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Plantlet Regeneration of Potato Yam (*Dioscorea bulbifera* L.) through *in Vitro* Culture from Nodal Segments

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ABSTRACT

Nodal vine segments from 6-week-old plants of *Dioscorea bulbifera* L. were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP) and kinetin (Kn) together with α-naphthaleneacetic acid (NAA). Explants cultured in MS basal medium supplemented with 2.0 mg/l Kn + 1.0 mg BAP + 0.5 mg/l NAA showed highest number (8.5 ± 0.51) of multiple shoots. When excised, shoots raised *in vitro* were inoculated onto half-strength MS basal media supplemented with 2.0 mg/l NAA, where rooting was profuse (5.8 ± 0.39 roots per plantlet). Rooted shoots were transplanted to the greenhouse for hardening and; survival was 90% in field conditions without any visible morphological variation.

Keywords: micropropagation, MS medium, nodal vine cutting, plant growth regulator

INTRODUCTION

Yam is a monocotyledonous natural group of tuber-forming tropical vines, which belongs to the order *Liliflorae*, family *Dioscoreaceae*, and genus *Dioscorea.* The *Dioscoreaceae* is considered to be the most primitive family among the angiosperms and contains over 600 species, of which only about 10 are considered as edible and cultivated as vegetable crops (Coursey 1967). *D. bulbifera* is one of the cultivated species with medicinal importance. It is cultivated in Southeast Asia, West Africa, and South and Central America. The wild form also occurs in both Asia and Africa (Martin 1974). The plant is characterized by the production of considerable numbers of aerial tubers or bulbils per plant and consumed in the Eastern part of Nigeria. Orissa is an important center of wild and different cultivars of *Dioscorea*. The species *D. bulbifera* is locally named as Pita kanda, Pita alu, Banalu, Kukuralu, Goicha alu, etc., found both in wild and domestic habitats throughout the state and mainly eaten by tribal peoples in times of famine after boiling and with much preparation. It is a left-twining species with simple alternate leaves including distinct male and female flowers of panickled spikes. It is termed air potato or potato yam in English and is grown chiefly for its edible aerial tubers. Bulbils are used externally for treating sores and paste from the tuber is used as a cure for snake bite (Sreeja *et al.* 2005; Behera 2009).

Plants of the genus *Dioscorea* have been cultivated for centuries as a food crop; the underground storage organs and aerial bulbils, where present, provide a good source of nutrition (Ogbuagu 2008). In recent years, however, these storage organs have been increasingly exploited for their considerable yields of diosgenin, which is the main precursor for the synthesis of steroidal drugs. Diosgenin, a steroidal sapogenin, is used for the synthesis of cortisones, corticosteroids, sex hormones (progesterone) and oral contraceptives (Aradhana and Kale 1992; Mandal 2006). Dhawan *et al*. (1977) reported that *D. bulbifera* has diuretic and anti-inflammatory activity. It is also one of the major Indian medicinal plants used in indigenous medicine (Kamboj 2000). Traces of diosgenin have also been detected in this species (Quigley 1978).

A major problem in the cultivation of *Dioscorea* species for commercial purposes is the lack of a rapid propagation technique. Both sexual and asexual methods are fraught with problems. Since plants are dioecious, the chances of finding fertile seed are automatically reduced. Additionally, these plants are characterized by low flowering rates, very low rates of fruit setting and poor seed germination (Abraham *et al.* 1986). Vegetative propagation by tuber fragmentation is the normal means of multiplication (Chaturvedi *et al.* 1982). Propagation by leaf and stem cuttings has been attempted, but rooting and survival was not more than 30% (Quamina *et al.* 1982). For vegetative propagation, most involves the tuber, so the efficiency of vegetative propagation is low and depends on breaking of seed dormancy. In addition, in the process of storage and cultivation, the materials used for vegetative propagation are susceptible to diseases that cause tissue senescence and degeneration. Plant tissue culture techniques can be used to conserve germplasm resources and even to rapidly multiply and breed elite varieties within short span of time, supply of planting material on a year-round basis (Han *et al*. 2000). Some researchers have studied organ (Sedigeh *et al*. 1998; Kohmura *et al*. 1995), callus (Chen *et al*. 2003; Yuan *et al*. 2005), and cell culture (Osifo 1988; Twyford and Mantell 1996) of other plants from the genus *Dioscorea*. Investigations on *D. bulbifera*, however, have not yet been described. Plant tissue culture and cell culture have a number of advantages and have been called the third mode of production after wild growth and cultivation (Breat and Carlv 1985).

Due to various anthropogenic activities like deforestation, pasturing, urbanization and conversation of denuded forest land into agricultural land the natural habitat of the species is lost at an alarming rate. Hence, urgent conservation strategies are required to preserve the genetic stock of the species before being threatened. An efficient tissue culture method would be a solution to the problem. Nodal stem segments *of Dioscorea* species have been successfully cultured *in vitro* (Forsyth and Van Staden 1982; Malaurie *et al.*

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1995; Okezie 2003). The present study describes a suitable protocol for *in vitro* plantlet regeneration in *D. bulbifera* through nodal vine culture. The methodology would definitely be helpful for obtaining large-scale disease-free plantlets to meet different purposes and conservation and domestication of wild species for sustainable development of food security.

MATERIALS AND METHODS

Explant source

Healthy vines with active buds were collected from 6-week-old plants of *D. bulbifera* maintained in the experimental garden of the P.G. Department of Botany, Utkal University and were cut into 1.5-2.0 cm long explants with intact single nodes. These nodal vine cuttings were washed with 5% (v/v) detergent solution (Teepol, Qualigen, Mumbai, India) for 10 min and rinsed several times with running tap water. They were surface sterilized with 0.3% (w/v) bavistin and 0.2% (w/v) streptomycin for 10 min each and then washed with sterile distilled water (SDW). In the laminar flow chamber the nodal segments were again treated with 70% alcohol for 30 sec to 1 min followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) treatment for 3-5 min. Finally, explants were washed thoroughly 3-4 times with SDW and dried with sterile blotting paper.

Culture medium and conditions

The sterilized blotted explants were inoculated onto Murashige and Skoog (1962) agar-gelled medium fortified with various concentrations/combinations of plant growth regulators (PGRs). For shoot induction, the medium was supplemented with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l kinetin (Kn) + 0.25, 0.5, 1.0, 1.5 mg/l 6-benzylaminopurine (BAP) and 0.25, 0.5, 1.0 mg/l α-naphthalene acetic acid (NAA), either individually or in combination with ascorbic acid 100 mg/l as an antioxidant. For root induction, *in vitro* raised shoots ~4–5 cm in height in multiplication medium were excised and cultured on half-strength MS basal medium supplemented with either NAA or IBA (indole-3-butyric acid) at 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l. Routinely, 25 ml of molten medium was dispensed into culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The pH of the medium was adjusted to 5.8 with using 0.1 N NaOH or 0.1 N HCl before autoclaving (1.04 kg/cm^{-2} at 121°C) for 15-20 min. All cultures were maintained in a 16-h photoperiod at 25 ± 3°C in diffused light (PHILIPS LFEMAX 18W/546500 °K fluorescent light; 30 µmol m^{-2} S^{-1}) and 60-70% relative humidity (RH) in the culture room. Each treatment was comprised of 20 culture tubes and the experiment was repeated in triplicate. The cultures were maintained by regular subcultures at 4-week intervals on fresh medium of the same composition and under the same culture environment.

Acclimatization

Rooted micropropagules were removed from culture tubes and roots were washed under running tap water to remove agar. The plantlets were transferred to 2.5 cm sterile poly pots (each pot holding one plantlet) containing 200 g vermiculite (TAMIN, India) inside a growth chamber at 28°C and 70-80% RH. After 3 weeks they were transplanted to earthen pots (8 cm in diameter and 10 cm in height) containing a mixture of soil, sand and manure (1: 1: 1) and kept under a shade-house for a period of 3 weeks to acclimatize. The potted plants were irrigated with Hoagland's (Hiregoudar *et al*. 2006) solution (50 ml per pot) every 3 days for a period of 3 weeks. The survival rate of plantlets was recorded after 3 weeks (Behera *et al.* 2009).

Experimental design and data analysis

A set of 20 cultures tubes were used per treatment and each experiment was repeated at least 3 times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and rooting were statistically calculated by row mean/total analysis using Graphpad Prism 5 statistical software.

RESULTS AND DISCUSSION

Shoot proliferation and multiplication

The response of *D. bulbifera* nodal vine explants cultured on different shoot proliferation media over a period of 6 weeks is presented in **Table 1**. Culture medium devoid of PGRs (control, T1) failed to stimulate the bud break response in the cultured explants even when the cultures were maintained beyond the normal observation period of 4 weeks. MS medium with PGRs produced better results in

Table 1 Shoot formation in nodal explants of *Dioscorea bulbifera* L. cultured on semi-solid MS medium supplemented with various concentrations of Kn + BAP and NAA.

Treatments	Plant growth regulators (mg/l)			% of explant response	Days to bud break	Mean No of shoots/explant (± SEM)	Mean shoot length (cm) (± SEM)	Mean No of nodes/shoot (± SEM)
	Kn[a]	BAP[b]	NAA[c]					
T1	0	0	0	-	-	-	-	-
T2	0.25	0.25	0	5	12-15	1.2 ± 0.06*	1.0 ± 0.09*	0.98 ± 0.09*
T3	0.5	0.25	0	10	12-15	1.2 ± 0.19*	1.2 ± 0.14*	0.96 ± 0.12*
T4	1.0	0.25	0	10	12-15	1.3 ± 0.16*	1.3 ± 0.09*	1.0 ± 0.09*
T5	1.5	0.25	0	15	12-15	1.5 ± 0.21	1.5 ± 0.18	1.0 ± 0.13
T6	2.0	0.25	0	20	12-15	2.0 ± 0.48	1.8 ± 0.23	1.0 ± 0.22
T7	2.5	0.25	0	20	10-12	2.5 ± 0.32	2.2 ± 0.26	1.6 ± 0.33
T8	3.0	0.25	0	20	12-15	2.0 ± 0.34*	2.2 ± 0.21*	2.0 ± 0.32*
T9	0.25	0.5	0.25	30	10-12	3.0 ± 0.33	2.8 ± 0.22	2.2 ± 0.28
T10	0.5	0.5	0.25	35	10-12	3.2 ± 0.19	3.2 ± 0.39	2.2 ± 0.26
T11	1.0	0.5	0.25	45	8-10	3.3 ± 0.26*	3.4 ± 0.42*	2.3 ± 0.29*
T12	1.5	0.5	0.25	64	8-10	4.4 ± 0.28	4.0 ± 0.41	2.4 ± 0.15
T13	2.0	0.5	0.25	71	6-8	5.5 ± 0.41	4.8 ± 0.35	3.6 ± 0.35
T14	2.5	0.5	0.25	62	7-9	3.2 ± 0.14	2.7 ± 0.37	1.4 ± 0.15
T15	3.0	0.5	0.25	43	8-10	2.5 ± 0.18*	2.4 ±0.34*	1.3 ± 0.18*
T16	0.25	1.0	0.5	28	10-12	3.0 ± 0.52	2.4 ± 0.25	1.0 ± 0.32
T17	0.5	1.0	0.5	34	10-12	3.5 ± 0.30*	3.2 ± 0.22*	2.2 ± 0.21*
T18	1.0	1.0	0.5	40	8-10	4.0 ± 0.29*	3.4 ± 0.16*	2.1 ± 0.33*
T19	1.5	1.0	0.5	78	7-9	6.3 ± 0.14	3.8 ± 0.16	2.3 ±0.22
T20	2.0	1.0	0.5	88	6-8	8.5 ± 0.51	5.9 ± 0.44	4.4 ± 0.24
T21	2.5	1.0	0.5	52	10-12	2.5 ± 0.31*	2.3 ± 0.28*	1.0 ± 0.64*
T22	3.0	1.0	0.5	46	12-15	2.3 ± 0.23*	2.2 ± 0.20*	1.1 ± 0.09*

[a] Kinetin; [b] 6-benzylaminopurine; [c] α-naphthalene acetic acid; * Callusing at the basal end

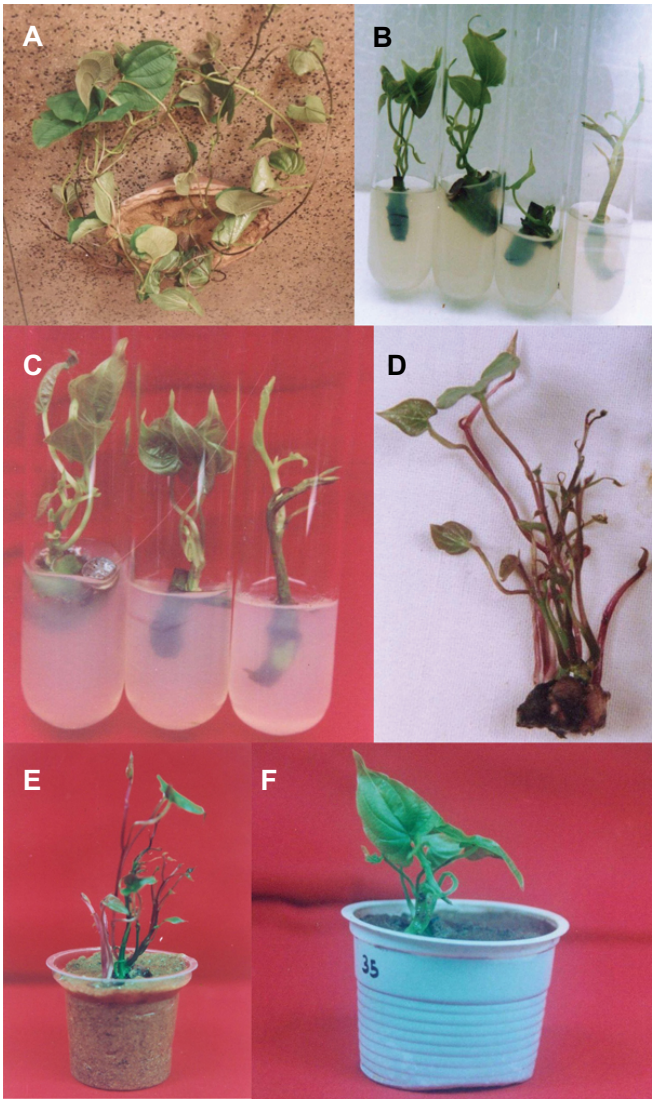

Fig. 1 ***In vitro*** **regeneration and plant establishment of** ***D. bulbifera*** **L.** (**A**) Potted plant from where explants are collected. (**B**) Shoot emergence in MS + 2.0 mg/l Kn + 1.0 mg/l BAP + 0.5 mg/l NAA + 100 mg/l ascorbic acid. (**C**) Multiple shoot emergence in MS + 2.0 mg/l Kn + 1.0 mg/l.0 BAP + 0.5 mg/l NAA + 100 mg/l vitamin C. (**D**) Shoots rooted in ½-MS + NAA (2.0 mg/l) and removed from culture for hardening. (**E, F**) Hardening of rooted plantlets in plastic pots.

terms of percentage explants response, shoots/explant, average shoot length and average number of nodes produced per shoot. In such media combinations bud break was noticed within 6-8 days of culture (**Fig. 1B; Table 1**). Of the combination tested MS + Kn (2.0 mg/l) + BAP (1.0 mg/l) + NAA (0.5 mg/l) elicited an optimal response with an average number of small shoots = 8.5 ± 0.51 (**Fig. 1B, 1C; Table 1**) with a mean shoot length of 5.9 ± 0.44 cm per explant in T20 treatments. The second best shoot multiplication (6.3 ± 0.14) was on MS + 1.5 mg/l Kn +1.0 mg/l BAP + 0.5 mg/l NAA medium with a mean shoot length of 3.8 ± 0.16 cm in T19 treatment. A higher concentration of Kn (2.5 and 3.0 mg/l) + BAP (1.0 mg/l) with NAA (0.5 mg/l) resulted in callusing of explants with fewer shoots. Similarly, at lower PGR concentrations and combination (T2-T11) explants shows increased callusing at the basal ends. In such cultures shoots were stunted and some produced compact callus at the base of explants. Prolonged culture on proliferation and multiplication media resulted in the blackening of basal ends of developing shoots.

Induction of rooting from *in vitro* shoots

The well developed elongated shoots were excised from shoot clumps and transferred to half-strength MS medium containing NAA or IBA. The rooting responses of shoots in terms of rooting percentage, days required for root initiation mean number of roots/shoot and mean root growth over a period of three weeks were different (**Fig. 1E; Table 2**). There was no rooting of shoots planted on auxin-free basal medium in T1 (control). Similarly, at lower levels of NAA (0.25, 0.5 mg/l, i.e., T2, T3) cultured shoots hardly rooted in the 4 weeks of observation. However, NAA at higher concentrations (1.5 and 2.0 mg/l, T6 and T5) and IBA at all concentrations (T9-T15) responded well. Rooting was highest in ½MS + 2.0 mg/l NAA medium in which ~90% (**Table 2**) of cultures responded with an average number of 5.8 ± 0.39 roots/plantlet and an average root length 4.0 ± 0.36 cm (**Fig. 1D; Table 2**). The second highest response (76%) was recorded with 1.5 mg/l of NAA (**Table 2**; T5). Emergence of root primordia was observed from the shoot base from 6 to 8 days after inoculation followed by rapid root growth. NAA was more effective than IBA in root induction as the number of days required to induce roots was only 6-8 vs 10-15 in the case of IBA.

Acclimatization and field establishment

About 90% of the rooted plantlets could be established in the greenhouse within 2-3 weeks of transfer. The plants grew well and reached 6-8 cm in height within 4 weeks of transfer (**Fig. 1E, 1F**). The acclimatized plants were established in field conditions and grew normally without any morphological variations.

In vitro shoot formation

The dependence of cultured explants on bud break response and shoot multiplication has already been established and extensively discussed (George and Sherrington 1984). This has also been recently reported in the case of micropropagation of other yams like *D. abyssinica* (Martine and Cappadocia 1991), *D. bulbifera* (Uduebo 1971), *D. composita* (Alizadeh *et al.* 1998), *D. floribunda* (Sengupta *et al.* 1984), and *D. oppositifolia* (Behera *et al.* 2009). In the present study, nodal vine explants of *D. bulbifera* showed a significantly higher response in medium with a combination of 2.0 mg/l Kn +1.0 mg/l BAP +0.5 mg/l NAA. The quality of shoots and the overall growth response in terms of average shoot length was better at this PGR combination. A comparatively lower response was recorded when BAP or Kn were added alone to the medium. Generally, the addition of either IAA or NAA to the culture medium improves shoot growth. For example, when *Spathiphyllum floribundam* cultured on medium with BA supplemented alone, limited proliferation of explants with a maximum of average of 1.8 shoots/cultured explant was observed while the addition of IAA increased the average number to 11.6 shoots/explant (Ramírez-Magon *et al.* 2001). A similar observation was reported in *Hovenia dulcis* nodal culture (Echeverrigaray *et al.* 1998). In our study two cytokinins were required for maximizing shoot multiplication. Some authors also suggested that the combination of two cytokinins were needed for better production of multiple shoots of *Aristolochia bracteolate* (Remeshree *et al.* 1994) and *Lavandula* sp. (Jordan *et al.* 1998).

In vitro rooting and plant establishment

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Kumar and Nair 1979). Most roots formed on ½MS with 2.0 mg/l NAA than with 2.0 mg/l IBA. Our observations are similar to those of Behera *et al.* (2009) in *D. oppositifolia* in which the same PGR combination (1/2 MS + 2.0 mg/l NAA) was used for profuse rooting of *in vitro*

Table 2 Influence of different levels of NAA and IBA in half-strength MS medium on rooting response of *in vitro* generated shoot lets of *Dioscorea bulbifera* L.

Treatments	PGR (mg/l)		% of rooting response	Days to root initiation	Mean number of roots ± SE	Mean root length (cm) ±SE
	NAA[a]	IBA[b]				
T1	0	0	-	-	-	-
T2	0.25	0	-	-	-	-
T3	0.5	0	24	8-10	1.6 ± 0.26*	1.0 ± 0.12*
T4	1.0	0	35	8-10	3.2 ± 0.34*	1.6 ± 0.20*
T5	1.5	0	76	7-9	4.3 ± 0.36	3.2 ± 0.22
T6	2.0	0	90	6-8	5.8 ± 0.39	4.0 ± 0.36
T7	2.5	0	38	10-12	2.1 ± 0.33*	2.4 ± 0.14*
T8	3.0	0	33	10-12	1.5 ± 0.22*	1.5 ± 0.28*
T9	0	0.25	18	12-15	1.0 ± 0.12*	1.2 ± 0.16*
T10	0	0.50	25	12-15	1.0 ± 0.23*	2.0 ± 0.04*
T11	0	1.0	48	10-15	2.2 ± 0.28	2.3 ± 0.30
T12	0	1.5	62	10-12	2.3 ± 0.35	2.6 ± 0.28
T13	0	2.0	72	10-12	2.4 ± 0.26	2.8 ± 0.34
T14	0	2.5	54	10-15	1.8 ± 0.28*	2.2 ± 0.28*
T15	0	3.0	48	10-15	1.6 ± 0.18*	1.7 ± 0.26*

[a] α-naphthaleneacetic acid; [b] indole-3-butyric acid; * Basal callusing at the cut end

plantlets. Well-rooted plantlets, when hardened under controlled conditions (**Fig. 1E, 1F**), produced new shoots and roots after 1 week. These were transferred to field conditions and the survival rate was 90% which is similar to the previous findings of Yan *et al.* (2002) in *Dioscorea zingiberensis*, Dave *et al.* (2003) in *Chlorophytum borivilianum* and Behera *et al.* (2008) in *D. hispida*.

The efficient micropropagation technique described here may be useful for raising disease-free *D. bulbifera* planting material for commercial and off-season cultivation which might help the socioeconomic development of farmers and also fulfill the food value and market demand of the species.

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