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Effective medium for *in vitro* sprouting of the buds and multiplication of the plantlets, and identification of the fungal contaminant associated with the explants of *Gnetum*

Abwe Mercy Ngone [1,*], Lawrence Monah Ndam [1], Rita Mungfu Njilar [1], Doungous Oumar [2] and Thomas Eku Njock [1]

[1] *Department of Agronomic and Applied Molecular Sciences, University of Buea, PO Box 63 Buea, Cameroon.*
[2] *Jay P. Johnson Biotechnology Laboratory, Ekona Regional Research Centre, Institute of Agricultural Research for Development (IRAD), PMB 25, Buea, Cameroon.*

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Abstract

Plant tissue culture requires the optimization of growth media. *Gnetum,* known locally in Cameroon as "Eru" is an indigenous gymnospermous vegetable with diverse medicinal, nutritional, cultural and socio-economic values. This resource is over-exploited and expected to neighboring countries, resulting to increased scarcity in the forest. Preliminary work on the *in vitro* culture of nodal cuttings was faced by the problem of fungal contamination. It was therefore necessary to isolate and identify the fungal contaminant, optimize the surface sterilization of field material and compose an appropriate medium for sprouting.

Pure cultures of the fungus were obtained and grown on Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA). The identification was based on the appearance of the fungal growth on plates and also on the microscopic view. This was affected by the use of keys. *Gnetum* explants were disinfected with the various concentrations of disinfectants, preceded in some instances by pre-treatments, as well as incorporating fungicides in the culture medium. Two different culture media were employed: the Woody Plant Medium (WPM) and the Murashige and Skoog (MS) based establishment medium (Y-1).

Gnetum was found to live in association with a complex of *Microsporum* species. The level of contamination of cultures was reduced from 100% to 40% when pre-treated before disinfection and even lower to 10% by incorporating fungicides in the medium. Sprouting was observed in WPM.

This study provides baseline information on the *in vitro* propagation of *Gnetum* and thus opens up avenues for more research to be carried out in this field.

Keywords: Micropropagation; *Gnetum; Microsporum* species; Sterilisation; Biotechnology

1. Introduction

Gnetum africanum Welw. And *G. buchholzianum* Engl. known locally in Cameroon as "Eru" are indigenous gymnospermous vegetables with diverse medicinal, cultural, nutritional and socio-economic values. Both species are understory lianas, though in some cases they are found to scramble into the crowns of trees. These species are very similar in appearance and can only be distinguished in leaf shape and the reproductive organs [1, 2].

* Corresponding author: Abwe Mercy Ngone
Department of Agronomic and Applied Molecular Sciences, University of Buea, PO Box 63 Buea, Cameroon.

The two *Gnetum* species occur throughout the tropics, Asia, South America and Central Africa [3]. They are distributed in the humid tropical forest from Nigeria through Cameroon, Central African Republic, Gabon and Democratic Republic of Congo to Angola [4, 5]. Although, historically and primarily consumed by specific ethnic groups, increased movements of people between Central African nations together with continuing trade have increased consumption of *Gnetum* dramatically such that it is now probably the most commonly consumed plant found growing wild in Central Africa [6]. (Fig 1 and 2)

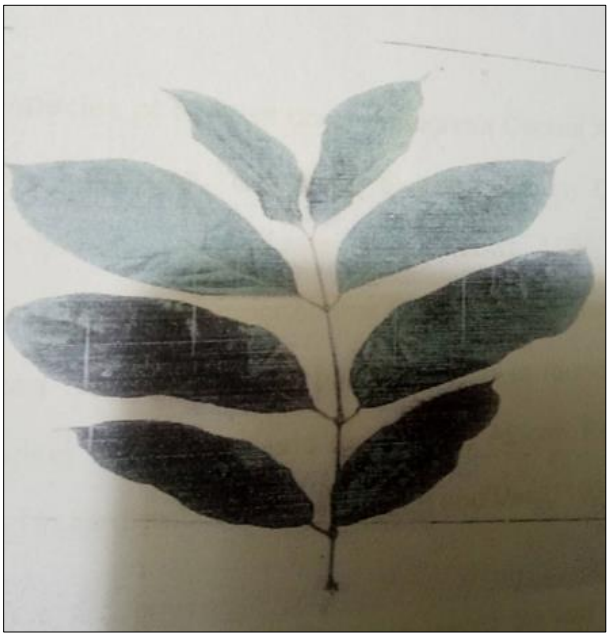

Figure 1 Leaves of *Gnetum*

Figure 2 Ripe fruits of *Gnetum*

The combination of increased demand, destructive harvesting, habitat degradation, use of bushfires to clear the forest for agricultural purposes and over-exploitation all contribute to the increasing scarcity of the plant. Moreover, this has resulted in almost the total extinction of the species in Nigeria and the need to travel further into the forest in search of this valuable vegetable in Cameroon [7]. It is estimated that 600 tons of eru a year are exported to neighboring countries [8]. To meet the needs of consumers and to conserve these species from going to extinction, there is a need to develop a rapid propagation method that guarantees the production of planting materials on a large scale.

Advances have been made in the domestication of *Gnetum* species in Cameroon using the conventional "propagation by cutting" technique [9, 10]. Successful domestication requires the development of techniques for rapid multiplication to produce large quantities of planting material. Several methods for genetic transformation of vegetables and fruits have been described in the literature, but so far the most effective are those using *Agrobacterium*-mediated transformation of juvenile materials, such as zygotic embryos, hypocotyls, epicotyls, and cotyledons [11, 12]. Plants regenerated from these explant sources have long juvenile stages before initial production, and many years are necessary before evaluating the horticultural and commercial traits introduced into the transgenic plants. An ideal protocol for *Gnetum* plant propagation should be based on the use of tissues as explants sources, circumventing the juvenile phase and permitting the growth regulators, culture media and antibiotics [13].

For successful propagation of *Gnetum* species, the first step is the establishment of an efficient plant regeneration system, since *in vitro* culture using mature tissue as explants is still far from routine [14]. Reasons for this include the relatively low responsiveness of vegetables to exogenous growth regulators and the failure of standard surface sterilization techniques [15]. Media compositions have been formulated for the specific plants and tissues. In general, the choice of medium is dictated by the purpose and the plant species or variety to be cultured. A culture medium with optimal mineral supply and a combination of plant growth regulators will increase the success for the recovery of *Gnetum* plants from cultured cells [16]. Furthermore, the addition of antibiotics to the culture medium may help to eliminate contamination by bacteria and fungi, which hinder the *in vitro* establishment of explants from mature tissues.

In micropropagation, the health status of the donor mother plant and of the plants multiplied from it are among the most critical factors, which determine the success of a tissue culture operation. The indexing of the mother plants for freedom from viral, bacterial, and fungal diseases is a normal procedure in large-scale plant propagation through tissue culture. Plants not originating from pathogen-tested material must be screened for the presence of pathogens. Most of the fungal diseases are eliminated during surface sterilization and culture ([17, 18]. This study therefore aimed to evaluate the effective medium for *in vitro* sprouting of the buds and multiplication of the plantlets, and identification of the fungal contaminant associated with the explants of *Gnetum*.

2. Material and methods

2.1. Source of *Gnetum* Material

Gnetum vines were obtained from the wild at Barombi-Kang, Southwest Region, and Cameroon. They were harvested about midway from ground level with a knife, wrapped in a moist plastic bag and transported to the Jay Johnson Biotechnology Laboratory of the Institute of Agriculture Research for Development (IRAD), Ekona Centre, Cameroon.

2.2. Isolation and identification of the Fungal Contaminant

The fungus, obtained by scooping the mycelium from contaminated *Gnetum* cultures was inoculated on potato dextrose agar using a wire loo pure cultures were isolated by sub-culturing in Sabouraud Dextrose Agar. Identification of the fungus was based on microscopic observation and the physical appearance, colour and texture of the fungal growth on plates. Prepared slides stained with lactophenol in cotton blue were viewed under the microscope. Characters such as nature of the mycelium, branching of hyphae and method of formation, and shape of spores were observed. The identification was affected by the use of keys and samples sent to the Commonwealth Mycological Institute (CMI), UK to confirm the identity.

2.3. Preparation of Culture Media

The media employed for this work included that based on Murashige and Skoog [19] referred to here as the Y-1 medium and the Woody Plant Medium (WPM) of McCown and Lloyd [20]. The Y-1 medium consisted of MS micronutrients, micronutrients and iron-EDTA, thiamine-HCL (0.1 mg/L), sucrose (30g/L), gelrite (2g/L), supplemented with kinetin (2mg/L) and indole-3- acetic acid (2mg/L).

The Woody plant medium (WPM) on the other hand consisted of McCown and Lloyd micronutrients, micronutrients, iron-EDTA, MS vitamins, sucrose (30g/L) and agar (8g/L). The medium was supplemented in some cases with 2-5 mg/L Kinetin and 2-6mg/L IAA and also with 0.1 mg/L NAA and 1.1 mg/L BA.

In preparing both the Y-1 and WPM media, the constituents (excluding gelrite and agar, respectively) were dissolved in a litre of distilled water and the pH adjusted to 5.8+ or - 2 using HCL OR NaOH. Gelrite or agar was then added and dissolved by bringing the medium to boiling point. Aliquots of the medium (10 mL) were then dispensed into culture vessels with an automatic dispenser. The macro- and micronutrients were prepared as stock solutions of 10-strength

or 100-strength and maintained under refrigeration. Aliquots of 100 mL and 10 mL respectively were taken from each stock solution per litre for medium to obtain the desired concentration. In order to hold moisture, reduce infection and allow for gaseous exchange, culture vessels were covered with polypropylene closures or caps. The media-containing vessels were then sterilised at 121°C for 15 minutes.

2.4. Pre-sterilisation Treatment

The vines were washed under running tap water for 1-2 hours to remove soil particles and reduce the level of superficial contaminants. Nodal explants of 2-3 cm long were excised made up of separate lateral buds each carried on a small piece of stem tissue (Fig.3).

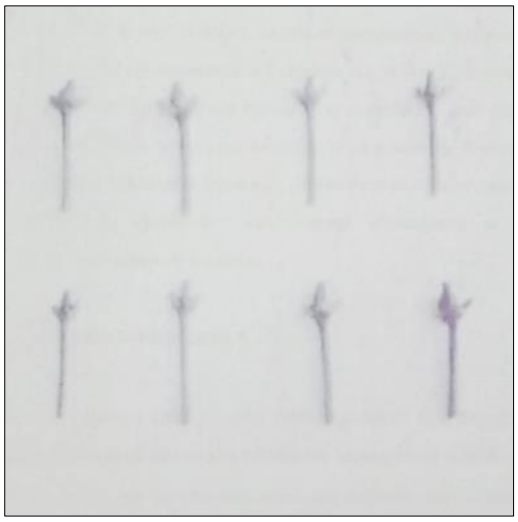

Figure 3 *Gnetum* nodal explants

The explants were immersed in distilled water containing 0.05mL of Tween 80 per 100mL of water, a wetting agent that made the microorganisms more likely to be killed by subsequent treatment with disinfectants. Explants were further immersed in a mild concentration of disinfectants (2%NaOCl) for 24-48 hours. In an attempt to reduce the rate of contamination, some explants were soaked in mild concentrations of fungicides for 24 hours. Another pre-treatment procedure used involved the spraying of about one-year-old stock plants with 5 g/l Aliette (a systemic fungicide) once a week for 5 weeks and explants collected 1 week after the last spray. All these pre-sterilisation treatments were followed by disinfectants at varied concentrations and exposure durations.

2.5. Disinfection Protocols

The general procedure used involved washing explants with distilled water containing Tween 80 for 30 minutes, followed by soaking in 70% ethanol for 3-5 minutes. Under the laminar flow hood, the explants were subjected to disinfection by the use of calcium hypochlorite, $Ca(OCl)_2$, (Containing 70 % available chlorine) and sodium hypochlorite (NaOCl) in the form of Clorox (6% available chlorine). Each sterilisation procedure was followed by 3 rinses in sterile distilled water to remove any traces of the disinfectant. The explants were then trimmed, blotted on sterile paper napkins before transferring into the culture medium.

2.5.1. Single Disinfection

The explants were exposed to varied concentrations of sodium hypochlorite ranging from 10%-50% for 10-30 minutes. Calcium hypochlorite solutions were also prepared by dissolving required quantities in distilled water, allowing it to settle and filtering the suspension through filter papers. Solution of 10%-50% $Ca(OCl)_2$ were employed for 10-30 minutes. In some cases, disinfection was preceded by various pre -treatments (Table 1).

Table 1 Summary of single disinfection procedure

Pre-treatment	**Disinfection**
No pre-treatment	NaOCl 10%, 15%, 20%, 25%, 30%, 50%, each for 10, 20 and 30 minutes
No pre-treatment	$Ca(OCl)_2$ 10%, 15%, 20%, 25%, 30%, 50%, each for 10, 20 and 30 minutes
Explants soaked in 0.1% $Ca(OCl)_2$ for 5 minutes before culturing	10%, 15%, 20%, NaOCl for 15 minutes
Explants soaked in 0.5% benlate for 5 minutes before culturing	10%, 15%, 20%, NaOCl for 15 minutes
Explants soaked in 0.05% Tween 80 for 24 hours	10%, 15%, 20%, NaOCl each for 15 minutes
Explants soaked in 2% NaOCl for 48 hours	10%, 15%, 20%, NaOCl each for 15 minutes

2.5.2. Double disinfection

After materials were treated with 70% ethanol, they were disinfected with Ca $(OCl)_2$ solutions of 8%-20%for 10 minutes, rinsed with sterile distilled water and later by solutions of 6% for 5 minutes. NaOCl solutions of 10%-20% were used for 10 minutes for the first disinfection and 8% for 5 minutes for the second. In some experiments, explants were pre-treated before disinfection (Table 2).

Table 2 Summary of double disinfection procedure

Pre-treatment	**1st disinfection**	**2nd disinfection**
No pre-treatment	$Ca(OCl)_2$ 8%, 12%, 16%, 20%, each for 10 minutes	$Ca(OCl)_2$ 6% each for 5 minutes
No pre-treatment	NaOCl 10%, 15%, 20%, each for 10 minutes	NaOCl 8% each for 5 minutes
Explants soaked in 0.1% $Ca(OCl)_2$ for 5 minutes before culturing	$Ca(OCl)_2$ 8%, 12%, 16%, 20%, each for 10 minutes NaOCl 10%, 15%, 20%, each for 10 minutes	$Ca(OCl)_2$ 6% each for 5 minutes NaOCl 8% each for 5 minutes
Explants soaked in 0.5% Benlate for 5 minutes before culturing	$Ca(OCl)_2$ 8%, 12%, 16%, 20%, each for 10 minutes NaOCl 10%, 15%, 20%, each for 10 minutes	$Ca(OCl)_2$ 6% each for 5 minutes NaOCl 8% each for 5 minutes
Explants soaked in 0.05% Tween 80 for 24 hours	Ca(OCl)2 8%, 12%, 16%, 20%, each for 10 minutes NaOCl 10%, 15%, 20%, each for 10 minutes	Ca(OCl) 6% each for 5 minutes NaOCl 8% each for 5 minutes
Explants soaked in 2 % NaOCL for 48 hours	NaOCl 10%, 15%, 20%, each for 10 minutes	NaOCl 8% for 5 minutes

Pre-treatment procedures also involved soaking in the following fungicide solutions for 24 hours:

- 0.5 Rovral, a contact fungicide with 500g/kg of fosetyl -AI as active ingredient.
- 0.5 Rovral, a systemic fungicide with 800g/kg of fosetyl-AI as active ingredient.

- 1% and 2% Benlate (50% Benomyl), a systemic fungicide.

This was followed by double disinfection with NaOCl (Table 3).

Table 3 Pre-treatment of explants with fungicide solutions

Pre-treatment solution	1st disinfection (NaOCl)	2nd disinfection (NaOCl)
0.5% Rovral	10%, 15%, 20%, each for 10 minutes	8% each for 5 minutes
0.5% Aliette	10%, 15%, 20%, each for 10 minutes	8% each for 5 minutes
1% Benlate	10%, 15%, 20%, each for 10 minutes	8% each for 5 minutes
2% Benlate	10%, 15%, 20%, each for 10 minutes	8% each for 5 minutes

2.5.3. Impregnation of culture medium

In one of the experiments, the woody plant medium was impregnated with 0.25% and 0.5% Benlate solutions before sterilising. In another case, antibiotic and/or fungicide solutions were incorporated into the medium after sterilising using sterile micropore filters. Treatments of 0.2 g/l streptomycin +1 g/L Benlate and 1 g/l Beate were employed. It is worth noting that in all the trials, some tubes were reserved that were not inoculated with the explants to serve as controls.

2.6. Data Analysis

Quantitative data were analysed using the Chi-square test of significance at 5% level of probability given by:

$$\chi^2 = \sum (O_i - E_i)^2 / E_i. \quad \text{Equation 1}$$

Where O_i is the observed value and E_i is the expected value

3. Results

3.1. Fungal Contamination

Gnetum explants when placed in culture media were found to be consistently contaminated by a fungus with a whitish, cotton-like mycelium, usually visible three days after culture (DAC) and resulting in 100% contamination of cultures. The fungal growth originated from the crevices of the node, growing very fast and colonizing the entire explant in the culture (Fig. 4).

3.2. Identification of the Fungal Contaminant

Pure cultures of the isolated fungus grown on plates revealed radial growth. A microscopic view of the slides showed clustered mycelia with branching hyphae.

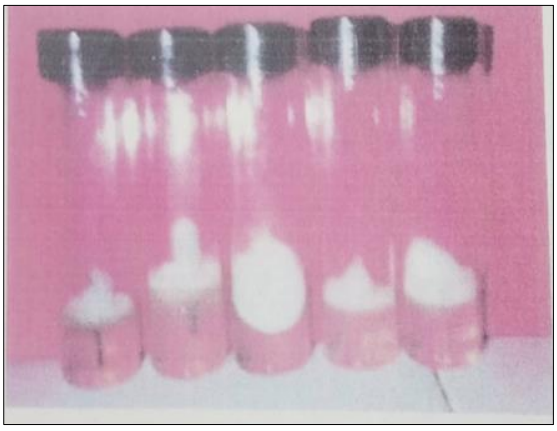

Figure 4 Fungal contamination of *Gnetum* explants in culture

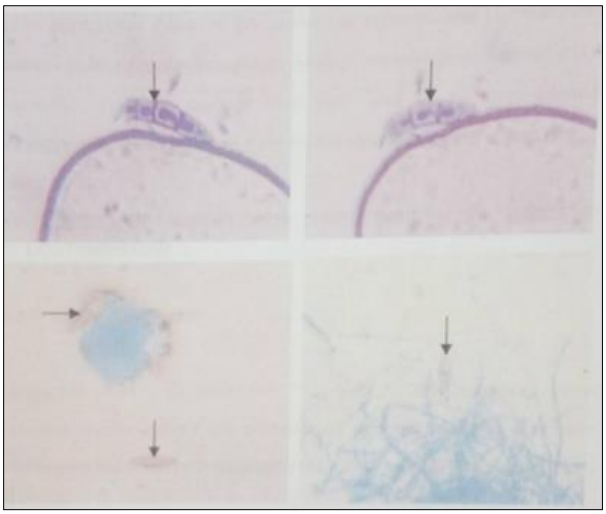

Figure 5 Micrographs of fungal spores (arrowed)

Four to five celled spores (macroconidia) were also observed under the microscope (Fig. 5). The spores were bulbous, hyaline and spindle-shaped to ellipsoidal in appearance. The above characteristics were diagnostic of fungi belonging in the genus *Microsporum* Gruby, family Moniliaceae, and order Moniliales of the class of the imperfect fungi *(Deuteromycotina*). Some of the plates viewed on the reverse side appeared bright orange-yellow, features of *Microsporum ferrugineum,* while others were pink, diagnostic of *M. audouinii*.

3.3. Effect of Disinfection Procedures on Rate of Contamination

3.3.1. Single Disinfection

All the 120 explants disinfected with 10%-50% NaOCl solutions for varied exposure duration without any pre-treatment were contaminated. The same results were registered for those disinfected with 10%-50% $Ca(OCl)_2$ solutions (Table 4).

Table 4 The effect of single disinfection of nodal cuttings of *Gnetum* with different concentrations of sodium hypochlorite and calcium hypochlorite without pre-treatment on contamination of explants

Disinfection	No. of explants cultured	No. of contaminated explants	% Contamination
NaOCl 10%, 15%, 20%, 25%, 30%, 50%, each for 10, 20 and 30 minutes	120	120	100
$Ca(OCl)_2$ 10%, 15%, 20%, 25%, 30%, 50%, each for 10, 20 and 30 minutes	120	120	100

Table 5 shows the contamination of explants under different disinfection regimes. The contamination rate ranged from 8 of 20 explants cultured in the combination of 20% NaOCl and 0.05% Tween 80 to 20 out of 20 in six other combinations. Pre-treatment with 5% Benlate resulted in complete contamination of explants while pre-treatment with 0.05% Tween 80 gave the best results. The difference in the rate of contamination for the different treatment combinations was, however, not significant at 0.05 probability level according to the chi-square test.

Some of the vines which were less contaminated were, however, adversely affected by the high concentration of the disinfectant solutions leading to the death of the explants *in vitro* (Fig. 6).

Table 5 The effect of single disinfection of nodal cuttings of *Gnetum* with different concentrations of sodium hypochlorite following different pre-treatments on the contamination of explants [a].

	Pre-treatment				
Disinfection	**0.1% Ca(OCl)2**	**0.5% Benlate**	**0.05% Tween 80**	**2% NaOCl**	**χ2**
10% NaOCl	20	20	10	20	4.285 ns
15% NaOCl	16	20	12	14	2.258 ns
20% NaOCl	20	20	8	14	6.386 ns
χ2	0.571 ns	0.000 ns	0.800 ns	1.500 ns	

a = values represent the number of contaminated explants out of 20 cultured for each treatment. ns = Chi-square value not Significant at 5% probability level.

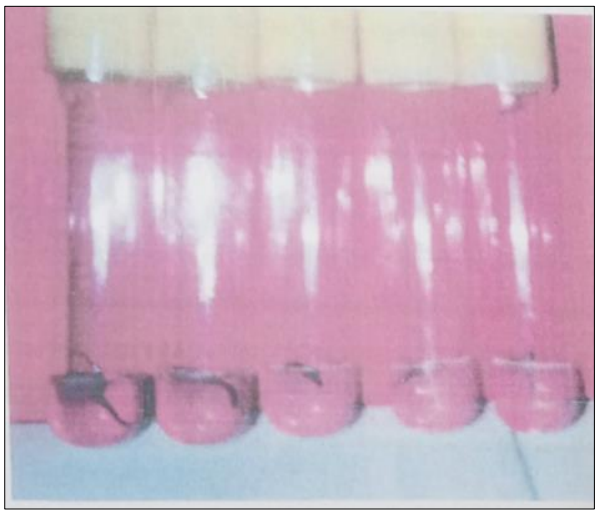

Figure 6 *Gnetum* explants killed in culture

3.3.2. Double Disinfection

Subjecting explants to two consecutive disinfecting solutions of varied concentrations without any pre-treatment registered high levels of contamination, ranging from 95%-100%. Pre-treating the explants before disinfecting with 10%-20% NaOCl followed by 8% NaOCl recorded 10-20 contaminated explants, out of the 20 explants cultured for each treatment (Table 6). Low levels of contamination were recorded for explants pre-treated with 0.05% Tween 80 for 24 hours, with the lowest of 8 contaminated explants registered for those disinfected with 15% NaOCl followed by 8% NaOCl solutions. The chi-square test, however, shows that there is no significant difference between the various pre-treatments as well as between the different disinfectant solutions.

Table 6 The effect of double disinfection of nodal cuttings of *Gnetum* with different concentrations of sodium hypochlorite following different pre-treatments on the contamination of explants [a]

	Pre-treatment				
Disinfection	**0.1% Ca (OCl)2**	**0.5% Benlate**	**0.05% Tween 80**	**2% NaOCl**	**χ2**
10%/8% NaOCl	18	18	10	14	2.937 ns
15%/8% NaOCl	18	16	8	18	4.534 ns
20%/8% NaOCl	20	18	10	20	3.999 ns
χ2	0.143 ns	0.155 ns	0.287 ns	1.077 ns	

[a] = values represent the number of contaminated explants out of 20 cultured for each treatment. ns = Chi-square value not Significant at 5% probability level.

On the other hand, pre-treating the explants before disinfecting with 8%-20% $Ca(OCl)_2$ followed by 6% $Ca(OCl)_2$ solutions recorded 12-20 contaminated explants (Table 7). The level of contamination again was low for those pre-treated with 0.05% Tween 80 for 24 hours, with the lowest of 10 contaminated explants for the 8%/6% and 12%/6% $Ca(OCl)_2$ treatments. Statistically, there is no significant difference between the pre-treatments and also the disinfectant solutions according to the chi-square test.

Table 7 The effect of double disinfection of nodal cuttings of *Gnetum* with different concentrations of calcium hypochlorite following different pre-treatments on the contamination of explants [a]

	Pre-treatment			
Disinfection	**0.1% Ca (OCl)2**	**0.5% Benlate**	**0.05% Tween 80**	**χ2**
8%/6% Ca(OCl)2	19	20	10	3.714 ns
12%/6% Ca(OCl)2	19	20	10	3.714 ns
16%/6% Ca(OCl)2	19	18	12	1.755 ns
20%/6% Ca(OCl)2	16	18	16	0.161 ns
χ2	0.370 ns	0.211 ns	1.999 ns	

[a] = Values represent the number of contaminated explants out of 20 cultured for each treatment, ns = Chi-square value not significant at 5% probability level.

When the explants were pre-treated by soaking in mild fungicide solutions, the level of contamination ranged from 2 to 20 explants out of 20 cultured for each treatment (Table 8). The lowest level of contamination was recorded for those pre-treated with Benlate. Disinfection regimes did not differ significantly from each other for each pre-treatment type. The pre-treatment means however showed significant differences for each of 15&/8% NaOCl (χ^2=16.351, P≤ 0.05) and 20%/8% NaOCl (χ^2=15.200, P ≤ 0.05).

Table 8 The effect of pre-treating nodal cuttings of *Gnetum* with different fungicides followed by double disinfection with NaOCl on contamination of explants [a].

	Pre-treatment				
Disinfection	**0.5% Rovral**	**0.5% Aliette**	**1% Benlate**	**2% Benlate**	**χ2**
10%/8% NaOCl	20	12	12	8	5.846 ns
15%/8% NaOCl	18	8	6	2	16.351*
20%/8% NaOCl	20	10	4	6	15.200*
χ2	0.138 ns	0.800 ns	4.727 ns	3.499 ns	

[a] = Values represent the number of contaminated explants out of 20 cultured for each treatment, ns = Chi-square Value not significant at 5% probability level. *= Chi-square value significant at 5% probability level.

3.3.3. Incorporation of fungicide in medium

The rate of contamination of explants cultured on media into which either 0.25% or 0.5% Benlate had been incorporated were low, ranging from 2 on 20 to 12 on 20 (Table 9). There were significant differences between the WPM + 0.05% Benlate (χ^2 =13.335, P≤ 0.05) and also for three of the disinfection regimes. When the medium was supplemented with antibiotic and/or fungicide solutions without prior pre-treatment, all the explants placed in culture were contaminated.

Table 9 Contamination of nodal cuttings of *Gnetum* cultured on media incorporated with different fungicides following different pre-treatments

Pre-treatments/Disinfection	Medium			
	WPM	WPM/0.25%w/v Benlate	WPM/0.5%w/v Benlate	χ2
0.5% Rovral				
15%/8% NaOCl	20	6	12	18.501*
25%/8% NaOCl	20	4	8	13.001*
0.5% Aliette				
15%/8% NaOCl	14	8	8	2.400 ns
25%/8% NaOCl	12	4	2	9.334*
0.5% Benlate				
15%/8% NaOCl	10	6	4	2.801 ns
25%/8% NaOCl	8	4	2	3.999 ns
χ2	9.142 ns	2.498 ns	13.335*	

[a] = Values represent the number of contaminated explants out of 20 cultured for each treatment, ns = Chi-square value not significant at 5% probability level. *= Chi-square value significant at 5% probability level

3.4. Effect of Media Composition on Sprouting

The two media employed in this study (Y-1 and WPM) were made up of varied constituents (Table 9 and 10).

Table 10 MS Establishment Medium (Y-1)

Macronutrients	Amount (g/L)	Micronutrients	Amount (g/L/
NH_4NO_3	16.5	H_3BO_3	0.620
KNO_3	19.0	$MnSO_4.4H_2O$	2.230
$CaCl_2.2H_2O$	4.4	$ZnSO_4.4H_2O$	0.083
$MgSO_4.7H_2O$	3.7	KI	0.083
KH_2PO_4	1.7	$Na_2MoO_4.2H_2O$	0.025
		$CuSO_4.5H_2O$	0.0025
		$CoCl_2.6H_2O$	0.0025

pH 5+/- 0.2

Table 11 Woody Plant Media (WPM)

Macronutrients	Amount (g/L)	Micronutrients	Amount (g/L)
NH_4NO_3	4.0	H_3BO_3	0.620
$Ca(NO_3)_2.4H_2O$	5.56	$MnSO_4.4H_2O$	0.0022
$CaCl_2.3H_2O$	0.96	$ZnSO_4.7H_2O$	0.860
$MgSO_4.7H_2O$	3.7	$Na_2MoO_4.2H_2O$	0.025
KH_2PO_4	1.7	$CuSO_4.5H_2O$	0.0025
K_2SO_4	9.9		

pH 5+/- 0.2

The Y-1 medium supplemented with growth regulators registered no growth of explant for both contaminated and uncontaminated tubes. Sprouting was, however, observed in WPM, which on the contrary contained no growth regulators.

4. Discussion

The success of *in vitro* propagation has been shown to depend on the mother plant from which explants were excised, which should be healthy and preferably grown in a greenhouse and treated in some way [21, 22]. Pre-treatment of plant material has been emphasized to alleviate contamination problems *in vitro*. This is evident here by the lower levels of contamination recorded by explants that were pre-treated before disinfection, particularly those that were soaked in 0.05% Tween 80 for 24 hours prior to disinfection. This is probably due to the fact that Tween 80 is a wetting agent that enhances the effectiveness of the sterilant by reducing the surface tension between the plant tissue and water, thereby establishing the best possible contact with the plant material [23, 24, 25].

Pre-treatment with mild fungicide solutions reduces the level of contamination but this has an adverse effect on the explants, contributing to the death of uncontaminated explants. However, the level of contamination as revealed by this study is still high compared to the case of microbial contamination in yam (*Dioscorea*) cultures that was reduced from 80% to 4% [26, 27]. All sterilization regimes used in this study were able to get rid of surface contaminants commonly encountered in *in vitro* culture [28] except for the *Microsporum* complex, which is being encountered for the first time. Most of the pre-treatment procedures only delayed the growth of this fungal complex which only became evident 6-8 DAC as compared to the 3 DAC for cases not pre-treated.

The results of this study also reveal that pre-treating explants with Benlate and Aliette, which are both systemic fungicides, led to a lower level of contamination by the fungus compared to Rovral, which is a contact fungicide. This suggests that the contaminating fungus is systemic in *Gnetum*. Systemic contaminants are generally more difficult to handle in *in vitro* culture than surface contaminants. Similar problems of systemic contaminants have been encountered in the *in vitro* culture of *Pennisetum purpureum* and *Cynodon dactylon* (Mih, Pers. Comm.) [29]. The incorporation of Benlate in the culture medium seemed to improve on the sterilizing effect of the fungicides. It will thus prove useful to include this as a routine practice for *in vitro* culture of *Gnetum* as has been the case for other plants [25].

The growth of explants on an unsupplemented medium suggested that they possessed some endogenous growth regulators. Therefore, placing them in a medium already containing these regulators led to inhibition of growth, considering the fact that growth regulators act best at concentrations between 0.001-10mg/L, which are usually low [30]. Plants with endogenous auxins may even because the formation of friable callus when cultured on reinforced medium as has been observed in soybean [31, 32].

Gnetum vines from which culture material was obtained showed no disease symptoms as a result of the presence of the *Microsporum*. The proliferation of the fungus when placed in culture was suggestive of the fact that it was in a natural association with *Gnetum* and the culture medium provided the necessary requirements for its growth. *Microsporum* has not previously been reported in *Gnetum*, but it is known to live on the surfaces of plants, animals and man. It is reported to cause dermatomycoses in animals and man [30]. There is thus a need to further elucidate the nature of the association.

The fungus could also be considered a fast-grower, colonizing the entire culture a few days after culture, as a result, choking the explant. Contaminated cultures appeared coloured several days after culture, indicating that substances were exuded into the medium. This may also have inhibited sprouting.

Cultures of the fungus revealed that a complex of different species of *Microsporum* probably occurred in association with *Gnetum*. Microscopic observations showed similar spores in all cases but visual observation of figures revealed slight differences in the colour of the fungal growth.

5. Conclusion

This work has made evident the general fact that plant materials obtained from the wild may be highly contaminated and not easily disinfected routinely unlike the case with other plants grown in the field or in greenhouses. The importance of pre-treating plant materials before disinfection and eventual culture cannot be over-emphasized. Pre-treatment by soaking *Gnetum* explants in 0.05% Tween 80 for 24 hours, followed by disinfection with 15%/8% NaOCl solutions for 10/15 minutes deals adequately with the surface contaminants while the incorporation of the Woody Plant

Medium (WPM) with 0.5% Benlate may be used to alleviate the situation of systemic contamination. *Gnetum* in particular, growing in association with the *Microsporum* complex poses a major problem of disinfection. It can, however, be concluded that the Woody Plant Medium is appropriate for sprouting of *Gnetum in vitro* and could only be optimized when need be. Considering the fact that no work has previously been reported on the *in vitro* propagation of this plant species, this study provides baseline information in that light and opens up avenues for more research in the field of disinfecting explants and mass propagation of the highly desired, endangered and threatened species.

It could therefore be recommended that to obtain more successful results subsequently, plants should be grown in a greenhouse, under controlled conditions from which explants would be excised for culture *in vitro*. This would greatly reduce the level of contamination. Detailed studies should be carried out on the association of the fungus with *Gnetum* so that appropriate measures can be taken to completely eliminate the fungus. It is equally necessary to consider initiating growth of *Gnetum in vitro* by using other tissue culture methods notably meristem culture, shoot-tip culture or somatic embryo culture.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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