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An Efficient Protocol For Medium - Term Conservation of the Aerial Yam, *Dioscorea Bulbifera* L. *in Vitro*

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Abstract
An efficient protocol for medium-term conservation of the aerial yam, *Dioscorea bulbifera* is hereby reported. Meristem and nodal segments from five-week old plants cultured in half strength MS or B5 medium containing 12 per cent sucrose and 0.5 mg l^{-1} abscisic acid [ABA] were successfully stored for 15 months at 25± 2°C without loss of morphogenetic capacity. Subculturing of the explants in full strength MS containing low levels of benzyl adenine (2.0-5.0 mg l^{-1}) and reduced sucrose concentration [2.0 per cent] resulted in vigorous shoot growth after 6 weeks in culture, whereas incorporating ABA (0.2-0.5mg l^{-1}) in the second passage culture medium resulted in the depression of shoot growth and shortening of the time to tuberization on transfer to the field after hardening.

Key Words: *Aerial yam, Dioscorea, Bulbifera, in vitro*

Introduction

The aerial yam, *Dioscorea bulbifera* L. produces edible tubers which serve both for food and medicinal purpose due to its high content of diosgenin [a saponin] and dioscorine [an alkaloid] [Coursey, 1967; Onwueme, 1978]. Although the flavour is distinctive, the bulbil [tuber] does not have the same appeal of the best varieties of *D alata* L. and *D rotundata* Poir (Martin, 1976) . Preference in Nigeria for the latter species which are more tasty and with much larger tubers has resulted in a rapid decline in *D. bulbifera* cultivation to the extent that it has now become an off-season crop grown more for its medicinal qualities than as a staple .To stem the decline of *D. bulbifera* cultivation and avert its possible extinction , there is a need for its conversation as this will help maintain its genetic supplies for breeding purposes.

In vitro methods involving tissue culture have proved to be much more efficient in germplasm conversation over traditional field maintenance [Ruredzo and Hanson, 1991] and have been extensively developed and applied in medium -term germplasm storage of many tropical species [Engelmann, 1991] which include cassava [Roca *et al.*, 1984] and some yam species [Ng, 1992]. Among the many advantages presented by the tissue culture systems

include aseptic systems (free from fungi, bacteria, viruses and insect pests), reduction in space requirement, reduction of genetic erosion, and reduction of expenses in labour costs and financial terms [Engelmann, 1991].

A variety of modifications in the environmental and chemical conditions of the culture medium have been successfully applied for purposes of medium-term conversation. These include the use of low temperatures [Mix-Wagner, 1996] which depends on the temperature-sensitivity of the species. Cassava, for instance, has to be stored at temperatures higher than 20°C [Roca *at al.*, 1984]. Low photon flux [under 30 mE m^{-2} S^{-1}] has been applied in potatoes [Veramendi *et al.*, 1998] and is often used concomitantly with temperature reduction. Chemical modifications of the culture media include reduction in the macro- and micro-element concentrations [Kartha *et al.*, 1981;Veramendi *et al.*, 1998], the use of growth retardants such as abscisic acid [Westcott, 1981; Powell and Caligary, 1989] or ancymidol [a synthetic growth retardant] Veramendi *et al.*, 1998]. However, these authors indicate that ABA is detrimental to some varieties. Other chemical modifications include the use of mannitol as osmotic factor [Dodds *et al.*, 1991] or high sucrose concentrations [Kwiatkowski *et al.* 1988; Hesky and Nagy, 1987]. Addition of activated charcoal [Roca *et al.*, 1984] was found to reduce defoliation, decreases shoot growth and limits chlorophyll degradation in cassava shoots. A combination of a few of the factors outlined above induces slow growth leading to an extension of the time needed between subcultures, and hence the possibility of storing cultures for up to 12 months as in Irish potato [Veramendi *et al.*, 1998] and other yam species such as D. *rotundata* [Ng., 1992] without loss of morphogenetic potential.

Earlier efforts at long-term storage of yams *in vitro* for conservation purposes have been directed at the more popular species [D. *rotundata*] cultivated mainly for their food value. The aim of the present study was to develop a reliable system for *in vitro* medium-term conversation of the aerial yam, *D. bulbifera* through the modification of culture conditions such as; a lowering of the mineral components, incorporation of a growth retardant [ABA], increase in carbon source [sucrose] concentration, and use of activated charcoal.

Materials and Methods

Preparation of Explants.

Shoot tip explants used in this study were excised from the most distal 10 nodes of 5-week old *Dioscorea bulbifera* plants maintained in the Botanical Garden of University of Nigeria, Nsukka. Two types of shoot tips, namely (i) the apical meristem [the meristematic dome + a few subtending leaf primordia], and (ii) defoliated nodal segment [the axillary complex comprising the primary, accessary, and bulbil primordia] served as the explants in this study. The explants [3-5 mm] were surface-sterilized by dipping first in 5% Teepol detergent [a wetting agent] for 5 min, rinsed in sterile distilled water, then treated with a fungicide/antibiotic solution, Bavistin [0.0% w|v] and streptocycline [0.02%w|v] for 20 min before immersion in 0.1% mercuric chloride [$HgCl_2$] for 2 min after which they were rinsed several times in sterile distilled water. The sterilized explants were then left immersed in sterile distilled water prior to inoculation in the appropriate media.

Establishment of Cultures.

For the conservation experiment , modified basal media of Murashige and Skoog [1962] and Gamborg *et al* (B5) [1968] were employed. The macro- and micro-elements of both media were used at half strength. In addition, both media were supplemented with 100 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine HCL, 0.5 mg l^{-1} niacin , and 0.5 mg l^{-1} pyridoxin HCL. Sucrose was employed as the carbon source and was tried at two levels (3.0 and 12.0% w/v) and the media were solidified with 0.8% w/v agar [Difco - Bacto] while activated charcoal [5.0% w/v] was also incorporated in all media to help absorb phenolic compounds characteristically produced by most *Dioscorea* species in culture. The plant growth retardant, abscisic acid [ABA] was incorporated in the media at 0.5 mg l^{-1} and the pH of each medium adjusted to 5.6 before dispensing in 20 ml portions in Pyrex test tubes capped with non-absorbent cotton wool wrapped in aluminum foil, prior to sterilization by autoclaving for 15 min at 121°C and 103 kN M^{-2} pressure.

The sterilized explants were cultured one per test tube and there were 60 replicate tubes per treatment and a total of eight treatments. All transfers were done under a laminar flow hood previously kept sterile by exposure to ultraviolet light for 30 min. Cultures were stored on racks in a growth chamber [Fisons 600/THTL] at 27±2°C under 12 h light / dark cycles and 5,000 to 6,000 lx intensity during the light hours.

By the 15th month from the day of culture, when many of the cultures were already browning and some of the leaves heavily chlorotic, 20 samples were randomly selected per treatment and scored for rooting, shoot production, and leaf chlorosis.

In order to test for the morphogenetic capacity of plantlets after the 15-month storage, explants were taken from treatments that yielded the healthiest - looking plantlets [i.e with shoots longer than 5.0 mm, and absence of necrosis] and subcultured on full strength MS supplemented with BA [0-7.0 mg l^{-1}] and/or ABA [0-0.7 mg l^{-1}] and reduced carbon source [sucrose, 2.0 mg l^{-1}]. There were 12 - 15 replicate explants per treatment and the cultures were maintained under the same conditions as for the conservation experiment, but for only 6 weeks.

At the end of the 6 weeks the cultures were scored for the following growth parameters: percentage of explants producing shoots more than 5.0 mm long, fresh weight of shoots produced per explant , and percentage of shoots with necrotic leaves. All the plantlets which were not included in the sampling for morphogenetic capacity at the end of the 6-week subculture were hardened and transferred to the Botanical Garden of the Department of Botany, University of Nigerian, Nsukka, to grow to full maturity.

In course of growth in the field, the plants were monitored for tuberization [time taken for the appearance of aerial bulbils]. At the end of 128 days in the field, by which time most of the plants had matured and started shriveling, they were then sampled for the following growth parameters: fresh weight of the organs [i.e roots, shoot, and tuber] produced as well as the % tuber / whole plant ratio under the various treatments.

Table 1: Influence of Sucrose and Abscisic Acid (ABA) on Explant Development after 15 Months of Storage at 27 ± 2°C

Treatment	Explants (%) producing:				Mean Fresh Weight (mg)				Mean Number		Mean Length (mm)	
	Roots	Shoots	Callus	Necrotic Shoots	Roots	Shoots	Callus	Whole Plantlet	Roots	Shoots	Roots	Shoots
MS + 3% Sucrose	100	100	12	46	31.7±5.0	83.8±11.6	18.9±2.2	134.4±18.8	6.9±1.8	2.3±0.3	4.2±0.9	6.6±0.8
MS + 12% Sucrose	100	100	16	62	27.1±3.6	62.0±7.7	25.0±4.6	114.1±15.9	5.6±1.0	1.8±0.1	3.9±0.7	6.0±0.6
MS + 3% Sucrose + 0.5mgl^{-1}ABA	100	94	0	11	23.1±3.2	55.5±7.0	0.0±0.0	78.6±10.2	4.9±1.5	1.6±0.1	3.3±0.7	6.8±0.8
MS + 12% Sucrose + 0.5mgl^{-1}ABA	100	91	0	0	26.6±4.8	58.1±6.8	0.0±0.0	84.7±11.6	4.4±1.2	1.2±0.1	3.0±0.6	4.9±0.9
B5 + 3% Sucrose	100	100	15	53	28.0±2.9	72.3±8.3	28.2±2.7	128.5±13.9	6.6±1.6	2.7±0.3	4.0±1.0	5.8±0.9
B5 + 12% Sucrose	100	86	12	58	25.5±3.4	63.9±6.8	16.3±3.2	105.7±13.4	5.9±1.6	2.0±0.3	3.9±0.8	5.3±1.2
B5 + 3% Sucrose + 0.5mgl^{-1}ABA	100	100	0	14	24.9±3.5	56.6±6.1	0.0±0.0	81.5±9.6	5.1±1.9	1.8±0.2	3.0±0.7	5.1±0.9
B5 + 12% Sucrose + 0.5mgl^{-1}ABA	100	100	0	0	22.0±3.3	53.9±7.0	0.0±0.0	75.9±10.3	4.9±1.6	1.8±0.3	3.7±0.8	5.1±1.0

MS and B5 were used at half strength

Results

Most of the explants had regenerated whole plantlets within 15 months from the day of establishment of cultures irrespective of the treatment given. However, the healthiest plantlets were noticed in media [2 strength MS and B5] containing high sucrose concentration [12%] in combination with ABA at 0.5mg l^{-1}. Under these treatments there was complete absence of necrotic leaves in the plantlets produced whereas different levels of leaf necrosis were observed among plantlets produced under all the other treatments [Table 1].

Vegetative growth was accompanied by callus production in the absence of ABA in the media [Table 1]. The callus consequently contributed substantially to the weight of plantlets produced under these treatments [Table 1]. Otherwise, there was no significant difference [P =0.05) in the root or shoot fresh weight between treatments. However, a consideration of the number and length of the roots and shoots produced showed that although more roots and shoots were produced in media that lacked ABA, these organs were not significantly longer [P=0.05] than those produced in media supplemented with ABA [Table 1].

The plantlets produced under 12% sucrose and 0.2-0.5mg l^{-1} ABA treatments retained a very high level of morphogenetic capacity after the 15 -month period of storage without subculturing . This was demonstrated by the fact that shoot tip explants taken from them regenerated complete plantlets [shoots and roots] within only 6 weeks in a full strength MS medium containing as low as 2% sucrose as carbon source [Table 2]. Growth of these explants was substantially improved when BA was incorporated in the medium at 2-5 mg l^{-1} as up to 4.1±0.6 and 4.8±0.9 shoots were produced when BA was used at 2.0 and 5.0 mg l^{-1} respectively as against 2.4±0.4 shoots per explant in the absence of BA in the medium. Supplementation of BA at levels above 5.0 mg l^{-1} caused a substantial depression of shoot growth as fewer shoots [1.1±0.3] were produced under this treatment. Superiority of shoot growth at the optimal BA levels [2-5mg l^{-1}] was also reflected in the length of shoot as well as fresh weight of shoots produced per explant [Table 2]. It was also observed that ABA substantially suppressed shoot growth at all the levels [0.2-0.7 mg l^{-1}] tested. While ABA depressed shoot formation by at least 40 percent when incorporated at 0.2 mg l^{-1} , there was absolutely no shoot development by any of the explants when ABA concentration was increased to 0.5 mg l^{-1} and beyond within the 6 weeks of growth [Table 2] .

Table 2: Effect of Reduced Sucrose Level (2.0%) on Shoot Growth of Explants of *Dioscorea bulbifera* Taken from 15 Month-old Plantlets and Subcultured in MS Medium Supplemented with Low Levels of BA and ABA for 6 Weeks

Treatment		Shoot Growth			
BA (mgl^{-1})	ABA (mgl^{-1})	Shoot>5mm (%)	No. of Shoots/Explant	Length (mm)	Fresh wt (mg)
0	0	31	2.4±0.4	29.2±4.8	19.3±2.7
0	0.2	12	0.2±0.1	5.3±0.8	8.1±1.6
0	0.5	0	0.0±0.0	0.0±0.0	0.0±0.0
0	0.7	0	0.0±0.0	0.0±0.0	0.0±0.0
2.0	0	100	4.1±0.6	53.8±12.6	63.8±9.9
2.0	0.2	60	0.8±0.2	19.0±4.3	28.4±3.9
2.0	0.5	0	0.0±0.0	0.0±0.0	0.0±0.0
2.0	0.7	0	0.0±0.0	0.0±0.0	0.0±0.0
5.0	0	100	4.8±0.9	57.7±11.9	73.1±11.2
5.0	0.2	62	0.8±0.3	18.6±4.0	31.3±3.7
5.0	0.5	49	0.5±0.2	9.0±1.1	16.0±4.7
5.0	0.7	0	0.0±0.0	0.0±0.0	0.0±0.0
7.0	0	86	1.1±0.3	23.3±8.1	29.9±4.2
7.0	0.2	32	0.5±0.1	14.8±3.6	19.8±2.3
7.0	0.5	25	0.3±0.1	7.9±1.2	11.6±2.0
7.0	0.7	0	0.0±0.0	0.0±0.0	0.0±0.0

Field growth of the plantlets was greatly influenced by the type and level of growth regulator [BA and ABA] applied during the 6-week subculture of the explants after the initial 15-month conservation period. Table 3 shows that shoot growth was greatly enhanced by the incorporation of BA [2.0-5.0 mg l^{-1}] in the subculture medium especially in the absence of ABA. Here, shoot fresh weights were as high as 202.7 and 231.0 g per explant when BA was incorporated at 2.0 and 5.0 mg l^{-1} respectively, in the absence of ABA. These values were significantly higher [$P = 0.05$] than 106.9 g obtained in the absence of BA [control] in the subculture medium. Incorporation of BA beyond 5.0 mg l^{-1} resulted in a significant depression of shoot growth [Table 3].

On the other hand, incorporation of ABA [0.2-0.5 mg l^{-1}] into the subculture medium resulted in earlier tuberization in the field. The earliest incidence of tuber formation in the field was recorded under 0.2 mg l^{-1} ABA/2.0 mg l^{-1} BA and 2.0 mg l^{-1} ABA/ 5.0 mg l^{-1} BA. Under these two treatments, tuberization was noticed 42 days from transfer of the plantlets to the field [Table 3]. Conversely, the absence of ABA in media containing BA delayed tuber formation substantially in the field. For instance, when BA was incorporated at 2.0 and 5.0 mg l^{-1} in the absence of ABA, tuberization occurred after 91 and 98 days respectively in the field.

A comparison of the % tuber / whole plant fresh weight after 128 days of growth in the field under each treatment showed that this parameter was higher for plants in which tuberization commenced earlier [Table 3] .

Table 3: Effect of Incorporating Low Levels of BA and ABA in the Second Passage Culture Medium on Vegetative Growth of Explants, and Eventual Tuber (bulbil) Yield after Growing for 128 Days in the Filed

Treatment		Fresh Weight (g)			Days to Tuberization	% Tuber/whole plant	
BA (mgl^{-1})	ABA (mgl^{-1})	Root	Shoot	Tuber	Whole Plant		
0	0	46.6	106.9	13.7	167.2	70	8.2
0	0.2	33.9	88.1	51.3	173.3	63	29.6
2.0	0	96.0	202.7	26.0	324.7	91	8.0
2.0	0.2	36.2	93.3	89.9	219.4	42	41.0
5.0	0	98.3	231.0	36.2	365.5	98	9.9
5.0	0.2	51.0	112.6	146.2	309.8	42	47.2
5.0	0.5	28.6	80.2	55.8	164.6	49	33.9
7.0	0	38.0	101.0	13.6	152.6	98	8.9
7.0	0.2	33.6	97.4	29.3	160.3	56	18.3
7.0	0.5	28.4	83.8	9.4	121.6	63	7.7

Discussion

Successful maintenance of *Dioscorea bulbifera* culture for as long as 15 months *in vitro* without subculture as reported in this study, has a lot of positive implications for its germplasm conservation. One of the greatest problems of *in vitro* culture of *Dioscorea* species in general is the ease with which phenolic compounds are produced in culture [Ammirato, 1984; Crompton and Preece, 1985]. These phenolics would normally impede growth and ultimately lead to death if the cultures are not transferred to fresh media, with the attendant risk of contamination during these transfers. The presence of nectrotic leaves under some of the treatments employed in this study might have been a direct consequence of interruption of growth processes by these phenolics. The regeneration and maintenance of healthy plantlets [i.e plantlets without necrotic leaves] for as long as 15 months in MS [Murashige and Skoog, 1962] or B5 [Gamborg *et al*, 1968] medium supplemented with 0.5 mg l^{-1} ABA with 12% sucrose as carbon source might have resulted in slow growth which was imposed on the culture by the presence of the growth retardant, ABA. This is consistent with some previous reports in which ABA was employed in the slow growth of similar tuberous crops such as *Solanum tuberosum* [Powell and Caligary, 1989, Westcott, 1981], *Ipomoea batatas* [Liu *et al.*, 2001; Gosukonda *et al.*, 1995] and over 1,000 accessions of the white yam, *Dioscorea rotundata* [Ng ,1992].The present study is the first report on successful prolonged conversation of *D. bulbifera* by the *in vitro* reduced [slow] growth method.

The need for high levels of sucrose as carbon source for long-term storage of cultures, as also found in this study [up to 12%] corroborates earlier reports for some other tuberous crops like Irish potatoes [8%] [Veramendi *et al.*, 1998; Dodds *et al.*, 1991] and white yams [Ng. 1988]. At such high levels it was possible to store the cultures for 15 months without subculturing in this study, an improvement over that reported for Irish potato [Veramendi *et al.*, 1998] and comparable to that reported for some other yam species [1-2years] [Ng, 1988]. Low sucrose levels [about 3%] as carbon source, on the other hand, resulted in culture deterioration [as shown by leaf necrosis] due to prolonged storage and hence the need for renewal of media. Ordinarily, under optimal conditions, this plant requires subculture every 4-6 weeks [Okezie, 2001] while Irish potato [another tuberous crops] requires 6-8 weeks per culture passage [Veramendi *et al*, 1998].

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