maintenance of asepsis: The mature seeds, under running water were washed to get rid of dirt and then sterilized by immersion in 70% absolute ethanol (v/v) as well as 10% (v/v) sodium hypochlorite (NaOCL) from Clorox for 10 min, followed by three rinses in sterile distilled water. Before inoculation in the sterile laminar air-flow hood, the seeds were removed from the seed coats and endosperm revealing the embryo with the use of sterile forceps and scalpels, on a sterile A4 paper in a Petri dish. Embryos were transferred one by one into culture vessels (test tube). The temperature, light cycle and intensity of the growth room were 25±2°C, 16 h light/8 h dark cycles and 2500 lux respectively.

Statistical analysis: There was a total of sixteen treatments which are listed below were used for statistical analysis:

T1 = (control) media+0.0 NAA/BA 0.0
T2 = Media+0.0 NAA/0.1 BA
T3 = Media+0.0 NAA/1.0 BA
T4 = Media+0.0 NAA/10.0 BA
T5 = Media+0.1 NAA/0.0 BA
T6 = Media+0.1 NAA/0.1 BA
T7 = Media+0.1 NAA/1.0 BA
T8 = Media+0.1 NAA/10.0 BA
T9 = Media+1.0 NAA/0.0 BA
T10 = Media+1.0 NAA/0.1 BA
T11 = Media+1.0 NAA/1.0 BA
T12 = Media+1.0 NAA/10.0 BA
T13 = Media+10.0 NAA/0.0 BA
T14 = Media+10.0 NAA/0.1 BA
T15 = Media+10.0 NAA/10.0 BA
T16 = 10.0 NAA/10.0 BA
All treatments in mg L^{-1}

Treatments 1-16 were composed of 200 mL of media supplemented with NAA and BA at different concentrations. Each treatment had 10 replicates. Embryos were cultured per vessel in each treatment. The experimental set up was done in a 2×2 x 4 (2 media, 2 growth regulators and 4 levels of concentration of each of the growth regulator) factorial in completely randomized design. Experiments comprised 16 treatments with 10 tubes for each of the treatments. To ascertain the reproducibility of the research outcome, each sets of treatments was repeated thrice within one year and embryos were cultured one per vessel. The data computation from germination studies was done using the one-way analysis of variance (ANOVA) from GENSTAT statistical package.

Regeneration studies: Regeneration of embryo explants of *Jatropha curcas in vitro* were monitored daily from time of inoculation. By the 10th day, when plantlets had been observed under some treatments and were gently removed from test tubes, culture medium was washed off from the roots and the following growth parameters were recorded thus: Leaf number, shoot length, length of roots (cm) and regeneration percentage. The lengths of shoots and roots were measured with a string and a metre rule while the numbers of leaves were determined by counting. The percentage of regeneration was estimated thus:

$$\text{Regeneration (\%)} = \frac{\text{Number of regenerated embryos}}{\text{Total number of embryos 1}} \times 100$$

RESULTS

***In vitro* studies of embryo explants:** It was observed that within two days of culture of the embryo explants of *Jatropha curcas*, the cotyledons which had a white colour at the beginning (Plate 3). Plantlets that emerged exhibited a healthy growth having been cultured on modified basal medium of B5 treated with hormones (Plate 4) and untreated (Plate 5). They turned green and opened up a little on both media (Plates 6a, 6b). By the fourth day, cotyledons opened fully and the green colour became prominent (Plate 7). There was a protrusion of the radicle which emerged from the base of the embryo axis. Embryos elongated both at the root and at the shoot axes: Five roots were observed: One tap root and four lateral roots (Plates 8, 9).

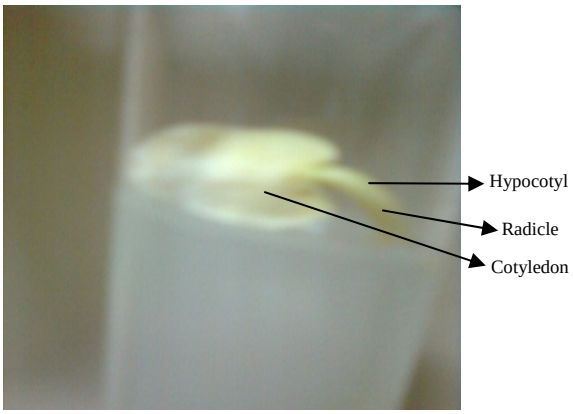

Plate 3: Two-day old embryo explant of *J. curcas* (showing elongation of hypocotyl and radicle. Cotyledon is gradually changing from white colour to green)

Plate 4: Ten-day old plantlet regenerated from embryo explant of *J. curcas* on B5 medium treated with 0.1 mg L^{-1} NAA 0.1 mg L^{-1} BA concentrations

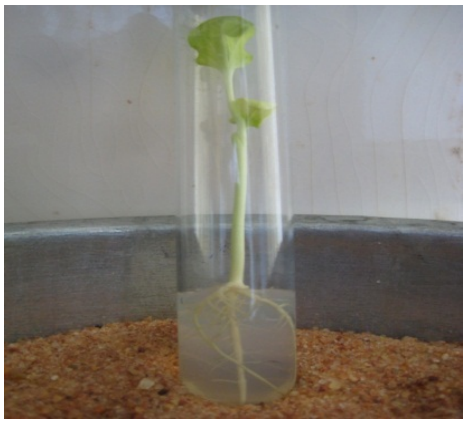

Plate 5: Ten-day old plantlet regenerated from untreated embryo explant of *J. curcas* and on B5 medium (control)

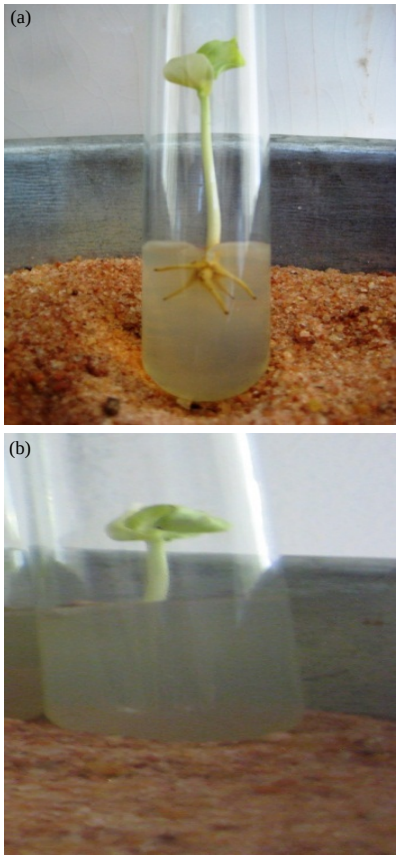

Plate 6(a-b): (a) Highest shoot-growth (29.9±0.32 mm) on B5 medium supplemented with 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA and 1.0 mg L^{-1} BA and (b) Least shoot-growth (2.6±0.11 mm) on B5 medium supplemented with 0.0 mg L^{-1} NAA

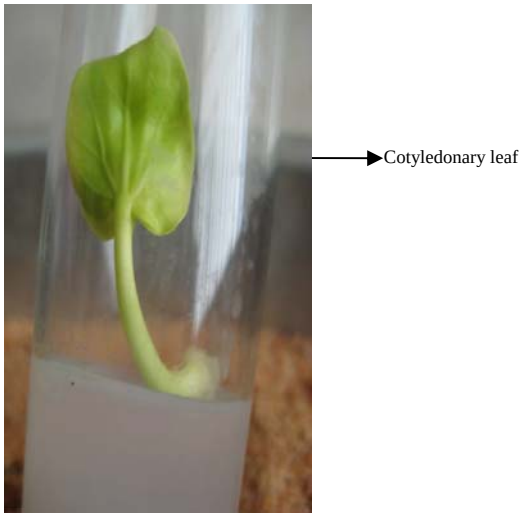

Plate 7: Four-day old plantlet of *J. curcas* showing prominent green colour of cotyledonary leaf

Plate 8: Plantlets arising from embryo explants of *J. curcas* showing roots

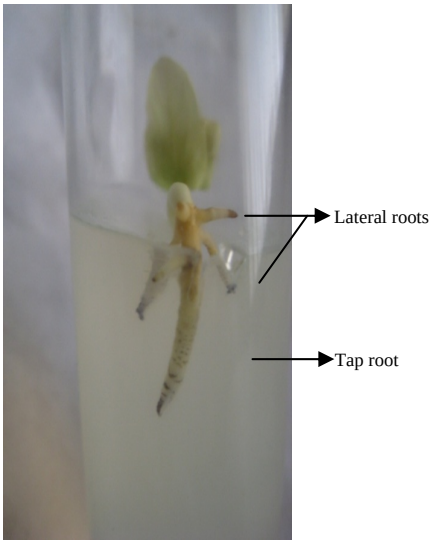

Plate 9: Plantlets arising from embryo explants of *J. curcas* showing lateral roots and tap

Sprouting embryo explants of *J. curcas* at two-day intervals (for 10 days) on MS and B5 media as affected by growth regulators: Figure 1 showed that in MS medium, embryo regenerated in the 16 growth-regulatory treatment combinations by the second day of culture. Embryos treated with growth regulatory substances in combi0nations of NAA at 0.0 mg L^{-1}/BA at 10.0 mg L^{-1} (T4) as well as NAA at 1.0 mg L^{-1}/BA at 0.1 mg L^{-1} (T10) recorded the highest

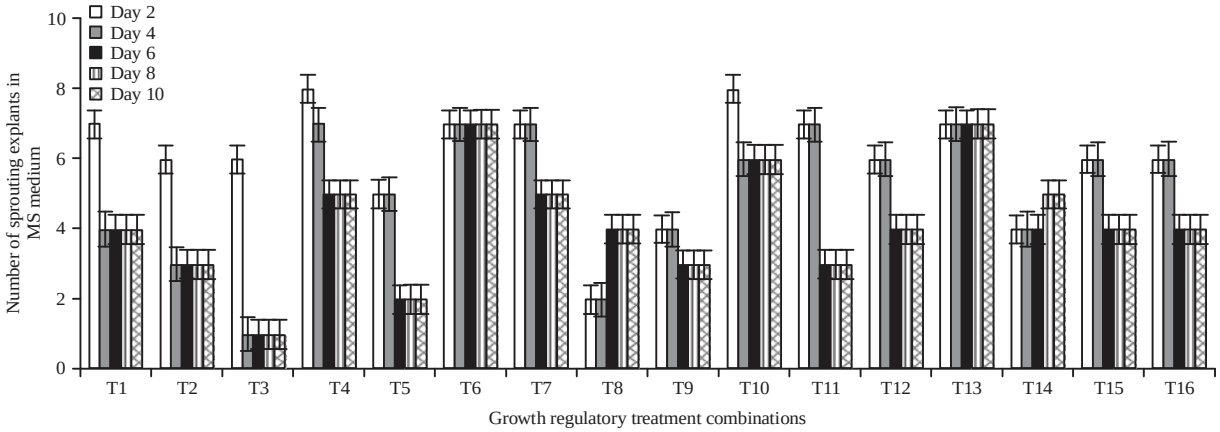

Fig. 1: Effects of growth regulatory substances (NAA/BA) on the number of sprouting embryo explants of *Jatropha curcas* at two day intervals for ten days on MS medium

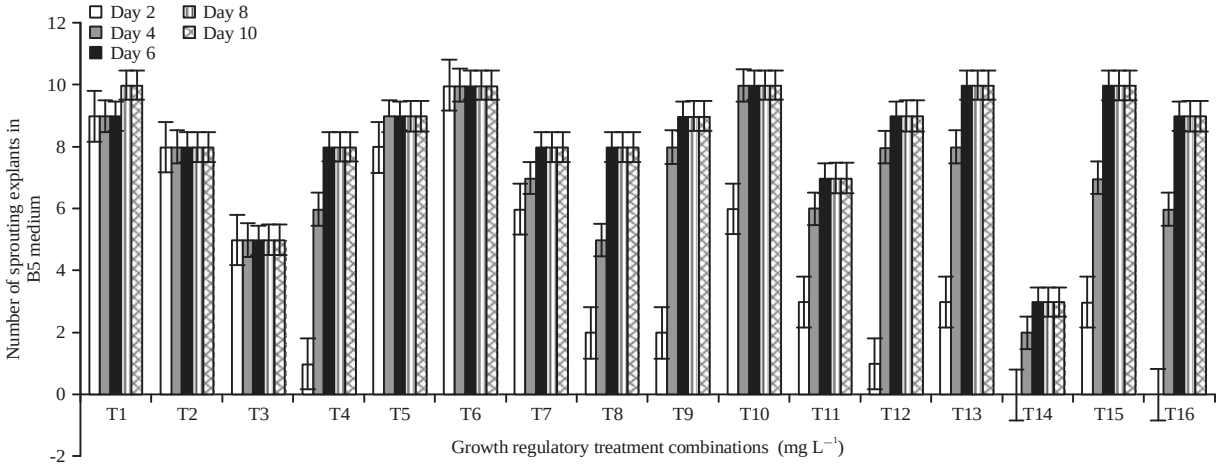

Fig. 2: Effects of growth-regulatory substances (NAA/BA) on the number of sprouting embryo explants of *Jatropha curcas* at two day intervals for ten days on B5medium

Table 1: Effects of basal media on growth parameters

Variables	MS	B5	$LSD_{(0.05)}$
Length of roots	0.039±0.01	0.142±0.03*	0.053
Length of shoots	0.412±0.06	1.282±0.09*	0.159
Number of leaves	0.575±0.07	1.387±0.07*	0.165

*Significant different at p<0.05

number of sprouts on day two followed by embryos treated with growth regulatory substances in combination of 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA (T6) as well as 10.0 mg L^{-1} NAA and 0.0 mg L^{-1} BA (T13).

In B5 medium, embryos regenerated but not in all treatments by the 2nd day of culture. Embryos treated with 10.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA (T14) as well as 10.0 mg L^{-1} NAA and 10.0 mg L^{-1} BA (T16) recorded no growth *in vitro* by the 2nd day. The highest number of regenerants was recorded for the treatment combinations of 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA (T6) (Fig. 2).

Comparative studies of the effects of basal media on parameters: From Table 1, the two media brought about the *in vitro* regeneration of the embryo explants of *Jatropha curcas*. The media had effects on the length of roots, length of shoots and number of leaves of the explants. The lengths of roots and shoots as well as numbers of leaves produced by the embryo explants, achieved on B5 medium were 0.142±0.03, 1.282±0.09 and 1.387±0.07 cm, respectively, while the same parameters achieved on MS medium recorded 0.039±0.01, 0.412±0.06 and 0.575±0.07 cm, respectively. The multiple comparisons of means (using LSD) showed that B5 medium differed significantly (p<0.05) from MS medium in all parameters (Table 1). This may suggest in this study that, B5 medium was found superior to MS medium for *in vitro* regeneration of *Jatropha curcas* through embryo culture.

Mean regeneration (%) of embryo explants of *Jatropha* Mean *curcas* as affected by NAA and BA treatment combinations on MS and B5: Results showed that MS medium had a significant ($p<0.05$) effect on the *in vitro* regeneration of embryo explants of *Jatropha curcas* (Table 2). This means that the medium contributed to its growth *in vitro*. Embryos treated with 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA as well as 10.0 mg L^{-1} NAA and 0.0 mg L^{-1} BA recorded the highest mean regeneration (%) of 70.00±0.00. This was followed by embryos treated with 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA which recorded a mean regeneration (%) of 64.00±4.00. Embryos treated with 0.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA gave the lowest mean regeneration (%) of 20.00±10.00. The multiple comparisons of treatment showed that treatments which recorded the highest regeneration percentage, differed significantly (5%) from some growth regulatory treatment combinations. Also it did not differ from some others (Table 2).

Results equally showed that B5 medium had a significant ($p<0.05$) effect on the regeneration of embryo explants of the physic nut. As in MS medium, embryos treated with the same concentration of NAA and BA at 0.1 mg L^{-1} recorded a hundred mean regeneration (%) (100.00±0.00). No embryo-mortality occurred in this treatment. This was followed by embryos that received no treatment (control) which recorded a mean regeneration (%) of 94.00±2.45. The lowest mean regeneration (%) of 22.00±5.83 was recorded for embryos treated with 10.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA. The multiple comparisons of treatments showed that the treatment which recorded the highest mean regeneration was significantly ($p<0.05$) different from some growth regulatory treatments. Also it did not differ significantly ($p>0.05$) from some others and the control (Table 2).

Length of roots: In B5 medium, the untreated explants (control) recorded the highest root length of 8.42±0.33 mm while the least root length of 0.000±0.00 mm was recorded for the growth regulatory treatment combinations of 0.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA (Fig. 3). ANOVA showed that the effects of the various treatments on the length of roots in B5 medium were significant at 5% level. This means that the application of plant growth regulators reduced the length of roots. The multiple comparisons ($LSD_{0.05}$ = 0.270) of the treatment means for length of roots showed that the control differed significantly ($p<0.05$) from all other treatments meaning that the elongation of roots of embryos of *Jatropha curcas* is possible even with no addition of plant growth regulators.

Similarly, in MS medium the control recorded the highest length of roots (1.12±0.05 mm) while the least (0.000±0.00 mm) was observed with the growth regulatory treatment combinations of 0.0 mg L^{-1} NAA and BA at 1.0 mg L^{-1} (Fig. 4). ANOVA showed that the effects of the various treatments on the length of roots in MS medium

Table 2: Mean regeneration (%) of embryo explants of *Jatropha curcas* as affected by NAA and BA treatment combinations on MS and B5 media

Hormonal treatments (mg L^{-1})		Germination (%)	
NAA	BA	MS	B5
0.0	0.0	46.00±6.00*	94.00±2.45^{ns}
0.0	0.1	36.00±6.00*	80.00±0.00^{ns}
0.0	1.0	20.00±10.00*	50.00+0.00*
0.0	10.0	60.00±6.32^{ns}	62.00±13.56*
0.1	0.0	32.00±7.35*	88.00±2.00^{ns}
0.1	0.1	70.00±0.00	100.00±0.00
0.1	1.0	58.00±4.90^{ns}	74.00±4.00^{ns}
0.1	10.0	32.00±4.90*	62.00±12.00*
1.0	0.0	34.00±2.45*	74.00±13.64^{ns}
1.0	0.1	64.00±4.00^{ns}	92.00±8.00^{ns}
1.0	1.0	46.00±9.80*	60.00±7.75*
1.0	10.0	48.00±4.90*	72.00±15.62*
10.0	0.0	70.0±0.00	82.00±13.56^{ns}
10.0	0.1	44.00±2.45*	22.00±5.83*
10.0	1.0	48.00±4.90*	80.00±13.78
10.0	10.0	48.00±4.90*	66.00±17.49*
$LSD_{(0.05)}$		15.95	28.45

*Mean values significantly ($p<0.05$) different from the treatment combination with the highest percentage regeneration of embryos explants on both media, ^{ns}Mean values not significantly ($p>0.05$) different from the treatment combination with the highest percentage regeneration of embryos explants on both media, $^{Ns: Non-significant}$Mean values will perform as good as treatments that elicited the best response which also implies that embryo explants of *Jatropha curcas* can germinate and grow to maturity even when they are not treated with any growth regulator especially on a B5 medium

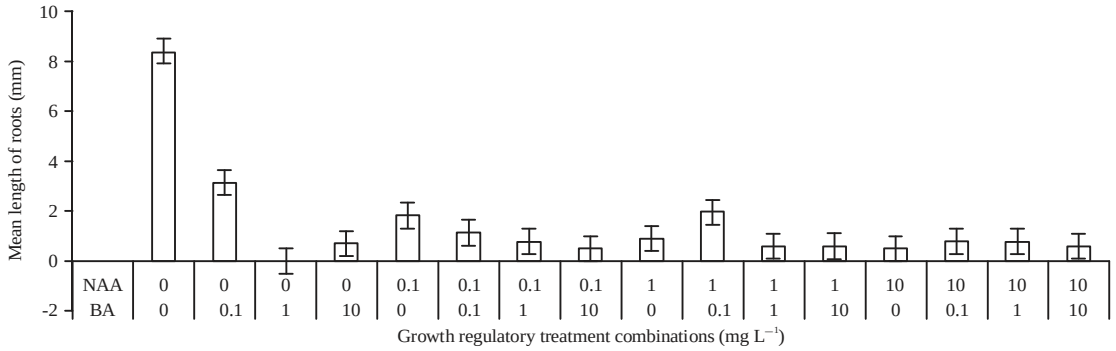

Fig. 3: Effects of growth regulatory substances (NAA/BA) on the length of roots of plantlets produced from embryo explants of *Jatropha curcas* on B5 medium

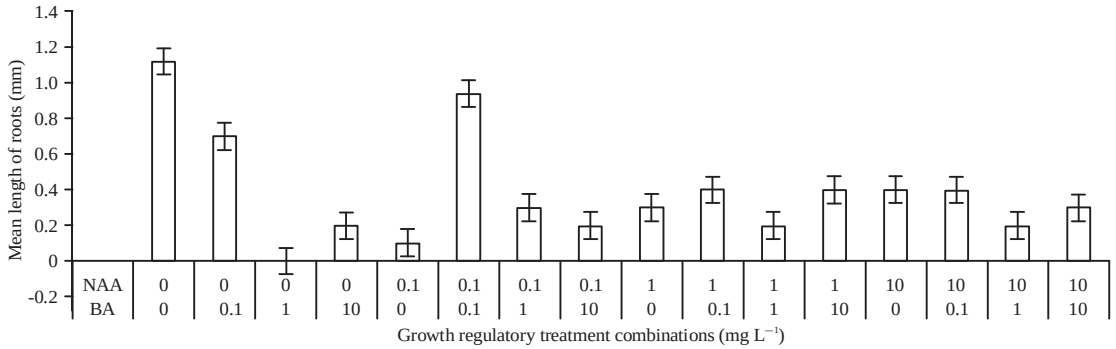

Fig. 4: Effects of growth regulatory substances (NAA/BA) on the length of roots of plantlets produced from embryo explants of *Jatropha curcas* on MS medium

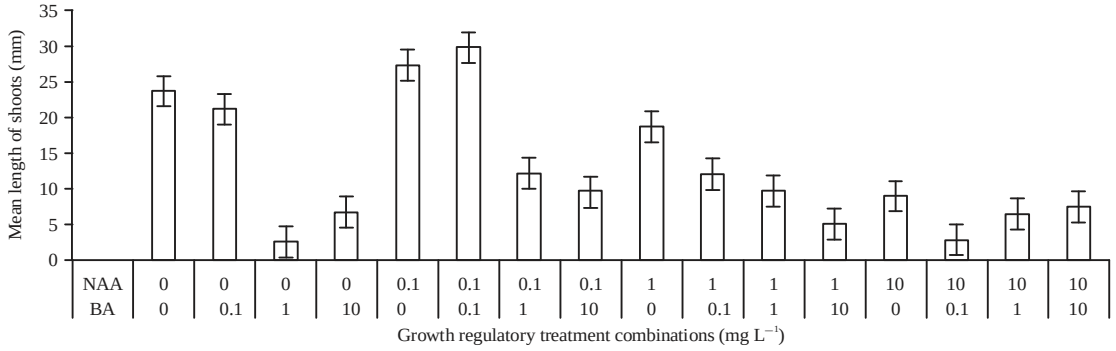

Fig. 5: Effects of growth regulatory substances (NAA/BA) on the length of shoots of plantlets produced from embryo explants of *Jatropha curcas* on B5 medium

were not significant ($p>0.05$). The multiple comparisons ($LSD_{0.05} = 0.074$) of the treatment means for length of roots showed that the control differed significantly ($p<0.05$) from some of the treatments.

Length of shoots: The results of the length of shoots of embryo explants of *Jatropha curcas* recorded after 10 days of culture on B5 medium showed that the growth regulatory treatment combinations of NAA at 0.1 mg L^{-1} and BA at 0.1 mg L^{-1} had the highest shoot length of 29.9±0.32 mm (Plate 7a). The least shoot length of 2.6±0.11 mm (Plate 7b) was recorded for the growth regulatory treatment combinations of NAA and BA at 0.0 and 1.0 mg L^{-1}, respectively (Fig. 5). ANOVA showed that the effects of the

various growth regulatory treatment combinations on the length of shoots were significant ($p<0.05$). This means that the application of plant growth regulators (taking the control as a standard) either reduced or increased the length of shoots. The multiple comparisons ($LSD_{0.05} = 0.761$) of the treatment means showed that the treatment with the highest shoot length differed significantly ($p<0.05$) when compared with some treatments. It did not differ significantly ($p>0.05$) from the control and the treatment of NAA at 0.1 mg L^{-1} and 0.0 mg L^{-1} BA, meaning that the control, as well as its counterpart can be as useful as the treatment that elicited the best response.

It was equally observed that in MS medium, the same growth regulatory treatment combinations of NAA at 0.1 mg L^{-1} and BA at 0.1 mg L^{-1} recorded the highest shoot length of 12.9±0.37 mm. The least shoot length of 1.2±0.06 mm was recorded for the growth regulatory treatment combinations of 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA (Fig. 6). ANOVA showed that the various growth regulatory treatments had a significant effect on the length of shoots. The multiple comparisons ($LSD_{0.05} = 0.593$) of the treatment means showed that treatment with the highest shoot length was not significantly ($p>0.05$) different from the control but differed significantly ($p<0.05$) from other treatments.

Number of leaves: Results showed that in B5 medium, the control and two other growths regulatory treatment combinations (NAA at 0.1 mg L^{-1} and BA at 0.1 mg L^{-1} as well as 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA) recorded the highest number of leaves of 20.0±0.10 mm. The least leaf number of 2.0±0.13 mm was recorded for the growth regulatory treatment combinations of NAA and BA at 0.0 mg L^{-1} and 1.0 mg L^{-1}, respectively (Fig. 7). ANOVA showed that the effects of the various growth regulatory treatment combinations on the number of leaves were significant ($p<0.05$). The multiple comparisons ($LSD_{0.05} = 0.683$) of treatment means showed that treatments that recorded the highest number of leaves differed significantly but not from all of the other treatments.

Also, the same growth regulatory treatment combinations of 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA in MS medium had the highest number of leaves of 12.0±0.32 mm (Fig. 8). The

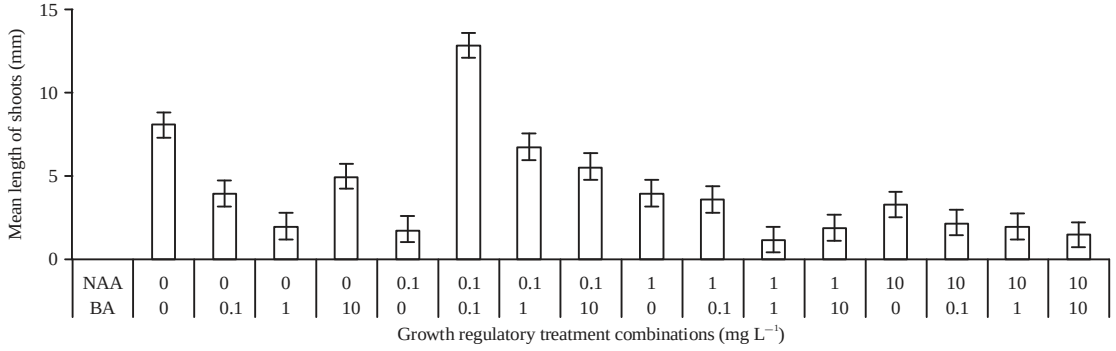

Fig. 6: Effects of growth regulatory substances (NAA/BA) on the length of shoots of plantlets produced from embryo explants of *Jatropha curcas* on MS medium

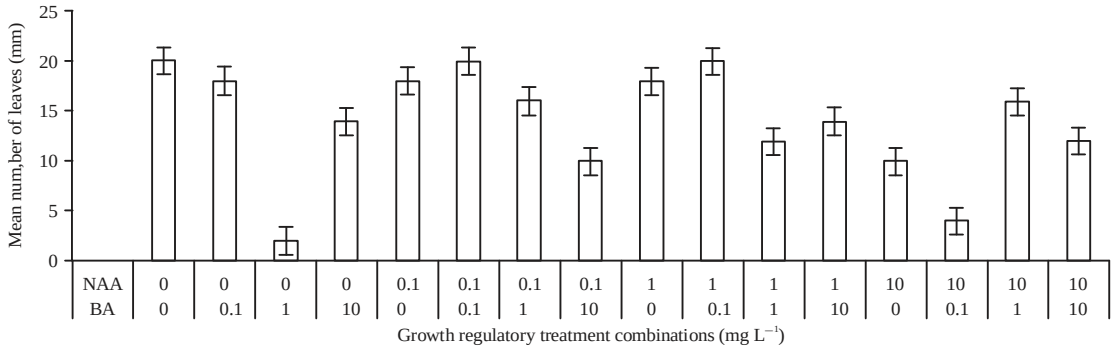

Fig. 7: Effects of growth regulatory substances (NAA/BA) on the number of leaves of plantlets produced from embryo explants of *Jatropha curcas* on B5 medium

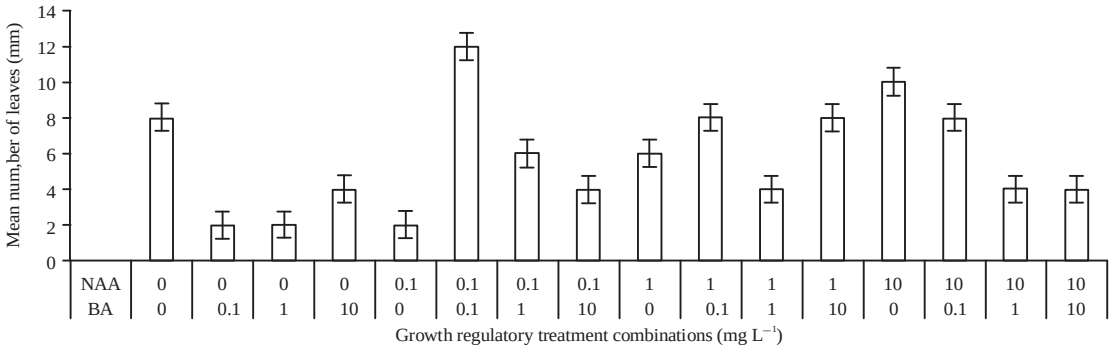

Fig. 8: Effects of growth regulatory substances (NAA/BA) on the number of leaves of plantlets produced from embryo explants of *Jatropha curcas* on MS medium

growth regulatory treatment combinations of 0.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA among two other treatments recorded the least leaf number of 2.0±0.20 mm (Fig. 8). ANOVA showed that the effects of the various growth regulatory treatment combinations on the number of leaves were not significant. The multiple comparisons ($LSD_{0.05}$ = 0.798) of treatment means showed that treatments with the highest number of leaves differed significantly but not from all of the other treatments.

DISCUSSION

The colour change of the cotyledon observed in the *in vitro* regeneration of embryo explants of *Jatropha curca* as observed this study from white to green about two days from time of inoculation may be an indication of autotrophy. The observation that the radicle and hypocotyl of the embryo explants which later gave rise to roots and shoot is consistent with the reports of Mohan *et al.*[10] in their studies on seed propagation and zygotic embryo culture of *Jatropha curcas in vitro*. The earlier response of the embryo explants employed here might have been as a result of the fact that mature embryos were employed in this study whereas Mohan *et al.*[10] employed immature embryos. The rooting system of *J. curcas* observed by Shah *et al.*[15] is also in conformity with these findings. The initiation of radicle either occurred when the osmotic potential of the cells in the radicle became more negative due to the metabolism of storage reserves or cell walls were more flexible to allow cell expansion[16].

In this study also, there was neither callus formation nor proliferation of shoots. This could either be as a result of the type of explants cultured or the type and combination of growth regulators employed. Also, the result obtained in this study might have been due to the fact that levels of growth regulators added might not have been optimum to elicit such responses[17]. Warakagoda and Subasinghe[2] reported a significantly ($p<0.05$) higher number of shoots achieved using 1 mg L^{-1} each of BA and Kinetin as well as 2 mg L^{-1} of NAA.

In this study, the maximum *in vitro* growth with low survival frequency observed in MS medium against a slow but steady regeneration with high survival rate observed in B5 medium could be attributed to media composition[18-19]. Also, the delay and slow emergence of the embryos observed in some treatments of B5 medium might have been caused by the inability of the embryos to overcome the external osmotic potential[16].

In this study, two media were compared (MS and B5) and both of them supported the *in vitro* regeneration of embryo explants of *Jatropha curcas* but B5 medium was found to be significantly ($p>0.05$) superior to MS medium in all growth parameters studied (mean regeneration (%), length of roots, length of shoots and number of leaves). This finding may suggest that B5 is better than MS for micropropagation of *J. curcas* through embryo culture. Also, these growth parameters helped us to determine the best growth regulatory treatment combinations for *in vitro* regeneration of the explants.

Bhojwani and Razdan[20] has maintained that the basic difference in basal media could be as a result of the amount of ions present in them. Similarly, George[21] considered the concentration of nitrogen and the fraction of nitrate to ammonium as two important factors useful in considering media formulations peculiar for different plant species. According to latter, a pronounced level of ammonium ion and the total nitrogen are much higher in MS medium than in the majority of media commonly used. Ions and the forms in which they are supplied are important in eliciting particular i*n vitro* responses in plants[12].

Murashige and Skoog[12] noted that the *in vitro* nutritional requirement for an ideal growth rate of a tissue from

different species or various tissues within a species may differ significantly in its nutritional conditions for adequate growth. It is emphasized that no sole basal medium can be sufficiently satisfactory for all plant part[20-22] . In addition, in most media, it is usually observed that a particular type of ion may be contributed by more than one salt in a media. For example, in MS medium, nitrate ions (NO_3^-) are contributed by ammonium nitrate (NH_4NO_3) as well as potassium nitrate (KNO_3), while potassium ions (K^+) are contributed by KNO_3 and potassium dihydrogen phosphate (KH_2PO_4). In B5 medium, nitrate and potassium ions are contributed by potassium nitrate while sulphate ions are contributed by ammonium sulphate (NH_4SO_4). Pierik[22] asserted that the most important factors in media comparison are ammonium and potassium ions. Toosi and Dilmagani[23] also suggested that the concentration should be calculated in millimolar (mM) in order to determine the final concentration of each ion whenever two different media are to be compared.

Comparing (MS/B5) basal media, taking into account the total concentrations of ions in them, the nitrate and ammonium ions for Murashige/Skoog medium recorded 39.41 and 20.62 mM, respectively, with total nitrogen (N) of 60.03 mM while B5 medium recorded 25.00 and 2.00 mM, respectively, with total nitrogen of 27.00 mM. Potassium ions recorded 20.05 and 25.00 mM for MS and B5 media, respectively[20]. From this analogy, it can be deduced that an increase in potassium (K) in B5 medium might have resulted in a marked increase in yield at low nitrogen levels[21]. Also, it is possible that B5 medium performed better than MS medium due to low quantity of ammonium ions found in B5. This finding may be in line with the report of Gamborg *et al.* [13]. where they mentioned that ammonium ions depressed the growth of soya bean cells when the concentration exceeded 2 mM.

Results obtained from the mean percent *in vitro* regeneration when embryo explants of *J. curcas* were cultured on MS and B5 media showed that treatments with the highest percentage regeneration differed significantly ($p>0.05$) from some treatment combinations but did not differ from others including the control in B5. This means that those that did not differ significantly ($p<0.05$) will perform as well as those that elicited the best response. It equally suggests that embryo explants of *Jatropha curcas* can regenerate (though slowly and irregularly) *in vitro*, to mature plantlets even in the absence of growth regulators especially on a B5 medium. This would have been due to the presence of endogenous hormones within the plant, expressed after a lag phase during which a reasonable level of autotrophy had been attained. This may imply that mature embryo explants of *Jatropha curcas* are hormone-autonomous: cells, tissues and organs that are able to grow without additions of growth regulatory substances. This means that the effects on explants in terms of regeneration and survival rates due to mineral salts alone constitute what is known as "basal growth rate"[21].

Okafor *et al.*[24] on the carbon source requirement for plantlet growth of *J. curcas* (*in vitro*) using mature zygotic embryos as explant on the effects of sucrose on embryo rescue of the same plant are consistent with this present report. The findings here may also be in line with the findings of Bhojwani and Razdan[20] who emphasized that excised embryo whether mature or immature may not really need the addition exogenous growth regulators especially auxins for the normal development. Instead, they are inhibitory for excised embryo growth and enhances dormancy breaking in some plant. In the same vein, Okafor and Okezie[18] recommended that plant growth hormones should not be included to the embryo culture media since they results in structural deformity when applied in the medium.

In this study also, media supplemented with NAA and BA showed visible signs of explant differentiation especially at the lowest concentrations[6]. The MS and B5 media supplemented in combination with 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA (Plate 7a) gave the best response when compared to other treatments virtually in all growth parameters studied, while the least was observed with the growth regulatory treatment combinations of 0.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA (Plate 7a). The observation that NAA at 0.1mg L^{-1} in combination with 0.1mg L^{-1} BA induced root and shoot formation, is an indication that only certain levels of plant growth regulators are effective in inducing embryogenesis. This finding is in line with the reports of Mineo[25] where 0.1 mg L^{-1} NAA represented the formula with the highest physiological auxin activity when compared with 1.0 and 10.0 mg L^{-1} concentrations. Also, Wei *et al.*[26] maintained that the combination of 0.1 mg L^{-1} IBA and 0.5 mg L^{-1} BA on MS basal medium could produce the highest shoot regeneration from epicotyl explants of *Jatropha curcas*. In the same way, regeneration of shoot was reported to be more frequent when both BAP (0.2 mg L^{-1}) and IAA (0.1 mg L^{-1}) were combined than on BAP (0.2 mg L^{-1}) alone with endosperm tissues of *Emblica officinalis*[27].

Results equally showed that growth regulatory treatment combinations of 0.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA produced no root when cultured on both MS and B5 media. This may have been due to inappropriate concentration of cytokinin[17]. It was also observed that embryos treated with high concentrations of plant growth regulators like 10.0 mg L^{-1} NAA/10.0 mg L^{-1} BA were stunted in growth. Shah *et al.*[15]

reported that concentrations above 2mg L^{-1} of BAP showed a decreasing trend in emergence and length of shoots in *J. curcas*. The decreasing trend in emergence and length of shoot may be attributed to the BAP in the media which may have stimulated lateral growth but prevented apical dominance as confirmed by Nogueira *et al.*[28]. Similarly, George[21] maintained that high level auxin is directly proportional to ethylene production and ethylene which accumulates in culture tubes may impede the growth and development of cultured plants.

CONCLUSION

The micropropagation protocols for *J. curcas* described in this study have the potential for the mass production of plantlets within a short time without the inclusion of plant growth regulatory substances. Mature zygotic embryos of *J. curcas* have sufficient innate hormone in a potassium ion rich and low ammonium B5 medium that is necessary to drive the morphogenesis and growth of *J. curcas* plantlets. Results obtained in this study indicated that Gamborg's medium (B5) was the most suitable for *in vitro* micropropagation of *J. curcas* using embryo explants.

SIGNIFICANCE STATEMENT

Plant growth regulatory substances (PGRs) are seen to be necessary for the plantlet regeneration of zygotic embryos (mature and immature) of various plants. In this study, therefore, *J. curcas* mature zygotic embryos regenerated into healthy plantlets *in vitro* without the supplementation of any PGRs used. On the other hand, MS basal medium is the most commonly used basal medium for various cultures *in vitro* but in this study, explants in B5 medium produced healthy plantlets relative to MS medium. In future, it is necessary to discover, isolate and characterize the type of innate hormone found in this plant and study the interaction between synthetic PGRs and basal media ions as they affect the morphogenesis of *J. curcas in vitro*.

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