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Full Length Research Paper

In vitro propagation of wild yams, *Dioscorea oppositifolia* (Linn) and *Dioscorea pentaphylla* (Linn)

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***In vitro* propagation of two wild yams, *Dioscorea oppositifolia* and *Dioscorea pentaphylla*, is reported. Multiple shoots were initiated from nodal explants on Murashige and Skoog (MS) medium supplemented with 8.8 µM 6-benzylaminopurine (BAP) and 0.3% (w/v) activated charcoal. Root induction was also achieved simultaneously from the base of the shoots in the same medium. Individual shoots with a minimum of one node were excised and rooted *in vitro* on MS medium with 2.67 µM α-naphthaleneacetic acid (NAA) or *ex vitro* rooted on by treatment with 49 µM indole-3-butyric acid (IBA) for 30 min. Regenerants acclimatized in soil-rite showed vigorous shoot growth (within 2 weeks) and after 5 - 6 months were suitable for planting. Plantlets also developed tubers on MS medium with 8.8 µM 6-benzylaminopurine (BAP).**

Key words: *Dioscorea*, diosgenin, microtuber, nodal culture.

INTRODUCTION

The genus *Dioscorea* includes over 600 species (Ayensu, 1972), and is of considerable economic importance. A number of *Dioscorea* wild species are the source of compounds used in the synthesis of sex hormones and corticosteroids (Coursey, 1967) and cultivated species are the source of food in some tropical countries (Coursey 1976). These true yams are the source of agents used to treat such varied conditions as inflammation, joint pain, diabetes, infections and dysmenorrhea. The pharmacologically active components of the *Dioscorea* species include diosgenin, which is a steroidal saponin, and dioscin, a form of diosgenin with sugars attached (Ramberg and Nugent, 2002)

Plantlet regeneration *in vitro* for vegetative propagation of some economically important *Dioscorea* species has been achieved using nodal cuttings (Chaturvedi, 1975; Lakshmisita et al., 1976; Mantell et al., 1978; Alizadeh et al., 1998; Yan et al., 2002; Chen et al., 2003), bulbils (Asokan et al., 1983), zygotic embryos (Viana and Mantell, 1989), meristem tips (Malaurie et al., 1995a, b), immature leaves (Kohmura et al., 1995) and roots (Twyford and Mantell, 1996). Attention has been paid to the clonal propagation through *in vitro* production of microtubers in *D. abyssinica* (Martine and Cappadocia, 1991), *D. alata* (Mantell and Hugo, 1989; Martine and Cappadocia, 1991; John et al., 1993; Jasik and Mantell, 2000), *D. batatas* (Koda and Kikuta, 1991), *D. composita* (Alizadeh et al., 1998) and *D. floribunda* (Sengupta et al., 1984)

The tubers of *D. oppositifolia* are used as an herbal tonic. It stimulates the stomach and spleen and has an effect on the lungs and kidneys. The tuber has been eaten for the treatment of poor appetite, chronic diarrhea, asthma, dry coughs, frequent or uncontrollable urination, diabetes and emotional instability. Externally, the tuber has been applied to ulcers, boils and abscesses. Leaf juice from *D. oppositifolia* can be used to treat snake bites and scorpion stings (Mandy, 2002).

In this paper, we describe the cultural conditions required to provide maximal *in vitro* shoot growth of two wild yams *D. oppositifolia* and *D. pentaphylla* and also to induce microtubers.

MATERIALS AND METHODS

Field grown plants of *D. oppositifolia* Linn and *D. pentaphylla* Linn, propagated from a wild tuber were used as source of explants for *in vitro* study. The nodal segments were kept in running tap water for 45 min. A few drops of Tween 20 were added followed by fungicide

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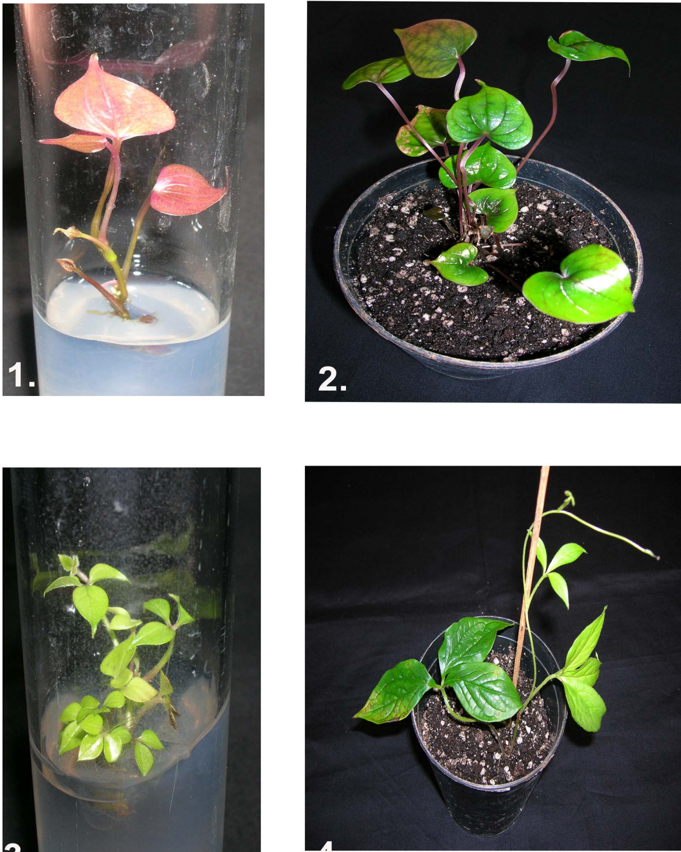

Figure 1. 1). *Dioscorea oppositifolia* aseptic culture obtained from single segment of field grown vine. 2) 90 days old plantlet regenerated from single node cutting. 3). Shoot development of *Dioscorea pentaphylla* from nodal culture after 6 weeks. 4). Regenerated *Dioscorea pentaphylla* established in pot.

treatment with 0.3% (w/v) Bavistin (Carbendazim 50% W/P), a systemic fungicide, for 2 h. The shoots were disinfected with 0.1%(w/v) mercuric chloride for 5 min, followed by thorough rinsing in sterile distilled water for at least 4 or 5 times. The surface sterilized explants were sized to 1 - 1.5 cm length containing a single node with an axillary bud. The explants were placed vertically on the MS (Murashige and Skoog, 1962) medium with or without growth regulators. The cytokinins 6-benzylaminopurine (BAP) and kinetin (KN) were used to examine their effect on axillary bud break. Activated charcoal (AC; 0. 3% W/V; LR Ranbaxy, India) or soluble polyvinylpyrrolidone (PVP; 40. 0.1% Sigma Chemical Co., St. Louis, MO. USA) was incorporated into the media to control phenolic oxidation and blackening of the explants. Sucrose (3%) was added before adjusting the pH to between 5.7 and 5.8, and the media were gelled with (0.8% w/v) agar (Regular grade, SRL, Bombay, India). Media (20 ml) were dispensed into tubes (15 X 2.5 cm) and to screw capped bottles (10 X 8.5 cm) prior to autoclaving at 121℃ at 105 Kpa for 20 min. All cultures were maintained at 25±2℃ under a 16 h photoperiod provided by cool-white fluorescent tubes (50 $\mu molm^{-2}s^{-1}$). Twelve replicates were used for each treatment. All the experiments were repeated twice.

The efficacy of both *ex vitro* and *in vitro* rooting were compared. For *in vitro* rooting, microshoots from subcultures measuring between 1.5 and 4 cm were cultured on either full-strength solid MS basal medium with 0.3% (w/v) activated charcoal and sucrose (3%, w/v) or MS liquid medium supplemented with α-naphthaleneaceticacid (NAA) and indole-3-butyric acid (IAA). For *ex vitro* rooting, the microshoots were washed with sterile water to remove traces of agar adhering to the shoot followed by immersing in 0.3% Bavistin solution for 30 min. The base of the shoot was dipped for 30, 60 or 120 min into concentrated solutions (49 µM) of IBA and immediately transferred into plastic pots containing sterile soil rite (equal proportions of decomposed coir and peat moss, Karnataka Explosives, Bangalore, India). The pots were covered with polythene bags, having been punched with holes, and maintained in the growth chamber at 80% relative humidity. After 5 weeks the number and length of roots were recorded.

Statistical analysis

Twelve explants were used per treatment on each multiplication media and rooting medium. Experiments were repeated twice by employing a completely randomized design. Collected data were analyzed by ANOVA and variations among means were compared using post hoc Duncan's multiple range test at $P<0.05$.

RESULTS AND DISCUSSION

Nodal explants of both the species were more responsive in terms of rapid bud break. The frequency and the rate of multiplication depended on the cytokinin and its concentration either alone or in combination. Enlargement and subsequent break of axillary buds was the initial response of nodal explants cultured on MS media supplemented with 8.8 µM BAP and 0.3% activated charcoal (Figure 1, Table 1). In both the species the nodal cuttings remained quiescent for about 20 days, after which swelling appeared at the site of axillary bud denting the formation of new tuberous tissue, from which roots came out first followed by the development of one or two shoots, as observed in *D. floribunda* (Chaturvedi, 1975; Lakshmisita et al., 1976). New shoots (six to eight) developed in this medium attained a mean length of 3 – 4 cm and two to three nodes within a span of 30 days in *D. pentaphylla*. While in *D. oppositifolia*, eight to ten nodes could be observed on the longest shoots.

A few cultures growing on medium containing BAP and kinetin initiated lateral shoots in the axils of leaves. All newly produced shoots in 8.8 µM BAP augmented media exhibited characteristic growth. Shoot multiplication was significantly improved by sub-culturing into same shooting medium, while the growth response of the cultured nodes to exogenous kinetin varied with the concentration of kinetin. At the lower concentrations, growth was normal while at higher concentration callus formation was induced as recorded in *D. bulbifera* (Uduebo, 1971), *D. alata* and *D. rotundata* (Mantell et al., 1978). Growth inhibitory effect of kinetin on shoot numbers of *D. oppositifolia* and *D. pentaphylla* microplants was observed. Lakshmisita et al. (1976), however, reported that the kinetin supplied at either 11.6 or 46.4 µM significantly increased the shoot development in *D. floribunda* shoots cultures. Also the promotive effects of kinetin (46.4 µM) on plantlet growth for *D. bulbifera*, which increased the number of shoots per plantlet, were shown by Forsyth

Table 1. Response of nodal explants of *Dioscorea oppositifolia* and *Dioscorea pentaphylla* on different concentrations of cytokinins after 6 weeks.

Species	Growth regulators (µM)	No. of shoots/nodal explant	Shoot formation (%)
D.oppositifolia	MS+Kn (2.32)	1.50±0,15[b]	63.5
	MS+Kn (4.65)	1.83±0.27[b]	58.3
	MS+Kn (9.29)	3.41±0.31[a]	25.0
	MS+BAP(2.22)	2.41±0.22[c]	65.5
	MS+BAP(4.44)	5.16±0.50[b]	78.0
	MS+BAP(8.87)	7.50±0.79[a]	89.3
D.pentaphylla	MS+Kn (2.32)	1.25±0.13[a]	56.8
	MS+Kn (4.65)	1.41±0.14[a]	43.6
	MS+Kn (9.29)	1.44±0.18[a]	33.3
	MS+BAP (2.22)	1.41±0.14[b]	68.6
	MS+BAP (4.44)	2.50±0.19[b]	79.5
	MS+BAP (8.87)	5.16±0.61[a]	87.8

Values represent mean ±SE (n=12).

and Van Staden (1982). In our present study, we found that BAP was more responsive than kinetin in inducing multiple shoot formation. In most cases, however, shoots were formed on media supplemented with BAP (Chaturvedi, 1975; Mantell et al., 1978; Forsyth and Van Staden, 1982; Heble and Staba, 1980).

Activated charcoal was observed to be more effective than PVP in reducing phenolic exudation. About 60% of the explants inoculated in medium supplemented with PVP exhibited browning after incubating cultures for more than 30 days. Better growth responses of plant tissues have been associated with addition of activated charcoal as it removes inhibitory substances from the media produced either on autoclaving (Weatherhead et al., 1978) or by the tissue itself (Fridborg et al., 1978)

Microshoots that attained a height of 3 cm with at least one node were individually excised from the shoot cluster and selected for rooting. *In vitro* rooting was efficiently obtained within a period of 30 days on MS medium with 2.67 µM NAA and MS basal medium. Simultaneous rooting was induced from the shoots by culturing them in both shoot initiation and shoot growth media. Similar responses have been reported in the shoot culture of *D. floribunda* (Chaturvedi, 1975; Lakshmisita et al., 1976; Borthakur and Singh, 2002). Roots were 5 – 6 cm long, white and slender (Figure 1).

Ex vitro rooting proved to be superior to *in vitro*, with 90% transplantation success. IBA is generally known to induce rooting in plant cutting either *in vitro* or *in vivo*, but IBA had negative effects on root growth *in vitro* as roots disintegrated during hardening. However, pulses of 30 min with 49 µM IBA were adequate to stimulate rooting from the basal portion of the shoot in 91.7% in *D. oppositifolia* and 89.9% in *D. pentaphylla* cultures after 5 weeks (Table 2). All *ex vitro* rooted shoots exhibited vigorous root systems. The plantlets were transplanted to plastic pots containing soil rite and hardened by exposing them gradually to an increased duration of daylight and temperature. No morphological abnormalities were visible in the transplanted plants.

Microtubers developed at the base of their rooted shoots. 60% of the cultures produced one to three tubers at the base of their rooted shoots. Using nodal cutting as explants, the phenomenon of *in vitro* tuberization has been observed in *D. bulbifera* (Uduebo, 1971; Ammirato, 1976, 1984; Mantell et al., 1987; Forsyth and Van Staden, 1982), *D. alata* (Ammirato, 1976; Mantell et al., 1987; Jean and Cappadocia, 1991; Alhassan and Mantell, 1994), *D. rotundata* (Mantell et al., 1987; Ng, 1988), *D. abyssinica* (Jean and Cappadocia, 1991), *D. opposita* (Mantell and Hugo, 1986) and *D. cayenensis* (Ng and Mantell, 1996). Both *D. oppositifolia* and *D. pentaphylla* nodal shoots produced tubers on the shooting media. These tubers were found to be bigger and more uniform in size in comparison with other treatment. A combination of 20 g l^{-1} sucrose and 8.8 µM BAP added to the MS basal medium induced tubers. However, these tubers were smaller and not uniform in size. Effect of sucrose on microtuberization has also been observed in *D. bulbifera* (Forsyth and Van Staden, 1984; Forsyth, 1982), *D. rotundata* (Ng, 1988), *D. alata* (Mantell and Hugo, 1989) and *D. opposita* (Kohmura et al., 1995). The nodal shoot cultures of *D. alata* and *D. bulbifera* showed maximum microtuber formation with 2% sucrose (Mantell and Hugo, 1989). When Kohmura et al. (1995) compared sucrose concentrations (3 and 6%) in *D. opposita* with 8.9 µM BAP alone in the medium, 6% sucrose was found to be more efficient for tuberization. In the present study, 8.87 µM BAP alone with 3% sucrose can successfully induce *in vitro* microtuber formation (Table 3). In *D. rotundata*, a decrease in the percentage of microtuberization with 8 or 10% sucrose and 2.5 µM

Table 2. Rooting of shoots OF *D. oppositifolia* and *D. pentaphylla*.

Species	Method	Growth regulators (µM)	No of roots/shoot	Root length (cm)	Rooting (%)
D. oppositifolia	*In vitro*	MS+NAA (0.0)	2.91±0.28[b]	4.08±0.31[b]	83.8
		MS+NAA (0.54)	2.66±0.18[b]	3.50±0.26	89.6
		MS+NAA (2.69)	5.25±0.44[a]	7.83±0.84[a]	76.9
	Ex vitro	IBA (49) 30 min	6.75±0.41[a]	6.33±0.35[a]	91.7
		IBA (49) 60 min	2.33±0.22[b]	3.50±0.28[b]	82.2
		IBA (49) 120 min	2.08±0.19[b]	2.25±0.25[c]	67.0
D. pentaphylla	*In vitro*	MS+NAA (0.0)	3.33±0.25[b]	3.08±0.28[a]	87.9
		MS+NAA (0.54)	2.41±0.22[c]	2.08±0.19[b]	82.3
		MS+NAA 2.69)	5.25±0.39[a]	3.50±0.28[a]	78.4
	Ex vitro	IBA (49) 30 min	4.66±0.28[a]	4.50±0.23[a]	89.9
		IBA (49) 60 min	2.50±0.26[b]	2.50±0.19[b]	83.3
		IBA (49) 120 min	2.0±0.17[b]	1.75±0.17[c]	54.8

Values represent mean ± SE (n = 12). Data recorded after 60 days.

Table 3. Influence of sucrose concentration on *in vitro* tuber production.

Species	Sucrose (gl^{-1})	Tuber number	Tuber weight (mg)
D. oppositifolia	20	1.33±0.14[c]	16.6±1.88[b]
	30	2.41±0.14[c]	55.8±7.92[a]
D. pentaphylla	20	1.58±0.19[c]	28.25±1.96[b]
	30	2.83±0.20[c]	65.83±5.56[a]

Values represent mean ±SE (n = 12). Data recorded after 60 day.

kinetin was reported by Ng (1988), which contrasted with the results obtained in the current study on *D. oppositifolia* and *D. pentaphylla*. Increase in the sucrose amount in culture media from 2 to 8%, in the presence of higher levels of kinetin (23.2 to 46.4 µM), raised micro-tuber frequencies in *D. bulbifera* (Forsyth and Van Staden, 1984).

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