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In Vitro Stimulation of the Auxiliary Nodal Complex for Enhanced Propagule Production In Five Edible *Dioscorea* (Yam) Species

Okezie, C.E.A. and Nwokolo, C.B.
Laboratory for Plant Tissue Culture and Biotechnology, Department of Botany, University of Nigeria, Nsukka

Abstract

Five species of edible yams, namely; *Dioscorea alata, D. bulbifera, D. cayenensis, D. dumetorum* and *D. rotundata* were screened on full strength MS media supplemented with a cytokinin (benzyl adenine) and an auxin (indolebutyric acid) for their morphogenetic capacity using axillary nodal explants. Shoot organogenesis was obtained for all the species, in which all the primordia within the axillary complex differentiated into shoots, except for *D. bulbifera* in which only three primordia showed shoot proliferation while the bulbil primordium proliferated into a profuse callus. Shoots arising from the explants were excised singly and rooted within 10 days in media containing 5.0 μM NAA. The resulting plantlets were hardened, and grown for 16 weeks into plants that yielded tubers (weighing between 39.2 ± 4.7 and 53.6 ± 8.2 g at harvest), which served as propagules for subsequent field planting.

Key Words: *In vitro* stimulation, Enhanced propagule production, *Dioscorea* species

Correspondence: carlokezie@unn.edu.ng

Introduction

Yams (Dioscorea spp.) are a major carbohydrate staple in the tropical and subtropical regions of the world, where they rank second in importance only to cassava. Some species, namely; *Dioscorea bulbifera* and *D. dumetorum* produce tubers which, in addition to serving as food, also contain high levels of pharmaceutically important secondary metabolites, notably alkaloids and diosgenins (Coursey, 1967; Degras, 1993).

New technologies for enhanced propagule production have involved regeneration by minisetts (Iwueke *et al.,* 1983), propagation by seed (Sadik and Okereke, 1975) propagation by nodal vine cuttings (Akoroda and Okonmah, 1982; Okezie *et al.,* 1999). Propagation by nodal vine cuttings involves the stimulation of the axillary complex and subsequent emergence of one or more shoots from the axillary buds. Studies involving the anatomy of the axillary complex have shown the presence of three to five bud primordia in the axillary complex of five edible *Dioscorea* species (Nwoke, 1987). Usually only one (the primary primordium) proliferates, developing into a new shoot during regeneration, while the rest (the subsidiary primordia) remain quiescent. However, Okezie (2003) was able to induce three of the four primordia in *D. bulbifera in vitro* with consequent proliferation of multiple shoots from this species.

The present study reports the successful induction of shoot proliferation from both the primary shoot primordium and all the subsidiary primordia in four other edible *Dioscorea* species, namely: *D. alata*, *D.bulbifera, D. cayenensis* and *D. dumetorum*, as well as *D. rotundata*. The implications of such proliferation for supplemental propagule production in seed yam production are also discussed.

Materials and Methods

Source of Explants: Whole, healthy, non-dormant underground tubers of four edible yam species, namely: *Dioscorea alata* Linn., *D. cayenensis* Lam., and aerial tubers of *D. bulbifera* L. were used to raise seedlings from which the explants (axillary buds) were sourced. Whole tubers (seed yams) weighing between 35 and 40 g were randomly selected and sown in well irrigated, potted, loamy soil in a screenhouse in the Botanical Garden of the Department of Botany, University of Nigeria, Nsukka, and grown for six weeks. Luxuriantly growing healthy seedlings were selected for their visual uniformity from which axillary buds were collected from the

ten uppermost nodes from each of the five experimental species to serve as explants for the studies.

Explant Preparation: To prepare the explant, an incision was made 5mm below and 2mm above each node along the vine axis by means of a sterile scalpel, thereby severing the node with its bud from the main vine. The subtending leaf at the node, in whose axil lay the axillary bud, was then carefully severed at the base of the petiole thereby freeing the bud (explant) as much as possible from the physiological control of the leaf. Each bud which measured between 3 and 5 mm in length (depending on the species), with its proximal and distal stumps was surface – sterilized by immersion for 10 min in 0.01 per cent (w/v) mercuric chloride containing 7.5 per cent (v/v) Teepol (a surfactant), rinsed five times in sterile distilled water, and then kept covered in a sterile beaker containing sterite double–distilled water prior to inoculation into the test media.

Establishment and Maintenance of Cultures: Basal media comprising macro and micro salts and vitamins of MS (Murashige and Skoog, 1962) and its modifications were screened at full strength for the growth of the experimental explants. Factorial experiments were designed in which these media were supplemented with an auxin (∝ - naphthaleneacetic acid) and a cytokinin (6 – benzylaminopurine) at 0, 2.0 and 5.0 μM either singly or in several combinations. Sucrose was used at 3.0 per cent (w/v) as carbon source while activated charcoal was employed at 1.0 per cent (w/v) in order to adsorb phenolic compounds characteristically produced in yam tissue cultures capable of retarding explant growth (Crompton and Preece, 1986). The pH of the media was adjusted to 5.8 before the addition of 0.7 per cent (w/v) agar (Difco-Bacto). All chemicals used were of analytical grade (Sigma and Merck). Molten medium was dispensed at 100 ml per flask into 250 ml Erlenmeyer flasks. The culture flasks containing the media were sealed with parafilm and sterilized by autoclaving at 121^0C and 1.05 kg cm^{-2} pressure for 20 min. The surface – sterilized explants were implanted vertically with the proximal vine stub of the nodal segment dipping into the semi-solid agar at four explants per flask and five replicate flasks per treatment. All transfers were done in a Laminar flow hood previously kept sterile by exposure to ultraviolet light for 30 min. All cultures were maintained on racks in a growth room at 27 ± 2^0C less than 16h photoperiod of 45-50 μmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Phillips, Made in England). After 28 days, the cultures were scored for shoot organogenesis. Only shoot buds and prominent shoots measuring at least 3mm in length were scored.

Shoot Elongation and Rooting of Shoots: Shoots formed *in vitro* were carefully excised by means of a sterile scalpel and cutured singly in fresh MS media fortified with 10 μM BA or kinetin and devoid of auxin. They were grown for a further 14 days after which they were rooted by subculturing for 10 days in fresh media containing 5.0 μM ∝-naphthaleneacetic acid or indolebutyric acid.

Acclimatization and Transfer of Plantlets to the Field: Well rooted plantlets were removed from the culture and the agar adhering to the roots washed off under running tap water, before transfer to 15 cm – diameter plastic planting buckets containing "soilrite". These were kept in plastic mist chambers initially at 80 – 85% relative humidity in a shade in the Botanical Garden, University of Nigeria, Nsukka. They were watered twice daily by means of a spray gone for the first 5 days. Subsequently, perforations were made on the plastic cover of the mist chambers white the frequency of watering was gradually reduced, thereby reducing the relative humidity to ambient level by the 14^{th} day. The hardened plantlets were then transferred to soil and grown to full maturity and thereafter scored for the fresh weight of the harvested tubers produced by each of the five species.

Statistical Analysis: The experimental design was random and factorial with auxins and cytokinins as independent variables. Data pertaining to number, types, length and fresh weight of organs formed were subjected to analysis of variance (ANOVA). Mean values were separated using Duncan's New Multiple Range test. There were twenty replicate samples per treatment and all the experiments were repeated twice.

Results

Explant proliferation commenced within 7 days and by the 10^{th} day, all the organs (shoot, S; root, R; and callus, C) were proliferated depending on the strength of hormonal combination (Table 1). Table 1

also shows that whereas only shoots were proliferated within 14 days when NAA and BA were combined at 0 –2.0 μM and 0-10.0 μM, respectively, among all the species, *D. bulbifera* always produced callus in addition to shoots and roots.

Optimum shoot production was obtained when the basal medium (MS) was supplement with 2 μM NAA and 10 μM BA (Table 2). At these hormonal combinations, number of shoots produced ranged from a minimum of 5.2 ±1.1 to a maximum of 6.8 ±1.6 in media supplemented with 2 μM NAA and 10 μM BA, among all the species *D. bulbifera* in which only three shoots (3.2 ± 0.9) were produced under the most effective hormonal combination (Table 2). It is apparent that all the primordia within the axillary complex of *D. alata, D. cayenensis, D. dumetorum,* and *D. rotundata*, were successfully induced to produce shoots whereas only three out of the four (i.e. 75%) *D. bulbifera* were successfully induced (Table 3), in which the bulbil primordium differentiated into a bulbous mass of callus.

Shoots and shoot buds produced by the nodal explants were substantially elongated within 14 days in fresh media supplemented with 10μM BA. Shoot elongation as well as fresh weight of shoots varied with species. Among the species in which shoot organogenesis was 100 per cent (see Table 3), the longest shoots were obtained for *D. dumetorum* (41.8 ± 9.6 mm) and down to 33.6 ± 6.1 mm for *D. cayenensis* (Table 4).

On the other hand, *D. bulbifera*, in which there was only 75 per cent shoot induction, recorded a significantly lower shoot elongation (19.9± 4.2 mm) relative to the other species (Table 4). Shoot fresh weight, on elongation, showed the same trend as shoot length except that the highest value for this parameter was recorded for *D. alata* (Table 4).

All the elongated shoot explants were successfully rooted in MS media supplemented with 5.0 μM NAA (Table 5). While the number of roots varied from 7.1± 1.4 to 10.3± 2.4 and fresh weight of roots ranged from 4.7 ± 0.8 to 6.6 ± 1.3 mm and from 55.7 ± 6.9 to 63.9 ± 11.3 mg, respectively, among the species, there were no significant differences (P = 0.05) among the species in these three growth parameters at 5 μM NAA supplementation of the basal MS media (Table 5)

Fresh weight of tuber obtained at harvest (after a 16 – week growth in the field) was consistently lower than the original weight of tuber used to generate the seedlings from which nodal explants were taken. These values ranged between 73% for *D. alata* and 88% for *D. rotundata* (Table 6).

Table 1: Types of Organs or Callus Produced by Nodal Explants of Five *Dioscorea* Species after 14 Days in MS Supplementedwith BA and ∝-NAA

Treatments		Types of Dioscorea				
NAA(μM)	BA(μM)	D. alata	D.bulbifera	D. cayenensis	D. dumetorum	D.rotundata
0	0	-	c	1	-	-
0	2	S	C	S	S	S
0	5	S	CS	S	S	S
0	10	S	CS	S	S	S
2	0	R	CR	R	R	R
2	2	SR	CSR	SR	SR	S
2	5	SR	CSR	S	S	S
2	10	SR	CS	S	S	S
5	0	R	CR	R	R	R
5	2	SR	CR	R	R	R
5	5	SR	CSR	SR	SR	SR
5	10	SR	CSR	SR	SR	SR

S=Shoot; R=Root; C=Callus

Table 2: Number of Shoots Produced after 14 Days by Leafless Nodal Explants of Five Edible Species of *Dioscorea* in Basal MS Media Supplemented with ∝-NAA and BA

Treatments		Types of Dioscorea				
NAA(μM)	BA(μM)	D. alata	D.bulbifera	D. cayenensis	D. dumetorum	D.rotundata
0	0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
0	5	2.6±0.7	0.8±0.2	3.2±0.8	3.9±1.0	2.2±0.6
0	10	1.9±0.5	0.6±0.3	3.0±1.0	2.4±0.5	2.0±0.8
0	20	0.9±0.2	0.2±0.1	0.9±0.4	0.9±0.3	0.7±0.3
2	0	0.3±0.1	0.0±0.0	0.2±0.1	0.5±0.2	0.3±0.1
2	5	5.3±0.9	2.3±0.7	5.2±1.1	6.6±1.8	4.8±0.9
2	10	6.2±1.8	3.2±0.9	6.8±1.6	5.8±0.9	5.2±1.1
2	20	3.9±0.9	2.7±0.6	4.1±1.2	4.6±1.1	5.3±1.4
5	0	0.8±0.2	0.6±0.2	1.1±0.4	0.9±0.3	1.3±0.6
5	5	2.1±0.7	0.9±0.4	3.1±0.9	2.8±0.9	2.2±0.9
5	10	1.7±0.7	0.4±0.1	1.3±0.4	1.9±0.7	1.4±0.6
5	20	1.5±0.8	0.4±0.2	1.1±0.5	1.7±0.7	1.6±0.6

S=Shoot; R=Root; C=Callus

Table 3: Per Cent Shoot Induction in Relation to Number of Primordia in the Axillary Complex

Dioscorea Species	Number of Primordia n Axillary Bud	Number of Shoots Produced	Per cent of Induction
D.alata	5	6.2 ±1.8	100
D.bulbifera	4	3.2±0.9	75
D. cayenensis	4	6.8±1.6	100
D.dumetorum	6	6.6±1.8	100
D.rotundata	4	5.3±1.4	100

Table 4: Shoot Elongation and Fresh Weight of Shoots Produced after 14 Days by *Dioscorea* Shoot Explants in MS Media Supplemented with 10μM BA

Dioscorea Species	Length (mm)	Fresh Weight (mg)
D.alata	39.±5.3	114.2±24
D.bulbifera	19.9±4.2	62.2±7.8
D. cayenensis	33.6±6.1	102.9±18
D.dumetorum	41.8±9.6	98.8±13.9
D.rotundata	28.8±6.8	76.0±13.7

Table 5: Number, Length and Fresh Weight of Roots Produced by *Dioscorea* Shoot Explants after 14 Days in MS Media Supplemented with 5.0 μM NAA

Dioscorea Species	No. of Roots	Length (mm) of Roots	Fresh Weight(mg) of Roots
D.alata	8.8±2.1	5.2±0.8	60.8±7.6
D.bulbifera	10.3±2.4	6.6±1.3	63.9±11.3
D. cayenensis	7.5±1.9	5.3±0.9	57.3±7.0
D.dumetorum	8.2±2.5	5.6±1.0	63.8±9.7
D.rotundata	7.1±1.4	4.7±0.8	55.7±6.9

Table 6: Comparative Weight of Tuber at Harvest to Initial Propagule Tuber Weight

Dioscorea Species	Initial wt (g)	Final wt (g)	% Yield
D.alata	73.4±9.9	53.6±7.8	73
D.bulbifera	56.8±6.3	39.2±5.7	69
D. cayenensis	62.4±7.7	41.8±6.1	67
D.dumetorum	59.5±7.9	45.2±7.2	76
D.rotundata	49.5±6.4	43.6±7.6	88

Discussion

The employment of nodal explant for *in vitro* yam propagation exploits the fact that if properly induced,the axillary bud within an excised node is capable of regenerating new shoot that could be rooted, thereby yielding a seedling that could serve as propagule, rather than the

consumable tuber (the erstwhile conventional propagule). This was first demonstrated with leafy nodal explants of *D. bulbifera* (Njoku, 1963), and later in *D. rotundata* (Akoroda and Okonmah, 1983), as with many other plant species including tobacco(Ramage and Williams, 2004) and *Ilex dumosa* (Luna *et al*., 2003). Generally, axillary bud explants are known to possess pre-existing meristem that could develop into shoots while maintaining clonal fidelity (Kyesmu *et al*., 2004; Cao *et al*., 2007).

Anatomical studies of the axillary complex of the five edible *Dioscorea* (yam) species employed in this study, revealed a variability in the number of shoot primordia among them . *D. alata* and *D. dumetorum* possess five shoot primordia each (one primary and four accessory); *D. cayenensis* and *D. rotundata* possess four (one primary and three accessory), while *D. bulbifera* has one bulbil primordium and three shoot primordia. Pioneer workers (Njoku, 1963; Nwoke, 1987) on shoot organogenesis from yam nodal explants reported that only one (the primary bud primordium) is involved in *in vitro* shoot formation in the *Dioscorea,* while the accessory primordia remain quiescent. Our current study in which all the shoot primordia in the five species hereby reported were successfully induced, resulting in the production of up to three shoots in *D. bulbifera,* five in *D. cayenensis* and *D. rotundata,* and as many as six in *D. dumetorum,* runs contrary to the earlier view. Our studies involved additional external supplementation of a cytokinin (benzyl adenine) in Murashige and Skoog's (1962) basal media at 2 to 10 μM for up to 10 days. Such supplementary hormonal level employed in our study, is most likely to have been optimal for such high level of shoot organogenesis in these edible yam species. Such high frequency shoot formation obtained with increasing BA concentration between 2 and 10 μM is consistent with some earlier reports on some other tuberous crops such as potatoes (Gosukonda *et al.,* 1995). The lesser degree of shoot organogenesis exhibited by *D. bulbifera* nodal explants, relative to the other four species employed in this study, can only be accounted for by its having the lowest number of inducible shoot primordia. Coupled to this is the fact that unlike the rest, *D. bulbifera* also possesses a bulbil primordium which rather differentiated into a callus at hormonal concentrations that enhanced only shoot formation in the other four species. This is not surprising as only *D. bulbifera* is known to produce edible aerial bulbils which have been suggested (Njoku, 1963; Degras, 1993) to result from the differentiation of the bulbil primordium.

The level of shoot organogenesis from the nodal explants reported here for the five edible *Dioscorea* species is remarkable from the point of view of supplemental propagule production for yam propagation. Although the tubers produced at harvest were considerably lower in fresh weight (between 39.2 ± 5.7 g and 53.6 ±7.8 g) as against a minimum fresh weight of 49.5 ±6.4 g and maximum weight of 73.4 ± 9.9 g, used for raising the seedlings from which the explants were taken, it should be borne in mind that each seedling bears several nodes each of whose axillary complex bears between three and five potential progagules, depending on species. Therefore, the potential number of propagules that could be yielded by each species is enormous.

Conclusion

In methods described here, most (if not all) of the shoot meristems of the axillary complex have the potential to yield shoots and shoot buds that could be elongated and rooted to yield seedlings to serve for propagating (at least for seed yam production) these edible *Dioscorea* (yam) species. Successful deployment of this technology would save most of the harvested yam tubers for consumption rather than plough at least, thirty per cent of it (the tuber) back into the ground during the next planting season, as is currently practiced.

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