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STUDIES ON KOLA TISSUE CULTURE I: Protocols for Establishing Kola Tissues *in vitro*

Obembe, O. O.
Department of Botany, Obafemi Awolowo University, Ile-ife, Nigeria.

Abstract

The micropropagation of *Cola nitida* (Vent.) Schott and Endlicher by means of tissue culture was investigated to provide baseline information on the requirements for the survival of kola tissues and organs *in vitro*. Investigations were conducted on development of sterilization protocols, and medium selection and modification. The best sterilization procedure was established with the step-wise treatment of 70% ethanol, for 20 seconds and 10% (w/v) CaOCl, for 10 minutes..The use of modified Murashige-Skoog (MS) medium (without Zn and Cu elements) as basal medium was found as appropriate as the original MS medium, for explants' survival. The appropriate antioxidant technique was also established with $10mgl^{-1}$ ascorbic acid.

Introduction

The kola tree is a tree crop of tropical and subtropical countries and belongs to the genus *Cola* in the family Sterculiaceae. There are about 90 species, *C. nitida* and *C. acuminata* (P. Beauv.) Schott and Endl. are cultivated as plantation crop strictly and only in West Africa countries because of the importance of their nuts.

Kola nut is rich in caffein (1,3,7-trimethyl-xanthine), theobromine (3,7-dimethyl-xanthine) and a glycoside, kolannin (Eijnatten, 1969) and becuase these alkaloids have stimulatory effects on man, kola nut is used as a masticatory and stimulant (Eijnatten 1969, 1973). Thus, it could be grouped under non-alcoholic caffein beverage crops with coffee (*Coffea* spp.) and cocoa (*Theobroma cacao* L.). Kola nut is used for traditional activities especially rituals and social ocassions such as naming, marriage and funneral ceremonies (Eijnatten, 1969). In Europe and the United States of America, kola nut is used to produce several pharmaceutical drugs, liqueurs and confectioneries (Egbe and Oladokun, 1987).

Kola trade has increased over time since its inception as early as the nineteenth century. The sustained increase in demand of kola has led to greater interest in it as a tree crop. A survey of available literature revealed that research work on the plant has been uncoordinated and inconclusive. As far as I know, there is no previous report on kola *in vitro* studies as there are for cocoa, tea and coffee. Recently, Esan (1992) stressed the need for the application of tissue culture techniques to all economic tree crops for the conservation of genetic resources and for the multiplication of superior plant individual and the progenies. However, before such techniques are exploited fully for commercial purposes, it is essential that baseline information on the responses of different tissue types and explants on various growth media be assembled. It is therefore the aim of this work to investigate the requirements for the survival of kola tissues and organs *in vitro* The term "kola" is commonly used to refer both to the "nut" which is the embryo as well as to the tree. This is adopted in this paper.

Materials and Methods

Source of explant

Plant materials were collected from shoots of fully-grown kola trees located in the Biological Garden and of seedlings raised in the screen-house of Botany Department, both of Obafemi Awolowo University, Ile-Ife, Nigeria. Young leaves and single node cuttings from seven day-old flushes of both sources were used as explants in this work. Preliminary investigation has shown that older or more mature plant materials from field-grown plants were not suitable because they could be too mature and be colonized by microbial epiphytes which are very difficult to eliminate in order to produce sterile explants.

The choice of *C. nitida* as the sole source of explants used for the investigation was based on its economic importance. It is of much more economic importance than *C . acuminata* (Eijnatten, 1973).

Sterilization Protocols

The use of vegetative shoot is based on the fact that it serves as a ready source of explants. Young leaves and nodal explants were washed under running tap water for one hour. All other steps of the sterilization procedure were carried out under sterile conditions inside a Laminar Flow Chamber. Explants were washed in (a) 10% calcium hypochlorite (CaOCl) for 10 minutes (b) 10% CaOCl for 15 minutes and (c) 15% CaOCl for 10 minutes. A 20 second pretreatment with 70% ethanol prior to CaOCl treatments were also investigated. A drop of Tween 20 per 100ml of freshly prepared calcium hypochlorite was used as the surfactant. Plant materials were rinsed four times with sterile distilled water. Leaf discs (10 mm diameter) were cut using a sterilized and flamed stainless steel cork borer. The explants were cultured on unmodified MS medium (leaf discs, cultured face down), and incubated at 25 ± 2^0C in the dark. The implanted materials were kept under observation for 2 weeks.

Media Selection

Two nutrient media tested were Murashige-Skoog (MS) medium (Murashige and Skoog, 1962 and its modification for banana and plantatin meristem culture *in vitro* and reported in the proceedings of the First Conference of the International Plant Biotechnology, Network (IPB Net) held in Fort Collins, Colorado, Oct. 21-25, 1985 (Table I). The micronutrients excluded were $CuS0_4.5H_20$ and $ZnS0_4.7H_20$.

Determination of appropriate antioxidant/adsorbent

The effectiveness of an adsorbent, activated charcoal (AC) and an antioxidant, ascorbic acid (AS) on the prevention of accumulation of toxic products of phenolic oxidation were compared. Various concentrations of AC; 0.1, 0.3, 0.5, 0.7 and 1.0% and 2.0, 4.0, 6.0, 8.0 and 10.0 mgl^{-1} AS were added separately to each of the culture solutions of the two nutrient media prior to autoclaving. The medium without antioxidant/adsorbent served as the control. No plant growth regulator was added to any of the culture media. The agar was maintained at 0.7% and the pH was adjusted to 5.7 ± 0.1. The culture flasks were covered with non-adsorbent cotton wool and autoclaved at 121^0C and $15b/in^2$ pressure for 15 minutes.

Statistical analysis

All experiments were repeated three times. Data on sterilization protocol and antioxidant techniques were statistically analyzed by the SAS software using a completely randomized design and means were compared at the $p=0.05$ level of significance using Turkey's studentized range (HSD) test.

Table I: Chemical composition of concentrated stock solutions and molar concentration of nutrients in the modified MS medium used for kola tissue culture.

Constituents	Concentration In Stock Solution (Gl^{-1})	ml Stock Solution Per 1l Medium	Concentration Medium ($Mg\ l^{-1}$)	In Final MM
Macronutrient I				
$KN0_3$	95.0		1900.00	18.8
NH_4N0_3	82.5	20	1650.00	20.6
$CaCl_2'2H_20$	22.0		440.00	3.0
Macronutrient II				
$MgSO_4.7H_20$	11.5	20	370.00	1.5
Macronutrient III				
KH_2PO_4	8.5	20	170	1.25
Micronutrients *				
$MnS0_4.4H_20$	22.3		22.3	0.1
H_3B0_3	6.2		6.2	0.1
KI	0.83	1	0.83	0.005
$Na_2Mo0_4.2H_20$	0.25		0.025	0.001
$CoCl_2.6H_20$	0.25		0.025	0.0001
Fe-Solution				
$FeS0_4.7H_20$	5.57	1	27.85	0.1
Na_2EDTA	7.45		37.25	0.1
Vitamins				
Glycine	2.0		2.0	0.025
Nicotinic acid	0.5	1	0.5	0.004
Pyridoxine HCL	0.5		0.5	0.002
Thiamine HCL	0.4		0.4	0.001
Ascorbic acid	10		10.0	
Carbon source				
Sucrose			30,000.00	87.7

*Excluded as basal salt modification

(i) $ZnS0_4.7H_20$ at 8.6 mgl^{-1} medium
(ii) $CuS0_4.5H_20$ at 0.025 mgl^{-1} medium.

Results and Discussion

Sterilization protocol

No significant difference was observed among treatments which involve quick dip in 70% ethanol followed by different treatments with calcium hypochlorite in either explant types from the juvenile plants (Table II). The complementary effect of the pre-treatment with 70% ethanol could have been more effective if combined with the treatment with Ca0Cl. However, tissue damage due to the sterilization was observed in some explants treated with either 10% Ca0Cl for 15 minutes or 15% Ca0Cl for 10 minutes. Subsequently, a step-wise treatment with 70% ethanol for 20 seconds followed by 10% Ca0Cl for 10 minutes was used for all the experiments. Significant interaction was observed between the treatments and age of the plant materials. The kola leaf tissues and nodal sections obtained from the screen house-grown seedlings responded well to treatments than explants from matured field-grown plants (Table II). This may be largely due to the exposure of the field-grown plant to microbial spores and colonies. However, there was no significant interaction between treatments and the two explant types (Table II, data not shown). Hence, the choice of young leaves and single node cuttings obtained from kola seedlings as the explants for subsequent stages of the work.

Table II: Responses of explants from *C. nitida* seedlings and explants from mature kola, to sterilization treatments.

Sterilization procedure	Clean explants (percentage # mean± SD)			
	kola seedling		mature kola	
	young leaf	single node cutting	young leaf	single node cutting
10%CaOCl (10 mins.)	00.0±0.0^{c}	00.0±0.0^{b}	00.0±0.0^{c}	00.0±0.0^{b}
70% ethanol (20 secs.)+ 10%Ca0Cl (10 mins.)	97.0±0.6^{a}	90.7±0.9^{a}	55.7±2.6^{a}	5.7±1.5^{a}
10% Ca0Cl (15 mins.)	17.0±2.1^{b}	15.7±2.0^{b}	7.7±1.5^{c}	00.0±0.0^{b}
70% ethanol (20 secs.)+ 10% Ca0Cl (15 mins)	19.7±1.2^{a}	91.0±0.6^{a}	56.3±2.9^{a}	5.7±0.7^{a}
15% Ca0Cl (10 mins)	19.7±0.9^{b}	16.3±1.7^{b}	8.3±1.2^{c}	00.0±0.0^{b}
70% ethanol (20 secs)+ 15% Ca0Cl (10 mins)	96.7±0.3^{a}	91.7±0.9^{a}	37.7±0.9^{b}	6.3±1.5^{a}

Means within a column having the same letter are not statistically significant (p = 0.05) according to Tukey's studentized range (HSD) test.

Antioxidant techniques and medium selection

All explants in the control experiment turned brown after the second day. No significant difference was observed in the effectiveness of AC at 0.3-1.0% in either media (Table III). However, explants on 0.5% and above were observed to be curling up, appeared flaccid but retained their freshness after the seventh week. As for AS, there was significant difference among the various concentrations tested. AS at 10mgl^{-1} gave the highest number of viable explant (Table III). However there was no significant difference among treatments with respect to the two basal media. Even though there was no callus induction, most of the explants on the media with 10 mgl^{-1} AS and 0.3% AC still appeared viable. AC could remove plant growth hormones and some media components from the medium alongside the harmfuful phenolics and carboxylic compounds (Weatherhead 1979). Owing to the

foregoing, AS at 10 mgl^{-1} has been adopted as the appropriate antioxidant for preventing accumulation of phenolics in *C. nitida.* Other antioxidants like Polyvinylpyrrolidone (PVP) and 1/2 strength MS medium (with 1 g myo-inositol), for the reduction of phenolic oxidation and prevention of necrosis of explants, respectively have been reported (Egnin *et. al.*, 1998).

There was no significant difference among treatments with respect to the two basal media (Table III). Hence, the modified MS medium was forthwith adopted for other subsequent studies. This adoption does not mean an aberration, other suitable media excluded Cu and Zn elements in their formula (Morel, 1964). This would reduce, to some extent, the cost of medium preparation. Holme-Hansen *et. al.* (1954) also stressed that Cu and Mo are optional addenda of nutrient medium for plant growth.

Table III. Effect of activated charcoal and ascorbic acid on the prevention of accumulation of phenolic products on cut surfaces of kola leaf explants.

Antioxidant	Viable explants (percentage # mean +SD)	
	MS[1]	mdMS[2]
Activated charcoal		
0.0%	00.0 ± 0.0^{c}	00.0 ± 0.0^{c}
0.1%	72.3 ± 2.5^{b}	72.6 ± 2.9^{b}
0.3%	96.3 ± 1.5^{a}	96.0 ± 1.0^{a}
0.5%	96.6 ± 1.2^{a}	96.3 ± 0.6^{a}
0.7%	96.3 ± 1.5^{a}	96.6 ± 1.5^{a}
1.0%	97.6 ± 0.6^{a}	98.0 ± 1.0^{a}
Ascorbic acid (mgl^{-1})		
0.0	00.0 ± 0.0^{f}	00.0 ± 0.0^{f}
2.0	49.0 ± 1.7^{e}	49.6 ± 2.1^{e}
4.0	64.3 ± 1.5^{d}	64.0 ± 1.0^{d}
6.0	70.3 ± 2.5^{C}	71.0 ± 2.0^{C}
8.0	89.3 ± 1.2^{b}	88.3 ± 2.1^{b}
10.0	98.3 ± 5.8^{a}	98.3 ± 1.2^{a}

Means under a treatment having the same letter are not statistically significant ($p = 0.05$) according to Tukey's studentized range (HSD) test.

[1]Murashighe-Skoog (1962) medium basal salt mixture including vitamins.
[2]Modified Murashige-Skoog (1962) medium which lacks Cu and Zn elements.

Conclusion

The findings of this investigation have provided baseline information upon which future *in vitro* studies on kola can be based. Inspite of the inherent accumulation of phenolics which constitutes the problem facing kola tissue culture, appropriate tissue types and appropriate package of medium requirements for their survival and callus induction have been successfully established.

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