THE INTERPLAY BETWEEN EXPLANT DEVELOPMENTAL STAGES AND PHYTOHORMONE TYPE IN CALLOGENESIS OF SHEA TREE (*Vitellaria paradoxa* C. F. Gaertn)

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ABSTRACT

The long gestation period of the Shea tree (Vitellaria paradoxa C.F Gaertn) is one of the limitations in its domestication and genetic improvement. Application of in vitro clonal procedure presents several advantages over the conventional propagation methods. This study was an attempt to investigate the interplay between the effects of developmental stages of leaf explants obtained from Shea tree and phytohormones used for media supplementation on callogenic responses. Three developmental stages (1, 2 and 3) of explants and the phytohormones: 2,4-dichlorophenoxyacetic acid (2,4-D) and Benzyl amino purine (BAP) were used. Leaf cultures were initiated using Murashige and Skoog medium, supplemented with 2,4-D (0, 0.1 and 0.5mg/l) and BAP (0, 0.1,1.0 and 1.5 mg/l), in combination or alone. Callus was obtained after 2 weeks of culture initiation in all the treatments, except in cultures supplemented with BAP alone or without growth regulators. The Stage 2 explant (intermediate explant age) seemed to be most suitable for callus proliferation, which was followed by the Stage 1 explant (youngest), while the Stage 3 explant (oldest) was least preferred. The optimal concentrations of 2,4-D and BAP when used in combination for media supplementation for callogenesis of Shea leaf tissue were 0.5mg/l and 1.5mg/l respectively.

Key words: Auxin, Callogenesis, Cytokinin, Explant, Shea tree, *Vitellaria paradoxa*

INTRODUCTION

The Shea tree (*Vitellaria paradoxa* C.F Gaertn) which belongs to the family *Sapotaceae,* grows widely in the savannah region of West African countries (Maranaz and Wiesman, 2003). It is a deciduous dicotyledonous crop that has a gestation period varying from 15 to 20 years. It takes about 45 years to attain maturity but after this, it may continually produce Shea nuts for up to 200 years. The matured trees vary considerably in height with some reaching a height of over 14 m and a girth over 1.75 m (Yidana, 2004). The Shea tree is of high economic importance with high value attributed to its butter, obtained from dried Shea nuts, which is a ready source of fat in local diets (Lamien *et al*., 1996). Adomako (1985) described the butter to have characteristics similar to that of cocoa butter. It's use as a cocoa butter equivalent (CBE) in the manufacture of confectionary and as an important ingredient in pharmaceutical and cosmetic industries has greatly increased its global demand. Naturally, Shea tree grows and regenerates itself in the wild but it's slow and poor natural regeneration pattern due to long gestation period, impacts of bush fires and desertification have limited the domestication and genetic improvement of this crop. These limitations have necessitated the need for an alternative method of conserving this plants genetic resource outside the natural habitat. The propagation of Shea tree by *in vitro* clonal technique presents such alternative method.

The term "*in vitro*" culture is used broadly to refer to the culture of living materials such as seeds, embryos, organs, tissues, cells and protoplasts in culture vessels such as test tubes under sterile conditions (Pierik, 1999). *In vitro* technique is rapidly becoming a commercial method for propagating new cultivars, rare species and difficult-to-propagate plants (Preece and Read, 1993). In *in vitro* culture, plant growth regulators are basic components of nutrient media which is critical in determining the developmental pathway of plant cells. The most widely used phytohormones are auxins and cytokinins. The types, concentrations and the ratio of these growth regulators used for media supplementation are essential for the type of culture responses obtained (Anca, 2009; Ball *et al.,* 1993), with 2,4-dichlorophenoxyacetic acid (2,4-D) inducing callus formation while indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA) promotes direct embryogenesis (Armstrong *et al*., 1987). Callus refers to rapidly proliferating undifferentiated mass of cells arising from an isolated differentiated tissue (explant) cultured on a nutrient medium supplemented with specific growth hormones (Skoog and Miller, 1957). Eke *et al.* (2010) presented a system for callus induction, root formation and shoot organogenesis from cotyledon explants of Shea.

The aims of this study were to investigate the developmental stage of Shea tree leaf explant most suitable for callogenic response and also determine the optimal concentration of 2,4-D and BAP (Benzyl amino purine) media supplementation, when used singly or in combination.

MATERIALS AND METHODS

Source of explants: Leaf tissues of Shea tree (*Vitellaria paradoxa*) were obtained from a single plant in the Nigerian Institute for Oil Palm Research (NIFOR), Benin City. Three stages of young Shea leaves as described in Table 1 and shown in Plate 1 were used.

Table 1: Description of leaf explant stages

Explant stage	Average length of leaf (cm)	Average width of leaf (cm)	Average length of leaf petiole (cm)
Stage 1	7.55	1.35	2.60
Stage 2	11.95	2.68	4.78
Stage 3	17.45	3.70	6.60

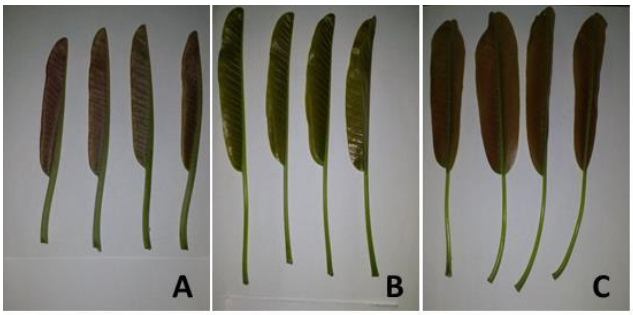

Plate 1: Shea tree leaves at developmental stages (A) 1; (B) 2 and (C) 3

Media preparation and hormonal supplementation: In this study, Murashige and Skoog (MS) medium (which consists of macro nutrients, micro nutrients, iron, vitamins, casein hydrolysate and other supplements) was used. The medium was prepared based on the laboratory protocol (Murashige and Skoog, 1962). The basal MS medium was supplemented with two growth regulators: 2,4-D (an auxin) and BAP (a cytokinin) and the pH was adjusted to 5.8. The medium was properly dispensed into test tubes and solidified with plant agar. These were covered with foil paper and sterilized along with forceps, petri dishes and foil papers by autoclaving at 121ºC, 15psi for 30 minutes. After autoclaving, the media were taken to the cooling room and allowed to cool. Each stage of leaf was subjected to five different treatments as shown in Table 2. One treatment consisted of BAP alone (Treatment 1), three treatments had combinations of BAP and 2,4-D in different concentrations (Treatments 2-4), while the last treatment had no hormone (control). The whole experiment was replicated four times.

Table 2: Combinations of phytohormones used for MS media supplementation

Growth regulator	**Hormonal distribution (mg/l)**				
	Trtm. 1	**Trtm. 2**	**Trtm. 3**	**Trtm. 4**	**Control**
2,4-D	0.0	0.1	0.1	0.5	0.0
BAP	0.1	0.1	1.0	1.5	0.0

MS: Murashige and Skoog; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzyl amino purine; Trtm.: Treatment

Culture initiation: The Shea tree explants were washed under running water and then sterilized using 0.2% mercuric chloride containing 5 drops of tween 20 as a wetting agent. This was followed by thorough rinsing in sterile distilled water. Each leaf was cut into 10-14 small pieces depending on the leaf size. These were cultured on MS medium supplemented with different combination of growth regulators (Table 2). The cultures were incubated in the growth room under dim light (110 lux) at 25^0C.

RESULTS

In vitro morphological changes were observed in the leaf cultures 4 days after inoculation. The initial rust red coloured leaves turned light green with curling of surfaces above medium. This observation was more prominent in the Stage 2 explants. In all the three stages, leaf explants inoculated in MS medium without hormone and medium fortified with only BAP had the least curling. Callus initiation started in some cultures after two weeks of explants inoculation. After 28 days of culture initiation, callus was not observed

in explants at all developmental stages on MS medium devoid of growth regulators. Similarly, callus was also not induced when BAP was used alone.

There were differences among the calli observed with combination of 0.1mg/l 2,4-D and 0.1mg/l BAP at the different developmental stages. In this combination, the Stage 2 explants (Plate 2d) had the best callogenic response with no original leaf explants attached to the calli. This was followed by the Stage 1 explants (Plate 2a) with about 70 % callogenic response on the surface of the explants, while the minimum callogenic response was recorded for the Stage 3 explants (Plate 2g). The combination of 0.1mg/l 2,4-D and 1mg/l BAP yielded the best callogenic response in the Stage 2 explants (Plate 2e) when compared to Stages 1 and 3 explants (Plates 2b and 2h, respectively), which still had original pieces of explants attached to their calli. The highest amount of callus production was observed in the Stage 2 explants, followed by the Stage 1, while those at Stage 3 gave the least response. The treatment with 0.5mg/l 2,4-D and 1.5mg/l BAP combinations yielded calli in all the three stages of Shea leaf explants. Stage 1 (Plate 2c) showed complete callusing of the explants as opposed to the Stages 2 (Plate 2f) and 3 (Plate 2i) explants, which had bits of original explants attached to their calli. Callus proliferation, was however more in the Stage 2 explants, followed by the Stage 1, while the Stage 3 explants had the least amount of calli.

Table 3 shows the effect of 2,4-D and BAP on the Stage 1 leaf explants. From the result obtained, the highest callus weight was obtained when 0.5mg/l 2,4-D was used in combination with 1.5mg/l BAP. This combination also yielded the highest callus weight for the Stage 2 leaf explants (Table 4), but for those of the Stage 3 explants, the best callus weight was obtained when 0.1mg/l BAP was used in combination with 0.1mg/l 2,4-D (Table 5).

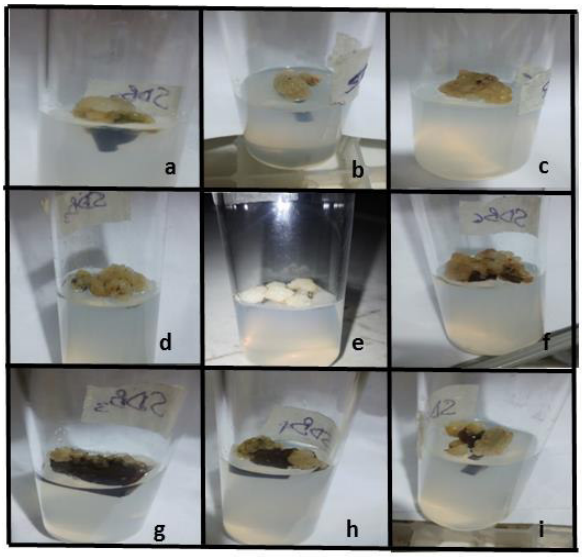

Plate 2: *In vitro* responses of leaf explants in different developmental stages
Stage 1 explant in MS supplemented with (a) 0.1mg/l 2,4-D and 0.1mg/lBAP; (b): 0.1mg/l 2,4-D and 1mg/lBAP; (c): 0.5mg/l 2,4-D and 1.5mg/l BAP; Stage 2 explant in MS supplemented with (d) 0.1mg/l 2,4-D and 0.1mg/lBAP; (e): 0.1mg/l 2,4-D and 1mg/lBAP; (f): 0.5mg/l 2,4-D and 1.5mg/l BAP; Stage 3 explant in MS supplemented with (g) 0.1mg/l 2,4-D and 0.1mg/lBAP; (h): 0.1mg/l 2,4-D and 1mg/lBAP; (i): 0.5mg/l 2,4-D and 1.5mg/l BAP; MS: Murashige and Skoog medium; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzyl amino purine.

Table 3: Effects of different concentrations of 2,4-D and BAP MS media supplementation on callogenesis of the Stage 1 leaf explants from *Vitellaria paradoxa*

Concentration of phytohormone (mg/l)		**Fresh weight of callus (assigned)**	**Intensity of Callus**
2,4-D	BAP		
0	0	-	-
0	0.1	-	-
0.1	0.1	1.04	+++
0.1	1.0	0.76	++
0.5	1.5	1.13	+++

-: No callus formation; **+:** Not profuse; **++:** Slightly profuse; **+++:** Profuse callus; **++++:** Very profuse; **+++++:** Highly profuse; MS: Murashige and Skoog; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzyl amino purine.

Table 4: Effects of different concentrations of 2,4-D and BAP MS media supplementation on callogenesis of the Stage 2 leaf explants from *Vitellaria paradoxa*

Concentration of Phytohormones used(mg/l)		**Fresh weight of callus(assigned)**	**Intensity of cells formed**
2,4-D	**BAP**		
0	0	-	-
0	0.1	-	-
0.1	0.1	1.39	+ + + +
0.1	1.0	1.25	+ + +
0.5	1.5	1.58	+ + + + +

-: No callus formation; **+:** Not profuse; **++:** Slightly profuse; **+++:** Profuse callus; **++++:** Very profuse; **+++++:** Highly profuse; MS: Murashige and Skoog; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzyl amino purine.

Table 5: Effects of different concentrations of 2,4-D and BAP MS media supplementation on callogenesis of the Stage 3 leaf explants from *Vitellaria paradoxa*

Concentration of phytohormones (mg/l)		Fresh weight of callus(assigned)	Intensity of cells formed
2,4-D	**BAP**		
0	0	-	-
0	0.1	-	-
0.1	0.1	0.23	+
0.1	1.0	0.91	+ + +
0.5	1.5	0.83	+ + +

-: No callus formation; **+:** Not profuse; **++:** Slightly profuse; **+++:** Profuse callus; **++++:** Very profuse; **+++++:** Highly profuse; MS: Murashige and Skoog; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzyl amino purine.

DISCUSSION

The presence of growth regulators (auxin and cytokinin) in culture medium is an important factor in callus proliferation, growth and differentiation of cultured cells (Kalidass *et al.,* 2010). The use of phytohormones is critical in callogenesis of Shea tree explant. This is shown in this study as the media without growth regulators were ineffective in inducing callus. In the study of Namrata and co-workers (2014) on the influence of hormones and explants towards *in vitro* callusing and shoot organogenesis in a commercially important medicinal plant, callus was also not induced in media without growth regulators. Liu *et al*. (2006) reported that 2,4-D had been used singly and in combination with cytokinins to enhance callus induction and maintenance.

In the present study, 2,4-D was used in combination with BAP. Callus induction was observed after two weeks of inoculation. Adu-Gyamfi *et al*. (2012) also observed callus after the same duration though the explant used was cotyledon. Callogenesis was not stimulated in leaf explants inoculated on media supplemented with BAP alone during the course of this study. This finding is in agreement with that of Fotso *et al*. (2008) where they compared the first stages of somatic embryogenesis in *Baillonella toxisperma* and *Vitellaria pardoxa* using leaf fragments. It is also similar to the observation of Sakpere *et al*. (2014) where initiation of callus using cytokinin alone was not promising in their study to induce callus from different explants of *Telfairia occidentalis*. Kinetin alone was ineffective in the *Sauropus* sp. callus induction study of Arivalagan *et al.* (2012).

In all the three developmental stages of leaf explants, irrespective of the phytohormonal concentrations, curling of leaf was observed four days after inoculation. This was more prominent in the developmental Stage 2 explants, which produced the highest quantity of callus. From the results obtained in Tables 3-5, it can be deduced that 2,4-D and BAP concentrations of 0.1mg/l each was not effective for

stimulation of profused calli in the leaf developmental Stage 3 explants (17.45 cm). The Stages 1 and 2 explants of lengths 7.55 cm and 11.95 cm respectively were more suitable for callogenesis of Shea leaf tissues. This observation was in line with the work of Głowacka *et al*. (2010) on the effects of inflorescence developmental stage on callus induction and plant regeneration in two *Miscanthus* species. It was observed that explants from the youngest inflorescences (0.1–2.5 cm in length) showed a significantly higher callus induction rate than those from more developed inflorescences (2.6–5 cm in length). This trend was also noticed in the results of Eke and co-workers (2010) in their work on *in vitro* morphogenesis from cotyledon explants of Shea tree, where the highest amount of callus obtained was from developmental Stages 2 and 3 when compared with a lower Stage 1 and higher Stages 4 and 5 explants. Explant taken from juvenile plant tissue are usually highly responsive; they are more capable of growth and organogenesis *in vitro* (George *et al.,* 2008). This may be as a result of the actively growing cells present in juvenile tissues.

The 2,4-D and BAP combination of 0.5mg/l and 1.5mg/l respectively was more effective in callus proliferation in the developmental Stages 1 and 2. This is evident in the results obtained in Tables 3 and 4. Phytohormone concentration of 0.1mg/l 2,4-D and 1.0mg/l BAP was optimal for callus formation from the Stage 3 explants (Table 4). This is in line with the work of Eke *et al*. (2010) where higher callus weight was obtained on media containing 5, 3 and 1mg/l BAP in combination with low levels of 2,4-D (0.1-1.5mg/l). The highest fresh weight of callus (1.58) obtained in the present study was from the leaf developmental Stage 2 cultured on MS medium supplemented with 0.5mg/l 2,4-D and 1.5mg/l BAP.

CONCLUSION

The findings from this study indicated that younger Shea leaves (developmental Stages 1 and 2) were more effective for callogenesis. The best hormonal combination of 2,4-D and BAP for callus induction was 0.5mg/l and 1.5mg/l, respectively. Callus was not observed in plant growth hormone free culture or MS medium fortified with BAP alone. The Stage 3 explants (the oldest) were not suitable for callogenesis of Shea leaf tissues.

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