Standardization of Callus Induction and Shoot Regeneration in Twelve Species of *Dioscorea*

KI Asha[1] and GM Nair[2]

[1] *NBPGR Regional Station, Vellanikkara, Thrissur-680656, Kerala, India*

[2] *Department of Botany & Biotechnology, University of Kerala, Thiruvananthapuram*

Protocol was standardised for callus induction and shoot regeneration from leaf/stem/petiole/bulbil or root explants in 12 species of *Dioscorea* namely *Dioscorea bulbifera, D. pentaphylla, D. hispida, D. oppositifolia, D. belophylla, D. wightii*, *D. spicata, D. intermedia, D. hamiltonii* and *D. pubera*. While in 2 species no callus could be induced. Type of explants as well as the type, concentrations and combinations of hormones used influenced callus induction and regeneration. The most proliferative callus was obtained from leaf on MS + 2,4-D (1-3 mg/l) in all the species. In *D. intermedia*, leaf explants cultured in MS medium with NAA (8 mg/l) and BA (2 mg/l) resulted in high callusing. 2,4-D in conjunction with BA in *D. spicata* and Kinetin in *D. intermedia* induced callus. Distinct variation in colour and texture of callus was noticed. White friable/powdery callus was produced in medium containing 2,4-D alone or in combination with BA. In *D. wightii* and *D. belophylla*, leaf explant in 2,4-D (3 mg/l) produced brownish highly regenerative compact callus. 2,4-D derived callus gave the maximum callus regeneration. In *D. bulbifera* and *D. pentaphylla*, callus regeneration occurred in MS basal medium itself, while, addition of Kn (2 mg/l) was essential for shoot induction (1-3 shoots/callus) in five species. In *D. belophylla* 4 shoot buds regenerated in BA (4 mg/l). In *D. hamiltonii* TDZ (1 mg/l) gave a maximum of 22 shoots. Synergistic effect of BA (2 mg/l) and Kn (1 mg/l) induced 6 shoots in *D. bulbifera,* 4 in *D. hispida* and 5 in *D. wightii*. Single shoot was regenerated in *D. belophylla* in combination with BA and IBA, while Kn with IBA produced 2 shoots in *D. pentaphylla* and 4 in *D. hispida*. A combination of BA and Kn along with NAA also resulted in shoot regeneration in *D. pentaphylla* (2 shoots/callus), D. *oppositifolia* (2 shoots/callus) and *D. intermedia* (2-3 shoots/callus).

Key Words: *Dioscorea*, **Callus induction, Callus regeneration, Caulogenesis**

Introduction

Dioscorea containing 620 species, producing tubers, bulbils or rhizomes of considerable economic importance (Ammirato, 1984; Purseglove, 1972), constitute the staple carbohydrate food having high protein and minerals for millions in tropical and subtropical countries (Onwueme, 1978; Ayensu and Coursey, 1972). Many wild species have medicinal and pharmacological value (Coursey, 1967), being source of steroidal sapogenin diosgenin, the precursor of sex hormone and corticosteroids. The present experiment was aimed to standardize protocols for the rapid clonal multiplication of the species of *Dioscorea* through indirect organogenesis.

Callus regeneration and indirect organogenesis have been reported in *D. alata* (Belarmino, *et al*., 1991; Acedo, 1994; Paneque *et al*., 1995; Nair and Chandrababu, 1996; Amoroso, 1999), *D. esculenta* (Belarmino *et al*., 1991), *D. rotundata* (Esenowo, 1986; Belarmino *et al*., 1991; Nwachukwu *et al*., 1996), *D. cayenensis* (Viana and Mantell, 1989), *D. floribunda* (Sengupta *et al*., 1984), *D. composita* (Rao, 1982; Viana and Mantell, 1989) and *D. deltoidea* (Mascarenhas *et al*., 1976; Singh, 1978; Chaturvedi and Chowdury, 1980).

In the present experiment, callus induction and shoot regeneration was achieved from leaf explants in ten species of *Dioscorea,* namely *Dioscorea bulbifera* (IC202349), *D. pentaphylla* (IC202367), *D. hispida* (IC202370), *D. oppositifolia* (IC202386), *D. belophylla* (IC248181), *D. wightii* (IC214855), *D. spicata* (IC202383), *D. intermedia* (IC202384), *D. hamiltonii* (IC202328) and *D. pubera* (IC202382). *D. wallichii* and *D. tomentosa* failed to initiate callus in any of the hormonal combinations tried.

Materials and Methods

One accession each from the 12 species of *Dioscorea* 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 explant source. Leaf, stem, petiole, root and bulbil segments from vines of healthy and disease free 4-month-old plants 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 sterilized inside the laminar airflow chamber using

Author for correspondence: E-mail: ashakarthy@gmail.com and ashakarthy@rediffmail.com

aqueous 0.1% $HgCl_2$ for 5-20 minutes and then rinsed in sterile double distilled water 4-5 times. The explants were then trimmed to appropriate sizes by removing off the cut ends after blotting in sterile filter paper before inoculation.

For the induction of callus, single explant each of leaf and bulbil segments (0.5 cm^2), stem, petiole and root explants (0.5-1.0 cm) were inoculated onto MS basal medium (Murashige and Skoog, 1962) augmented with varying concentrations and combinations of hormones, *viz.,* 2,4-D, NAA, IAA and IBA (0.5-10 mg/l) alone or in combination with cytokinins BA/Kn/2-iP (0.5-5 mg/l). The cultures were then kept in the culture room maintained under 12 h photoperiod at a light intensity of 3000 at 25±1^{o}C and at 70-80% RH. The callusing responses were recorded after every two weeks. The primary calluses after 30-45 days of inoculation were subcultured to fresh media containing similar or varied hormonal combination for further proliferation, maintenance and organogenesis. The growth of the calluses was evaluated by measuring the callus proliferated per unit area of the samples.

For callus regeneration, the initiated and proliferated calli were transferred to basal MS or to the regeneration media, containing BA/Kn/TDZ/2-iP (0.5-4 mg/l) alone or in combination with 2,4-D, IAA, IBA or NAA (0.5-4 mg/l).

Results

Callus Induction

Various explants like leaf, stem, petiole, root and bulbil segments from species of *Dioscorea* were tested for callus induction from species of *Dioscorea* on MS medium supplemented with different concentrations and combinations of phytohormones. Surface sterilization for 8-10 min. for leaf and petiole and 12-15 min in case of stem, root and bulbil explants was adequate to establish 85-90% contaminant free cultures. Callus induction was achieved in these explants in MS medium containing auxins *viz*. 2,4-D, IAA, IBA and NAA (0.5-10 mg/l) either alone or in combination with BA/Kn (0.5-3 mg/l). Explants inoculated on to 2,4-D resulted in induction of callus in 7-10 days of culture. Initial responses like curling of the leaf explants and inflation of the petiole segment tip started from the 4th day itself. The type and concentration of hormones used exhibited significant influence on callogenesis. In most of the cases white or cream friable callus was formed. Of the various auxins used for callus induction, 2,4-D gave the best callus initiation and proliferation followed by NAA, IAA and IBA in all the species. Likewise callus morphology also varied with explant. Young explants showed better response giving high callus proliferation. Mature explants showed mild response in callusing, which was arrested after 15-25 days and dried off. Among the different explants used, leaf segments gave the most proliferative callus followed by petiole, stem and bulbil segments. Due to high phenolic exudation in species like *D. pentaphylla* and *D. hispida*, browning of the calli and death of the explant was observed in some cultures upon ageing. In order to overcome this, frequent subculturing of such calli was done to maintain their regenerative capacity.

In *D. bulbifera*, of the various explants tried, leaf explants responded early and produced white callus from cut ends within 7 days in 2,4-D (1 mg/l) (Fig. 1). The callus is yellowish and nodular at 2-3 mg/l of 2,4-D (Fig. 2). From stem explants, white powdery callus was developed in 2-3 mg/l while petiole, irrespective of the concentration of 2,4-D tried (0.5-3 mg/l), produced powdery white callus (Table 1). Bulbil explants, at higher concentrations of 2,4-D (5 mg/l), produced powdery white callus (Fig. 3), while, at 3 mg/l black callus was formed (Table 1).

In *D. pentaphylla*, leaf explants in 3 mg/l 2,4-D induced callus. At 5 mg/l, black glutinous callus was produced. When medium was augmented with BA (3 mg/l) + Kn (1 mg/l) + NAA (1 mg/l), cream, compact organogenetic callus was produced (Table 2). Petiole explants in 2,4-D (5 mg/l) alone or in combinations of cytokinins and auxins, *viz*., BA (3 mg/l), Kn (1 mg/l) and NAA (1 mg/l), initiated brown powdery callus from cut ends (Fig. 4). Bulbil segments in 2,4-D (5 mg/l) initiated black callus

Table 1. Effect of 2,4-D on callus induction in *Dioscorea bulbifera*

Explant	Conc. (mg/l)	Callus morphology	Roots	Rate of callusing*
Leaf	1.0	White	–	+++
	2.0	Yellow	–	+
	3.0	Yellow nodular, compact	–	++
Stem	1.0	White	–	+
	2.0	White powdery	+	++
	3.0	White powdery	–	++
Petiole	0.5	White powdery	–	+
	1.0	White powdery	–	+
	2.0	White powdery	–	+
	3.0	White powdery	–	++
Bulbil	1.0	White powdery	–	+
	2.0	White powdery	–	+
	3.0	Black	–	+
	5.0	White powdery	–	++

* Rate of callusing, n = callus proliferated per unit area of the samples

Fig. 1 – 20: Callus induction in species of *Dioscorea*

Table 2. Effect of phytohormones on callus induction in *D. pentaphylla* and *D. hispida*

Species	Explant	Hormones conc. (mg/l)				Callus morphology	Rate of callusing*
		2,4–D	BA	Kin	NAA		
D. pentaphylla	Leaf	1	–	–	–	Brown powdery	++
		3	–	–	–	Nodular compact	+++
		5	–	–	–	Black glutinous	+
		–	3	1	1	Cream compact organogenetic	++
	Petiole	1	–	–	–	Brown powdery	+
		3	–	–	–	Brown powdery	+
		5	–	–	–	Brown powdery	++
		–	3	1	1	Brown powdery	++
	Bulbil	1	–	–	–	Brown	+
		3	–	–	–	Brown	+
		5	–	–	–	Black	++
D. hispida	Leaf	0.5	–	–	–	Black glutinous	+
		1	–	–	–	Black glutinous	+
		3	–	–	–	Compact proliferative	++
		5	–	–	–	Compact	+

* Rate of callusing, n= callus proliferated per unit area of the samples

(Fig. 5). In *D. hispida*, black glutinous callus was formed from leaf explants in medium containing 2,4-D (0.5-1 mg/l). At 3 mg/l 2,4-D, compact proliferative callus was induced (Table 2).

In *D. oppositifolia*, powdery white callus was initiated from cut ends of leaf segments in 2,4-D at 3 mg/l, while, at higher concentrations (5 mg/l), meager white powdery non regenerative callus was formed from the whole surface (Fig. 6). At still higher concentrations (> 5 mg/l), rhizogenesis alone was noticed (Table 3). Petiole in 2,4-D (3 mg/l) produced white compact callus (Fig. 7). Powdery white callus obtained in 2,4-D (3 mg/l) on transfer to medium containing 2,4-D (4 mg/l) and Kn (2 mg/l) produced compact light brown non-regenerative callus.

In *D. belophylla*, explants responded very well in cultures containing 2,4-D (0.5-3 mg/l) (Table 3). Leaf explants produced highly regenerative compact brown callus at 3 mg/l (Fig. 8), while, petiole explants produced creamy glutinous callus in 2,4-D (0.5 mg/l). Stem explants did not produce any callus. In *D. wightii*, leaf segments produced brown compact callus in 2,4-D (3 mg/l) (Fig. 9), which became regenerative on transfer to TDZ (2 mg/l) enriched medium. Callus was also induced from cut ends of leaf segments in medium containing Kn (3 mg/l) and 2,4-D (0.5 mg/l).

In *D. spicata*, root explants produced shiny globular callus in 2,4-D (3 mg/l), while, stem explants produced shiny glutinous callus (Fig. 10). In presence of BA (0.5-2 mg/l) and 2,4-D (0.5-1 mg/l), root explants produced powdery non-regenerative callus (Table 4). Petiole, in medium containing BA (2 mg/l) and 2,4-D (6 mg/l) produced creamy compact callus (Fig. 11). Root explants planted in 2,4-D (0.5 mg/l) also showed similar results on transfer to TDZ (3 mg/l). Mature leaf explants in 2,4-D (0.5-2 mg/l) gave rise to dull green callus, which also showed rhizogenesis (Table 4). At 3 mg/l 2,4-D, greenish powdery compact callus showing rhizogenesis was obtained (Fig. 12). Young explants in 2,4-D at 3 mg/l induced white nodular regenerative callus, which became brown lateron (Fig. 13).

In *D. intermedia*, leaf and petiole explants responded similarly in cultures. At 2,4-D (1 mg/l), maximum callusing was noticed (Table 5). The callus was proliferative and powdery white at 1 and 3 mg/l, while, at 2 mg/l, nodular callus was formed. At higher concentrations of 2,4-D (5 mg/l), brown nodular callus (Fig. 14) and at 10 mg/l, white friable callus was produced (Fig. 15). Young petiole explant produced shiny glutinous callus from the cut ends in 2,4-D (3 mg/l) (Fig. 16).

Apart from 2,4-D, the other auxins also induced callus in leaf explants of *D. intermedia* (Table 5). Leaf segments in IAA, IBA and NAA produced white compact callus at low concentrations (0.5-1 mg/l). At higher levels (5 mg/l), callusing along with rhizogenesis was noticed. NAA derived callus on transfer to TDZ (0.5 mg/l) produced proliferative nodular compact callus. Friable white callus was produced in a combination of 2,4-D (4 mg/l) and Kn (2 mg/l) (Fig. 17), while, creamy compact proliferative callus was produced in NAA (8 mg/l) and BA (2 mg/l) (Fig. 18). Lower concentration of NAA (0.5 mg/l) along with BA (4 mg/l) initiated nodular organogenetic callus (Table 5).

The most regenerative callus was obtained in *D. hamiltonii* where the leaf explants in 2,4-D (1 mg/l) developed compact regenerative callus (Fig. 19), while petiole explant yielded black and glutinous callus. In *D. pubera,* bulbil explant in 2,4-D (3 mg/l) induced white compact callus (Fig. 20). Explants from two other species namely, *D. wallichii* and *D. tomentosa* failed to initiate callus in any of the hormonal combinations tried.

Callus Regeneration

Organogenetic/morphogenetic calluses induced from leaf, stem, petiole, root and bulbil segments were subcultured on to basal MS or to lower concentrations of the initiation medium or to different regeneration media containing BA/Kn/2-iP alone or in combination with NAA/IAA/IBA/2,4-D, in order to get regeneration from the calli.

In *D. bulbifera*, yellowish nodular regenerative leaf callus initiated in 2,4-D (3 mg/l), on subculturing to a medium containing BA/Kn (2 mg/l) produced two shoots (Fig. 21), while, combination of BA (2 mg/l) and Kn (1 mg/l) in the regeneration medium resulted in the formation of 4 shoots (Fig. 22). The same callus on transfer to BA (2 mg/l) and then to BA (2 mg/l) and Kn (1 mg/l) developed 1-2 shoots. The same callus upon subculture to Kn (2 mg/l) instead of BA and then to BA (2 mg/l) and Kn (1 mg/l), developed 6 shoots (Fig. 23; Table 6).

Similarly, the yellowish nodular regenerative petiole callus raised in 2,4-D (3 mg/l) upon transfer to BA (2 mg/l) or BA (2 mg/l) and Kn (1 mg/l) also developed 2-3 shoots. 2,4-D (5 mg/l) raised callus in BA (2 mg/l) developed single shoot with 3 branches and roots, while, in medium containing Kn (2 mg/l) or a combination of BA (2 mg/l) and Kn (1 mg/l) developed 2 more shoots with 8 branches and roots (Fig. 24).

Table 3. Effect of 2,4–D on callus induction in (*D. oppositifolia, D. belophylla, D. hamiltonii, D. wightii* and *D. pubera)*

Explant		MS+ 2,4–D (mg/l)	Callus morphology	Roots	Rate of callusing*
D. oppositifolia	Leaf	1	White	–	+
		3	White powdery	–	+++
		5	White powdery non regenerative	–	+
		10	No callus formation	+	–
	Petiole	1	White	+	+
		3	White compact	+	+
		5	White	++	+
D. belophylla	Leaf	0.5	Brown	–	+
		1	Brown compact	–	++
		3	Brown compact highly regenerative	–	+++
		5	Black	–	+
	Petiole	0.5	Creamy glutinous	–	++
		1	White	–	+
		3	White nodular	–	+
		5	Brown powdery	–	+
D. hamiltonii	Leaf	1	Nodular compact regenerative	–	+++
		3	Compact	–	++
		5	Powdery	–	+
	Petiole	1	Black glutinous	–	++
		3	Black	–	+
		5	Black	–	+
D. wightii	Leaf	1	Brown	–	+
		3.0	Brown compact	–	+++
		5	Brown	–	+
D. pubera	Bulbil	1	White powdery	–	+
		3	White compact	–	++
		5	Brown powdery	–	+

Table 4. Effect of phytohormones on callus induction in *Dioscorea spicata*

Explant	Conc. (mg/l) 2,4–D	BA	Callus morphology	Roots	Rate of callusing*
	0.5	–	Chlorophyllous	+	+
	1	–	Chlorophyllous	+	+
	3	–	White nodular regenerative	–	+++
Leaf	5	–	Greenish powdery compact	–	++
	1	–	Powdery non– regenerative	+	+
	3	–	Powdery non regenerative	+	+
	5	–	No callus formation	+	–
Petiole	6	2	Creamy compact	–	+++
	1	–	Shiny	–	+
	3	–	Shiny glutinous	–	+++
Stem	5	–	Shiny	–	+
	1	–	Shiny	–	+
	3	–	Shiny glutinous globular	–	+
	5	–	Powdery	–	+
	0.5	0.5	Powdery non– regenerative	–	+
	0.5	1	Powdery non–regenerative	–	+
	1	1	Powdery non– regenerative	–	+
	0.5	2	Powdery non– regenerative	–	+
Root	1	2	Powdery non– regenerative	–	+

* Rate of callusing, n= callus proliferated per unit area of the samples

Table 5. Effect of phytohormones on callus induction in *D. intermedia*

Explant	Hormones conc. (mg/l)						Callus morphology	Roots	Rate of callusing*
	2,4–D	BA	Kin	IAA	IBA	NAA			
	1	–	–	–	–	–	Powdery white proliferative	–	++++
	2	–	–	–	–	–	Nodular	–	++
	3	–	–	–	–	–	White powdery proliferative	–	++
	5	–	–	–	–	–	Brown nodular	–	++
	10	–	–	–	–	–	White friable	–	+
	4	–	2	–	–	–	White friable	–	+
	–	2	–	–	–	8	Creamy compact	–	+++
	–	4	–	–	–	0.5	Nodular organogenetic	–	+++
	–	–	–	0.5	–	–	White compact	–	++
	–	–	–	1	–	–	White compact	–	++
	–	–	–	3	–	–	White	+	+
	–	–	–	5	–	–	White	+	+
	–	–	–	10	–	–	No callus	+	–
Leaf	–	–	–	–	0.5	–	White compact	–	++
	–	–	–	–	1	–	White compact	–	++
	–	–	–	–	3	–	White	+	+
	–	–	–	–	5	–	White	+	+
	–	–	–	–	10	–	No callus	++	–
	–	–	–	–	–	0.5	White compact	–	++
	–	–	–	–	–	1	White compact	–	++
	–	–	–	–	–	3	White	+	+
	–	–	–	–	–	5	White	+	+
	–	–	–	–	–	10	No callus	+++	–
	1	–	–	–	–	–	White compact	–	++
	2	–	–	–	–	–	White compact	–	++
Petiole	3	–	–	–	–	–	Shiny glutinous	–	++
	5	–	–	–	–	–	Crystalline	–	+
	10	–	–	–	–	–	White	+	+

* *Rate of callusing, n*= callus proliferated per unit area of the samples

Table 6. Effect of cytokinins (mg/l) alone or in combination on callus regeneration

Explant	Hormones conc. (mg/l)				Number of shoots induced									
	BA	Kin	2–iP	TDZ	1	2	3	4	5	6	7	8	9	10
Leaf	2	–	–	–	2	–	–	–	–	–	–	–	–	–
	–	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	1	–	–	6	–	–	–	–	–	–	–	–	–
Petiole	2	–	–	–	2	–	–	–	–	–	–	–	–	–
	2	1	–	–	3	–	–	–	–	–	–	–	–	–
	2	–	–	–	1		–	–	–	–	–	–	–	–
Bulbil	–	2	–	–	3	–	–	–	–	–	–	–	–	–
	2	1	–	–	3	–	–	–	–	–	–	–	–	–
	–	–	2	–	4	–	–	–	–	–	–	–	–	–
	–	3	–	–	–	2	–	–	–	–	–	–	–	–
	4	2	–	–	–	2	–	–	–	–	–	–	–	–
	–	–	–	0.5	–	1	–	–	–	–	–	–	–	–
	2	1	–	–	–	–	4	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	1	–	–	–	–	–	–
Leaf	3	–	–	–	–	–	–	1	–	–	–	–	–	–
	4	–	–	–	–	–	–	–	4	–	–	–	–	–
	–	–	2	–	–	–	–	–	1	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	3	–	–	–	–
	2	1	–	–	–	–	–	–	–	5	–	–	–	–
Petiole	–	2	–	–	–	–	–	–	–	–	1	–	–	–
	2	1	–	–	–	–	–	–	–	–	1	–	–	–
Leaf	–	–	–	1	–	–	–	–	–	–	–	–	22	–
Petiole	–	–	–	1	–	–	–	–	–	–	–	–	6	–
Bulbil	–	3	–	–	–	–	–	–	–	–	–	–	–	1

1. *D. bulbifera,* 2. *D. pentaphylla,* 3. *D. hispida,* 4. *D. oppositifolia,* 5. *D. belophylla,*
6. *D. wightii,* 7. *D. spicata,* 8. *D. intermedia,* 9. *D. hamiltonii,* 10. *D. pubera*

Bulbil callus raised from 2,4-D (5 mg/l), on transfer to Kn (2 mg/l) or a combination of BA (2 mg/l) and Kn (1 mg/l) developed 3 shoots (Table 6). Later on it produced 4-8 branches from each shoot and bulbils from each axils (Fig. 25). The same callus on transfer to BA (2 mg/l) and NAA (0.5 mg/l) or 2-iP (2 mg/l) developed 4 shoots with 3-5 branches each (Table 7). Black callus produced from bulbils in medium with 2,4-D (3 mg/l) on subculture to lower concentrations of 2,4-D (1 mg/l) and BA (0.5 mg/l) formed vitrified shoots (Fig. 26). Moreover, the basal callus formed in BA (2 mg/l) and NAA (0.5 mg/l) produced single shoot with 6 branches and bulbils from each axil on transfer to basal MS medium (Fig. 27).

Leaf, petiole and bulbil callus was regenerative in *D. pentaphylla* and *D. hispida*. The callus got blackened in these due to high phenol exudation and hence frequent subculturing was necessary to retain the regenerative capacity of the callus. In *D. pentaphylla*, brown compact BA (2 mg/l) and NAA (2 mg/l) derived callus from leaf developed 2 shoot buds in BA (4 mg/l) and Kn (2 mg/l) containing medium (Fig. 28; Table 6). Leaf callus in 2,4-D (3 mg/l) on transfer to Kn (3 mg/l) developed 2 shoots. Nodular, compact 2,4-D (3 mg/l) raised callus on transfer to TDZ (0.5 mg/l) developed a single shoot, while, in BA (2 mg/l) and NAA (0.5 mg/l), developed 2 shoots with branches (Fig. 29). Cream compact organogenetic leaf callus cultured in medium containing BA (3 mg/l), Kn (1 mg/l) and NAA (1 mg/l) gave 2 shoot buds (Table 7). Petiole derived glutinous callus developed in the same medium upon transfer basal MS, regenerated 2 shoot buds.

In *D. hispida*, black glutinous leaf callus obtained in 2,4-D (0.5 mg/l) gave rise to 4 shoots in medium containing BA (2 mg/l) and Kn (1 mg/l). Compact callus derived from 2,4-D (3 mg/l) on on transfer to medium with Kn (2 mg/l) and IBA (0.5 mg/l) resulted in the formation of 3-4 healthy shoots (Fig. 30; Table 7).

In *D. oppositifolia,* the 2,4-D derived leaf callus when subcultured to regeneration medium with BA (2-3 mg/l) produced a single shoot with roots. In presence of BA (3 mg/l) + Kn (1 mg/l) + NAA (1 mg/l), leaf callus from 2,4-D (3 mg/l) developed 2 shoots (Fig. 31).

In D. *belophylla*, brown compact organogenetic callus derived from leaf explants in 2,4-D (3 mg/l) on subculture to BA (4 mg/l) developed 4 - 8 shoots (Table 6). These shoots elongated and produced branches on transfer to basal MS medium (Fig. 32). Meanwhile, the black compact regenerative leaf callus derived in 2,4-D (0.5 mg/l) on transfer to BA (2 mg/l) and IBA (0.5 mg/l) developed a single shoot along with rhizogenesis (Fig. 33; Table 7). In 2-iP (2 mg/l) also a single shoot got differentiated. Petiole callus derived in 2,4-D (0.5 mg/l), upon transfer to medium containing Kn (2 mg/l) developed suppressed shoots with underdeveloped roots (Fig.34).

In *D. wightii*, the organogenetic leaf callus derived from 2,4-D (3 mg/l) produced 2-3 shoots in regeneration medium supplemented with BA (2 mg/l). When regeneration medium was complemented with Kn (1 mg/l) along with BA (2 mg/l), an increase in the number of shoots to 3 – 5 was noticed (Fig. 35; Table 6).

The cream compact nodular petiole callus of *D. spicata* induced in medium containing 2,4-D (6 mg/l) and BA (2 mg/l) on transfer to Kn (2 mg/l) or BA (2 mg/l) and Kn (1 mg/l) developed a single shoot (Fig. 36; Table 6). The powdery leaf callus derived in 2,4-D (3 mg/l) showed only rhizogenesis in BA (2 mg/l).

In *D. intermedia*, 2,4-D (5 mg/l) leaf callus on transfer to basal MS developed a single plant with roots, while, the same callus on subculture to BA (3 mg/l) + Kn (1 mg/l) + NAA (1 mg/l) developed 2 shoots (Table 7). Nodular callus developed in BA (4 mg/l) and NAA (0.5 mg/l) showed signs of regeneration and on transfer to BA (3 mg/l) + Kn (1 mg/l) + NAA (1 mg/l) developed 3 shoots and many shoot initials (Fig. 37).

In *D. hamiltonii*, black glutinous callus developed from petiole explant in 2,4-D (1 mg/l) on subculture to TDZ (1 mg/l) developed 6 shoots (Fig. 38), while, the nodular compact leaf callus raised in the same hormonal regime upon transfer to TDZ (1 mg/l) produced 22 shoots (Fig. 39; Table 6). In *D. pubera*, bulbil callus raised in 2,4-D (3 mg/l) upon subculture to Kn (3 mg/l) or BA (2 mg/l) and Kn (1 mg/l) developed single shoot (Fig. 40; Table 6).

Discussion

Callus Induction

The type, concentrations and combinations of hormones as well as the type of explants influenced callus induction in *Dioscorea* species. In the present study, a significant difference was recorded in the rate of callusing of the different explants. This difference in callusing potential may be due to the species variation, physiological status of the explant source, culture conditions and sterilization procedures (Dodds and Roberts, 1995). Among the different explants used *viz*., leaf, stem, petiole, root and bulbil, most proliferative callus was obtained from leaf.

Fig. 21-40: Callus regeneration in *Dioscorea* species

Table 7. Effect of auxins in combination with cytokinins on callus regeneration

Explant	Hormones conc. (mg/l)				Number of shoots induced									
	BA	Kin	NAA	IBA	1	2	3	4	5	6	7	8	9	10
Bulbil	2	–	0.5	–	4									
Stem	2	–	0.5	–	1	–	–	–	–	–	–	–	–	–
	2	–	0.5	–	–	2	–	–	–	–	–	–	–	–
	3	1	1	–	–	2	–	–	–	–	–	–	–	–
	–	2	–	0.5	–	–	4	–	–	–	–	–	–	–
Leaf	3	1	1	–	–	–	–	2	–	–	–	–	–	–
	2	–	–	0.5	–	–	–	–	1	–	–	–	–	–
	3	1	1	–	–	–	–	–	–	–	–	2	–	–

1. D. bulbifera, 2. D. pentaphylla, 3. D. hispida, 4. D. oppositifolia, 5. D. belophylla
6. D. wightii, 7. D. spicata, 8. D. intermedia, 9. D. hamiltonii, 10. D. pubera

Wernicke and Park (1993) reported leaf explants as the best source for callus induction in *D. bulbifera*.

In the present study, of the various auxins tried alone, 2,4-D was the best for callus induction and proliferation in *Dioscorea* species. According to George (1993), the most frequently employed auxin to initiate callus is 2,4-D. Similar results of 2,4-D as efficient for induction of callus were reported in *D. bulbifera* (Wernicke and Park, 1993), *D. alata* (Acedo, 1994; Paneque, *et al*., 1995) and D. *cayenesnsis* (Viana and Mantell, 1989). Moreover, amongst the various concentrations of 2,4-D tried, leaf explant in 1 mg/l is the best giving maximum proliferation. Effectiveness of 2,4-D (1 mg/l) in inducing highest amount of callus was reported in *D. alata* by Acedo, *et al*. (1994). Higher concentrations of 2,4-D (above 5 mg/l), inhibited callus formation. This may be due to the toxic effect of the hormone beyond an optimum level.

The other auxins NAA/IAA/IBA in lower concentrations (0.5-1.0 mg/l) resulted in callus formation whereas in higher concentration (3.0-10.0 mg/l) rhizogenesis was observed.

2,4-D in conjunction with BA in *D. spicata* and Kn in *D. intermedia* induced callus. Efficiency of 2,4-D along with BA/Kn in inducing callus was reported by Sengupta *et al*. (1984) in *D. floribunda*.

Addition of cytokinins BA/Kn along with auxins resulted in callus induction in some species. In *D. intermedia*, leaf explants cultured in medium with NAA (8 mg/l) in combination with BA (2 mg/l) resulted in high rate of callusing. Similar reports of profuse callus formation in NAA and BA containing medium was reported in *D. alata* (Acedo *et al*., 1994; Amoroso, 1999) and *D. praehensilis* (Malaurie *et al*., 1995).

The synergistic effect of auxin NAA along with cytokinins BA and Kn also evoked good results in *D. intermedia*. This observation is concomitant with that in *Salvia officinalis* (Bolta *et al*., 2000), where, a combination of NAA, BA and Kn produced compact callus.

Based on the hormonal type and concentrations used, calluses raised in cultures showed variation in morphology, texture, colour and quality in *Dioscorea* spp. Development of callus with varying morphology with respect to hormones was reported in *Azhadirachta indica* (Ramesh and Padhya, 1990). Macek (1989) observed difference in texture ranging from compact to friable texture with a range of colours in calluses induced simultaneously under the same hormonal conditions in *Solanum* species. White friable/powdery callus was produced in all in medium containing 2,4-D alone or in combination with BA. Similar results of white friable callus formation in presence of 2,4-D alone or in conjunction with BA/Kn was reported in *Solanum nigrum* (Shahzad and Hassan, 1999).

In *D. wightii* and *D. belophylla,* medium containing 2,4-D at 3 mg/l induced brownish highly regenerative compact callus from leaf explants. The report on brown friable callus formation in presence of 2,4-D in *Hypericum brasilience* (Cardosa and de Oliveira, 1996) supports the present result. BA (3 mg/l), Kn (1 mg/l) and NAA (1 mg/l) produced compact organogenetic callus from leaf and petiole explants of *D. pentaphylla*. Nodular compact regenerative callus was formed in all in presence of 2,4-D (1-3 mg/l). The influence of 2,4-D in inducing nodular compact callus was reported in species such as *Sorghum bicolor* (Syamala and Devi, 2003).

Callus Regeneration

Callus regeneration was obtained by transferring the callus to basal MS medium or regeneration medium containing varying concentrations and combinations of cytokinins and auxins. Callus mediated adventitious shoot regeneration has the advantage of generating somaclonal variations having desirable qualities which can be selected and propagated.

In almost all the cases of callus regeneration, 2,4-D derived callus gave the maximum callus regeneration on transfer to basal MS medium or to the regeneration medium containing cytokinins (BA/Kn/TDZ/2-iP) alone or their combination or in combination with auxins (NAA/IBA). In *D. bulbifera* and *D. pentaphylla*, callus regeneration occurred in MS basal medium itself. Nair and Chandrababu (1996) obtained regeneration from callus cultures of *D. alata* and *D. esculenta* on MS medium.

Of the various cytokinins tried alone, Kn (2 mg/l) was found to be a potent cytokinin for shoot induction (1-3) from callus in all the species studied. The potentiality of Kn in inducing shoot bud formation from callus was reported in *Asparagus racemosus* (Daniel *et al*., 1999).

Considering the number of shoots regenerated from the callus, BA was found to be better than other cytokinins. In *D. belophylla* 4 shoot buds developed from callus in BA at 4 mg/l. Positive effect of BA on organogenesis from callus was also reported in a wide range of genera like Niger (Ahmed and Pandey, 1988), *Ocimum* spp.(Patnaik and Chand, 1996) and *Piper nigrum* (Bhat *et al*., 1995).

However, in *D. hamiltonii* TDZ when used alone at 1 mg/l gave a maximum of 22 shoots from the callus. TDZ has been shown to have high cytokinin activity by influencing endogenous cytokinin biosynthesis (Hutteman and Preece, 1993).

Regeneration medium containing a combination of cytokinins (BA+Kn) gave better results in all. Synergistic effect of the two (BA at 2 mg/l + Kn at 1 mg/l) produced 6 shoots in *D. bulbifera,* 4 in *D. hispida* and 5 in *D. wightii*. Synergistic effect of cytokinins in callus regeneration was reported by Mascarenhas *et al*. (1976) in *D. deltoidea.*

Synergistic effect of BA/Kn with NAA/IBA favoured shoot regeneration from callus in all. Promotion of shoot bud regeneration by BA in combination with NAA has been reported in species of *Dioscorea* such as *D. composita* (Datta *et al*., 1982), *D. floribunda* (Sengupta *et al*., 1984), *D. rotundata* (Belarmino *et al*., 1991; Nwachukwu *et al*., 1996), *D. alata* (Belarmino *et al*., 1991; Nair and Chandrababu, 1996; Amoroso, 1999) and *D. esculenta* (Belarmino *et al*., 1991; Nair and Chandrababu, 1996).

Synergistic effect of BA and IBA resulted in caulogenesis (single shoot) in *D. belophylla*, and Kn with IBA produced 2 and 4 shoots in *D. pentaphylla* and *D. hispida*. Moreover, the cytokinins BA and Kn along with NAA also resulted in shoot regeneration in *D. pentaphylla* (2 shoots), *D. oppositifolia* (2 shoots) and *D. intermedia* (2-3 shoots). Similar results of cytokinin-auxin combination inducing shoot regeneration were reported in *Plumbago zeylanica* (Rout *et al*., 1999) and *Catalpa ovata* (Lisowska and Wysokinska, 2000).

The present study results on callus induction and regeneration in wild species of *Dioscorea* from various explants achieved in MS medium under varied hormonal concentrations and combinations will be of much importance in large-scale multiplication of quality plants and effective management of the wild germplasm of Dioscoreas.

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