

Full Length Research Paper

***In vitro* callus initiation of a ‘threatened’ Nigerian leafy vegetable, *Gnetum africanum* (WILW)**

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Gnetum africanum is a green leafy vegetable found in Nigeria, where it is a highly valued food source. Stocks of this plant in the wild are increasingly threatened by land transformation and harvesting methods are unsustainable. *In vitro* callus initiation of *G. africanum* has been developed. The surfaces of 3 to 4 day old leaf explants were sterilized before exposure to a range of different concentrations of plant growth regulators. *In vitro* callus initiation of explants in single concentrations of auxins (2,4D: 2-4-dichloroxyphenoxyacetic and pichloram) did not initiate callus after 3 months of culture. However, combinations of cytokines (BAP: 6-benzylaminopurine, and kinetin) and auxins initiated callus. The highest percentage callus initiation response of 100% was observed in the combination of BAP (1.0 mg/l) + 2,4D (7 mg/l). There was a significant difference ($P < 0.05$) in callus production of explants in response to different callus induction and initiation media. However, there was no significant difference ($P > 0.05$) between the degree of callus response and callus size to these different media.

Key words: BAP (6-benzylaminopurine), callus initiation, *Gnetum africanum*, kinetin, 2,4D (2-4-dichloroxyphenoxyacetic), pichloram.

INTRODUCTION

Green leafy vegetables are plant bioresources that constitute an indispensable part of the human diet in Africa generally and West Africa in particular (Oguntona, 1988). Eneobong (1997) has defined bioresources as the total biological variation manifested as individual plants, animals or their genes which can be taken by man for use as drugs, food, live stock feed as well as environmental protection.

Gnetum is a leafy vegetable with lone genus in the family *Gnetaceae*; with about thirty species in the genus, occurring throughout the tropics in Asia, South America and in Central Africa up to Nigeria (Mialoundama and Paulet, 1986). The majority of the species of *Gnetum* are lianas or climbing vines, with two species native to Africa; *Gnetum africanum* and *G. buchholzianum*. These species are distributed in the humid tropical forests of Nigeria (10°N 8°E) to Cameroon (3° 52'N 11°31'E), Central African Republic (7°N 21°E), Gabon (1°N 11°45'E), DR of Congo (4°31'N 15°32'S) to Angola (12°30'S 18°30'E) (Lowe, 1984). Both species are understory lianas,

although in some cases individuals have been found to scramble into the crowns of emergent trees. Both species are very similar and can only be distinguished by the shape of the leaves and characteristics of the male reproductive parts (Mialoundama, 1993).

Both *Gnetum* species have significant value to many forest-based communities and have a number of vernacular and trade names such as Koko, Eru, Okok, Okazi and Afang (Bahuchet, 1990). *G. africanum* (Wilw) is a ceremonial delicacy in many parts of Eastern Nigeria up to the Southern part of Nigeria; its use has spread within Nigeria to towns and cities like Lagos, Abuja, Ibadan, and Makurdi. It is one of the vegetables in great demand by Nigerians in the Diaspora, although it is very expensive.

The plant's nutritional and ethno-medical effects are well documented by Okafor (2003), Okafor et al. (1996), Burkill (1994) and Watt and Breyer-Brandwijk (1962). It is for its food value that *Gnetum* is most prized. The leaves are either eaten raw or are finely shredded and added to soups and stews (Burkill, 1994). Okazi soup is popular to the people of Imo, Abia, Anambra and some parts of the Delta states and Afang soup to the people of Cross Rivers and Akwa Ibom; *Gnetum* is the only and major vegetable component in these soups.

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The leaves of *G. africanum* are a very important article of trade in the central African region, particularly close to Cameroon. These leaves are harvested on a daily basis and sold in local and regional markets. As the leaves are evergreen, they are available all year round. The volume of export trade in these leafy vegetables has significantly increased in recent years (Shiembo, 1997). Irrespective of the high economic value of *Gnetum*, the plant is increasingly threatened as a result of industrialization and urbanization characterized by rapid deforestation, uncontrolled logging, burning and search for food.

In the wild, *Gnetum* grows and forms underground tubers or roots that store plant food reserves. These tubers can remain alive for many years when the vegetation and the *Gnetum* vines above ground are cleared and soil surface is laid bare. It has been reported that some local tribes in East Cameroon and Congo eat these tubers as wild yams, particularly during lean seasons (Bahuchet, 1990).

During vine collection, the trees on which *Gnetum* climbs are often felled, creating widespread damage. In the course of harvesting the vines, the buds on the tubers are damaged and it may take a long time for new buds to develop into vines. In some cases the forceful pulling of vines creates wounds on the tubers /roots, leaving them vulnerable to fungal attack that can cause rot disease. As a result, harvesting methods do not ensure the growth and supply of future *Gnetum* leaves, and are not sustainable (Shiembo, 1997). The unsustainable harvest increases the threat of genetic erosion of the species. For this reason, there is a great need to protect this plant.

Since man cannot do without exploiting plants and other available bioresources, there has to be a balance between use of these resources and their conservation. In this way an ecosystem, although altered, could retain its bioresources and provide food, as well as perform vital environmental functions on a long-term basis (Uyoh et al., 2003).

Biotechnology provides valuable gains in the research on conservation of plant bioresources (De-Smet, 1995). Although modern biotechnology is a newly introduced science, its impact has greatly excited the imagination and provoked the concern of almost every part of society worldwide (Eneobong, 2003). Many of our "orphan (Considered to be 'minor crops' that, although having high economic, livelihood and food value in developing countries, receive little attention from researchers or investors.)" crops are hard to propagate due to their production of seeds with little or defective endosperms, or the fact that they are frequently polyploids or aneuploids. Plant tissue culture (TC), a tool in biotechnology, is an option that provides a method for their mass clonal propagation, as well as serving as a tool for their germplasm collection and conservation (Uyoh et al., 2003; Opabode and Adeboye, 2005). The richness of plant and animal diversity in developing countries is a major asset in agricultural and sustainable development. Biotechnology can

thus play an important role in the propagation and conservation of many plant bioresources.

In the light of these considerations, we report preliminary callus initiation studies of *G. africanum* as a stage in the development of a regeneration protocol for *G. africanum*.

MATERIALS AND METHODS

Collection of donor plants

G. africanum plants were collected from the wild in Issele-Azagba, Aniocha North Local Government Area in Delta state (6°12'00"N 6°43'12"E), by carefully uprooting them. Uprooted plants with forest soil were placed in buckets and an artificial forest condition was created for the plants to acclimatize: plants were shaded from the direct rays of the sun using oil palm fronds and were watered three times daily to maintain moist conditions of the forest understorey according to the method of Okafor (2003).

Those surviving acclimatized plants were then transferred to the Biotechnology Advanced Laboratory Sheda Science and Technology Complex, Abuja. These plants served as sources of leaf explants for the experiments (Plate 1).

Explants preparation and surface sterilization

Three to four day old young leaves were pruned off the mother plants using a sterile knife, for use as explants. In order to sterilize the surfaces of explants, they were washed in the liquid detergent Tween 20 (2 drops per 100 ml solution) for 3 min, rinsed in sterile distilled water for 20 min, soaked in 70% ethanol for 2 min and, lastly, soaked in 1% sodium hypochlorite for 20 min. Explants were rinsed twice with sterile water after ethanol treatments and three times after the sodium hypochlorite treatments, following the method of Gopi and Vatsala, (2006). All explants were then trimmed to small sizes of about 1 cm by 1 cm, after which they were cultured on culture medium.

Plant growth regulators (PGR)

Murashige and Skoog (1962) culture medium was supplemented with different concentrations of auxins (2,4D and Pichloram). In order to encourage callus initiation, low concentrations of cytokines (BAP and Kinetin) were combined with high concentrations of auxins according to the methods of Suahersan and Aboel- Nil (2002), Thomas and Puther (2004) and Balogun et al. (2007) as represented in Table 1.

In vitro callus initiation

Murashige and Skoog (1962) culture medium (MS medium) was supplemented with 30 mg/l sucrose, 0.1 mg/l inositol and 5 ml/l ascorbic acid. pH was adjusted to 5.8 using sodium hydroxide (1 mM) or dilute hydrochloric acid (1 mM). Agar (Phytigel) was added as gelling agent to the medium at a concentration of 3 g/l before the medium was sterilized at 121°C for 15 min. At the end of the sterilization, the medium was allowed to cool. It was aseptically poured into sterile Petri-dishes in the laminar flowhood and allowed to set for 24 h. Twenty sterile explants were cultured for each class of experiment by placing them about 2-4 cm apart on the culture medium. Petri dishes were sealed using parafilm "M" according to Sudhersan and Aboel- Nil (2002). All Petri dishes were wrapped in foil and stored in the dark in the growth room at 28°C. Cultures were moni-



Plate 1. *Gnetum africanum* plants in the screen house.

Table 1. Showing concentrations and combinations of PGR.

24D Mg/l						Pichloram mM				
	0	1	3	5	7	0	0.01	0.05	0.1	0.5
BAP	CT₁	D₁	D₂	D₃	D₄	CT₂	P₁	P₂	P₃	P₄
0										
0.5			BD ₁		BD ₂				BP ₁	BP ₂
1.0			BD ₃		BD ₄				BP ₃	BD ₄
Kinetin	CT₃					CT₄				
0										
0.5				KD ₁	KD ₂				KP ₁	P ₂
1.0				KD ₃	KD ₄				KP ₃	P ₄

Key: **CT**; Control, **D**; 2,4D, **P**; Pichloram, **BD**; BAP + 2,4D, **BP**; BAP + Pichloram, **KD**; Kinetin + 2,4D, **KP**; Kinetin + Pichloram.

Cultures were monitored for callus initiation once every 2 weeks.

Measurement of callus response and size

Callus initiation response and size were evaluated visually. The criteria for scoring explants for callus according to Amoo and Ayisire (2005) was based on the number of explants producing callus per media. This is represented in Table 2. The percentage of explants that showed callus initiation was also calculated.

The degree of callus response and callus size was compared using the student's T-Test at 0.05 level of significance using the SPSS statistical software. The mean variance of degree of callus response, percentage callus response and callus size were also compared using SPSS software at 0.05 level of significance.

RESULTS AND DISCUSSION

The results of the *in vitro* callus initiation study are summarized in Table 3.

Although, callus production could be initiated using different auxins as reported by Taurus et al. (1991), Amoo and Ayisire (2005), Eke et al. (2005), Suaherasn and Aboel-Nil (2002) and Puchooa (2004), it was not observed in this study on *G. africanum*. However, with the use of a combination of auxins and cytokines, callus production was initiated.

There was a significant difference ($P < 0.05$) in the degree of response of explants and the size of callus pro-

Table 2. Showing callus criteria for scoring explants for callus response and size.

	Response	Size
	0	0
	1	I (1-3 mm)
Callus	2	III (3-6 mm)
	3	V (6-9 mm)
	4	

1-4: Order of callus response; I-V: Order of callus size.

Table 3. The results of callus initiation for various concentrations and combination of PGR.

PGR concentration	Number of explants	Degree of callus response ^{#\$} Mean±SD	% Callus response*	Callus size ^{#Z} Mean±SD
2,4-D				
1mg/l	20	0.0±0.0	0	0.0±0.0
3 mg/l	20	0.0±0.0	0	0.0±0.0
5 mg/l	20	0.0±0.0	0	0.0±0.0
7 mg/l	20	0.0±0.0	0	0.0±0.0
Pichloram				
0.01mM	20	0.0±0.0	0	0.0±0.0
0.05mM	20	0.0±0.0	0	0.0±0.0
0.1mM	20	0.0±0.0	0	0.0±0.0
0.5mM	20	0.0±0.0	0	0.0±0.0
BAP + 2,4-D				
0.5mg/l + 5mg/l	20	0.0±0.0	0	0.0±0.0
0.5mg/l + 7mg/l	20	1.0±0.0	20	0.5±0.6
1.0mg/l + 5mg/l	20	2.75±0.5	70	2.5±1.0
1.0mg/l + 7mg/l	20	4.0±0.0	100	4.5±1.0
Kinetin + 2,4-D				
0.5mg/l + 5mg/l	20	0.0±0.0	0	0.0±0.0
0.5mg/l + 7mg/l	20	1.0±0.0	20	0.5±0.0
1.0mg/l + 5mg/l	20	2.0±0.0	65	2.5±1.9
1.0mg/l + 7mg/l	20	1.0±0.0	50	1.5±1.0
BAP + pichloram				
0.5mg/l + 0.1 mM	20	1.0±0.0	50	1.5±1.0
0.5mg/l + 0.5 mM	20	1.0 ±0.0	50	1.0±0.0
1.0mg/l + 0.1 mM	20	2.0±0.0	20	3.0±0.0
1.0mg/l + 0.5 mM	20	1.75±0.5	20	3.0±0.0
Kinetin + pichloram				
0.5mg/l + 0.1mM	20	2.0±0.0	70	3.0±0.0
0.5mg/l + 0.5mM	20	1.75±0.5	50	2.0±1.2
0.5mg/l + 0.1mM	20	1.25±0.5	50	1.0±0.0
1.0mg/l + 0.1mM	20	1.25± 0.5	20	2.0±1.2
Control No PGR	20	0.0±0.0	0	0.0±0.0

#: P>0.05: There is no significant difference between callus response and callus size; \$: P<0.05: There was significant difference between the degree of callus response and other parameters tested; *: P<0.05: There was significant difference between the % callus response and other parameters tested.; Z: P<0.05: There was significant difference between callus size and other parameters tested.

duction for the different callus initiation media. However, there was no significant difference ($p > 0.05$) between the degree of callus response and callus size, showing that the composition of the different media did not affect the callus size produced.

Explants of *G. africanum* cultured on MS medium formed callus at their cut end., Callus formation at the proximal end of the explants in this study conforms to reports on *Peganum harmala* (Saini and Jaiwal, 2000) as well as *Holostemma ada-kodien* (Martin, 2000).

Marks and Simpson (1994) suggested that callus formation may be due to the action of accumulated auxins at the basal cut ends which stimulates cell proliferation, especially in the presence of cytokines. This hypothesis seems to hold true for *G. africanum*, where callus was initiated with a combination of a cytokines and an auxin. Preece et al. (1991) reported that formation of callus at the basal cut explant on cytokines-enriched medium is frequent in species with strong apical dominance. This synergistic effect of a cytokine and an auxin has been demonstrated in many plants including *Santolina canescens* (Casado et al., 2002), *Bupleurum fruticosum* (Fraternale et al., 2002) and *Acacia nilotica* (Sane et al., 2001). Furthermore, a high degree of callusing was reported in single node explants of fluted pumpkin, cultured in medium containing both a cytokine (Kinetin) and an auxin (NAA) (Balogun et al., 2002). High callus formation from a combination of cytokine (BAP) and auxin (NAA) has also been reported by Balogun et al. (2007). What these observations imply is that the combination of auxin and cytokine can be used as an effective initiation medium for plant regeneration via somatic embryogenesis.

One of the serious constraints of this study was the browning of the callus and explants which led to the death of the cells. According to Mantel et al. (1998), browning can be attributed to the presence of secondary metabolites (Phenolic compounds). These compounds are oxidized when in contact with oxygen to form toxic compounds after wounding of leaf explants (Aliyu, 2005). Phenolic compounds are mostly secreted by woody plants which may explain why the explants and callus of *G. africanum* were able to produce these toxic metabolites. Death due to browning may have prevented formation of callus in the single PGR concentration experiment. However, explants in some media where callus was initiated showed necrosis after 5 days, Death of callus due to browning has been reported in cashew by Aliyu (2005), Sturt's Desert Pea (Suaherasn and Aboel-Nil, 2002) and *Parkia biglobosa* (Amoo and Ayisire, 2005).

Conclusion

There is no doubt that the exploration of plant bio-resources in the wild is having a negative impact on their natural populations and is threatening their survival. The provision of alternative sources of cultivation could go a long way in reducing the heavy dependence on these

wild populations. Plant tissue culture, an indispensable part of biotechnology, has provided a sure pathway for the rescue of many "orphan" crops which are found in the wild and are extremely difficult to propagate. Their response to various plant growth regulators is a positive sign that a protocol for *in vitro* culture can be achieved, thereby helping to alleviate the economic exploitation of wild plants and enabling their conservation.

This study has successfully formulated a protocol for callus initiation in *G. africanum*. The reported results are expected to contribute to the scientific baseline data necessary for the somatic embryogenesis protocol of *G. africanum*; a sine qua non for its micropropagation and conservation.

DEDICATION

In memory of a great scientist, Dr. Danladi Dada KUTA.

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