IN-VITRO CALLUS INDUCTION AND SHOOT REGENERATION FROM LEAF AND NODAL EXPLANTS OF *STERCULIA TRAGACANTHA* LINDL.

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ABSTRACT

In this study, the effects of Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) alone and in combination with Benzyladenine (BA) were investigated on callus production and shoot regeneration from leaf and nodal explants of *S. tragacantha*. Callus was induced by the various concentrations of NAA, 2,4-D and their separate combinations with BA from the leaf and nodal explants. With the leaf explants, 10.8 μM NAA + 4.4 μM BA induced the largest amount of callus; nodular/bud-like structures were observed on calli induced by 2,4-D/BA mixtures. Multiple shoots were developed from nodal explants cultured on media containing mixtures of NAA and BA, maximum number of shoots/explants (3.4 ± 0.36) occurring on Murashige and skoog's (MS) media supplemented with 2.7 μM NAA/8.8 μM BA mixture. These shoots when subcultured in the same medium reached mean height of 4.2 ± 0.56 cm after five (5) weeks. Only 28% of the regenerated shoots produced short thickened roots of mean length 2.1 ± 0.42 cm after 4 weeks of culture in MS medium containing 4.9 μM indole-3-butyric acid.

INTRODUCTION

Sterculia tragacantha, commonly called African Tragacanth belongs to the family Sterculiaceae. It is a medium-sized deciduous tree growing up to about 28 m high with bole of diameter 12-14 cm. Widespread in tropical Africa, it is commonly found in open parts or edges of forest. Several parts of the plant have wide applications in medicine, agriculture and phytochemistry. The gum obtained from the bark and fruit capsule has official status in many pharmacopoeia: a binding agent for pills, a suspension agent in mixtures and an adhesive in dental fixative powders (Oliver, 1960). It is claimed that the leaf can be used as antidotes for venomous stings and bites, arthritis and rheumatism while the leaf combined with the bark are useful in the treatment of diarrhea and dysentery (Buckhill, 1985). The wood of *S. tragacantha* which is very useful in carpentry and related applications can also be burnt to ashes for soap production or crushed to produce wood pulp or paper. In addition, like most trees in West Africa, it is exploited for fuel wood energy.

Although *S. tragacantha* regenerates readily (Aubreville, 1950), the seeds exhibit dormancy probably due to its two protective coats. The fruits and seeds are often attacked by some insects. These two factors no doubt make the seedlings of this plant species rare in their habitat.

The deforestation rate in West Africa is generally very high, an average of about 1% annually compared to most other parts of Africa and South America where the deforestation rate is between 0.5 and 0.8% annually (Brink, 2008). This is because of the continuous use of the existing forests for urban, agricultural and recreational purposes aside from the forest serving as a constant source of fuel wood and drugs for the rural poor.

The consumption of fuel wood accounts for 85% of West Africa's total energy consumption (FAO, 2000). It is, therefore, not far-fetched to say that many tree species in the region are threatened and endangered (IUCN, 2011) because their natural regeneration processes lag behind the deforestation rate. To prevent extinction and to sustain the benefits obtained from multipurpose and economic trees such as *S. tragacantha,* the germplasm must be conserved. Today, *in vitro* propagation techniques are an integral component in conservation or tree improvement programmes. To our knowledge there has been no biotechnological work on *S. tragacantha* reported in scientific publications. The paper reports protocol for *in vitro* induction of callus and shoot production using explants of leaf and nodal cuttings of *S. tragacantha.*

MATERIALS AND METHODS

Plant Materials

Dehisced mature brown fruit of *S. tragacantha* with the seeds intact were plucked from tree stands variously located on the campus of Obafemi Awolowo University, Ile-Ife (Longitude: 04°33' E and Latitude 08°28' N). The seeds were obtained by simply detaching them from their funicles and, after scarification using sand paper, they were washed and germinated as described by Ayisire *et al.* (2009).

Basal Medium and Culture of Explants

The basal medium consisted of full strength MS (Murashige and Skoog, 1962) salts containing 3% sucrose. The pH of the medium was adjusted to 5.7 with 1N HCl or 1N NaOH, solidified with 0.8% agar and autoclaved at 121°C and 103 KPa for 15 minutes. For callus induction, the basal medium was supplemented with 2.7, 5.4 and 10.8 μM Naphthaleneacetic acid (NAA), 2.25, 4.5 and 6.75 μM 2,4-dichlorophen-oxyacetic acid (2,4-D) or different concentrations of NAA or 2,4-D separately combined with 4.4 μM or 8.8 μM Benzyladenine (BA). The explants with or without callus or shoot buds were sub-cultured on MS media with the same composition for further development. Developed shoots were separated and transferred to MS media containing 4.9 μM indole-butyric acid (IBA) for rooting.

Leaf and nodal explants that were used were excised from 3-5 week-old seedlings; they were disinfected with 10% NaOCl containing two drops of Tween 20 per 100 ml for 15 minutes after which they were rinsed in three changes of sterile water. The leaf explants cut to about 25 mm^2 size and the nodal explants to about 10mm in length were cultured in either 15 cm^3 test tubes or 100 cm^3 Erlemenyer's flasks, each containing 10 ml or 25 ml of medium respectively. The leaf explants were inoculated on the medium with the adaxial surface in contact with the medium while the nodal cuttings were inoculated vertically on the medium.

After inoculation, the culture vessels were covered with non-adsorbent cotton wool, wrapped with aluminum foil and incubated at 25±2°C under 16 hours photoperiod provided by 30-40 μmol m^{-2} S^{-1} cool white fluorescent tubes.

After 4-5 weeks the responses of the cultures, in terms of callus production, to the different treatments were scored rather than accurately measured (Cousins and Saeger, 2002); the frequency of callus formation was also determined. The frequency of shoot formation and number of shoots/explants induced by the different treatments were also determined after 4-5 weeks of sub-culturing.

Twenty-five explants were used for each treatment and all experiments were repeated three times. The effects of the different treatments were quantified accordingly and the data subjected to statistical analyses using "standard error (S.E) of the mean". Computations were carried out using SPSS.

RESULTS

No callus formation was observed with the leaf explants cultured on MS medium alone. The explants, however, enlarged during the period of culture. Both the 2,4-D and NAA separately added to the MS medium or in combination with BA induced callus formation in the leaf explants to various degrees. Callus initiation occurred between 3-4 weeks in the leaf explants cultured in NAA or 2,4-D alone while it occurred between 2-3 weeks in the media containing NAA/BA or 2,4-D/BA mixtures. The largest callus size was formed in the culture medium containing a mixture of 10.8 μM NAA and 4.4 μM BA after 5 weeks of culture (Plate 1A) and the response was observed in 92 ± 6.1% of the cultures. The colour of the calli induced by NAA, a mosaic of green and brown was rather hard and compact. The callus formed on MS medium containing 2,4-D alone or mixed with BA was light brown in colour soft and fragile. Plate 1B shows callus formed from the leaf explants after 4 weeks of culture in MS medium supplemented with 4.5 μM 2,4-D and 4.4 μM BA.

When the calli formed by the leaf explants were sub-cultured in media with the same hormonal composition, the rates of callus formation increased steadily within the first two weeks. In the cases where the media was supplemented with either NAA or 2,4-D alone, the rate of callus formation from the third week of culture gradually reduced and the calli turned brown and

died without producing shoots. In the 3rd week of subculture, the calli induced by the 2,4-D/BA mixtures revealed white patches on the surface which became nodular in appearance and then bud-like by the 5th week. The greatest nodulation producing multiple shoot buds was observed on MS medium in which 8.8 μM BA/2.25 μM 2,4-D mixtures had been included (Plate 1C). When the segments of these buds were transferred to MS media alone or MS media supplemented with BA, no further development occurred, rather they turned brown and became necrotic. The cultures in which mixtures of NAA and BA were present increased in callus formation.

The callus induced by combinations of NAA and BA was light brown and rather friable and showed no evidence of nodular formation throughout the period of sub-culture.

The nodal explants that were cultured on MS medium alone showed both longitudinal and lateral growth without producing callus at the cut surfaces during the period of culture. Nodal explants cultured on BA alone produced callus at the base, callus initiation beginning between 4-9 days of culture. Subculturing the explants with the callus in media with the same composition resulted in increased callus formation at the base with the formation of shoot buds. Plate 2A shows nodal explants with callus production and shoot regeneration after subculturing nodal explant and callus in MS medium plus 8.8 μM BA for 3 weeks.

A mean number of 1.5±0.34 shoots/culture (with 92±8.4% response) and 1.1±0.43 shoots/culture (with 85±6.8% response) developed on MS media supplemented with 8.8 μM BA and 4.4 μM BA respectively. Within six weeks of culture, the shoot buds developed to mean shoot height of 3.0 ± 0.40 cm on MS media supplemented with 8.8 μM BA; the shoots developed on MS medium supplemented with 4.4 μM BA attained a mean height of 3.5 ± 0.45 cm (Plate 2B). Nodal cultures grown on MS medium plus various concentrations of either NAA alone (with exception of 10 μM) or mixture of NAA/BA produced callus and multiple shoot buds (Plate 2C) within two weeks of culture, the shoot buds being generated earlier in the NAA/BA mixtures. When the shoot buds were separated and transferred to media of the same composition, cell proliferation continued at the base of the shoot buds which developed to various heights. MS medium supplemented with a combination of 8.8 μM BA plus 2.7 μM NAA induced the highest number of shoots/explants (3.4 ± 0.36) with a generation response of 58.2± 5.8% and separated shoots reached a mean height of 4.2 ± 0.56 cm within 5 weeks (Plate 2D). The mean number of shoots/explants induced by the various concentrations of NAA or NAA/BA mixtures and the percent response of the explants are given on Table 1.

Developed shoots induced by various treatments produced singular thick roots that were about 2.1± 0.4 cm within 5 weeks of transfer into MS medium supplemented with 4.9 μM indole-3-butyric acid (IBA); the percent response was 42.2 ± 8.1%. Plate 2D shows nodal cutting with shoot and root i.e plantlet) on MS medium containing 5.4 μM NAA + 4.9 μM IBA. Addition of 2,4-D alone or combined with BA induced soft brown callus at the base of the nodal explants without shoot formation. When the explants with the calli were transferred to MS medium with the same concentrations of 2,4-D or to the mixtures of 2,4-D/BA, the calli formed were light brown and friable.

Plate 2E shows a 3-week old subcultured basal callus of nodal explants on MS medium to which 6.75 μM 2, 4-D had been added.

Table 1: Percent response of callus formation, degree of calli formation and morphology of callus produced from leaf of *S. tragacantha* grown on different concentration of Naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid or their separate mixtures with Benzyladenine.

Phytohormone Combination (µM)					
BA	**NAA**	**2,4-D**	**Degree of Callus Formation**	**Percent Explants Response**	**Morphology of Callus**
-	-	-	No callus formed	-	-
4.4	-	-	+	65±5.60	Green, nodular
4.4	2.70		++	69±8.20	Brown/green hard
4.4	5.40		++	80±4.30	Brown/green hard
4.4	10.80		+++	9.2±5.10	Brown/green hard
4.4	-	2.25	++	89±3.80	Brown/green hard
4.4	-	4.50	++	70±4.20	Brown/green hard
4.4	-	6.75	+	34±6.50	Brown/green hard
8.8	-	-	++	75±5.30	Nodular, green hard
8.8	2.70	-	+++	85±6.10	Brown, hard
8.8	5.40	-	+++	81±3.50	Brown, hard
8.8	10.80	-	++	62±4.80	Brown, hard
8.8	-	2.25	++	68±6.40	Light brown, nodular
8.8	-	4.50	+	54±5.30	Light brown, nodular
8.8	-	6.75	+	34±8.10	Light brown, nodular
-	2.70	-	-	0	-
-	5.40	-	+	6.5±8.00	Brown, hard
-	10.80	-	++	70±6.00	Brown, hard
-	-	2.25	++	55±9.00	Light brown, friable
-	-	4.50	+	64±7.00	Brown, friable
-	-	6.75	+	30±6.00	Brown, friable

+ = Low callus formation
++ = Moderate callus formation
+++ = Massive callus formation
** The values represent the means of triplicate determinations ± standard error.

Table 2: Effects of various concentrations of Naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid or their mixtures with Benzyladenine on shoot regeneration from nodal explants of *S. Tragacantha*

Phytohormone Combination (μM)					
BA	**NAA**	**2,4-D**	**No. of Shoots/ Explant (± S.E)**	**Percent Response (± S.E)**	**Mean Shoot Length (cm) (± S.E)**
-	-	-		-	
4.4	-	-	1.1±0.43	85±6.80	3.5±0.45
4.4	2.70	-	2.4±0.64	65±5.40	
4.4	5.40	-	1.4±0.56	4.8±3.80	
4.4	10.80	-	No shoots	0	
4.4	-	2.25	No shoots	0	
4.4	-	4.50	No shoots	0	
4.4	-	6.75	No shoots	0	
8.8	-	-	1.5±0.34	92±8.40	3.0±0.40
8.8	2.70	-	3.4±0.36	58.2±5.80	4.2±0.56
8.8	5.40	-	2.1±0.47	41± 6.80	3.2±0.24
8.8	10.80	-	1.5±0.25	34±8.20	2.5±0.18
8.8	-	2.25	-	-	-
8.8	-	4.50	-	-	-
8.8	-	6.75	-	-	-
-	2.70	-	1.0±0.55	56±10.60	
-	5.40	-	0.8±0.22	48±5.00	
-	10.80	-	No shoots	-	-
-	-	2.25	No shoots	-	-
-	-	4.50	No shoots	-	-
-	-	6.75	No shoots	-	-

*Data were obtained after 5 weeks of subculture of explants and callus in media of the same composition as the induction medium.
** The values represent the means of three independent experiments, at least 25 explants in each.

DISCUSSION

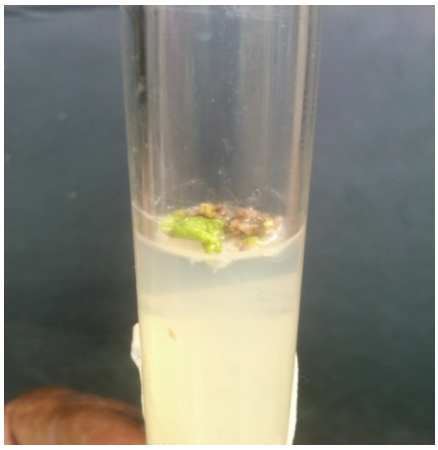
The use of *in vitro* approaches to propagation, conservation and genetic improvement of forest trees in West Africa has become a necessity in the light of the great demand placed on them for fuel wood, timber, drugs and several industrial purposes. Callus induction and shoot regeneration have been used to propagate a good number of important tree species. Different concentrations of NAA, 2,4-D or their combinations with BA induced callogenesis from explants of leaves or nodal cuttings of *S. tragacantha*. The Combination of 4.4 μM BA and 10.8 μM NAA induced the highest amount of callus in leaf explants of *S. tragacantha*. Isikalan *et al*., (2010) reported that the best callus formation from leaf explants of *Amygdalus communis* L. CV Yallinski was found on MS media supplemented with 1mg/L NAA combined with 1mg/L BA. Callus induction from cotyledon explants of *Zuglano nigra* L. (Newman *et al.,* 1993), *Cassia anguistifolia* (Agrawal and Sardar, 2007) and *P. biglobosa* Jacq. & Benth (Amoo and Ayisire, 2005) had been reported. MS medium supplemented with 1mg/L BA and 0.2mg/L NAA was the optimum growth regulator combination for callus induction in leaf and hypocotyl explants of *P. biglobosa* Benth. (Ntui *et al.,* 2012).

Shoot organogenesis occurred in leaf-derived calli subcultured on MS media supplemented with NAA combined with BA. The highest shoot regeneration was observed in MS medium containing 8.8 μM BA combined with 2.7 μM NAA with a mean number of 4.0± 0.8 shoots per explants. The use of 2,4-D combined with BA did not induce organogenesis but rather enhanced callus formation. Adventitious shoot induction from leaf-derived callus has been reported by several workers. Rahman *et al.,* (2010) in their study found that adventitious shoots were induced from leaf-derived callus of *Lagerstroemia speciosa* (Banaba or Queens Crapemyrtle) grown on MS medium that was supplemented with 5 μM BA, 3 μM NAA, 10% coconut milk and 568 μM ascorbic acid. Similarly, calli that were obtained from root and leaf fragments of *Swietenia mycrophyla* King were induced to form shoots on modified medium with three-quarter-strength nutrients and vitamins supplemented with 4.4 μM BA and 0.54 μM NAA or 8.9 μM BA and 0.11 μM or 0.54 μM NAA (Rocha Quoirin, 2004).

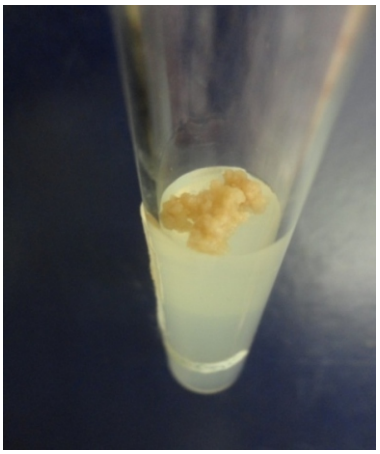
In the present study, nodal explants of *S. tragacantha* cultured on MS medium containing various concentrations of NAA, 2,4-D or their combination with BA, all favoured callogenesis at the basal cut surfaces. Rate of callus formation was quite faster than the rate of callus formation in the leaf explants and callus initiation was a matter of 5-10 days compared to 3-4 weeks in the leaf explants. Best callus formation was obtained from nodal explant cultured on MS medium supplemented with 8.8 μM BA + 2.7 μM NAA. Subculturing the explants with the callus produced the highest average number of shoot/ explants (3.4± 0.36 shoots/explants). The clonal propagation of plants from shoot tips, meristem tips or nodal explants usually has an accelerated proliferation of (axillary) shoots during subcultures (Pijut *et al.*, 2011). The best medium for shoot regeneration from nodal explants of *Azadiracta indica* was MS medium to which 13.32 μM BA had been added (Rodriguez and Ortiz, 2001). Similarly, Rodriguez-Sahagun *et al.*,(2007) in their study produced shoots from nodal cuttings of *Enterolobium cyclocarpum* cultured on MS medium supplemented with 2.2 μM BA and 10.7 μM NAA. Shoot proliferation from nodal explants was optimized for *Melaleuca alternifolia* (Tea tree) when cultured either in liquid or on an agar-based MS medium containing 1.11 μM BA or 0.55 μM BA (Oliveira *et al.*, 2010). Multiple shoots were also obtained from nodal explants of 18-day old *in vitro* seedlings of *Pterocarpus marsupium* Roxb. cultured on MS medium with 4 μM BA, 0.5 μM IAA and 20 μM adenine sulphate (Husain *et al.*, 2008).

In this study, we have developed a protocol for the micropropagation of *S. tragacantha* which if optimized will be useful in the establishment of the tree plantations that would complement the plant's natural regeneration and ensure the supply of the plant species in the future. Further work will, however, be required to develop the nodular/bud-like structures induced by 2, 4-D in the leaf explants of *S. Tragacantha.*

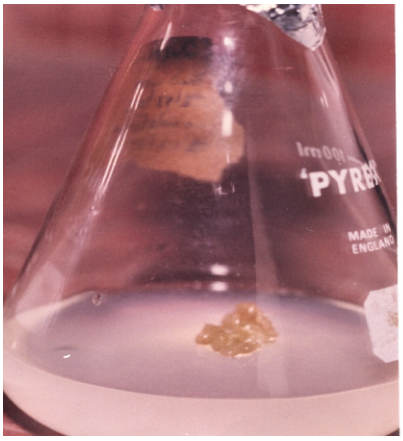
Plate **1:** callus induction from leaf



(1A): Callus formation in MS supplemented with 10.8µm+ 4.4µm BA after 5 weeks



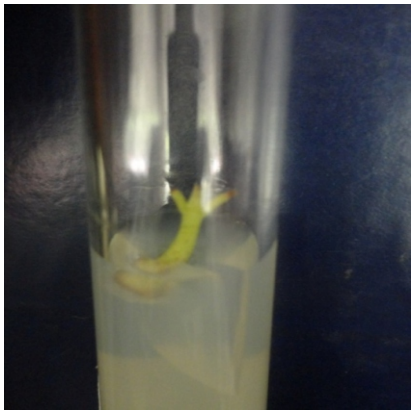
(1B): Callus formation in MS + 4.5µm 2, 4-D + 4.4µm



(1C): Shoot bud formation in leaf explants subcultured on MS medium + 2.25µm 2, 4-D + 8.8µm BA after 5 weeks.weeks

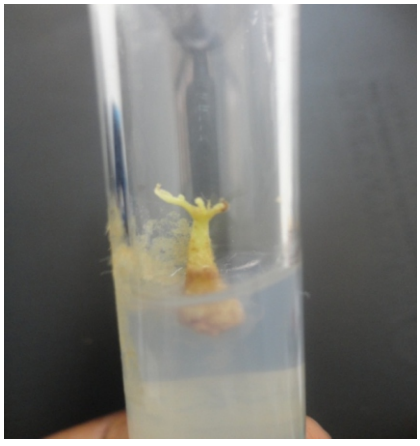
Plate 2: Callus Induction and Shoot Regeneration from Nodal Explants of *S. Tragacantha*

(2A): callus formation and shoot regeneration after culturing for 4 weeks in 8.8µm BA and subculturing in MS medium of same composition for 3 weeks.

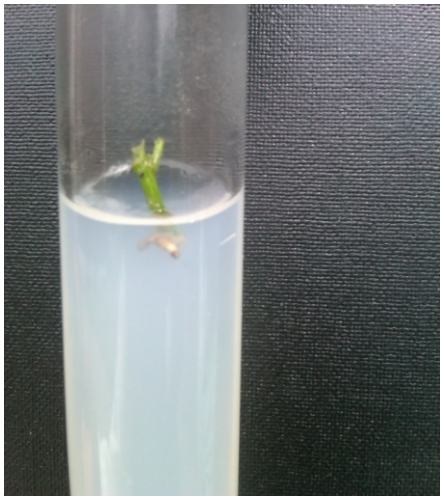


(2B): Callus formation and shoot regeneration after culturing for 4 weeks in 4.4µm BA and subculturing in MS medium of same composition for 6 weeks.

(2C): Multiple shoot formation on MS medium + 8.8µm BA+2.7µm NAA.



(2D): callus formation and shoot regeneration after culturing for 4 weeks in MS medium + 8.8µm BA + 2.7µm NAA and subculturing on MS medium of same composition for 5 weeks.



(2E): Root formation from shoot developed on MS medium + 5.4µm NAA + 4.9µm IBA.

(2F): Callus formation on MS medium + 6.75µm 2, 4-D + 8.8µm BA.

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