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Research Article

Tissue Culture Propagation of *Afzelia africana* – A Potential Candidate for Biofuel

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ABSTRACT

Afzelia africana is a medicinal and multipurpose tree that is under permanent pressure from its continuous exploitation for medicine, food and timber products. Adding to being under threat of extinction, its seeds are dormant and recalcitrant with a low rate of seed germination thus posing a challenge on propagation through conventional planting. This study, the first of its kind, reports an *in vitro* shoot regeneration protocol in *Afzelia africana*. *In vitro* propagation method is appropriate for propagating *A. africana* since it can boycott the use of seeds. Leaf, nodal, cotyledonary node (CN), shoot tip, stem and hypocotyl explants were cultured on Murashige and Skoog (MS) medium 1962 supplemented with different concentrations and combinations of plant growth regulators (PGR) and phloroglucinol. Two sets of experiments were done; (1) effect of PGRs and explant types on callus and shoot induction; (2) effect of PGRs on shoot multiplication and elongation from cotyledonary node explant. Both nodal explant and the cotyledonary node induced multiple shoots, 20% of nodal explants formed multiple (3) shoots on MS supplemented with 20mg/L BA and 40% of cotyledonary nodes formed multiple (10) shoots on MS supplemented with 15 mg/L BA and 15 mg/L PG. The response was better in cotyledonary explants, hence the shoot multiplication protocol was maximized using cotyledonary nodes where multiple (10.00 ± 0.00) shoots resulted from CN cultured on both MS supplemented with 15 mg/L BA,15 mg/L PG and 5 mg/L TDZ and MS supplemented with 15 mg/L BA, 15 mg/L PG and 10 mg/L TDZ. This study has paved the path for rapid regeneration of *A. africana* through *in vitro* propagation.

Keywords: African mahogany; multiple; shoots; *in vitro.*

INTRODUCTION

Forest trees are a major trap for Carbon IV Oxide; thus, they play a key role in protecting the earth against global warming. However, the rate of deforestation now is beyond reconciliation, calling for quick and consistent action from experts, policy makers as well as the public. Plant tissue culture method provides a means for mass clonal propagation of trees and serves as a tool for conserving their germplasm (Tefera, 2019). In tree species such as *Pongamia pinnata,* WPM supplemented with 30µM BAP and 1 mM phloroglucinol (PG) induced multiple shoots from nodal explants (Tan et al., 2018). Also, DCR medium supplemented with TDZ promoted bud breaking and adventitious bud formation in *Pinus pinaster* (Humánez et al., 2011).

Afzelia africana is known as African mahogany or African oak and it is a large deciduous tree in the family of Fabaceae. The seeds contain about 31% fat and may be a source of seed oil for domestic and industrial uses. The leaves of *A. africana* are consumed (Mefoh et al., 2013) and ruminants feed on the leaves as forage during the dry season (Oduguwa et al., 1997). Oedema can be treated with a mixture of the decoction of the leaves of *A. africana* with *Syzygium guineensis* leaves and *Xylopia* fruit (Orwa et al., 2009). The nitrogen rich leaves help improve the soil during mulching (Orwa et al., 2009).

Afzelia africana is propagated by seeds, and through budding and cuttings. However, seed predation by animals in the wild is usually high; thus, constraining natural regeneration (Amusa, 2010). The seeds of *Afzelia africana* are dormant and they become recalcitrant when stored (Orwa et al., 2009). Also, the rate of seed germination in the wild is low and its seedlings rarely develop into saplings. This is because the seeds of *A. africana* have hard seed coat, placing a mechanical barrier on the embryo; thus, limiting germination. Animals like birds and rodents feed on the seeds even while still on the parent plant. Indigenous people of the eastern part of Nigeria use the seed as an important condiment in meals, hence the seeds are heavily harvested when they are still on trees. Also, because *A. africana* is high grade timber, it is harvested for construction in some parts of Africa (Ajayi and Arowosoge, 2018). In addition to all these, the IUCN has listed *A. africana* as a threatened species (IUCN, 2024). Furthermore, the seeds of *A. africana* are used as an alternative to wheat flour. This has led to permanent pressure on its natural populations. As a result of this, conservation measures have been recommended (Amusa, 2010).

One of the numerous applications of plant tissue culture is germplasm conservation and transgenic plant production for crop improvement. Propagation of *A. africana* through tissue culture technique is appropriate and recommended because it bypasses the use of seeds with hard seed coat constraint as starting material for its propagation. In addition to its usefulness in propagation, it is only through the tissue culture approach that *A. africana* can be subjected to transgenic manipulations, which will be necessary to study the metabolic pathways involved in oil production, as well as characterize and modify the genes responsible for oil production in its seeds. Again, because *A. africana* seeds contain 31% of oil, it is a potential candidate for biofuel or oil production in the near future. Molecular studies to identify genes and mechanisms responsible for oil production, and thus allowing their modification for increased oil production, will only be possible through a transgenic approach. To date, there is paucity of research on the *in vitro* regeneration of *A. africana,* as an alternative means of propagating and conserving this potential biofuel plant. Hence this study was carried out to explore its regeneration *in vitro* and to establish a protocol for its mass propagation, conservation purposes and for future genetic engineering studies on it.

MATERIALS AND METHODS

Seed collection and culture media and conditions

Viable seeds were obtained from Obafemi Awolowo University, Nigeria (7° 32ˈ N 4° 31ˈ E). All the seeds collected were placed in a transparent jar containing water, and the viable seeds among them were identified as those that sank to the bottom of the water jar. These were sorted and kept safely in a well-labelled container for the study, while the others were removed. Freshly collected seeds of *A. africana* were disinfected properly by subjecting them to soaking in 100g/L mancozeb (C4H6MnN2S4.C4H6N2S4Zn) for 120 minutes, soaking in 100ºC water for 30 minutes and finally in 10% sulphuric acid for 30 minutes (Ogbimi et al., 2023). 5 seeds were used per treatment and the experiment was repeated. Murashige and Skoog (1962)

medium supplemented with 3% w/v sucrose and 0.2% w/v phytagel prepared according to standard protocols was used throughout the study. Cultures were maintained in a growth room under a temperature of 25 ± 2 º C and 16/8 -hour photoperiod.

Callus and shoot induction

The responsiveness of *A. africana in vitro* was tested by culturing the leaf, node, cotyledonary node, shoot tip, stem, and hypocotyl explants on MS supplemented with different concentrations and combinations of plant growth regulators. Explants were collected from 4-week-old *in vitro* grown seedlings. The Percentage Response of Callus Induction (PCRI), Percentage Response of Shoot Initiation (PRSI), were calculated using the below formula, Morphology of Callus (MOC) was recorded by visual observation while number of shoots per explant (NSPE) was counted.

$$PCRI = \frac{Number\ of\ explants\ showing\ callus\ induction\ response\ on\ a\ given\ media\ combination}{Number\ of\ replicates\ for\ that\ media\ combination} \times 100$$

$$PSRI = \frac{Number\ of\ explants\ showing\ shoot\ initiation\ response\ on\ a\ given\ media\ combination}{Number\ of\ replicates\ for\ that\ media\ combination} \times 100$$

Shoot multiplication and elongation

In other to maximize the shoot initiation potential of cotyledonary node explants, cotyledonary nodes were obtained from *in vitro* grown seedlings of *A. africana.* They were cultured on MS media supplemented with different concentrations and combinations of plant growth regulators. Five explants were used per treatment and explants cultured on MS basal media alone was kept as the control. The cultures were maintained in a growth room at a temperature of 25 ± 2 ºC and 16 / 8 - hour photoperiod. The number of multiple shoots initiated were recorded. In the elongation experiment, multiple shoots initiated from cotyledonary nodes were transferred into elongation media (MS + 15 mg/L BA+ 15 mg/L PG) after which number of elongated shoots and length of shoots were recorded.

Statistical analysis

The number of shoots, the number of elongated shoots and the length of shoots recorded from the shoot multiplication and elongation experiment were subjected to one-way ANOVA using SAS version 9.2 and the means were separated using Duncan's multiple range test at 0.05 alpha level.

RESULTS

Effect of PGRs on callus induction and shoot initiation

Varying responses resulted from the culture of different explants on MS basal media alone and MS supplemented with different concentrations and combinations of plant growth regulators. Leaf explants was subjected to MS supplemented with 2,4-D (4,10) mg/L in combination with BA (0.1 to 1.0) mg/L. Nodal explants was subjected to MS supplemented with BA (1, 10, 15, 20 and 25) mg/L NAA (0.5 and 5) mg/L. Cotyledonary node was subjected to MS

supplemented with BA (1, 3, 15, 20 and 25) mg/L, NAA (0.5 and 5) mg/L, IAA (5 and 10) mg/L and PG (5, 10 and 15) mg/L. Shoot tip explant was subjected to MS supplemented with BA (0.1, 2, 5, 15, 20 and 25) mg/L in combination with NAA (5 mg/L). Stem explant was subjected to MS supplemented with BA (2, 3, 5, 15 and 20) mg/L in combination with NAA (0.5 mg/L) and PG (15 mg/L), and hypocotyl explant was subjected to MS supplemented with BA (1, 5, 15 and 20) mg/L in combination with NAA (0.5 mg/L) and PG (10, 15 and 20) mg/L. Leaf explants cultured on MS basal media alone showed no response (Table 1). However, when the basal media was supplemented with varying concentrations of cytokinin and auxin, callus response was elicited with varying morphology observed (Table 1). Dark-brown compact callus resulted from leaf explants cultured on MS supplemented with 4 mg/L 2,4 – D and 0.1 mg/L BA (Fig. 1A, Table 1) with the highest callus response - 100%, and off-white embryogenic callus formed when leaf was cultured on MS supplemented with 4 mg/L 2,4 – D and 0.7 mg/L BA (Fig. 1B, Table 1) with 60% callus response.

Table 1: Response of leaf explants of *Afzelia africana* on MS supplemented with 2, 4 – D in combination with BA.

2,4 -D (mg/L)	BA (mg/L)	DOCF	PRCI (%)	MOC
0	0	NR	0	_
4	0	NR	0	_
4	0.1	++	100	White/dark brown, compact hard
4	0.2	++	60	White/brown, compact hard/soft
4	0.3	+	60	White/light brown, compact hard
4	0.4	+++	80	White/cream/light brown/dark brown, compact hard/soft
4	0.5	++	40	Cream/dark brown, compact hard/soft
4	0.6	NR	0	_
4	0.7	+	60	White/ friable
4	0.8	++	40	White/dark brown, compact hard
4	0.9	++	20	Light brown/compact soft
4	1	+++	60	Light brown/compact soft
10	0	NR	0	_
10	0.1	NR	0	_
10	0.2	+++	20	White/brown, compact hard
10	0.3	NR	NR	_
10	0.4	+	40	White/compact hard
10	0.5	NR	NR	_
10	0.6	+	30	White/compact hard
10	0.7	+	40	White/compact hard
10	0.8	NR	0	_
10	0.9	+	30	White/transparent soft
10	1	+	20	White/compact hard

Keys: 2, 4 – D – 2, 4 – Dichlorophenoxy acetic acid, BA – Benzyladenine, DOCF – Degree of callus formation, MOC – Morphology of callus, PRCI – Percentage response of callus induction, NR – No response

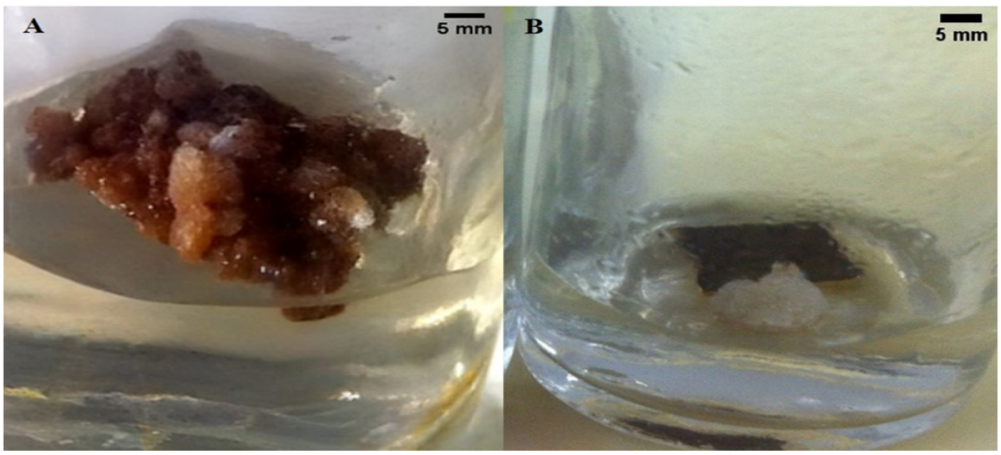

Figure 1: Leaf explant *of Afzelia africana* (A). Dark-brown compact callus on MS + 4 mg/L 2,4 – D + 0.1 mg/L BA. (B). Off-white embryogenic callus on MS + 4 mg/L 2,4 – D + 0.7 mg/L.

Nodal explants also did not show any visible response on MS basal media alone. However, 20% of nodal cultures responded by initiating a bud on MS supplemented with 1 mg/L BA (Fig. 2A, Table 2). The inclusion of NAA to the media – MS + 1 mg/L BA + 0.5 mg/L NAA suppressed shoot initiation and induced callus formation from 40% of nodal cultures. As the concentration of BA was further increased in the media, shoot initiation was inhibited (0%) and callus induction increased to 100% with massive callus production when media was MS + 10 mg/L BA + 0.5 mg/L NAA. On MS + 15 mg/L BA, only shoot initiation was stimulated with one shoot initiating from 50% of the cultures. 20% of the cultures formed 3 shoots on MS supplemented with 20mg/L BA (Fig. 2B, Table 2). The addition of auxin NAA (5 mg/L) to the media containing 20 mg/L BA inhibited shooting and enhanced the formation of friable callus (Fig. 2C, Table 2), while the addition of auxin IAA (5 mg/L) to the same media suppressed shooting (one thick shoot formed) and also enhanced the formation of compact callus (Fig. 2D, Table 2).

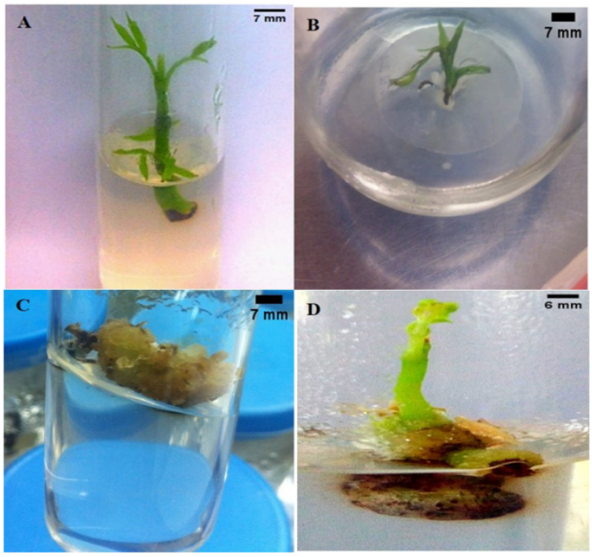

Figure 2: Nodal explant of *Afzelia africana* (A). Young developing bud formed on MS + 1 mg/L BA. (B). Multiple shoots formed on MS + 20 mg/L BA. (C). Friable callus formed on MS + 20 mg/L BA+ 5 mg/L NAA. (D). A thick shoot and basal callus formed on MS + 20 mg/L BA + 5 mg/L IAA.

Table 2: Effect of plant growth regulators on callus induction and shoot initiation from nodal explants of *Afzelia africana*.

BA (mg/L	NAA (mg/L)	IAA (mg/ L)	PRCI (%)/ DOCF	PRSI (%)/ NSPE
-	-	-	-	-
1	-	-	NR	20 / 1
1	0.5	-	40 / +	-
10	-	-	50 / +	40/ 1
10	0.5	-	100 / +++	-
15	-	-	100 / ++	50 / 2
15	5	-	25 / +	-
15	-	5	NR	NR
15	-	10	100 / +	-
20	-	-	20 / ++	20 / 3
20	5	-	25 / +	-
20	-	5	60 / +	40 / 1
20	-	10	75 / ++	50 / 2
-	-	20	50 / +	50 / 1
25	-	-	60 / +	100 / 1
25	-	5	100 / +	-
25	-	10	25 / +	75 / 2
-	-	25	100 / +	-

Keys: - PRCI – percentage response of callus induction, DOCF – degree of callus formation, PRSI – percentage response of shoot induction, NSPE – number of shoots per explants, NR – No response

Cotyledonary nodes of *A. africana*, similar to the response of leaf and nodal explants, also did not show any observable response on MS basal media alone. The addition of 1 mg/L BA to the MS media induced callusing response from 40% of the cultures (Table 3). However, when NAA was added to this media where callusing was initially observed – 0.5 mg/L NAA, the callusing response was inhibited and callus formed only from 20% of the cultures. At higher concentrations of BA; on MS + 15 mg/L BA, shooting response was first observed with two shoots initiating from 67% of the cultures. Shooting response was also inhibited on MS + 15 mg/L BA + 5 mg/L NAA, a shoot being induced from 33% of the cultures. On another media, shooting response was completely inhibited and callusing enhanced from 67% of the cultures, while on MS + 20 mg/L BA, number of shoots increased to three (Fig. 3A Table 3). Again, the addition of NAA to media containing high concentration of BA – MS + 15 mg/L BA+5 mg/L NAA inhibited shoot organogenesis but enhanced callus induction. Callus resulted from 67% of the cultures. The addition of another type of auxin to the media; MS + 15 mg/L BA+5 mg/L IAA, completely inhibited both the callusing and shooting response. Increasing the concentration of BA to 20 mg/L initiated more shoots – 3 from 20% of the cultures (Fig. 5A, Table 3). Cotyledonary node (CN) explants cultured on MS and 25 mg/L BA and 5 mg/L IAA formed friable callus from 75% of the cultures (Fig. 3B, Table 3). 10 shoots were formed from 40% of CN cultured on MS supplemented with 15 mg/L BA and 15 mg/L PG (Fig. 3C, Table 3). The number of shoots were observed to reduce at a higher concentration of PG - MS + 15 mg/L BA + 20 mg/L PG, 8 shoots were formed from 20% of the cultures.

Table 3: Effect of different plant growth regulators on shoot induction from cotyledonary node explants of *Afzelia africana.*

BA (mg/L)	NAA (mg/L)	IAA (mg/ L)	PG (mg/L)	PRCI(%)/ DOCF	MOC	PRSI (%)/ NSPE
-	-	-	-	-	-	-
1	-	-		40 / +	Cream, soft friable/light brown	0 / 0
1	0.5	-		20 / +	Green friable	0 / 0
3	-	-		40 / ++	Cream soft	0 / 0
15	-	-		NR	-	67 / 2
15	5	-		67 / +		0 / 0
15	-	5		NR	-	0 / 0
-	-	10		NR	-	0 / 0
20	-	-		67 / ++	Light brown, soft	20 / 3
20	5	-		75 / +	Cream friable	0 / 0
20	-	5		100 / +	Cream friable	0 / 0
25	-	-		NR	-	50 / 1
25	5	-		100 / ++	Cream, soft friable	100 / 3
25	-	5		75 / +	Cream friable	0 / 0
-	-	-	5	NR	-	20 / 1
-	-	-	20	NR	-	0 / 0
15	-	-	15	NR	-	40 / 10
15			20	NR	-	20 / 8

Keys: PRCI – Percentage Response of Callus Induction, DOCF – Degree of Callus Formation, PRSI – Percentage Response of Shoot Induction, MOC – Morphology of Callus, NSPE – Number of Shoots Per Explant, PG – Phloroglucinol, IAA – Indole Acetic Acid, NAA – Naphtalene Acetic Acid, BA – Benzyladenine, NR – No response

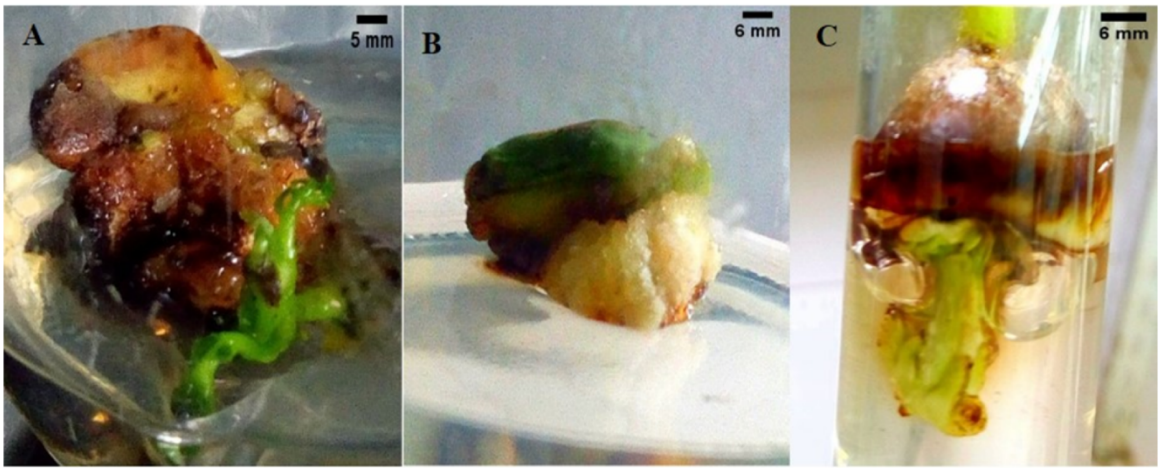

Figure 3: Cotyledonary node *of Afzelia africana* (A). Multiple shoots formed on MS + 20 mg/L BA. (B). Cream friable callus formed on MS + 25 mg/L BA+ 5 mg/L IAA. (C). Multiple buds formed on MS + 15 mg/L BA + 15 mg/L PG.

Shoot tip explants cultured on media with different plant growth regulators showed both shoot organogenesis and callogenesis. One shoot was initiated from shoot tip cultured on MS basal media alone (Fig. 4A, Table 4). At a very high concentration of BA supplemented media, 2 shoots were initiated from 40% of the cultures (Fig. 4B, Table 4). Also, soft cream callus resulted from 40% of cultures in MS media supplemented with 25 mg/L BA and this was the highest recorded.

Table 4: Organogenesis and callogenesis from shoot tip explants of *in vitro* grown seedlings of *Afzelia africana*.

BA (mg/L)	NAA (mg/L)	PRCI (%)/ DOCF	MOC	PRSI (%)/ NSPE
-	-	-	-	20/1
0.1	-	NR	-	20/1
2	-	NR	-	20/1
5	-	NR	-	20/1
15	-	NR	-	20/1
15	5	NR	cream, soft callus	20/1
20	-	20/+	cream, compact brown	20/1
25	-	20/+	cream, soft callus	40/2

Keys: BA – Benzyladenine, NAA – Naphtalene Acetic Acid, PRCI – Percentage Response of Callus Induction, MOC – Morphology of Callus, PRSI – Percentage Response of Shoot Induction, NR – No response

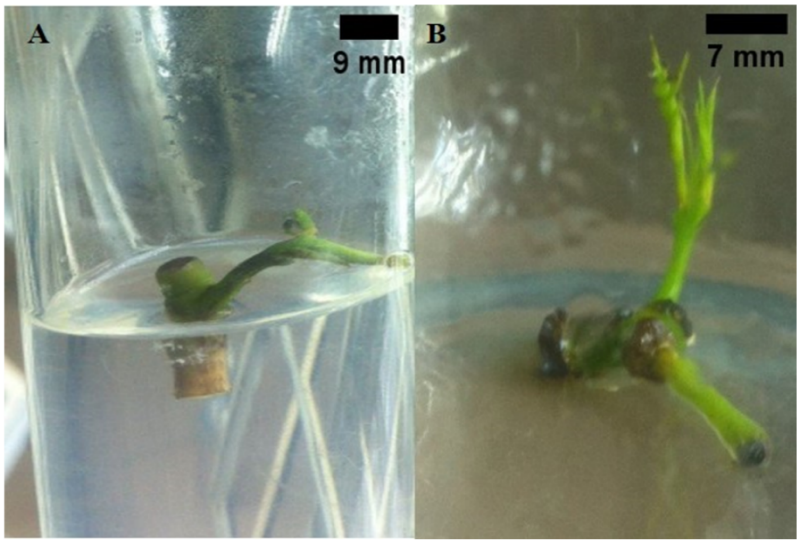

Figure 4: Shoot tip of *Afzelia africana* (A). Young developing buds formed on MS alone. (B). Two shoots formed on MS + 25 mg/L BA.

Stem (Table 5) and hypocotyl (Table 6) explants of *A. africana* were also responsive *in vitro* showing shoot initiation and callus formation, however multiple shoot formation was not recorded in these explant types.

Table 5: Organogenesis and callogenesis from stem explants of *in vitro* grown seedlings of *Afzelia africana.*

BA (mg/L	NAA (mg/L)	PG (mg/L)	PRCI (%)/ DOCF	MOC	PRSI (%)/ NSPE
-	-	-	NR	-	-
2	-		NR	-	-
3	-	-	40 / ++	Soft cream/Dark brown	20 / 2
5	0.5		NR	-	-
15		15	100 / ++	Off white/dark brown, compact	- / -
20	-		60 / ++	Creamy white, soft	20 / 1

Keys: BA – Benzyladenine, NAA – Naphtalene Acetic Acid, PG – Phloroglucinol, PRCI – Percentage Response of Callus Induction, MOC – Morphology of Callus, PRSI – Percentage Response of Shoot Induction, NR – No response

Table 6: Organogenesis and callogenesis from hypocotyl explants of *in vitro* grown seedlings of *Afzelia africana*.

BA (mg/L)	NAA (mg/L)	PG (mg/L)	PRCI(%)/ DOCF	MOC	PRSI (%)/ NSPE
-	-	-	NR	-	20 / 1
1	0.5	-	NR	-	NR
	-	10	NR		20 / 1
5	0.5		NR	-	NR
15	-	10	80 / +	Compact hard, dark	NR
15	-	15	80 / ++	Off white / cream,	20 / 1
15	-	20	20 / +	Soft cream, friable	NR
20	-	-	100 / +	Soft cream	NR

Keys: BA – Benzyladenine. NAA – Naphtalene acetic acid, PG – Phloroglucinol, PRCI – percentage response of callus induction, DOCF – degree of callus formation, MOC – morphology of callus, PRSI – percentage response of shoot initiation, NSPE – number of shoots per explants

Shoot multiplication and elongation from cotyledonary node of *Afzelia africana*

The preliminary experiment showed that CN explants were more responsive *in vitro*, hence shoot multiplication experiment was maximized using them. However, variations in shoot response were recorded due to different environmental conditions under which this replication and shoot maximization experiment occurred (Table 7). In the second experiment, cultures were subjected to a more humid environment and this accounted for the variation in response with respect to the preliminary experiment. Multiple shoots were formed from the culture of CN explant on MS + 15 mg/L BA + 15 mg/L PG (4.33 ± 0.33) (Fig. 5A, Table 7) and also on MS + 15 mg/L BA + 20 mg/L PG (4.00 ± 0.00) (Fig. 5B, Table 7). Significant multiplication of shoot was recorded on thidiazuron-containing media with 10.00 ± 0.00 shoots forming on both MS + 15 mg/L BA + 15 mg/L PG + 5 mg/L TDZ (Fig. 5C, Table 7) and MS + 15 mg/L BA + 15 mg/L PG + 10 mg/L TDZ (Fig. 7D, Table 7). The multiple shoots formed (Fig. 5C, Fig. 5D) were then transferred back to the media without thidiazuron (15 mg/L BA + 15 mg/L PG) for elongation which occurred within 22 days. (Fig. 5E, Table 8). The media containing equal concentration of BA and PG - 15 mg/L BA + 15 mg/L PG enhanced the longest shoots (5.63 ± 1.10), while the highest number of elongated shoots was formed on MS + 15 mg/L BA + 20 mg/L PG (2.33 ± 0.21).

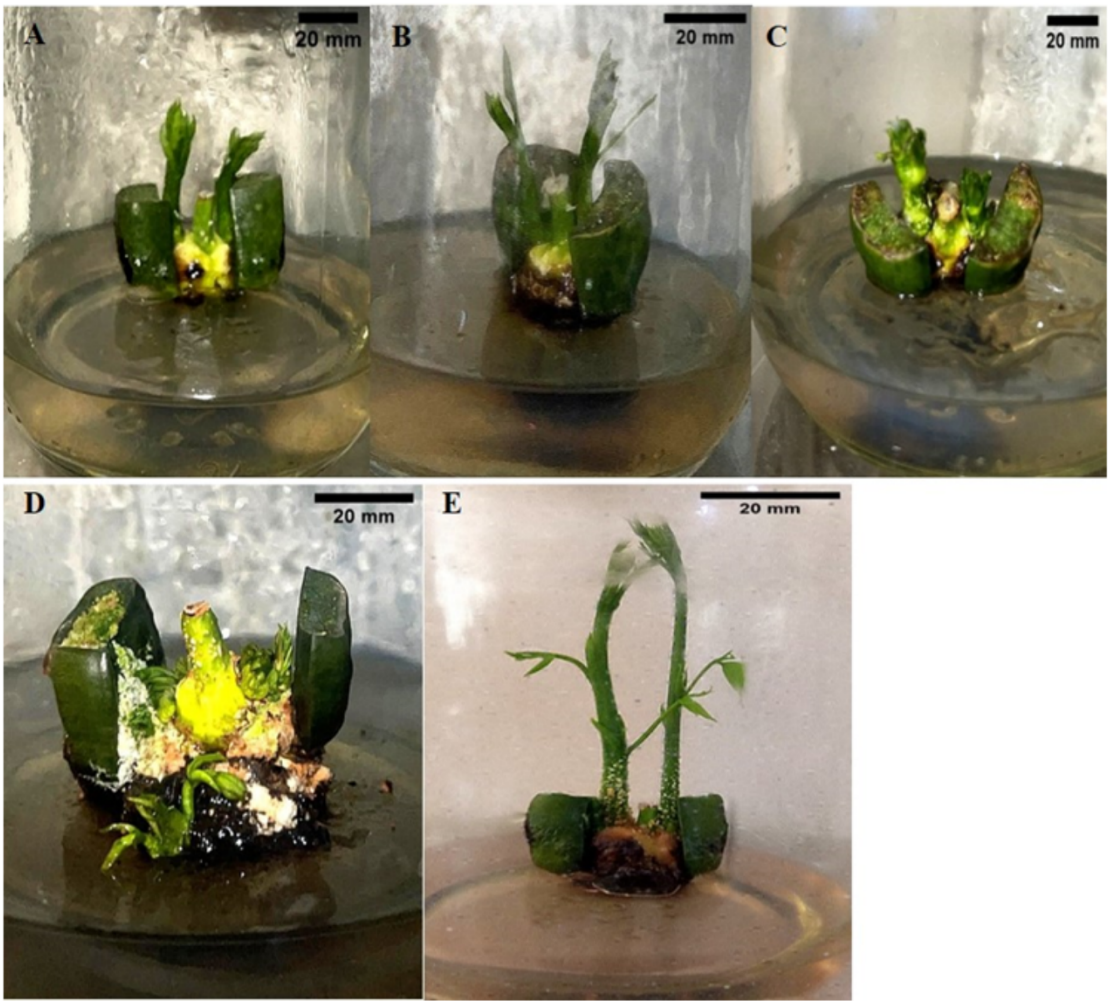

Figure 5: Shoot multiplication and elongation from cotyledonary node of *Afzelia africana* (A). Multiple shoots formed on MS + 15 mg/L BA + 15 mg/L PG (B). Multiple shoots formed on MS + 15 mg/L BA + 20 mg/L PG (C). Multiple shoots formed on MS + 15 mg/L BA + 15 mg/L PG + 5 mg/L TDZ (D). Multiple shoots formed on MS + 15 mg/L BA + 15 mg/L PG + 10 mg/L TDZ (E). Elongated shoots on MS + 15 mg/L BA + 15 mg/L PG

Table 7: Shoot multiplication from cotyledonary node of *Afzelia africana.*

Media combination	BA (mg/L)	PG (mg/L)	TDZ (mg/L)	Number of Shoot Initiated
1	-	-	-	0.00 ± 0.00^{c}
2	-	15	-	0.00 ± 0.00^{c}
3	15	15	-	4.33 ± 0.33^{b}
4	15	20	-	4.00 ± 0.00^{b}
5	15	15	5	10.00 ± 0.00^{a}
6	15	15	10	10.00 ± 0.00^{a}
7	15	15	15	0.00 ± 0.00^{c}

Means with different letter along the column are significantly different from each other at $p \leq 0.05$.
Keys: BA – Benzyladenine, PG – Phloroglucinol, TDZ – Thidiazuron

Table 8: Elongation of multiple shoots induced from cotyledonary node of *Afzelia africana*.

Media combination	BA (mg/L)	PG (mg/L)	Number of Shoots Transferred From TDZ-containing media	Number of Elongated Shoots	Length of Shoot (cm)
1	-	-	10.00 ± 0.00^{a}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
2	-	15	10.00 ± 0.00^{a}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
3	15	15	10.00 ± 0.00^{a}	2.00 ± 0.00^{b}	5.63 ± 1.10^{a}
4	15	20	10.00 ± 0.00^{a}	2.33 ± 0.21^{a}	3.48 ± 0.05^{b}

Means with different letter along the column are significantly different from each other at $p \leq 0.05$.
Keys: BA – Benzyladenine, PG – Phloroglucinol, TDZ – Thidiazuron

DISCUSSION

No response was observed from leaf explants cultured on MS media devoid of PGR, however on the inclusion of PGR, varying responses resulted. Raspor et al. (2021) reported that PGR especially auxins and cytokinins play a key role in the differentiation process in *in vitro* culture systems. Callus response was initiated from the leaf explants of *A. africana* when media was supplemented with a high concentration of auxin (2, 4–D) in combination with a low concentration of cytokinin. Usually, the addition of exogenous auxin to the media stimulates the synthesis of endogenous auxin for regeneration (Nic-Can and Loyola-Vargas, 2016) and in this case the callusing response. 2, 4–D alone or in combination with cytokinin has been widely used in callus cultures (Atta et al., 2009), where its concentration affects the callus culture and its morphology. Similar to previous findings that embryogenic or friable callus is produced from low concentrations of 2, 4 – D and inhibited by high concentration 2, 4-D, our finding showed the same observation where 4 mg/ L 2, 4 – D + 0.7 mg/L BA produced friable callus while 10 mg/ L 2, 4 – D + 0.9 mg/L BA produced compact callus. 2 mg/L 2, 4-D was reported to be optimal for embryogenic callus induction in Poaceae plants (Çabuk and Özgen, 2016). At lower concentrations of 2, 4-D, the percentage response of leaf explants to callus induction was high while at higher concentrations, the percentage response was low, which can be an inhibitory effect of PGR at higher concentrations. The best callus response (100%) was observed on MS + 4 mg/ L 2, 4 – D + 0.1 mg/L BA, and this concentration is optimal for callus induction from leaf explants of *A. africana*. In *Zigyphus jujuba*, Ye et al. (2012) reported that the best callus induction response (95%) was obtained from leaf explants cultured on MS + 2 mg/L 2, 4 – D + 0.5 mg/L BA.

Similarly, as varying responses resulted from the culture of leaf explants, nodal explant cultures also resulted in different responses. Observable responses did not occur from the culture of nodal explants on the basal media alone, however addition of BA (1 mg/L) initiated organogenesis with a shoot bud arising from 20% of the cultures. BA is a cytokinin, and cytokinins are known to initiate shoot development (Pernisova et al., 2009). In addition, BA is a widely used cytokinin for promoting cell division and organ differentiation (Phillips and Garda, 2019). The inclusion of exogenous cytokinin (BA) in the medium increased the concentration of cytokinin available, hence increasing the competence of nodal explants for organogenesis. Irina (2008) reported that the optimum level of BA needed for organogenesis is dependent on the concentration of endogenous cytokinin available in the plant species. The media containing either BA alone or in combination with IAA elicited shoot and callus response simultaneously while only callus resulted in media containing BA and NAA. A similar observation was reported by Zafarullah (2013) where IAA in addition to BA enhanced

shooting while NAA in addition to BA inhibited it in *Chrysanthemum Indicum* L. Shoot initiation was inhibited from nodal explants on MS + 1 mg/L BA +0.5 mg /L NAA while this media enhanced callus induction from 40% of the cultures. Callus induction is usually favored in media containing equal (or almost equal) concentrations of cytokinin and auxin (Rahman et al., 2010), hence the observed callusing response with this media combination. A similar response of inhibition of shoot initiation was observed with MS + 10 mg/L BA + 0.5 mg/L NAA and MS + 15 mg/L BA + 5 mg/L NAA. Shoot organogenesis occurred in these media combinations when NAA was excluded, hence showing that NAA in the media suppressed the shoot-stimulating effect of BA. The optimal media for shoot initiation from nodal explant is MS + 20 mg/L BA where 3 shoots were simultaneously produced with an average-sized white friable and transparent callus. BA-containing media has been used to induce shoot emergence and development in Jerusalem artichoke (Abdalla et al., 2021; Kim et al., 2016).

Other explants apart from nodal explants such as shoot tip, stem, and hypocotyl explants also initiated shoots *in vitro*. However, cotyledonary node explants of *A. africana* responded best with multiple shoots production *in vitro*. Cotyledonary node explants cultured on MS + 25 mg/L BA + 5 mg/L NAA produced 3 shoots from 100% of cultures. On MS + 15 mg/L BA + 15 mg/L PG; 10 shoots were produced from 40 % of the cultures and finally on MS + 15 mg/L BA + 20 mg/L PG - 8 shoots from 20% of the cultures. PG-containing media was best for initiating the multiple shoots. In the initial part of the study, MS + 15 mg/L BA + 15 mg/L PG was optimal for multiple shoot initiation in *A. africana*. PG has growth-promoting properties and acts in synergism with auxin and cytokinin as well as acting like cytokinin and auxin (Teixeira da Silva et al., 2013). In the further experiment to maximize shoot multiplication protocol, a significant difference was recorded in the number of shoots initiated on MS + 15 mg/L BA + 15 mg/L PG when compared to what was recorded on it during the initial experiment. Environmental differences from varied humidity conditions can explain this variation in the result as both experiments were undertaken at different times. On all media tested, shoot multiplication was highest in MS + 15 mg/L BA + 15 mg/L PG + 5 mg/L TDZ and MS + 15 mg/L BA + 15 mg/L PG + 10 mg/L TDZ producing 10.00 ± 0.00 number of initiated shoots, which however did not elongate while they remained in the media containing TDZ. A similar response was observed in the continuous culture of explants of *Syzygium cumini* on TDZ-containing media, the multiple shoots induced did not elongate until they were transferred to a media without TDZ (Naaz et al., 2021). In this study also, shoots present on TDZ devoid media were shown to elongate significantly, hence multiple shoots produced on TDZ containing media were further transferred back to MS alone, MS supplemented with PG alone, and MS supplemented with BA and PG for elongation. MS media containing BA and PG was optimum for elongation and a significantly higher number of elongated shoots (2.33 ± 0.21) were produced on MS + 15 mg/L BA + 20 mg/L PG while on MS + 15 mg/L BA + 15 mg/L PG, the highest elongation was recorded with shoots growing up to 5.63 ± 1.10 cm. Usually, auxin function is promoted by PG (Petti, 2020), hence the PG in the media has enhanced endogenous auxins within the shoots resulting in stem elongation - a known functional role of auxin. In addition, Manokari et al. (2021) reported the role of PG not only in inducing new shoots but also in the development and elongation of the formed shoots.

CONCLUSIONS

This study has confirmed that regeneration of *A. africana* is feasible through *in vitro* propagation. In addition, it has provided a shoot multiplication and elongation protocol from cotyledonary node explants of *A. africana* for its massive propagation. MS + 15 mg/L BA + 15 mg/L PG + 5 mg/L TDZ and MS + 15 mg/L BA + 15 mg/L PG + 10 mg/L TDZ were optimal for the production of the highest number of multiple shoots. Hence, this media can be utilized for massive propagation of *A. africana*, and for transformation studies during genetic modification. Again, it can be useful for raising seedlings *in vitro* for afforestation, reforestation and ex-situ conservation purposes such as gene banks.

Genetic modification for crop improvement studies to increase the seed oil content of *A. africana* is recommended for future research. These studies will identify and characterize the genes and the metabolic pathways responsible for oil accumulation in seeds and attempt to upregulate these genes for higher expression resulting in higher oil accumulation. Plant tissue culture techniques remain the only means through which transformed cells can be grown into whole plants. In addition, through Plant Tissue Culture techniques, resulting transformed plants can be massively propagated using the protocol developed in this study. However, the protocol should be optimized to eliminate the effect of environmental factors. Further studies to root the shoots regenerated *in vitro* and acclimatize them in the field are ongoing. In conclusion, this study, which is the first of its kind, has provided information on how *A. africana* - a potential biofuel plant, can be regenerated through *in vitro* propagation methods, hence reducing the pressure on its wild stands as well as conserving its germplasm for future purposes.

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