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Explant establishment for callus initiation of a Nigerian "endangered" leafy vegetable, *Gnetum africanum (WILLD)*

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A prerequisite for successful *in vitro* culture is the establishment of an aseptic technique, thus the experiment was to investigate suitable sterilization regimes for the leaf explants of *Gnetum africanum*, an endangered green leafy vegetable. Three sterilization regimes were tested to establish the best regime using three to four days old leaves. The surface sterilized explants were later aseptically introduced onto the surfaces of sterile Murashige and Skoog agar media, incubated at 25°C for three weeks in the growth chamber. 100% sterility was observed from the regime which was significantly different (P<0.05) from the other two regimes thus the best regime adopted for further experiments was; washing in two drops of Tween 20/100 mls of sterile water, soaking in 70% ethanol for 2 min and later in 1% sodium hypochlorite for 20 min. Fungal contaminants responsible for *in vitro* contaminations was also investigated and possible isolates were identified as *Asperigilus niger* (28.71%); *A. flavus* (26.73%); *Rhyziopus* spp. (24.75%) and *Mucor* Spp (19.81%) respectively.

Key words: *Gnetum africanum, A. niger*, *in vitro* culture, green leafy vegetable.

INTRODUCTION

Gnetum a leafy vegetable and plant bioresources is the lone genus in the family Gnetaceae; there are about 30 species in the genus, which occurs 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. There are two species of *Gnetum* in Africa, *Gnetum africanum* and *Gnetum buchholzianum* and they are distributed in the humid tropical forests from Nigeria through Cameroon, Central African Republic, Gabon, DR of Congo to Angola (Mialoundama, 1993). Both species are understorey lianas, although in some cases some individuals have been found to scramble into the crowns of emergent trees. These two species are very similar and can only be distinguished by the shape of the leaves and characters of the male reproductive parts (Lowe, 1984).

Both *Gnetum* species have significant value to many forest based communities and have a number of vernacular and trade names. In the Central African Republic, Gabon, Congo, DR of Congo and Angola, the two species are locally called Koko, in the Anglophone Cameroon, they are know as Eru, while in Francophone Cameroon the name Okok is applied. In Nigeria, the two *Gnetum* species are called Okazi by the Igbo tribe while the Efik/Ibibio tribes call them Afang (Bahuchet, 1990).

G. africanum (Willd) is a ceremonial delicacy in many parts of Eastern Nigeria up to the southern part of Nigeria. The use of this plant has spread beyond the borders of these places to far away towns and cities like Lagos, Abuja, Ibadan, and Makurdi etc. It is one of the

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highly demanded Nigerian vegetables for Nigerian's in the Diaspora although it is also very expensive. *Gnetum* according to Hinialoeudama (1993) is an endangered plant with a great risk of lost due to industrialization and urbanization characterized by rapid deforestation, uncontrolled logging, burning and uncontrolled search for food thus a need to conserve them. Biotechnology provides valuable gains in the research on conservation of plant Bioresources (De-Smet, 1995).

The method of plant tissue culture (TC), a tool in Biotechnology is an option that provides a method for their Mass clonal propagation as will as serving as a tool for their germ-plasm collection and conservation (Uyoh et al., 2003). However, there are little or no reports to show *in vitro* propagation of *Gnetum* thus a need to establish a tissue culture protocol for the plant. Since the prerequisite for successful *in vitro* culture is the establishment of an aseptic technique, we therefore developed a suitable sterilization regime for the leaf explants of *Gnetum africanum.*

MATERIALS AND METHODS

Collection of donor plants

Mother plants of *G. africanum* were collected from the wild in Issele-Azagba, Aniocha North LGA, Delta state. The plants were gotten by digging deep into the soil to uproot them. Uprooted plants with forest soil were put into buckets and an artificial forest condition was created for the plants to acclimatize. This artificial forest condition included shading off the plants from the direct rays of the sun using oil palm fronds and watering them three times daily to maintain moist conditions of the forest under stories according to the method of Okafor (2003). Few 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.

Explants preparation and surface sterilization

In order to surface sterilize the explants, three to four days old young leaves were used. These leaves were pruned off the mother plants using a sterile knife. Three different sterilization regimes were tested according to the method of Puchooa (2004), which was as follows:

ST_1: Explants were surface wiped with ethanol and later soaked in 1% sodium hypochlorite for 15 min
ST_2: Explants were soaked in 70% ethanol for 1 min and then soaked in 1% sodium hypochlorite for 15 min
ST_3: Explants were soaked in 70% ethanol for 2 min and then in 1% sodium hypochlorite for 20 min.

Before the surface sterilization, explants for ST_2 and ST_3 experiments were washed in the liquid detergent Tween 20 (two drops per 100 ml solution) for 3 min and then rinsed in water for 20 min (Suaherasn and Aboel-nil, 2002).

Explants were rinsed two times with sterile water after ethanol treatments and three times after the sodium hypochlorite treatments (Gopi and Vatsala, 2006). All explants were then trimmed to small sizes of about 1 by 1 cm after which they were cultured on culture medium.

Culturing of sterile explants

The culture medium devised by Murashige and Skoog (1962) supplemented with sucrose 30 g/L and 0.1 mg/L inositol was used in all the sterilization studies. The pH of the media was adjusted to 5.8 using 1 milli-mole sodium hydroxide (NaOH) or hydrochloric acid (HCL). Agar (phytagel) was added into the media at a concentration of 3 g/L. The medium was sterilized at 121°C for 15 min. At the end of sterilization, the medium was allowed to cool; it was aseptically poured into sterile Petri-dishes in the laminar flowhood. This was then allowed to set for 5 h in the flowhood. The 20 trimmed sterile explants were each aseptically transferred into the

Petri-dishes and placed 2 to 4 cm apart using sterile forceps. Petri-dishes with explants were sealed using parafilm "M" according to the study of Sudhersan and Aboel-nil (2002) and cultured, covered with black polyethylene in the growth rooms at 28°C and monitored every week for contaminations. Results for contamination were taken for three weeks and from these experiments a sterilization regime was adopted and used for all future experiments.

The levels of contamination were determined by physical examination of the culture plates and were scored according to the study of Amoo and Ayisire (2005) as follows: +++ (3): Heavy contaminated, ++ (2): For mildly contaminated, + (1): For lightly contaminated and - (0): For no contamination.

Percentage sterility was also calculated as follows:

$$\frac{\text{Number of Uncontaminated explants}}{\text{Total number of Cultured explants}} \times \frac{100}{1}$$

Isolation and identification of fungi contaminants

After three weeks of incubation, several fungal contaminants were observed. Using a sterile loop, organisms were picked and plated on potato dextrose agar (PDA) which was supplemented with chloramphenicol. The plates were then incubated at room temperature (32±2°C) for 72 h.

Resulting mycelia growth was sub-cultured on fresh PDA plates for growth and identification carried out by physical examination of plates as well as microscopic examination of the type of sporangia, type of hyphae, presence or absence of rhizoids. These parameters were adopted for identification according to the study of Barnett and Hunter (1972).

Determination of the frequency of occurrence of the isolated fungi

The frequency of isolations of the different fungi associated with explants contamination was determined. The number of times each fungus was encountered was recorded. The percentage frequency of occurrence was then calculated using the formula below according to the study of Ilondu and Iloh (2007)

$$\frac{\text{Number of fungus colony encounter}}{\text{Total number of fungal colonies}} \times \frac{100}{1}$$

Statistical analysis

The Genstart Statistical software was used to test the mean variance of the surface sterilization experiments at 0.05 level of significance.

Table 1. Results of surface sterilization experiment on leaf explants *Gnetum africanum.*

Sterilant experiment	Time (min)	Level of contamination (Mean ± SD)	% Sterility of explants
$S+_1$	Leaves surfaced wiped with 70% ethanol +1% sodium hypochlorite for 15 min.	3.0±0.0	40
$S+_2$	Leaves washed in Twen20 (2 drops/100 ml sterile water) + Explants dipped in 70% ethanol for 1 min + 1% sodium hypochlorite for 15 min.	1.3±1.0	70
$S+_3$	Leaves washed in Twen20(2drops/100ml sterile water) + Explants dipped into 70% ethanol for 1 min +1% sodium hypochlorite for 20 min.	0.0±0.0	100

P<0.05: There was significant difference among the levels of contamination of three sterilization regimes.

Table 2. Frequency of occurrence of fungal contaminants associated with explants in the growth media.

Name of organism	Total no. of isolates	% isolate frequency
Asperigillus niger	29	28.71
A. flavus.	27	26.73
Rhyziopus spp.	25	24.75
Mucor spp.	20	19.81
Total	101	100

RESULTS AND DISCUSSION

Table 1 summarizes the efficiency of the different sterilization regimes for surface sterilization of the leaf explants. Best sterility results were obtained using 70% ethanol for 2 min and 1% hypochlorite for 20 min which resulted in 100% sterility and its effects was significantly different (P<0.05) from the other regimes. A similar result had been reported by Puchooa (2004) and was subsequently used for this study.

However, although the above regime was very effective during decontamination, a high proportion of the explants became necrotic after three days in the culture. The explants were seen to have lost their original colour (bleached) thus markedly reducing explants vigor with shriveling and necrosis. This was also observed by Puchooa (2004) working with *(litchi chinensis)*. This bleaching effect according to the stuyd of Puchooa (2004) was probably due to the bleaching abilities of chlorine in the sodium hypochlorite. The difficulty to obtain survival of shoot explants from field-grow stock mature plants of cashew was due to the inability of the explants to survive the strong levels of surface sterilization required to decontaminate such materials (Rodrigues, 1995; Das et al., 1996)

In the experiments where Tween 20 was used to wash the leaves before surface sterilization, there was a reduction in percentage contamination. Tween 20 has being reported to be a surfactant that acts as a detergent binding its hydrophobic head with possible oil exudates, again helping to establish clean explants (Puchooa, 2004). Thomas and Puthru (2002) also reported low contamination percentages when drops of Tween 20 were used to wash explants before surface decontamination.

The fungal organisms isolated and identified as possible explants contaminants are *Rhyziopus* spp., *Mucor* S spp., *Aspergillus niger* and *A. flavus* respectively (Table 2). Fungal organisms disseminate their spores through the air and it is likely the spores of the contaminants were deposited on the leaves of the plant in the field which is consistent with the observation of Dongo and Ayodele (1997). Fungal spores liberated in the air can quickly settle on suitable substrates and germinate (Okhuoya and Ayanlola, 1986). They also reported *Rhiziopus; Aspergillus* spp. as often microbial pest in the laboratories. Since spores of these fungi are in the air, contamination of explants could also have come from unsterilized growth rooms as in the case of Puchooa (2004) who reported external contaminations due to unsterilized growth/ culture rooms in as much as decontamination was achieved using Sterilants.

In this study, we have successfully achieved *in vitro* explant establishment by developing a proper sterilization

regime, the success achieved in reducing the impact of contamination would help in future studies of the effect of growth regulators and establish an effective method for the micropropagation and conservation of *G. africanum.*

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