Shoot Multiplication in *Dioscorea* Species

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Standardized protocols for shoot induction and multiplication through axillary shoot proliferation from single node explants of nine species of *Dioscorea* namely, *Dioscorea bulbifera, D. pentaphylla, D. hispida, D. intermedia, D. hamiltonii, D. wightii, D. belophylla, D. spicata* and *D. oppositifolia* of the Southern Western Ghats. Cytokinins were effective in promoting shoot induction and multiplication in these species. Of the different cytokinins used, optimum results were obtained with BA (2-4 mg/l) followed by TDZ and Kinetin (Kn) in all the species. In *D. pentaphylla*, 12 shoots were obtained on MS medium supplemented with BA (4 mg/l) and Kn (2 mg/l) whereas in *D. oppositifolia* up to 8 shoots were produced in BA (2 mg/l) and Kn (1 mg/l). BA (2 mg/l) along with NAA (0.5 mg/l) produced 8-10 shoots in *D. pentaphylla* and *D. intermedia*.

Key Words: *Dioscorea*, Nodal segment culture, Shoot multiplication

Introduction

The genus *Dioscorea* L., commonly called true yams, has about 620 species, which are both agriculturally and pharmaceutically important (Purseglove 1972; Coursey 1967). Of these, 23 species occur in the Indian subcontinent and 44% of these are endemic. Many wild species are of medicinal and pharmacological importance as they are the source of steroidal sapogenins especially diosgenin, a precursor for commercially synthesized sex hormone and corticosteroids. Moreover, they have industrial, aesthetic and ritualistic improtance. Yams are vegetatively propagated due to heterozygosity/shy flowering/non-synchronization in flowering/non-flowering or due to failure of seed set. These, therefore, need biotechnological interventions for their clonal multiplication as well as conservation. Reports on the *in vitro* propagation of species of *Dioscorea* using meristem tip or nodal explants of vines or tuber sprouts offer the prospects of germplasm storage by means of minimal-growth or cryopreservation, will be of application in the national and international exchange of germplasm.

Micropropagation is popular due to large-scale multiplication of high quality clonally propagated plants and it provides a means of germplasm storage for maintenance of disease-free stocks (Withers 1989). Ammirato (1984) reported that plants which are produced by nodal or meristem culture show a high degree of uniformity. The present experiments were carried out with the objective of formulating micropropagation protocols through nodal segment culture in *Dioscorea* species for their large-scale multiplication, conservation and exchange.

The *in vitro* propagation of cultivated species of *Dioscorea* using meristem tip or nodal explants of vines or tuber sprouts has been attempted by various workers (Mantell *et al.*, 1978; Ammirato, 1982; Forsyth and van Staden, 1982; Malaurie *et al.*, 1988, 1993; Nair and Chandrababu, 1996). Clonal propagation using nodal segments or meristems has also been reported in wild species like *D. deltoidea* (Grewal *et al.*, 1977), *D. floribunda* (Chaturvedi 1975; Sita *et al.*, 1976), *D. composita* (Ammirato 1982; Alizadeh *et al.*, 1998), *D. abyssinica* and *D. mangenotiana* (Lauzer *et al.*, 1992).

Materials and Methods

One accession each from 12 species of *Dioscorea* (Table 1) having distribution in Southern Western Ghats, procured from NBPGR Regional Station, Thrissur and grown in the green house of the Department of Botany, University of Kerala, Thiruvananthapuram served as the source of explants for the experiments.

Single node segments from vines of healthy mother plants of 4-months were used. The explants were initially washed in running tap water for 30-60 minutes, then in aqueous 10% Labolene for 15-20 minutes and again in tap water and in sterile distilled water to remove the adhering surface contaminants. They were then treated with 0.1% $HgCl_2$ for 5-20 minutes followed by rinsing with sterile double distilled water 4-5 times inside the laminar airflow. The explants were then trimmed to approximate size (1-2 cm) by removing off cut ends after blotting in sterile filter paper before inoculation.

For shoot multiplication, surface sterilized single node segments (1-2 cm) were inoculated onto culture

Table 1. Materials used

#	Species name	IC number	Locality of collection
1	*Dioscorea bulbifera* L.	202349	Peechi, Thrissur, Kerala
2	*Dioscorea pentaphylla* L.	202367	Peechi, Thrissur, Kerala
3	*Dioscorea hispida* Dennst	202370	Nilambur, Malappuram, Kerala
4	*Dioscorea tomentosa* Heyne	202379	South Canara, Karnataka
5	*Dioscorea pubera* Bl.	202382	Begur, Wynad, Kerala
6	*Dioscorea intermedia* Thw.	202384	Santhanpara, Idukki, Kerala
7	*Dioscorea spicata* Roth.	202383	Thirthahally, Coorg, Karnataka
8	*Dioscorea oppositifolia* L.	202386	Valpara, Palghat, Kerala
9	*Dioscorea wightii* Hook. f.	214855	Mundanthurai, Tamil Nadu
10	*Dioscorea belophylla* Voight	248181	Devakolly, Coorg, Karnataka
11	*Dioscorea wallichii* Hk. f.	202312	Courtallum, Tirunelveli, Tamil Nadu
12	*Dioscorea hamiltonii* Hk.	202328	Vadakkancherry, Thrissur, Kerala

tubes containing 15 ml of MS basal medium (Murashige and Skoog, 1962) augmented with cytokinins like BA, Kn, TDZ (0.5-5.0 mg/l) alone or in combination with auxins like IAA, IBA or NAA (0.5-2.0 mg/l). Combinations of BA and Kn were also tried (0.5-5.0 mg/l). The cultures were then kept in the culture room maintained under 12 h photoperiod at a light intensity of 3000 lux at a temperature of 25±2°C and at 70-80% RH. The responding explants were transferred to fresh media either of the same composition or to lower concentrations for further proliferation and elongation. The shoot induction was recorded for seven replicates per treatment, each repeated three times.

Results and Discussion

Effect of Cytokinins Alone

Dioscorea species, belonging to the family Dioscoreaceae of the order Dioscoreales, were micropropagated through shoot multiplication. Single node segments showed various responses in accordance with the plant growth regulators supplied in the medium. About 80-90% contaminant free cultures were obtained following treatment of nodal segments with 0.1% $HgCl_2$ for 12 or 15 minutes.

Following 10 days of inoculation, sprouting of the axillary bud was observed. Elongation and multiplication of the shoot was observed after 30 days of culture. Response of different species has been presented below.

Multiple shoots were induced by cytokinins when used alone. The cytokinins BA, Kn and TDZ induced axillary bud proliferation at various concentrations (0.5–5 mg/l). It was observed that lower and higher levels of cytokinins, (less than 1 mg/l and above 5 mg/l) failed to induce shoot bud proliferation in all the species of *Dioscorea*. BA at 4 mg/l gave the best result in inducing 4 shoots in *D. pentaphylla* (Fig. 1) while Kn (3 mg/l) induced 3 shoots (Fig. 2). In *D. hispida*, TDZ at 3 mg/l produced 3 shoots (Fig. 3), while a single shoot was developed in BA at 1 mg/l (Fig. 4).

In *D. bulbifera*, BA (2 mg/l) initiated 3 shoots after 30 days of culture (Fig. 5; Table 2). When sub-cultured

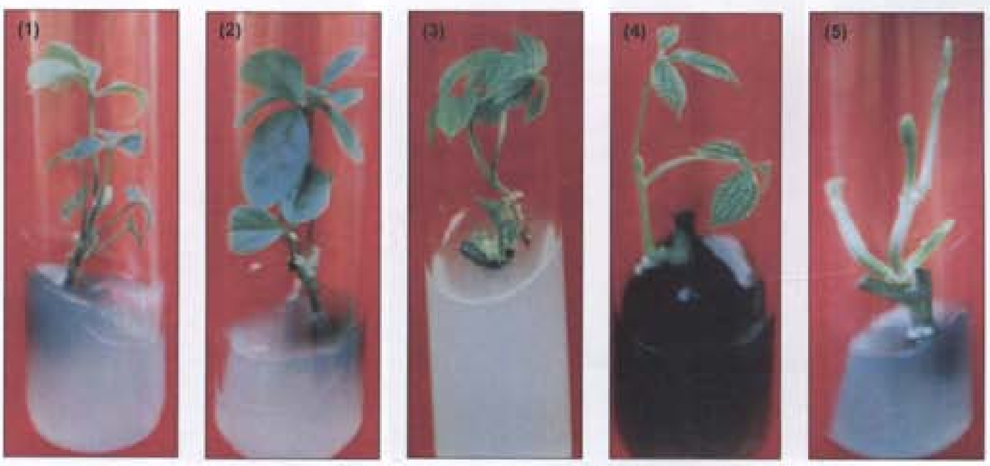

Fig. 1-5: Effects of cytokinins in shoot multiplication in different Dioscorea species

Table 2. Effect of cytokinins alone on shoot multiplication

Species	BA (mg/l)	Kn (mg/l)	TDZ (mg/l)	No. of shoots*(30 days)
D. bulbifera	2.0	–	–	3 ± 0.22
	1.0	–	–	2 ± 0.18
	–	2.0	–	2 ± 0.13
	–	3.0	–	2 ± 0.18
D. pentaphylla	1.0	–	–	1 ± 0.18
	2.0	–	–	3 ± 0.13
	4.0	–	–	4 ± 0.29
	–	3.0	–	3 ± 0.13
D. hispida	–	–	3.0	3 ± 0.20
	–	1.0	–	1 ± 0.10
D. pentaphylla	–	–	1.0	3 ± 0.22
	–	–	2.0	2 ± 0.18
	–	–	3.0	4 ± 0.29
D. intermedia	1.0	–	–	3 ± 0.18
	2.0	–	–	5 ± 0.29
D. hamiltonii	3.0	–	–	3 ± 0.22
D. wightii	4.0	–	–	2 ± 0.13
	5.0	–	–	2 ± 0.22
	–	2.0	–	1 ± 0.18
	–	3.0	–	3 ± 0.22
	–	4.0	–	1 ± 0.18
D. wightii	–	–	0.5	2 ± 0.12
	–	–	1.0	5 ± 0.25
D. belophylla	–	–	1.0	22 ± 1.25
	–	–	2.0	3 ± 0.22
D. spicata	–	–	3.0	3 ± 0.18
	–	–	4.0	3 ± 0.22
	–	–	5.0	3 ± 0.22

* Values represent mean of seven replicates ± SE

on to the same medium, multiplication occurred at the same rate. The shoots were further subjected to elongation in basal MS medium.

A maximum of 5 shoot buds were obtained in *D. intermedia* in BA at 2 mg/l (Fig. 6), while at 1 and 3 mg/l, 3 shoots got developed in *D. hamiltonii* (Fig. 7). At BA (4-5 mg/l), 2 shoots got differentiated in *D. wightii* (Fig. 8). The shoots produced in 5 mg/l were suppressed and did not elongate in *D. oppositifolia* (Fig. 9). When Kn (0.5-5 mg/l) was used instead of BA, the shoot development was inhibited. At 3 mg/l, a maximum of 3 stunted shoots was formed in *D. wightii* (Fig. 10). In this species, when TDZ was used at different concentrations (0.5-5 mg/l), multiple shoot development was noticed. A maximum of 5 shoots were initiated in *D. wightii* (Fig. 11), whereas, 22 shoots were produced in

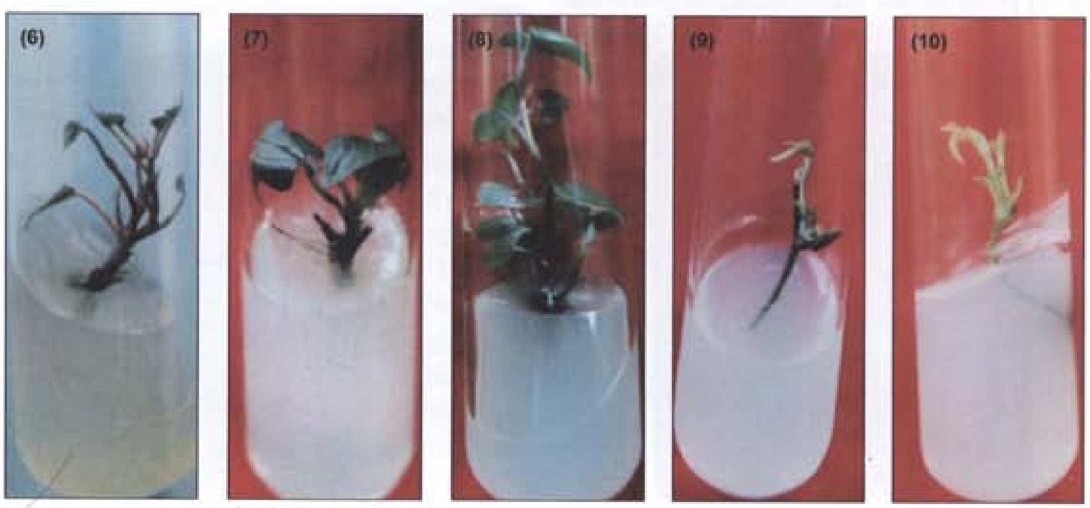

Fig. 6-10: Rsponse of different Dioscorea species to shoot multiplication media

D. belophylla in TDZ at 1 mg/l, (Fig. 12; Table 2). At higher concentrations of TDZ (>3 mg/l), 3 shoots with lush dark green leaves were produced in *D. spicata* (Fig. 13), while, at lower concentration (0.5 mg/l), 2 shoots having branches were initiated in *D. belophylla* (Fig. 14).

Effect of Cytokinins in Combination

BA and Kn when used in combination resulted in better shoot multiplication compared to that when used singularly in *D. pentaphylla* and *D. oppositifolia*. Synergistic effect of BA (4 mg/l) and Kn (2 mg/l) resulted in 12 shoots in *D. pentaphylla* (Fig.15; Table 3), while, an increase in the Kn concentration to 4 mg/l reduced the number of shoots to 4 (Fig.16; Table 3). BA (2 mg/l) and Kn (1 mg/l) induced 8 shoots in *D. oppositifolia* (Fig.17; Table 3).

Effect of Cytokinins in Combination with Auxins

Cytokinins *viz*., BA/Kn (0.5-5.0 mg/l) in conjunction with NAA/IAA/IBA (0.5-2.0 mg/l) induced axillary bud proliferation in some species. In *D. bulbifera*, BA at 2-3 mg/l along with NAA/IAA (0.5 mg/l) resulted in single shoot formation (Fig. 18), with basal callusing. While in presence of IBA (0.5 mg/l), 2 shoots were induced (Fig. 19). The shoots produced in BA+IAA containing medium were long having 7-10 culturable nodes after 30 days of culture. When BA was replaced with Kn, in all combinations, a single shoot was induced (Table 4).

Addition of NAA (0.5 mg/l) along with BA (2 mg/l) induced a maximum of 8-10 shoots per node in *D. pentaphylla* (Fig. 20). Five shoots were obtained in 1 and 4 mg/l BA (Fig. 21) and 3 in 3 mg/l BA along with 0.5 mg/l NAA (Fig. 22). On the other hand, IBA (0.5 mg/l) when used in place of NAA, in conjunction with BA (2-3 mg/l), single shoot formation was noticed in *D. pentaphylla* (Table 4). Combinations of BA with IAA and Kn with IAA/IBA/NAA resulted in single shoot formation only (Table 4). In *D. hispida*, Kn (2 mg/l) along with NAA (0.5 mg/l) resulted in the formation of a single healthy shoot (Fig. 23).

Table 3. Synergistic effect of cytokinins (BA + Kn) on shoot multiplication

Species	BA (mg/l)	Kn (mg/l)	No. of shoots (30 days)*
D. bulbifera	2.0	1.0	2 ± 0.26
	2.0	1.0	1 ± 0.18
D. pentaphylla	4.0	2.0	12 ± 0.25
	4.0	4.0	4 ± 0.20
	2.0	2.0	3 ± 0.22
D. oppositifolia	2.0	1.0	8 ± 0.25
	4.0	4.0	2 ± 0.13
	2.0	2.0	1 ± 0.18
	4.0	2.0	1 ± 0.18

* Values represent mean of seven replicates ± SE

Table 4. Effect of cytokinins along with auxins on shoot multiplication

Species	BA (mg/l)	Kn (mg/l)	NAA (mg/l)	IBA (mg/l)	IAA (mg/l)	No. of shoots (30 days)
D. bulbifera	2.0	–	0.5	–	–	1 ± 0.18
	3.0	–	0.5	–	–	1 ± 0.18
	2.0	–	–	0.5	–	2 ± 0.13
	3.0	–	–	0.5	–	2 ± 0.13
		2.0	0.5	–	–	1 ± 0.22
		3.0	–	0.5	–	1 ± 0.18
D. pentaphylla	2.0	–	–	0.5	–	1 ± 0.18
	2.0	–	0.5	–	–	8 ± 0.25
	1.0	–	0.5	–	–	5 ± 0.25
	4.0	–	0.5	–	–	5 ± 0.25
	3.0	–	0.5	–	–	3 ± 0.22
D. hispida	–	2.0	0.5	–	–	1 ± 0.18
D. intermedia	2.0	–	0.5	–	–	10 ± 0.32
	3.0	–	0.5	–	–	5 ± 0.25
	3.0	–	–	0.5	–	5 ± 0.25
	–	2.0	0.5	–	–	1 ± 0.18
	3.0	–	–	–	0.5	1 ± 0.18
	–	3.0	–	–	0.5	1 ± 0.18

* Values represent mean of seven replicates ± SE

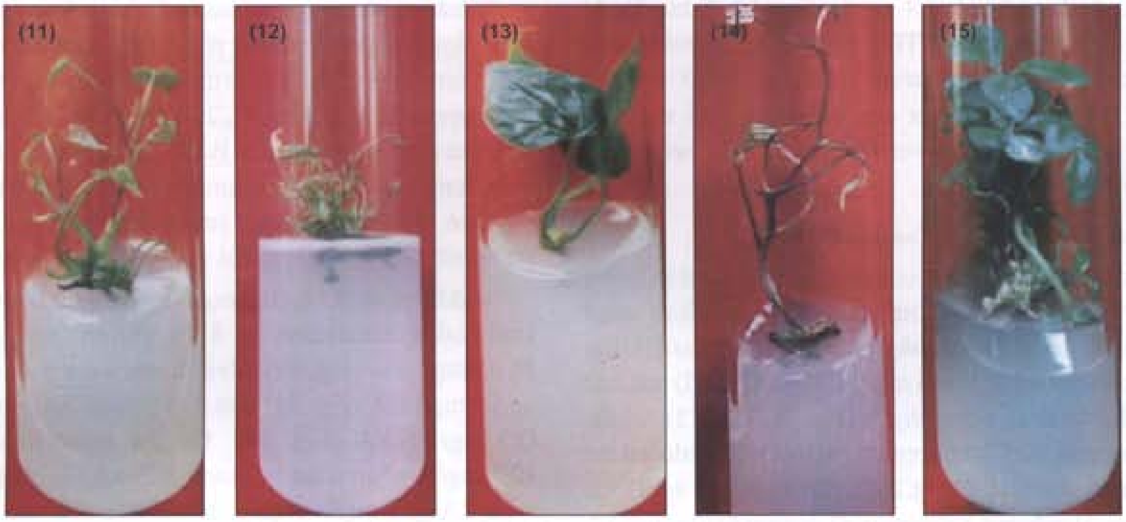

Fig. 11-15: Effect of varying TDZ concentrations in Dioscorea species

NAA (0.5 mg/l) along with BA (2-3 mg/l) induced 10 and 5 shoots, respectively in *D. intermedia* (Fig. 24, 25) as compared to 5-6 shoots at BA (3 mg/l) and IBA (0.5 mg/l) (Fig. 26; Table 4). However, BA along with IAA as well as all the combinations of Kn along with NAA/IAA/IBA resulted in single shoot development.

Selection of appropriate nutrient medium forms the initial and essential step for the success and fulfillment of all experimental systems of plant tissue culture. In the present study, micropropagation through shoot tip and nodal segment cultures was attempted in MS.

Of the different methods of *in vitro* propagation, multiple shoot induction is the most frequently used multiplication technique in plant micropropagation systems. The production of plants from axillary shoots has proved to be the most applicable and reliable method of *in vitro* propagation. According to George and Sherrington (1984), shoot tip/nodal segment culture depends on stimulating axillary shoot growth by overcoming the dominance of shoot apical meristem by the incorporation of growth regulators in to the medium.

The use of nodal segments as initial explants for the *in vitro* propagation of different *Dioscorea* species has been reported in *D. floribunda* (Chaturvedi, 1975; Sinha and Chaturvedi, 1979; Sita *et al.*, 1976), *D. bulbifera* (Forsyth and van Staden, 1982), *D. composita* (Datta *et al.*, 1982) and in *D. alata* and *D. rotundata* (Mantell *et al.*, 1978; Nair and Chandrababu, 1996).

In the present study, shoot multiplication was achieved in different species of *Dioscorea* by culturing single node segments in MS medium supplemented with different concentrations and combinations of cytokinins *viz.* BA/Kn/TDZ alone or in combination with auxins IAA/IBA/NAA. The number of shoots produced in different species varied in these hormonal regimes. Of the different cytokinins used for shoot multiplication, BA (2-4 mg/l) was found to be the best followed by TDZ and Kn in all the species of *Dioscorea*. The superiority of BA over other cytokinins for shoot proliferation has been established for plants such as *D. alata* (Mantell *et al.*, 1980), *D. bulbifera* (Forsyth and van Staden, 1982), *D. opposita* (Kobayashi, 1991) and *D. cayenensis* and *D. trifida* (Mitchell *et al.*, 1995b). However, in *D. belophylla*, 22 shoots were obtained in TDZ at 1 mg/l.

Cytokinins in combination showed better response than cytokinin alone for shoot multiplication in *D. pentaphylla*. In the present investigation, on BA+Kn, there was higher number of shoots in some species. A maximum of 12 shoots were produced in *D. pentaphylla* in presence of BA (4 mg/l) and Kn (2 mg/l), while up to 8 shoots in *D. oppositifolia* in presence of BA (2 mg/l) and Kn (1 mg/l). George and Sherrington (1984) were of the opinion that adding more than one cytokinin to the medium results in improved production of shoot or shoots of better quality. Mitchell *et al.* (1995a) also affirmed the potentiality of using more than one cytokinin in the medium for better shoot multiplication in *D. trifida*.

Addition of auxins along with cytokinins had a positive impact on shoot multiplication in some *Dioscorea* species. Of the various auxins used along with cytokinins, NAA/IAA/IBA, NAA at 0.5 mg/l gave the optimum results. BA at 2 mg/l along with NAA (0.5 mg/l) produced

Fig. 16-26: Effect of cytokinin concentrations in Dioscorea species

8-10 shoots in *D. pentaphylla* and 10 shoots in *D. intermedia*. Similar results have been reported in various *Dioscorea* species such as *D. floribunda* (Sita *et al.*, 1976), *D. bulbifera* (Forsyth and van Staden, 1982), *D. abyssinica* and *D. mangenotiana* (Lauzer *et al.*, 1992) and *D. alata, D. esculenta* and *D. rotundata* (Nair and Chandrababu, 1996).

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