

***In vitro* callus and shoot regeneration in *Enterolobium cyclocarpum* (Jacq.) Grised. - a fast timber yielding species**

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Abstract

This study was conducted to investigate the *in vitro* callus induction and rapid shoot regeneration potential in *Enterolobium cyclocarpum*, a plant native to central Mexico but widely introduced into Africa. The leaf, stem and nodal explants of *E. cyclocarpum* were cultured on full strength Murashige and Skoog (MS) medium supplemented with different concentrations of Cytokinins - Benzyladenine (BA) and/or Kinetin and Auxins - Naphthalene acetic acid (NAA) and/or 2,4-Dichlorophenoxyacetic acid (2,4-D) each alone and in combination. The leaf explants did not respond to these treatments. The Nodal explants were best for caulogenesis, while the explant responses were in the order- nodal > stem > cotyledon for callogenesis in MS medium supplemented with BA and/or Kin combined with NAA and/or 2,4-D. The varied combinations induced white compact callus. The highest callus production was observed on MS medium supplemented with 2.7 μM NAA + 2.2 μM BA and 5.4 μM NAA alone. Nodal and cotyledon explants developed callus and multiple shoots on MS supplemented with a combination of cytokinin (BA and/or Kin.) and auxin (NAA and/or 2,4-D). The maximum number of 3.98 ± 0.37 and 2.1 ± 0.11 shoots/explants were recorded for nodal and cotyledon explants on MS medium supplemented with a combination of 8.8 μM BA+2.7 μM NAA and 2.2 μM BA+2.7 μM NAA respectively. On the basal medium, 10% of the excised shoots rooted successfully. Thus, this *in vitro* method can be exploited for conservation and mass propagation of this fast timber yielding tree and also utilized for embryogenesis studies.

Keywords: callus; caulogenesis; callogenesis; explants; multiple shoots

Introduction

The anthropogenic deforestation occurring throughout the world has always been increasing, with an alarming rate in the last 3 decades in particular in West Africa primarily due to urbanization, unsustainable logging, agricultural farming and collection of fuel wood (FOMERCU, 1999; Sayer *et al.*, 2010). The gradual increase in deforestation practices for economic and/or social reasons without any simultaneous replanting is a global threat to the sustainability of the environment (FAO, 2005; Odediran *et al.*, 2013). Africa has the second highest rate of tropical deforestation in the world. The tropical forests in this region have declined at an annual rate of 3.4 million hectares between 2000 and 2010 because of degradation and deforestation processes (FAO, 2010; Eleanya, 2014).

Nigeria, alone lost a range between 3.5 to 3.7% of forest land per annum (Ladipo, 2010; UN-REDD, 2013;). Attendant consequences of deforestation are erosion, flooding, global warming, loss of habitat for earth's land animals and plants and not the least desertification (Farinola *et al.*, 2013). In order to mitigate these consequences, reforestation is urgently needed and this can be done by introducing fast-growing tree species such as *Enterolobium cyclocarpum*.

Enterolobium is an exotic species introduced to Nigeria and other tropical regions (Ezenwa, 1999; Rodríguez-Sahagún *et al.*, 2007). It is native to tropical America, where it is among the most invaluable and majestic tree tolerant of a wide range of rainfall level, temperature and soil condition. *E. cyclocarpum* is naturally distributed from western and central Mexico to the northern part of South America (Neimbro-Rocas *et al.*, 2003). *E. cyclocarpum* usually called monkey ear or ear pod tree belongs to the family Fabaceae. The genus *Enterolobium* contains only five species, all native to South and Central America. *E. cyclocarpum* is the only widely distributed and is the national tree of Costa Rica and prized for its ornamental, timber and large feathery shady relief from the intensity of the sun. It is a semi-deciduous, medium- to large-sized, and fast-growing tree species reaching about 35-50 m in height, wide spread of about 35 m or more (Rodríguez-Sahagún *et al.*, 2007). The trunk is branched with many stems, with light gray bark, and with prominent dark brown vertical fissure.

The tree crown is broader and widely spreading especially those that reach canopy (Figures 1 and 2). The bipinnate compound leaves are alternate, 15-40 cm in length, with 20-25 leaflets. The inflorescent consists of fragrant, white flowers, with about 20 filamentous stamens, as its main features, and a single pistil bounded together by a short green corolla and calyx. (Neimbro-Rocas *et al.*, 2003; Harmon, 2008; Pier, 2008).

The fruits are flattened, glossy, dark-brown indehiscent pod, shaped like an orbicular disk (Figures 3 and 4). The pod contains about 8-16 radially arrange seeds. The seed is hard-coated resembling small stone in strength and durability, which avoids germination until a structural modification (scarification) allows the hydration of the embryo. *Enterolobium* seeds are big, red-brown and marked with a conspicuous light brown ring. Seed are rich in protein (about 35%) and amino acid (Rodríguez-Sahagún *et al.*, 2007). They also contain iron, calcium, Phosphorus and ascorbic acid (Harmon, 2009). Also, seeds and bark contain tannins (Barwick, 2004).

The wood of *E. cyclocarpum* is lightweight and is resistant to termite attack (Uphof, 1959; Standley and Steyermark, 1976), which makes it feasible for house construction, furniture and shipbuilding (Niembro-Rocas, 2003; Rodríguez-Sahagún *et al.*, 2007). It is also useful as firewood due to its high caloric content (Rodríguez-Sahagún *et al.*, 2007). It is widely grown as a shade tree to shelter plantation, livestock, even its fruits and leaves are used as fodder directly from the tree or as a nutritional complement (Carranza-Montaño *et al.*, 2003; Mota *et al.*, 2005). Syrup and gum obtained from the bark and truck respectively are used in the treatment of colds and medicine for chest affections (Uphof, 1959; Burkil, 2004).

In Mexico, the boiled seeds are consumed in sauces, soups and as a coffee substitute, and several medicinal properties have been attributed to them (Carranza-Montaño *et al.*, 2003; Niembro-Rocas, 2003). Moreover, several biotechnological applications such as its gum as a fungi culture substrate or for the production of ice cream and yogurt have recently been proposed for this tree species (Rincón *et al.*, 2005, 2006).

The conventional method of propagating *E. cyclocarpum* is mainly sexual i.e from seeds. However, the regeneration rate of most leguminous trees in natural habitats is low due to seed dormancy (Nanda *et al.*, 2004). This limitation consequently affects the germination rate and the death of young seedlings under natural conditions. The conventional method of propagation cannot meet the need for forest restoration programs, hence micropropagation, is required.

To date, micropropagation offers a rapid means for reforestation, multiplying woody biomass and for conserving elite and rare germplasm (Xie and Hong, 2001). There is limited information on *in vitro* techniques of propagating *E. cyclocarpum*. Rodríguez-Sahagún *et al.* (2007) in Mexico has used nodal segments and apical segment from axenic seedlings were cultured on MS basal medium supplemented naphthaleneacetic acid

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(NAA) in combination with benzyl adenine (BA) or kinetin (KIN). They reported multiple shoot induction using nodal segment collected from *in vitro* germinated seedling when cultured on MS medium was supplemented with combination BA and NAA. However, *E. cyclocarpum* as an established exotic species to Nigeria, little or no studies had been done on it *in vitro* propagation.

Thus, the objective of this study was to evaluate the effects of Cytokinin-BA and Auxin- NAA on multiple shoot induction using *ex vitro* explants of leaf, stem and node of *E. cyclocarpum* - a protocol needed for the mass propagation as well as genetic improvement programs.



Figure 1. Habit of *Enterolobium cyclocarpum* tree

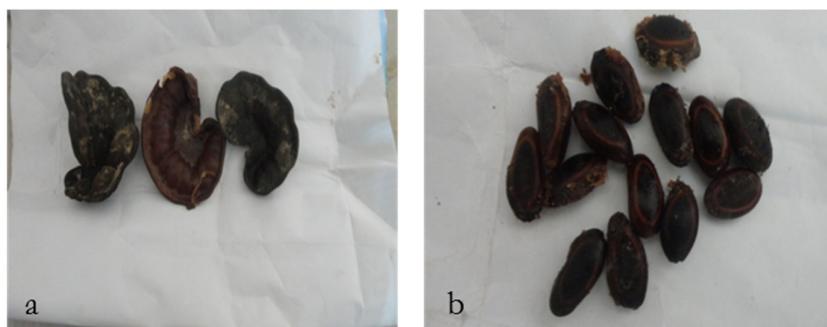


Figure 2. Ear pod fruits (a) and the hard-coated seeds (b)

Materials and Methods

Plant materials

The seeds of *E. cyclocarpum* were obtained from indehisced pods collected from an actively growing tree in Reforestation Garden of Botany, Department of Botany, Obafemi Awolowo University, Ile - Ife. Seeds were selected based on their non-dehisced seed coat, scarified using emery paper. The scarified seed was surface sterilized using 10% NaOCl for 15 minutes, and then kept under running tap water for 30 min. Then they were soaked in distilled water for 15 mins to allow for imbibition. Finally, the seeds were planted in petri- dishes for 2 weeks before they were transferred to top soil and were out in a randomized environment in which they can access various environmental factors.

Explant source and sterilization

The leaf, stem and node *ex vitro* explants of *E. cyclocarpum* were obtained from 3-8 weeks old seedlings and were kept under running water for 10-15 minutes before culturing.

Media and culture of explants

The explants were cultured on full strength MS (Murashige and Skoog, 1992) basal medium containing 3% sucrose per liter. The pH of the media was adjusted to 5.7 ± 0.02 with 1 N NaOH or 1 N HCl solutions, solidified with 0.8% agar (BDH) per liter prior to autoclaving at 121 °C and 103 kpa for 15 minutes. The basal medium alone served as control and was also supplemented with cytokinin separately and/or with auxins.

The leaf, stem and Nodal explants were disinfected with 70% ethanol for 3-4 minutes and surface sterilized by washing with 10% NaOCl containing two drops liquid detergent (tween-20) for 15 minutes after which they were rinsed in at least three changes of sterile distilled water. The leaf explants were sectioned to about 10 mm² in size while the stem and the node were about 10 mm in length. The explants were cultured in 15 cm³ test tube containing 8 ml of the culture medium. The leaf explants were cultured with the adaxial surface in contact with the medium while the nodes and stem cutting were slantingly explanted on the medium.

Culture conditions

The culture tubes were covered with non-adsorbent cotton wool and wrapped with aluminum foil, after which they were incubated at a temperature of 25 ± 2 °C and a photoperiod of 16 h light with cool white fluorescent light ($20 \mu\text{mol m}^{-2}\text{s}^{-1}$).

Statistical analysis

The data in terms of the number of induced shoot buds, shoot length, callus induction, morphology, and colour of callus were recorded after 4-5 weeks in culture. Each treatment consisted of 10 replicates and all experiments were repeated twice. The data were analyzed statistically using standard error (S.E) of the mean and were separated using Duncan Multiple Range Test (DMRT). All statistical analyses were performed with Statistical Package for Social Sciences (SPSS, version 11.5).

Results

There was no callus initiation with the cotyledon explants of *E. cyclocarpum* cultured in the MS medium alone i.e. the control. Initiation of callus was observed within 2 weeks in the cotyledon explants on MS media containing low concentrations of auxin alone or their various concentrations combined with the cytokinins. The proliferation rate of callus induced from the cotyledon explants was generally slow. Shoot bud formation became evident from the scanty callus induced by some auxin /cytokinin combinations after 3 weeks of culture (Figure 3a).

A maximum mean number of 2.1 shoots per explant was observed with cotyledon explants grown on MS medium containing 2.7 μM NAA + 2.2 μM BA which also induced the highest frequency ($58.47 \pm 1.19\%$) of callus formation (Tables 1 and 3). The concentration of 2.7 μM NAA + 4.7 μM kinetin in its case induced highest frequency ($51.57 \pm 1.88\%$) of off white callus (Table 5) and the highest mean number of 1.5 shoots/explant although this was not significantly ($p \leq 0.05$) different from 1.4 shoots/explant induced from cotyledon explants cultured on MS medium containing 2.7 μM NAA + 2.35 μM kinetin (Tables 2, 4 and 5). Little extension growth was shown by the shoot buds when subcultured. The shoot buds that formed from the cotyledon-derived callus on MS medium supported with 2.7 μM NAA + 4.7 μM BA developed further when subcultured, 40% of the shoots produced thick roots in MS hormonal free medium forming complete plantlets.

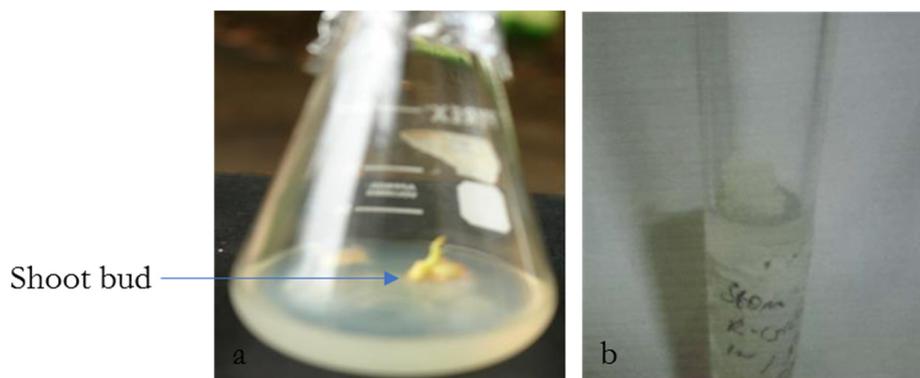


Figure 3. Scanty (with shoot bud) and compact callus induced from cotyledon and stem explant of *E. cyclocarpum* after three weeks of culture respectively on MS medium supplemented with $4.5 \mu\text{M}$ 2, 4-D + $9.4 \mu\text{M}$ Kinetin (a) and $5.4 \mu\text{M}$ NAA alone (b)

Table 1. The effects of various concentrations of NAA, 2,4-D alone or each separately combined with BA

S/N	MS medium + PGR concentration (μM)			% response of cotyledon explant (mean \pm S.E)	% response of nodal explant (mean \pm S.E)	% response of stem explant (mean \pm S.E)
	BA	NAA	2,4-D			
1.	-	-	-	0.00 \pm 0.00 ^a	0.000 \pm 0.00 ^a	0.00 \pm 0.00 ^a
2.	-	-	2.25	34.59 \pm 3.01 ^{cd}	48.47 \pm 1.099 ^{cd}	43.80 \pm 2.50 ^{defg}
3.	-	-	4.5	34.22 \pm 3.62 ^{cd}	43.40 \pm 1.47 ^{bc}	48.10 \pm 4.31 ^{eg}
4.	-	-	9.0	0.00 \pm 0.00 ^a	39.73 \pm 1.88 ^b	41.60 \pm 2.31 ^{def}
5.	-	2.7	-	33.23 \pm 3.50 ^{cd}	55.37 \pm 4.33 ^e	44.37 \pm 3.14 ^{de}
6.	-	5.4	-	42.34 \pm 4.00 ^{ef}	51.43 \pm 2.38 ^{de}	51.83 \pm 5.811 ^{efg}
7.	-	10.8	-	0.00 \pm 0.00 ^a	48.13 \pm 1.40 ^{cd}	32.17 \pm 5.97 ^b
8.	2.2	-	-	0.00 \pm 0.00 ^a	66.67 \pm 2.14 ^f	0.00 \pm 0.00 ^a
9.	2.2	-	2.25	29.76 \pm 0.68 ^{bc}	74.50 \pm 2.05 ^{ghijk}	45.83 \pm 4.54 ^{de}
10.	2.2	-	4.5	40.5 \pm 2.22 ^{def}	75.27 \pm 2.30 ^{ghijk}	33.63 \pm 3.80 ^{bc}
11.	2.2	-	9.0	35.23 \pm 3.30 ^{cdef}	68.67 \pm 1.54 ^{fgh}	25.97 \pm 2.41 ^b
12.	2.2	2.7	-	58.47 \pm 1.19 ^g	81.57 \pm 1.92 ^k	62.33i \pm 3.49 ^h
13.	2.2	5.4	-	43.09 \pm 2.14 ^f	80.60 \pm 2.19 ^{jk}	69.60 \pm 1.54 ^{ij}
14.	2.2	10.8	-	40.44 \pm 2.94 ^{def}	75.80 \pm 3.23 ^{hijk}	66.70 \pm 3.30 ^{hij}
15.	4.4	-	-	0.00 \pm 0.00 ^a	71.90 \pm 1.96 ^{fghi}	0.00 \pm 0.00 ^a
16.	4.4	-	2.25	31.14 \pm 3.45 ^c	80.57 \pm 1.25 ^{jk}	35.77 \pm 3.84 ^{bcd}
17.	4.4	-	4.5	34.24 \pm 3.56 ^{cd}	76.10 \pm 2.81 ^{hijk}	32.13 \pm 2.40 ^b
18.	4.4	-	9.0	22.32 \pm 2.71 ^b	75.20 \pm 2.84 ^{ghijk}	0.00 \pm 0.00 ^a
19.	4.4	2.7	-	41.24 \pm 3.49 ^{def}	76.10 \pm 2.81 ^{hijk}	73.03 \pm 3.58 ^{jk}
20.	4.4	5.4	-	21.49 \pm 6.32 ^{def}	79.83 \pm 1.91 ^{jk}	82.20 \pm 2.63 ^k
21.	4.4	10.8	-	23.54 \pm 2.10 ^b	73.23 \pm 1.8 ^{fghij}	66.17 \pm 3.27 ^{hij}
22.	8.8	-	-	0.00 \pm 0.00 ^a	70.43 \pm 2.77 ^{fghi}	0.00 \pm 0.00 ^a
23.	8.8	-	2.25	30.84 \pm 2.05 ^c	69.73 \pm 2.52 ^{fghi}	35.67 \pm 2.94 ^{bcd}
24.	8.8	-	4.5	28.54 \pm 4.44 ^c	68.90 \pm 2.59 ^{fghi}	42.77 \pm 3.71 ^{cde}
25.	8.8	-	9.0	0.00 \pm 0.00 ^a	67.50 \pm 2.04 ^{fg}	0.00 \pm 0.00 ^a
26.	8.8	2.7	-	29.53 \pm 3.54 ^{bc}	75.07 \pm 2.85 ^{ghijk}	56.40 \pm 3.79 ^{fgh}
27.	8.8	5.4	-	23.07 \pm 2.50 ^b	71.63 \pm 1.79 ^{fghi}	59.57 \pm 5.088 ^{ghi}
28.	8.8	10.8	-	22.34 \pm 2.15 ^b	76.67 \pm 3.11 ^{ijk}	51.93 \pm 6.058 ^{efg}

on callus formation from cotyledon, stem and nodal explants of *E. cyclocarpum*

PGR: Plant growth regulators; BA: Benzyl Adenine; NAA: Naphthaleneacetic Acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid

Table 2. The effects of various concentrations of NAA, 2, 4-D alone or each separately combined with kinetin on callus/shoot induction from cotyledon, stem and nodal explants of *E. cyclocarpum*

S/N	MS medium + PGR concentration (μM)			% response of cotyledon explant (mean \pm S.E)	% response of nodal explant (mean \pm S.E)	% response of stem explant (mean \pm S.E)
	KIN	NAA	2,4-D			
1.	-	-	-	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
2.	-	-	2.25	34.59 \pm 3.01 ^{cd}	48.47 \pm 1.10 ^{cd}	43.80 \pm 2.50 ^{defg}
3.	-	-	4.50	34.22 \pm 3.62 ^{cdc}	43.40 \pm 1.47 ^{bc}	48.10 \pm 4.31 ^{eg}
4.	-	-	9.00	0.00 \pm 0.00 ^a	39.73 \pm 1.88 ^b	41.60 \pm 2.31 ^{cdef}
5.	-	2.70	-	48.40 \pm 1.24 ^f	55.37 \pm 43 ^{dc}	45.8 \pm 3.06 ^{efg}
6.	-	5.40	-	41.03 \pm 2.48 ^{dc}	51.43 \pm 2.38 ^d	50.37 \pm 6.07 ^{gh}
7.	-	10.80	-	0.00 \pm 0.00 ^a	48.13 \pm 1.40 ^{cd}	45.67 \pm 2.83 ^{efg}
8.	2.35	-	-	0.00 \pm 0.00 ^a	70.90 \pm 0.86 ^{ghijk}	0.00 \pm 0.00 ^a
9.	2.35	-	2.25	44.50 \pm 4.75 ^{ef}	73.73 \pm 1.45 ^{jkl}	41.47 \pm 1.91 ^{cdef}
10.	2.35	-	4.50	41.23 \pm 1.37 ^{dc}	79.2 \pm 2.77 ^l	50.27 \pm 1.21 ^{gh}
11.	2.35	-	9.00	0.00 \pm 0.00 ^a	67.20 \pm 3.24 ^{ghij}	35.57 \pm 2.80 ^{bcd}
12.	2.35	2.70	-	49.43 \pm 2.048 ^f	79.87 \pm 2.43 ^l	42.83 \pm 2.70 ^{cdefg}
13.	2.35	5.40	-	48.67 \pm 4.50 ^f	78.13 \pm 1.24 ^{jkl}	43.97 \pm 2.53 ^{defg}
14.	2.35	10.80	-	24.90 \pm 2.25 ^b	71.00 \pm 3.27 ^{ghijk}	47.73 \pm 3.36 ^{fg}
15.	4.70	-	-	0.00 \pm 0.00 ^a	72.23 \pm 2.01 ^{hijkl}	0.00 \pm 0.00 ^a
16.	4.70	-	2.25	35.70 \pm 2.76 ^{cd}	74.87 \pm 2.71 ^{jkl}	61.73 \pm 1.84 ^{ij}
17.	4.70	-	4.50	35.47 \pm 2.71 ^{cd}	78.20 \pm 3.93 ^{jkl}	75.53 \pm 2.88 ^l
18.	4.70	-	9.00	0.00 \pm 0.00 ^a	64.60 \pm 3.18 ^{fgh}	71.67 \pm 2.02 ^{kl}
19.	4.70	2.70	-	51.57 \pm 1.88 ^f	73.43 \pm 1.35 ^{ijk}	62.90 \pm 2.80 ^{ij}
20.	4.70	5.40	-	47.50 \pm 4.62 ^{ef}	74.60 \pm 2.81 ^{kl}	67.567 \pm 2.39 ^{jk}
21.	4.70	10.80	-	0.00 \pm 0.00 ^a	69.70 \pm 2.98 ^{ghij}	56.10 \pm 4.80 ^{hi}
22.	9.40	-	-	0.00 \pm 0.00 ^a	62.77 \pm 2.98 ^{fg}	0.00 \pm 0.00 ^a
23.	9.40	-	2.25	29.63 \pm 2.14 ^{bc}	68.40 \pm 2.11 ^{ghij}	40.93 \pm 0.88 ^{cdef}
24.	9.40	-	4.50	33.73 \pm 2.64 ^c	68.03 \pm 1.87 ^{ghij}	41.30 \pm 2.45 ^{cdef}
25.	9.40	-	9.00	0.00 \pm 0.00 ^a	59.80 \pm 2.06 ^{ef}	29.10 \pm 0.81 ^b
26.	9.40	2.70	-	35.03 \pm 3.43 ^{cd}	68.83 \pm 2.39 ^{ghij}	39.70 \pm 5.11 ^{cdef}
27.	9.40	5.40	-	31.73 \pm 2.21 ^c	65.40 \pm 2.84 ^{fghi}	34.73 \pm 2.96 ^{bc}
28.	9.40	10.80	-	0.00 \pm 0.00 ^a	64.57 \pm 2.86 ^{fgh}	38.83 \pm 1.71 ^{cde}

PGR: Plant growth regulators; KIN: Kinetin; NAA: Naphthaleneacetic Acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid

Table 3. The effects of various concentration of NAA, 2, 4-D alone or each separately combined with BA on caulogenesis from stem, cotyledon and nodal explants of *E. cyclocarpum*

S/N	MS medium + PGR Concentration (μM)			Degree of cotyledon callus formation	No. of shoot per cotyledon explants	Degree of nodal callus formation	No. of shoot per nodal explants	Degree of stem callus formation
	BA	NAA	2,4-D					
1.	-	-	-	No callus		No callus		No callus
2.	-	-	2.25	+		+		+
3.	-	-	4.5	+		+		++
4.	-	-	9.0	No callus		+		++
5.	-	2.7	-	+		+		+
6.	-	5.4	-	+		+		+
7.	-	10.8	-	No callus		+		+
8.	2.2	-	-	No callus		++S	1.50 \pm 0.40 ^{abc}	No callus
9.	2.2	-	2.25	+ S	0.80 \pm 0.12 ^a	++S	1.70 \pm 0.30 ^{bcd}	++

10.	2.2	-	4.5	+ S	1.40±0.17 ^a	++		++
11.	2.2	-	9.0	+		++		+
12.	2.2	2.7	-	+ S	2.1±0.11 ^d	+++S	1.80±0.50 ^{cdc}	++
13.	2.2	5.4	-	+		++S	2.70±0.20 ^{efgh}	++
14.	2.2	10.8	-	+		+++		++
15.	4.4	-	-	No callus		++S	2.80±0.40 ^{efgh}	No callus
16.	4.4	-	2.25	+S	1.40±0.20 ^{bc}	++S	2.50±0.50 ^{dfg}	++
17.	4.4	-	4.5	+		++S	2.00±0.30 ^{def}	+
18.	4.4	-	9.0	+		++		No callus
19.	4.4	2.7	-	+ S	1.30±0.18 ^{ab}	++S	3.00±0.50 ^{fgh}	++
20.	4.4	5.4	-	+		++S	2.70±0.40 ^{efgh}	+++
21.	4.4	10.8	-	+		++	2.10±0.30 ^{def}	++
22.	8.8	-	-	No callus		++S	3.00±0.60 ^{fgh}	No callus
23.	8.8	-	2.25	+	1.60±0.23 ^{bcd}	++S	3.10±0.50 ^{fgh}	+
24.	8.8	-	4.5	+		+S	2.60±0.40 ^{efgh}	+
25.	8.8	-	9.0	No callus		+S	1.90±0.30 ^{cdc}	No callus
26.	8.8	2.7	-	+	1.90±0.17 ^{cd}	++S	3.98±0.50 ^{fghi}	+
27.	8.8	5.4	-	++		++S	2.70±0.50 ^{efgh}	+
28.	8.8	10.8	-	+		+++S	1.80±0.40 ^{cdc}	+

+ Slight callus formation; ++Moderate callus formation; +++ Massive callus formation

PGR: Plant growth regulators; BA: Benzyl adenine; NAA: Naphthaleneacetic Acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid; **S: Shoot initiation

Table 4. The effects of various concentrations of NAA, 2, 4-D alone or each separately combined with kinetin on caulogenesis from stem, cotyledon and nodal explants of *E. cyclocarpum*

S/N	MS medium + PGR Concentration (μ M)			Degree of cotyledon callus formation	No. of shoot per cotyledon explants	Degree of nodal callus formation	No. of shoot per nodal explants	Degree of stem callus formation
	KIN	NAA	2,4-D					
1.	-	-	-	No callus		No callus		No callus
2.	-	-	2.25	+		++		+
3.	-	-	4.50	+		++		++
4.	-	-	9.00	No callus		+		++
5.	-	2.70	-	+		++		+
6.	-	5.40	-	+		++		+
7.	-	10.80	-	No callus		+		++
8.	2.35	-	-	No callus		++S	1.40±0.40 ^{abc}	No callus
9.	2.35	-	2.25	+		++S	1.30±0.30 ^{ab}	+
10.	2.35	-	4.50	++		++		+
11.	2.35	-	9.00	No callus		+		
12.	2.35	2.70	-	+S	1.4±0.05 ^b	+S	2.30±0.11 ^{defgh}	++
13.	2.35	5.40	-	+S	0.8±0.11 ^a	+S	2.40±0.50 ^{efgh}	++
14.	2.35	10.80	-	+		+++		++
15.	4.70	-	-	No callus		+S	2.10±0.17 ^{cdfg}	No callus
16.	4.70	-	2.25	++S	0.17±0.11 ^{ab}	++S	2.30±0.11 ^{defg}	++
17.	4.70	-	4.50	+		++S	2.00±0.18 ^{bcdef}	+++
18.	4.70	-	9.00	No callus		+		++
19.	4.70	2.70	-	+S	1.5±0.12 ^b	++S	3.50±0.28 ^{ij}	++
20.	4.70	5.40	-	+		++S	3.0±0.23 ^{hi}	+++
21.	4.70	10.80	-	No callus		+		+

22.	9.40	-	-	No callus		+S	3.50±0.40 ^{ij}	No callus
23.	9.40	-	2.25	+		+S	3.70±0.29 ^j	+
24.	9.40	-	4.50	+S	1.20±0.23 ^{ab}	+S	2.50±0.40 ^{gh}	+
25.	9.40	-	9.00	No callus		+S	2.20±0.30 ^{defg}	+
26.	9.40	2.70	-	+		+S	3.30±0.50 ^{ghi}	+
27.	9.40	5.40	-	+		+S	3.60±0.50 ^{ij}	+
28.	9.40	10.80	-	No callus		+S	0.28±0.35 ^{gh}	+

+ Slight callus formation; ++ Moderate callus formation; +++ Massive callus formation

PGR: Plant growth regulators; KIN: Kinetin; NAA: Naphthaleneacetic Acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid; **S: Shoot initiation

Table 5. The effects of various concentrations of NAA, 2, 4-D alone or each separately combined with BA on morphology of callus from stem, cotyledon and nodal explants of *E. cyclocarpum*

S/N	MS medium + PGR concentration (μ M)			Morphology of cotyledon callus	Morphology of nodal callus	Morphology of stem callus
	BA	NAA	2,4-D			
1.	-	-	-	-	-	-
2.	-	-	2.25	Light brown and hard	White and friable	Light brown and rather hard
3.	-	-	4.5	Light brown and hard	White and friable	Light brown and rather hard
4.	-	-	9.0	-	White and friable	Light brown and rather hard
5.	-	2.7	-	Light brown and hard	White and friable	Creamy/ semi hard
6.	-	5.4	-	Light brown and hard	White and friable	Creamy/ semi hard
7.	-	10.8	-	-	White and friable	Creamy/ semi hard
8.	2.2	-	-	-	White and friable	
9.	2.2	-	2.25	Brown and granular	White and friable	Light brown and semi hard
10.	2.2	-	4.5	Light brown and hard	White and friable	Light brown and semi hard
11.	2.2	-	9.0	Brown and hard	White and friable	Light brown and semi hard
12.	2.2	2.7	-	Brown and hard	White and friable	Light brown and Friable
13.	2.2	5.4	-	light and loose yellow	White and friable	Light brown and Friable
14.	2.2	10.8	-	light and loose yellow	White and friable	Light brown and Friable
15.	4.4	-	-	-	White and friable	
16.	4.4	-	2.25	Light brown and hard	White and friable	Light brown and semi hard
17.	4.4	-	4.5	Light brown and hard	White and friable	Light brown and semi hard
18.	4.4	-	9.0	Light brown and granular	White and friable	
19.	4.4	2.7	-	Light and friable yellow	White and friable	Light brown and Friable
20.	4.4	5.4	-	Light yellow and friable	White and friable	Light brown and Friable
21.	4.4	10.8	-		White and friable	Light brown and Friable
22.	8.8	-	-		White and friable	
23.	8.8	-	2.25	Light brown and semi-hard	White and friable	Light brown and semi hard
24.	8.8	-	4.5	Hard	White and friable	Light brown and semi hard
25.	8.8	-	9.0		White and friable	
26.	8.8	2.7	-	Light yellow and rath. hard	White and friable	Light brown and semi hard
27.	8.8	5.4	-	Light yellow and hard	White and friable	
28.	8.8	10.8	-	Light yellow and hard	White and friable	Light brown and semi hard

PGR: Plant growth regulators; BA: Benzyl Adenine; NAA: Naphthaleneacetic Acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid

The stem explants of *E. cyclocarpum* explanted on MS medium alone grew in size, expanded longitudinally and laterally at first but later the cut edges began to turn brown and the explants died ultimately. The stem explants explanted on MS medium supplemented with either BA or kinetin alone did not show callogenesis. With MS medium containing the individual auxin, callus initiation appeared after 3 weeks

followed by a very slow proliferation of creamy/ off white hard callus (Figure 3b). The NAA/ BA or 2,4-D/ BA combinations brought about callus initiation in the stem explants within 2 weeks to produce off white friable or semi-hard (Tables 5 and 6). The largest callus size was produced in stem explants cultured on MS medium containing $5.4 \mu\text{M}$ NAA + $4.4 \mu\text{M}$ BA (Figure 4a), and $82.2 \pm 2.63\%$ response was obtained (Table 1). The combination of NAA/ kinetin and 2,4-D/ kinetin induced callus from the stem explants of *E. cyclocarpum* within 2 weeks, the calli were generally light brown and semi-hard. Massive callus induction was observed with stem explants of *E. cyclocarpum* cultured on MS medium supported with $4.5 \mu\text{M}$ 2,4-D + $4.7 \mu\text{M}$ kinetin with the highest frequency of $75.53 \pm 2.88\%$ (Table 2).

Nodal explants of *E. cyclocarpum* cultured on MS medium alone enlarged in size without either production of callus or axillary bud release. When the explants were grown on MS medium to which auxins alone were added, the only callus formed and with auxin/cytokinin combinations, both callus and shoot buds were observed within 2 weeks of culture. Calli formed at the basal cut ends of the explants in all the cases were cream-coloured or white and friable.

The highest frequency of callus formation (81.57 ± 1.92) was induced from nodal explants cultured on MS medium supplemented with $2.7 \mu\text{M}$ NAA + $2.2 \mu\text{M}$ BA (Table 1) and the maximum shoot/explants induced (4.0) was obtained from nodal explant cultured on MS medium supplemented with $2.7 \mu\text{M}$ NAA + $8.8 \mu\text{M}$ BA (Table 3). Massive callus formed from nodal explants with the highest frequency ($79.2 \pm 2.77\%$) when cultured on MS medium supplemented with $4.5 \mu\text{M}$ 2,4-D + $2.35 \mu\text{M}$ kinetin (Table 2). Callus and shoot buds induced by $4.5 \mu\text{M}$ 2,4-D + $2.35 \mu\text{M}$ Kinetin, while callus and short buds induced from nodal explants cultured on MS medium supported with $2.7 \mu\text{M}$ NAA + $2.2 \mu\text{M}$ BA after 4 weeks (Figure 4b and 5). On the subculture of shoot buds so induced from nodal explant-derived callus, they showed extended growth without root formation.

On the transfer of separated shoot buds to MS medium without hormones, less than 10% of them rooted after 6 weeks of culture. In MS medium containing $4.9 \mu\text{M}$ IBA, short and thick roots formed in over 80% of the shoots within 4 weeks of culture. Figure 6 shows the acclimatized plantlets induced from nodal explant of *E. cyclocarpum* with thick root in a rooting medium of $4.9 \mu\text{M}$ IBA.

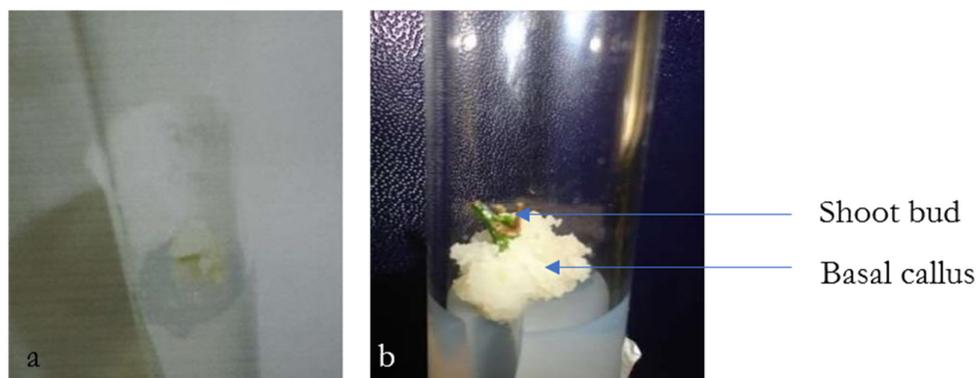


Figure 4. Callus and basal callus with shoot induced from stem and nodal explant of *E. cyclocarpum* after four weeks of culture respectively on MS Supplemented $5.4 \mu\text{M}$ NAA + $4.4 \mu\text{M}$ BA for the stem(a) and $4.5 \mu\text{M}$ 2,4-D + $2.35 \mu\text{M}$ Kinetin for the node(b)

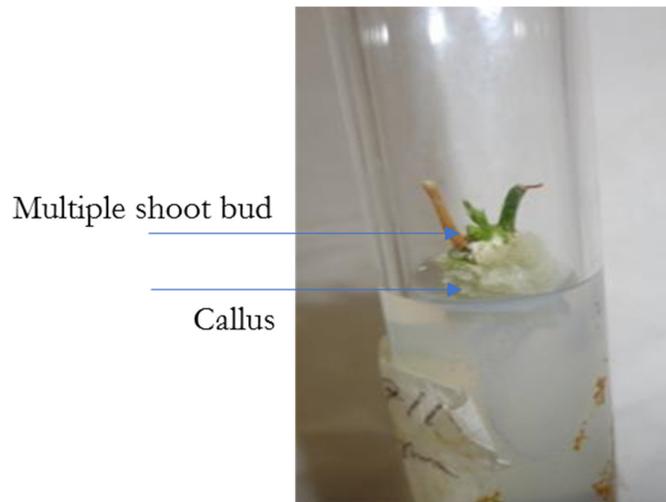


Figure 5. Basal callus with shoot buds obtained from nodal explant of *E. cyclocarpum* after four weeks of culture on ms supplemented with 2.7 μ M NAA+ 2.2 μ M BA



Figure 6: Acclimatized plantlets of *E. cyclocarpum* with thick roots

Table 6. The effects of various concentrations of NAA, 2, 4-D alone or each separately combined with kinetin on morphology of callus from stem, cotyledon and nodal explants of *E. cyclocarpum*

S/N	MS medium + PGR concentration (μ M)			Morphology of cotyledon callus	Morphology of nodal callus	Morphology of stem callus
	KIN	NAA	2,4-D			
1.	-	-	-	-	-	-
2.	-	-	2.25	Light brown and hard	White and friable	-
3.	-	-	4.50	Light brown and hard	White and friable	Light brown and rather hard
4.	-	-	9.00	-	White and friable	Light brown and rather hard
5.	-	2.70	-	Light brown and rather hard	White and friable	Creamy/semi hard
6.	-	5.40	-	Light brown and rather hard	White and friable	Creamy/semi hard
7.	-	10.80	-	Light brown and hard	White and friable	Creamy/semi hard
8.	2.35	-	-	-	Creamy and friable	-

9.	2.35	-	2.25	Light brown and hard	Creamy and friable	Light brown and semi hard
10.	2.35	-	4.50	Light brown and hard	Creamy and friable	Light brown and semi hard
11.	2.35	-	9.00		Creamy and friable	Light brown and semi hard
12.	2.35	2.70	-	Light brown and hard	Creamy and friable	Light brown and semi hard
13.	2.35	5.40	-	Light yellow and hard	Creamy and friable	Light brown and semi hard
14.	2.35	10.80	-	Light yellow and hard	Creamy and friable	Light brown and semi hard
15.	4.70	-	-	-	Creamy and friable	-
16.	4.70	-	2.25	Deep brown and hard	Creamy and friable	Light brown and semi hard
17.	4.70	-	4.50	Light yellow and hard	Creamy and friable	Light brown and semi hard
18.	4.70	-	9.00	-	Creamy and friable	Light brown and semi hard
19.	4.70	2.70	-	Light brown and hard	Creamy and friable	Light brown and semi hard
20.	4.70	5.40	-	Light yellow and hard	Creamy and friable	Light brown and semi hard
21.	4.70	10.80	-	-	Creamy and friable	Light brown and semi hard
22.	9.40	-	-	-	Creamy and friable	-
23.	9.40	-	2.25	Deep brown and hard	Creamy and friable	Light brown and semi hard
24.	9.40	-	4.50	Light yellow and hard	Creamy and friable	Light brown and semi hard
25.	9.40	-	9.00	-	Creamy and friable	Light brown and semi hard
26.	9.40	2.70	-	Light brown and hard	Creamy and friable	Light brown and semi hard
27.	9.40	5.40	-	Light brown and hard	Creamy and friable	Light brown and semi hard
28.	9.40	10.80		-	Creamy and friable	Light brown and semi hard

PGR: Plant growth regulators; KIN: Kinetin; NAA: Naphthaleneacetic Acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid

Discussion

The use of *in vitro* approaches to propagation, conservation and genetic improvement of forest trees has been of increased interest since the last three decades (Chalupa, 1981; Tomar and Gupta, 1988; Xia and Hong, 2001; Chalupa, 2002; Anis *et al.*, 2005; Faisal, 2007; Renukdas *et al.*, 2010 and Sajeevan *et al.*, 2013). Two methods of plant regeneration widely used in rapid and large scale micropropagation or plant transformation studies are organogenesis or embryogenesis. The success of these techniques requires good callus quality and quantity (Lin *et al.*, 2010).

The present studies showed that the stem, cotyledon, and nodal explants, but not leaf explants of *E. cyclocarpum* species were callogenic. The initiation and proliferation of callus were promoted by using NAA, 2,4-D alone or separately combined with Kinetin or BA to different extents in different explants. It was generally observed that the percentage of explants response in terms of callus induction was greater with auxin combination with BA than with Kinetin in the explants studied. The auxins, NAA and 2,4-D are commonly used with BA for callus induction in plant systems (Dhar and Joshi, 2005; Abbasin *et al.*, 2010; Isikalam *et al.*, 2010; Ntui *et al.*, 2012). Other research groups also induced callus, a consequence of wound reaction (Khal, 1983), *in vitro* without the use of plant growth regulators of several plant species (Handro and Floh, 2001; Martin, 2002).

Cotyledon explants of *E. cyclocarpum* were found to produce callus together with shoot buds when cultured on MS medium containing NAA combined with BA or Kinetin. The frequency of shoot induction appeared favoured by high cytokinin relative to auxin. A combination of 2.2 μ M BA + 2.7 μ M NAA induced the best shoot production from the cotyledon explants of *E. cyclocarpum* (2.1 shoots/explant). The influencing

effects of auxin and cytokinin combination on organogenic differentiation have been well documented in several plant systems. Rahman *et al.* (2010) found that adventitious shoots were induced from leaf-derived callus of *Lagerstroemia speciosa* grown on MS medium that was supplemented with 5 μM BA, 3 μM NAA, 10% coconut milk and 568 μM ascorbic acid. Similarly, cotyledon derived calli induced by 0.5 μM NAA + 0.5 μM BA produced high-efficiency shoot regeneration (4.2 shoots/explant) when subcultured on MS medium supplemented with 5 μM BA (Agrawal and Sadar, 2007). Salvi *et al.* (2001) who used various Neem explants including cotyledon, produced multiple shoots on MS medium containing 8.88 μM BA and 0.57 μM NAA. By addition of 3 mg/L Hymexazol to a modified MS medium containing 2.22 μM BA and 0.05 μM NAA, Yin *et al.* (2001) increased adventitious shoot development from cotyledon explants of *Albizia julibrissin*.

The leaf explants of *E. cyclocarpum* formed no callus when cultured on either MS medium alone or supplemented with various concentrations of either BA or NAA alone and in their combinations. The leaf explants, however, were found to have lost chlorophyll turning light green the end. This is contrary to wound reaction inducing mitosis in the cells from the cut surfaces and culminating in callus formation (Pérez-Francés *et al.*, 1995). The loss of response of the explants may suggest that the endogenous level of auxins/cytokinin or the exogenously applied growth regulators were insufficient to induce callus. It is also known that different tissues can respond in different ways during *in vitro* culture process (Jimenez, 2001; Banerjee *et al.*, 2011), and the requirements for plant growth regulators appear to be tissue-specific (Venkatachalam *et al.*, 1999; Banerjee *et al.*, 2011). Vidoz *et al.* (2012) also reported that the leaf explants of *Lotononis bainesii* did not respond on MS media to the absence of plant growth regulators. Conversely, leaf explants have been reported by several authors as the best for callus induction and shoot initiation. Jiancan *et al.* (2011) obtained 95% callus induction from leaf explants of *Zizyphus jujuba* in MS supplemented with 0.5 mg/L NAA.

In the absence of growth regulators in the culture medium, *E. cyclocarpum* stem explants neither formed callus nor shoot buds. The stem explants inoculated either on MS medium alone or supplemented with BA alone did not show callogenesis or caulogenesis but they first increased in size and gradually turned brown later. Cultures of stem explants on MS medium supplemented with the different concentrations of auxin and combined with BA started callus initiation after 2 weeks and proceeded with high proliferation.

Nodal explants exhibited the greatest tendencies to form callus and shoots under the influence of plant growth regulators compared to stem, hypocotyl, leaf and cotyledon explants of the plant species investigated. An average of 3.98 shoot / explant was generated from the nodal explant-induced callus of *E. cyclocarpum* by 2.7 μM NAA combined with 8.8 μM BA.

The nodal explants of several tree seedlings have been known to produce both callus and shoot buds *in vitro* under the influence of some plant growth regulators. Rahman *et al.* (1993) reported that NAA or 2,4-D separately combined with BA induced both callus and shoots within 3 weeks of culture of nodal explants of *Caesalpinia Pulcherima*. Sugla *et al.* (2007) induced multiple shoots *in vitro* from nodal explants through forced axillary branching in *Pongamia pinnata* using 7.5 μM BA while more recently, Sajeewan *et al.* (2012) reported inducing multiple shoots from nodal explants of *Morus alba* L. variety VI cultured on MS medium supplemented with 1.0 mg/L BAP, 0.1 mg/L TDZ and 0.25 mg/L NAA. Maximum shoot induction was obtained from the nodal cutting of *E. cyclocarpum* seedlings cultured on MS medium supplemented with a combination of 10.7 μM NAA and 2.2 μM BA (Rodriquez-Sahagun *et al.*, 2007). Multiple shoots were also obtained from nodal explants of 18-day-old *in vitro* seedlings of *Pterocarpus marsupium* Roxb. culture on MS medium containing 4 μM BA, 0.5 μM NAA and 20 μM adenine sulphate (Husain *et al.*, 2008). Shoot proliferation from nodal explants of *Melaleuca alternifolia* (Tee Tee) cultured in liquid or on agar-based MS medium containing 1.11 μM BA or 0.55 μM BA had also been reported in the literature (Yohana *et al.*, 2010).

Root induction was also achieved in the rooting medium (MS + 4.9 μM IBA). Stout short single roots were induced from the cotyledon and nodal explant-derived shoots of *E. cyclocarpum*. It appears that different species have a different critical concentration of hormones below which root initiation will occur and above

which there will be inhibition. Furthermore, organogenic differentiation of callus leading to production of shoots and roots are dependent on several factors aside from hormonal factors and these include level of salt (e.g. PO_4^{3-}), quality of light, and temperature, in addition to physiological state, size of explant, and orientation of the medium (Razan, 2003). Chevre (1985) opined that rooting is often more difficult with the ligneous plants than with the herbaceous plants. More recently, similar observations were reported by Lin *et al.* (2010) stating that in general, establishing an efficient tissue culture technique was difficult in woody plants compared to herbaceous plants. This in part is related to the phase change from juvenility to maturation that most woody plants undergo (Trevor *et al.*, 1990). The development of basal callus is one of the main physiological disorders that affect rooting competence of micro shoots, a situation which is more severe in woody species (Bairu and Kane, 2011). These authors explained that basal callus constitutes a sink trapping essential growth constituent and consequently affecting many physiological processes of the shoot.

Conclusions

The optimal concentration of NAA/BA that can induce stem callus in a potential source for plant regeneration (either by organogenesis or embryogenesis) and production of secondary metabolites were determined for *E. cyclocarpum*. Also, the optimal concentration of NAA/BA for shoot induction and plantlets formation of the plant species from cotyledon and nodal explants were also determined. Thus, it can be concluded by means of *in vitro* culture of the cotyledon and nodal explants of *E. cyclocarpum*, true clone plantlets can be produced. This plantlet can be employing for reforestation and/or forest restoration programs.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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