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Growth Evaluation of *In-Vitro* Propagated Embryo of *Morinda Citrifolia* L. Seeds

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ABSTRACT: The dormant nature of *Morinda citrifolia* seeds is a limitation to its efficient *in-vitro* plantlet multiplication. Hence, the use of embryo culture for successful in-vitro culture initiation. Matured embryo of freshly collected noni seeds were cultured on Murashige and Skoog basal medium supplemented with kinetin (Kn) and Benzyl amino purine (BAP) in the range of A: control (no addition); B: 0.5 mg/l Kn+1.0 mg/l BAP; C: 1.0 mg/l Kn+2.0 mg/l Bap; D: 1.5 mg/l Kn+3.0 mg/l BAP and E: 2.0 mg/l Kn+4.0 mg/l BAP. The results at 4 weeks after inoculation (WAI) showed that germination was faster from medium A without hormone whereas highest percentage germination was obtained from both medium D and E with 80 %. Medium B and C had 65 % each while medium A gave the least (40 %). The development of the plantlets showed that longest shoot (3.9 cm) from medium A was closely related to 3.58 cm from Medium B while root lengths (2.28 cm) and number of adventitious roots (26) from medium A were significantly higher than other media at 12 WAI. Highest number of nodes (2.25) obtained from medium D was comparable to Media C and B while medium A had the least at 12 WAI. Number of leaves obtained was similar between the media at 12 WAI. These results indicated that using embryo is reliable for fast in-vitro propagation and shoot development of noni plant with optimum cytokinins (0.5/1.0 mg/l Kn/BAP) application.

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Morinda citrifolia Linn most commonly known as noni or Indian mulberry is a fruit producing shrub of family Rubiaceae. The species originated from Southeast India and Asia to Australasia (Singh *et al.,* 2011). The species is of research interest because of its high medicinal and food value. Various parts of the species contained phytochemicals that made them suitable for treatment of different diseases and ailments (Assi *et al*., 2017). Most importantly, the species fruit is processed into juice of high economic value in the international market. The juice contains a lots of bioactive compounds such as anthraquinones, saponins, scopoletin, flavonoids, alkaloids and essential oil in addition to vitamin C, B1, B2, B3 and B12 which qualified the juice as medicinal supplement (Satwadhar *et al*., 2011). The species propagation has been achieved in the past by either conventional or plant tissue culture techniques (Shekhawat *et al*., 2015; Jayaprakash *et al*., 2017). However, of different in-vitro propagation protocols developed for the species, none employed the use of embryo for its culture. Seed embryos can be performed to overcome seed dormancy, shorten the period of germination and breeding cycle, determine seed viability, conduct micro-cloning of the source material for rapid multiplication, and conserve several medicinally important and endangered plants (Bhatia, 2015; Drewes-Alvarez, 2017). Noni seed is characteristically dormant and require seed pre-treatment for fast germination. Alternatively, the use of embryo can facilitate rapid and efficient multiplication of noni elite plantlets (Hussain *et al*., 2012). Hence, the present work aimed at assessing the in-vitro growth of matured noni embryo with a view to provide a reliable and effective culture initiation protocol for mass propagation of the species.

MATERIALS AND METHODS

Study site: The study was conducted in the Tissue culture laboratory of Biotechnology Section, Department of Bioscience, Forestry Research Institute of Nigeria (FRIN). The Institute is located on longitude 07°23'18′′ to 07°23'43′′N and latitude 03°51'20′′ to 03°23'43′′E (FRIN, 2018).

Treatments and Experimental design: The experiment comprised five treatments which are Murashige and Skoog (MS) basal medium supplemented with Benzyl Amino Purine (BAP) and Kinetin (Kn) in order of A: 0.0 mg/l Kn+0.0 mg/l BAP; B: 0.5 mg/l Kn+1.0 mg/l BAP; C: 1.0 mg/l Kn+2.0 mg/l Bap; D: 1.5 mg/l Kn+3.0 mg/l BAP and E: 2.0 mg/l Kn+4.0 mg/l BAP. These were prepared with ten replications each and laid out in a completely randomised design.

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Media preparation: MS basal medium was prepared by adding 34.43g of MS powder with Vitamin and sucrose supplements (M5501, SXS5501015A) per litre of distilled water. The steps include the addition of the MS powder to a volume of distilled water lesser than the final volume and stirring until completely dissolved. Then dividing the media into five treatments while adding growth regulators accordingly. Adjusting the pH of the media to 5.8 and making up to final volume. Addition of Agar at 9g/l, homogenising the agar and dispensing the media at 20 ml per tube. The media with forceps, petri-dish laid filter paper and bottle of distilled water were then sterilized in autoclave at 121 °C and 15 *psi* for 15 minutes.

Explant collection and sterilization: Matured, ripen and soft fruit of Noni were obtained from Central nursery of FRIN. The fruits were placed in a basket and mashed under flowing tap in order to extract the seeds. The extracted seeds were then sundried, wrapped with paper and kept in a dry place. Seeds of relatively uniform size were sorted and their embryo were removed with the aid of surgical blade. The embryo were surface sterilised by soaking in 70 % ethanol for 3 minute, rinsed 3 times and thereafter soaked in 10 % hypochlorite + 2 drops of Tween 20 for 10 minute. It was then rinsed four times with sterile distilled water and put on the sterile petri dish. The embryo were then inoculated into the media at one embryo per tube. The tubes were sealed and kept in the growth room under 16/8 hours light and dark photoperiod and 20 ± 2 °C.

Data collection and analysis: Data collected include germination percentage at 4 WAI while Shoot and Root lengths, Number of leaves, Number of adventitious roots and Number of nodes were collected at 4 weeks interval starting from 4 WAI. These were subjected to analysis of variance using GenStat (edition 4) and the significant means were separated with Fisher's protected Least Significant Different (LSD) at $p \leq 0.05$.

RESULTS AND DISCUSSIONS

Germination percentage: The results of the germination of inoculated Noni embryo at 4 Weeks after inoculation (WAI) is displayed in Figure 1. Though rapid germination was observed in medium A (control), maximum germination (80 %) was observed in each of MS media supplemented with 1.5 mg/l Kn+3.0 mg/l BAP (Treatment D) and 2.0 mg/l Kn+4.0 mg/l BAP (Treatment E). This was followed by 65 % from media added 0.5 mg/l Kn+1.0 mg/l BAP and 1.0 mg/l Kn+2.0 mg/l BAP (Treatment B and C respectively). The least (40 %) germination was from MS basal medium without the addition of any growth regulator (Treatment A). This results indicated that higher germination of cultured matured noni embryo was favoured by high levels of Kinetin and BAP combinations in the range of 1.5-2 mg/l Kn and 3-4.0 mg/l BAP. This was evident in downward germination rate towards the medium with no growth regulators. This results could be attributed to the dormant nature of the noni seed which could have made its embryos become inactive thereby requiring high level of cytokinins for better germination under 4 WAI. Naturally, it takes up to 6 months before noni seed germinate (Bhoomika, 2015). Hence, the obtained results underscore the positive role of growth regulators in the in-vitro response of the embryo culturing of the species. This results was related to that of Amaefule *et al*., (2018) where matured zygotic embryo of *Jatropha curcas* seed developed into plantlet better on MS medium supplemented with NAA and BA at 0.1 mg/l.

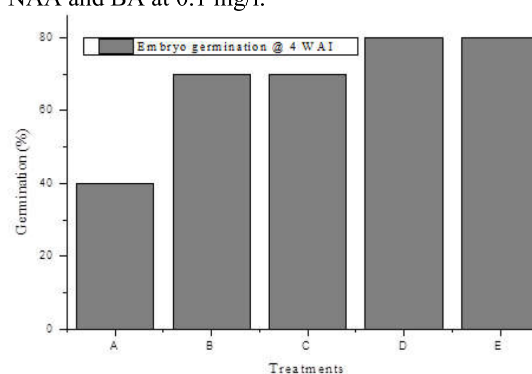

Fig. 1 Germination of Noni embryo at 4 weeks after inoculation (WAI) on MS medium supplemented with BAP and Kinetin
A: 0.0 mg/l Kn+0.0 mg/l BAP; B: 0.5 mg/l Kn+1.0 mg/l BAP; C: 1.0 mg/l Kn+2.0 mg/l Bap; D: 1.5 mg/l Kn+3.0 mg/l BAP; E: 2.0 mg/l Kn+4.0 mg/l BAP

Shoot and Root lengths (cm): The effect of kinetin and BAP combinations were assessed on the morphological growth of noni embryo. Analysis of variance showed that there was significant difference ($p \leq 0.05$) between the treatments means at 4 – 12 weeks after inoculation (WAI) (Table 1 and Plate 1). Shoot lengths from treatment A (0.0 mg/l Kn + 0.0 mg/l BAP) was significantly higher than other treatments at 4 and 8 WAI but similar to treatment B (0.5 mg/l Kn + 1.0 mg/l BAP) at 12 WAI. The least shoot length was observed from treatment E (2.0 mg/l Kn + 4.0 mg/l BAP) at 8 and 12 WAI. Moreover, root lengths from treatment A (1.3, 1.92 and 2.28) was higher than those from medium B at 4, 8 and 12 WAI respectively. Treatments C, D and E did not produce any root across successive weeks (Table 1 and Plate 1).

Table 1. Effect of kinetin and Bap combinations on shoot and root lengths of Noni embryo

Treatments	Shoot lengths (cm)			Root lengths (cm)		
	4WAI	8WAI	12 WAI	4 WAI	8WAI	12 WAI
A	1.9a	3.43a	3.9a	1.3a	1.92a	2.28a
B	0.93b	2.35b	3.58ab	0.28b	0.7b	1.2b
C	0.48b	2.15b	2.55b	0.0b	0.0c	0.0c
D	0.83b	2bc	2.52b	0.0b	0.0c	0.0c
E	0.58b	1.25c	1.35c	0.0b	0.0c	0.0c
LSD @ $P \leq 0.05$	0.49**	0.89**	1.16**	0.71**	6.0**	0.28**

***means difference in the same column were significant at p ≤ 0.01*
A: 0.0 mg/l Kn+0.0 mg/l BAP; B: 0.5 mg/l Kn+1.0 mg/l BAP; C: 1.0 mg/l Kn+2.0 mg/l Bap; D: 1.5 mg/l Kn+3.0 mg/l BAP; E: 2.0 mg/l Kn+4.0 mg/l BAP

Adventitious roots and Number of leaves: The results of adventitious roots showed that significantly higher ($p \leq 0.05$) number of adventitious roots (15, 23 and 26 at 4, 8 and 12 WAI respectively) were produced from treatment A without growth regulators higher than 0, 3.5 and 12.5 from treatment B at 4, 8 and 12 WAI respectively (Table 2 and Plate 1). Other treatments at the same period did not produce adventitious roots. There was no significant difference ($p > 0.05$) in the number of leaves produced between treatments except at 8 WAI (Table 2). Average number of leaves (8) from treatment E which similar to B and D (7 and 6.5 respectively) was higher than those of treatment A (control) at 8 WAI. Highest but not significant average number of leaves (8.5) obtained at 12 WAI was from treatment C (1.0 mg/l Kn+2.0 mg/l BAP) and E > D (8) > B (7.5) and A with the least (6).

Number of nodes: Table 3 showed the results of number of nodes produced by the noni plant at 8 and 12 WAI. All the media with kinetin/BAP additions produced comparable number of nodes which were higher than medium without growth regulators at 8 WAI. It was observed at 12 WAI that, treatment D (1.5 mg/l Kn+3.0 mg/l BAP) with average of 2.25 nodes was higher ($p \leq 0.05$) than 1.25 and 1.0 from treatments E (2.0 mg/l Kn+4.0 mg/l BAP) and A (control) respectively (Table 3 and Plate 1).

Table 2. Effect of kinetin and Bap combinations on number of leaves and adventitious roots of Noni embryo

Treatments	Adventitious roots			No of leaves		
	4WAI	8WAI	12 WAI	4WAI	8WAI	12 WAI
A	15a	23a	26a	2.25	4b	6
B	0b	3.5b	12.5b	1.5	7a	7.5
C	0b	0b	0c	1	6ab	8.5
D	0b	0b	0c	2.5	6.5a	8
E	0b	0b	0c	2	8a	8.5
LSD @ $P \leq 0.05$	4.19**	3.63**	6.57**	1.69	2.10*	2.94

and indicates means difference in the same column were significant at p ≤ 0.05 and p ≤ 0.01*
A: 0.0 mg/l Kn+0.0 mg/l BAP; B: 0.5 mg/l Kn+1.0 mg/l BAP; C: 1.0 mg/l Kn+2.0 mg/l Bap; D: 1.5 mg/l Kn+3.0 mg/l BAP; E: 2.0 mg/l Kn+4.0 mg/l BAP

Table 3. Effect of kinetin and BAP combinations on number of nodes of Noni embryo

Treatments	Number of Nodes	
	8WAI	12 WAI
A	0b	1c
B	1.5a	1.75abc
C	1a	2ab
D	1.5a	2.25a
E	1.25a	1.25bc
LSD @ $P \leq 0.05$	0.85**	0.80*

**and **indicates means difference in the same column were significant at p ≤ 0.05 and p ≤ 0.01*
A: 0.0 mg/l Kn+0.0 mg/l BAP; B: 0.5 mg/l Kn+1.0 mg/l BAP; C: 1.0 mg/l Kn+2.0 mg/l Bap; D: 1.5 mg/l Kn+3.0 mg/l BAP; E: 2.0 mg/l Kn+4.0 mg/l BAP

The observed better growth from medium A with no BAP and Kinetin in terms of longest shoot at 8 WAI (Table 1) and, root lengths (Table 1) and higher number of adventitious roots at 12 WAI (Table 2) implies that Noni embryo may not require external addition of cytokinins for these morphological development. This observation might be due to the production of natural and available endogenous growth substance in the species. These result correlated with that of Jayaprakash *et al*., (2017) who also observed root induction in cultured noni shoot tips without the exogenous supply of growth regulators. The growth similarity observed between media A (control) and B (0.5 mg/l Kn+1.0 mg/l BAP) in shoot length with increase in time could be attributed to degradation of the endogenous growth substance and

continuous availability of BAP and kinetin in medium B. This also explains the obtained higher number of leaves (Table 2) and nodes (Table 3) from other media at 12 WAI. These results also indicated that growth of noni embryo generated plantlets requires minimal cytokinins addition as there was no significant increase in number of leaves and nodes obtained at higher concentrations (Plate 1). Lack of better shoot elongation from medium E with highest Kinetin/BAP concentration (2.0 mg/l Kn+4.0 mg/l BAP) indicated that the level was too high. This was evident in more number of leaves produced with unusual shape and hyper-hydric shoot (Table and plate 1). Cytokinins are effective in removing apical bud dormancy and promoting multiple shoot formation however, higher concentration could cause the shoots formed to be too short for rooting and transfer (George and Debergh, 2008). This assertion explains the results obtained from medium E in this study.

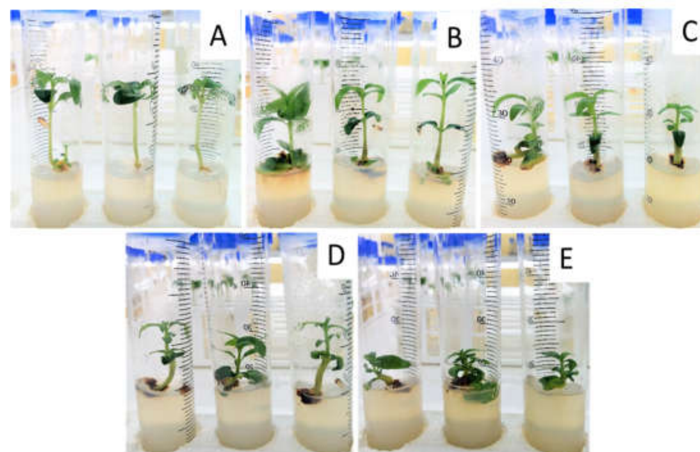

Plate 1. Growth of *M. citrifolia* embryo as affected by BAP and kinetin at 12 weeks after inoculation

Conclusion: Embryo culture is one of the numerous methods used in tissue culture to facilitate germination of dormant seeds. Growth assessment for in-vitro cultured matured noni embryo in the present study revealed that, germination took place under four weeks even in MS basal medium without growth regulators. Whereas, shoot growth and overall development of the plantlets required optimal addition of 0.5/1.0 mg/l Kinetin/BAP. This embryo technique is reliable and hence, the protocol is recommended for in-vitro culture initiation of noni plantlets.

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