Direct Organogenesis in *Dioscorea* Species

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Protocol was developed for direct adventitious shoot regeneration from leaf and petiole explants of six species of *Dioscorea* in MS medium augmented with varying concentrations and combinations of cytokinins and auxins. Leaf explants responded well in cultures of *Dioscorea pentaphylla, D. hamiltonii, D. wightii* and *D. intermedia* followed by petiole in *D. bulbifera* and *D. hispida*. Cytokinins are effective in promoting direct shoot initiation. Amongst different cytokinins, BA was effective in producing maximum bud sprouting from leaf explants of *D. pentaphylla*, where 3 bulbils and 8 shoot buds got regenerated in 2 mg/l while *D. wightii* in BA at 4 mg/l induced 3 shoots. Kinetin (2 mg/l) in the medium also induced 16 shoots from leaf explants of *D. hamiltonii*. Synergistic effect of BA (3 mg/l), Kin (1 mg/l) and NAA (1 mg/l) initiated the development of 12 shoots in *D. pentaphylla* from leaf explants.

Key words: *Dioscorea*, Diosgenin, Bulbil, Micropropagation, Direct Organogenesis

Yams, members of the genus *Dioscorea* L. producing edible tubers and bulbils are of immense economic importance (Ammirato, 1984). They constitute the staple carbohydrate food, which contains high protein and mineral contents for millions in many tropical and subtropical countries. Moreover, wild species are of medicinal and pharmacological importance as they are sources of steroidal sapogenins especially diosgenin, which is the precursor in the commercial synthesis of sex hormone and corticosteroids (Coursey, 1967). They being vegetatively propagated and producing highly heterozygous seeds and having problems of shy flowering, non-synchronization in flowering and even non-flowering needs biotechnological approaches for clonal multiplication. The present study was aimed to standardize protocols for the rapid clonal multiplication of the twelve species available in Southern Western Ghats through direct organogenesis. In the method of direct organogenesis, adventitious shoots arise directly from the tissues of the explant without a callus phase. In *Dioscorea floribunda*, from the excised leaves, cultures were initiated by Sinha and Chaturvedi (1979). The regenerative proliferation and development of multiple shoots occurred from the pulvinus tissue of the leaf explants devoid of axillary buds and were successfully transplanted into soil. Direct organogenesis has also been recorded in *D. opposita*, where immature leaves were used as the explant (Kohmura *et al.*, 1995). Similarly, cultivated forms also behaved in culture with direct organogenesis as in *D. rotundata* (Nwachukwu *et al.*, 1996) and *D. batatus* (Matsubara *et al.*, 1992).

In the present experiment, direct organogenesis was obtained from leaf or petiole explants of six species of *Dioscorea* in MS medium augmented with varying concentrations and combinations of cytokinins and auxins. Leaf explants responded well in cultures of *D. pentaphylla, D. hamiltonii, D. intermedia* and *D. wightii* followed by petiole in *D. bulbifera* and *D. hispida*. The other explants tried namely stem, root and bulbil did not give any positive response.

Materials and Methods

One accession each from the 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 explant source for the present experiments. Leaf, stem, petiole, root and bulbil segments from vines of healthy and disease free mother plants of 4-months-old 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 aqueous 0.1% $HgCl_2$ for 5-20 minutes and then rinsed in sterile double distilled water 4-5 times in order to remove the adhering sterilants.

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Table 1. Materials used and locality of collection

Species Name	IC Number	Locality of collection
Dioscorea bulbifera L.	202349	Peechi, Thrissur, Kerala
Dioscorea pentaphylla L.	202367	Peechi, Thrissur, Kerala
Dioscorea hispida Dennst	202370	Nilambur, Malappuram, Kerala
Dioscorea tomentosa Heyne	202379	South Canara, Karnataka
Dioscorea pubera Bl.	202382	Begur, Wynad, Kerala
Dioscorea intermedia Thw.	202384	Santhanpara, Idukki, Kerala
Dioscorea spicata Roth.	202383	Thirthahally, Coorg, Karnataka
Dioscorea oppositifolia L.	202386	Valpara, Palghat, Kerala
Dioscorea wightii Hook. f.	214855	Mundanthurai, Tamil Nadu
Dioscorea belophylla Voight	248181	Devakolly, Coorg, Karnataka
Dioscorea wallichii Hk. f.	202312	Courtallum, Tirunelveli, Tamil Nadu
Dioscorea hamiltonii Hk.	202328	Vadakkancherry, Thrissur, Kerala

The explants were then trimmed to approximate sizes by removing off the cut ends after blotting in sterile filter paper before inoculation.

For the induction of direct adventitious buds, leaf, stem, petiole, bulbil and root segments (0.5-1 cm) were inoculated onto sterile culture tubes containing 15 ml of MS basal medium (Murashige and Skoog, 1962) augmented with varying concentrations and combinations of cytokinins *viz.*, BA, Kin, TDZ and 2-iP (0.5-10 mg/l) or auxins *viz.*, NAA, IAA, IBA (0.5-5 mg/l) alone or combinations of the two (0.5-3 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±1°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 shoots developed were separated from the growing clusters and transferred to rooting medium.

Micro-shoots obtained through direct organogenesis were rooted within 15 days either in MS basal medium or medium containing auxins *viz.*, IAA, IBA or NAA (0.5–2 mg/l). The rooted plantlets were transferred to tubes containing basal liquid medium with reduced levels of sucrose (1-2%) for hardening. The hardened plantlets were transferred to plastic cups containing soilrite and covered with moistened plastic bags to maintain maximum humidity and were kept in culture room for 2-3 weeks. The plantlets were nourished with 1/10 dilute Hogland's solution (Epstein, 1972) on every 3rd day for 2 weeks. The plantlets with newly sprouted leaves were then transplanted to pots containing garden soil and sand mixture in 1:1 ratio under green house conditions. The survival rate of plants was recorded as 5 replicates per treatment, each repeated 3 times.

Results

Dioscorea species were successfully micropropagated through direct organogenesis. Response of the various explants to different hormones, *viz.* cytokinins and auxins, either alone or in combination was studied.

Micropropagation was achieved in six species namely *Dioscorea bulbifera, D. pentaphylla, D. hispida, D. intermedia, D. hamiltonii* and *D. wightii* through adventitious *de novo* organogenesis from leaf and petiole explants when inoculated on to MS medium supplemented with cytokinins *viz.* BA/Kin/TDZ/2-ip alone or in conjunction with auxins *viz.* NAA/IAA/IBA/2,4-D. Surface sterilization of different explants in aqueous mercuric chloride at 0.1% and 0.2% for various time exposures (5-20 min.) showed that 0.1% $HgCl_2$ in an exposure time of 5-10 min. in case of leaf and petiole and 12-15 min. in case of stem and bulbil and 20 min. for root explants was found to be the best to obtain 85-90% contaminant free cultures for all the 12 species.

In *Dioscorea bulbifera*, young petiole alone showed organogenetic response in 2,4-D (3 mg/l) in the medium developing a bulbil from one of the cut ends (Plate-1. 1) which on subculture to basal MS developed a shoot. In *D. pentaphylla*, leaf explants developed 3 bulbils and 8 shoot buds directly from the explant on BA (2 mg/l) (Table 2). Synergistic effect of BA (3 mg/l), Kin (1 mg/l) and NAA (1 mg/l) after 25 days of culture resulted in the development of 12 shoot buds in this species from leaf explants (Table 2; Plate-1, 2), while in 60 days, the number of shoots increased to 43 (Plate-1, 3). These shoot buds on transfer to MS basal medium resulted in elongation and were subsequently transferred to rooting medium.

In *D. hispida*, from the cut end of young petiole, a single shoot bud was developed in 2,4-D (1 mg/l) after 30 days of culture (Plate 1, 4), which on subculture to TDZ (3 mg/l) produced 2 more shoot buds from the base.

Leaf segments of *D. hamiltonii* inoculated on medium containing TDZ (1mg/l), on subculture to Kin (2 mg/l), developed 16 shoots (Table 2; Plate-1, 5). In *D. wightii*, BA (4 mg/l) initiated the development of 3 shoots from leaf explants, which, on subculture to MS

Table 2. Effect of hormones on direct organogenesis in six species of *Dioscorea*

Species Name	Conc. of hormones (mg/l)								No. of shoots /
(Explant)	BA	Kin	TDZ	2-ip	IAA	IBA	NAA	2,4-D	bulbils (30 days)
D. bulbifera									
(Petiole)	-	-	-	-	-	-	-	**3.0**	1 (bulbil)
D. pentaphylla	**2.0**	-	-	-	-	-	-	-	8 / 3 (bulbils)
(Leaf)	3.0	1.0	-	-			1.0	-	12 shoots
D. hispida									
(Petiole)	-	-	-	-	-	-	-	1.0	1 shoot
D. hamiltonii									
(Leaf)	-	-	1.0	-	-	-	-	-	16 shoots
D. wightii									
(Leaf)	4.0	-	-	-	-	-	-	-	3 shoots
D. intermedia									
(Leaf induced root)	-	2.0	-	-	-	0.5	-	-	1 shoot

produced 3 more shoots with the elongation of the existing shoots in 45 days of culture (Plate-1, 6). In *D. intermedia*, leaf explants on culture media failed to show shoot organogenesis but resulted in the development of feathery white roots in IBA (0.5 mg/l). However, in this species, leaf disc cultured in IAA, IBA and NAA (3-5 mg/l) initiated roots in 12 days. The IAA/IBA raised roots after 40 days developed shoot directly. IBA raised roots shooted in a combination of Kin (2 mg/l) and IBA (0.5 mg/l) and produced branches (Plate-1, 7).

D. bulbifera responded very slowly in culture with regard to rooting. In presence of NAA (0.5 mg/l) in the medium it produced healthy roots in 30 days (Plate-2, 1). The other species *viz.*, *D. hispida*, *D. hamiltonii* and *D. wightii* took only 10-15 days for healthy rooting in presence of NAA (0.5 mg/l) (Plate-2, 3, 4, 5). *D. pentaphylla* and *D. intermedia* produced roots without a separate rooting medium (Plate-2, 2, 6).

The rooted shoots were transferred to liquid MS medium supplemented with 2% sucrose over filter paper bridges and were transferred to plastic cups containing soilrite covered with plastic bags for hardening and acclimatization. Sprinkling them with Hogland's solution at intervals of 3 days for 2 weeks provided nourishment to the plantlets. After 15 days, the hardened plantlets were planted in pots containing garden soil and sand in the ratio 1:1 and were then shifted to green house conditions for establishment. Survival rate of the regenerants was from 85% in *D. hispida* and *D. wightii*, 88% in *D. hamiltonii* and *D. bulbifera* to 94% in *D. pentaphylla* and *D. intermdia*, which grew luxuriantly as the green house grown mother plants (Plate-3, 16).

Discussion

Members of the genus *Dioscorea* being vegetatively propagated possess unique problems in genetic conservation as well as improvement. Hence there is a need to employ non-conventional propagation method like tissue culture to propagate the different species of *Diosorea*. With the aim to standardize protocols for rapid clonal multiplication, twelve different species of *Dioscorea* of the southern Western Ghats, were selected, of which six of them yielded positive results in terms of *de novo* adventitious shoot organogenesis.

By several trials appropriate explant sterilization procedure was standardized for each species with minimal rate of microbial contamination and without damage to the tissues. 0.1% mercuric chloride and exposure to 8-10 minutes in case of leaf and petiole explants and 12-15 minutes for stem, bulbil and root explants was adequate to get 85-90% contaminant free cultures in all the experiments.

Selection of appropriate nutrient medium forms the initial and essential step in plant tissue culture. Earlier reports by Acedo, *et al.* (1994) and Nair, *et al.* (1994) also affirmed the potentiality of MS basal medium on micropropagation of edible yams.

In the present study, direct organogenesis was achieved from leaf and petiole explants of six species of *Dioscorea* in MS medium augmented with varying concentrations and combinations of cytokinins and auxins. Direct organogenesis in *Dioscorea* species varied according to the species, type of the explant and treatment with plant growth regulators.

Adventitious shoot formation directly from excised

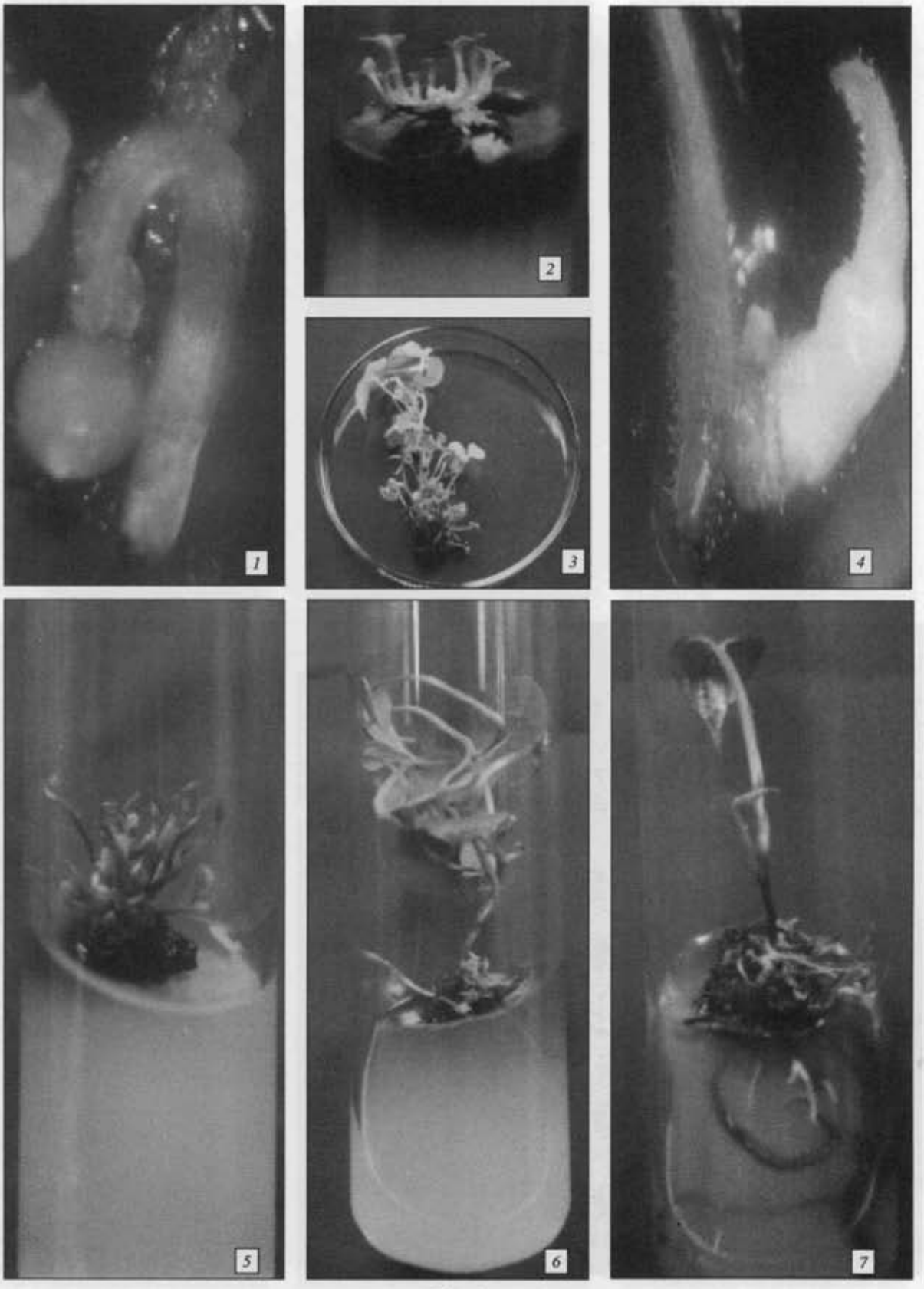

Plate 1

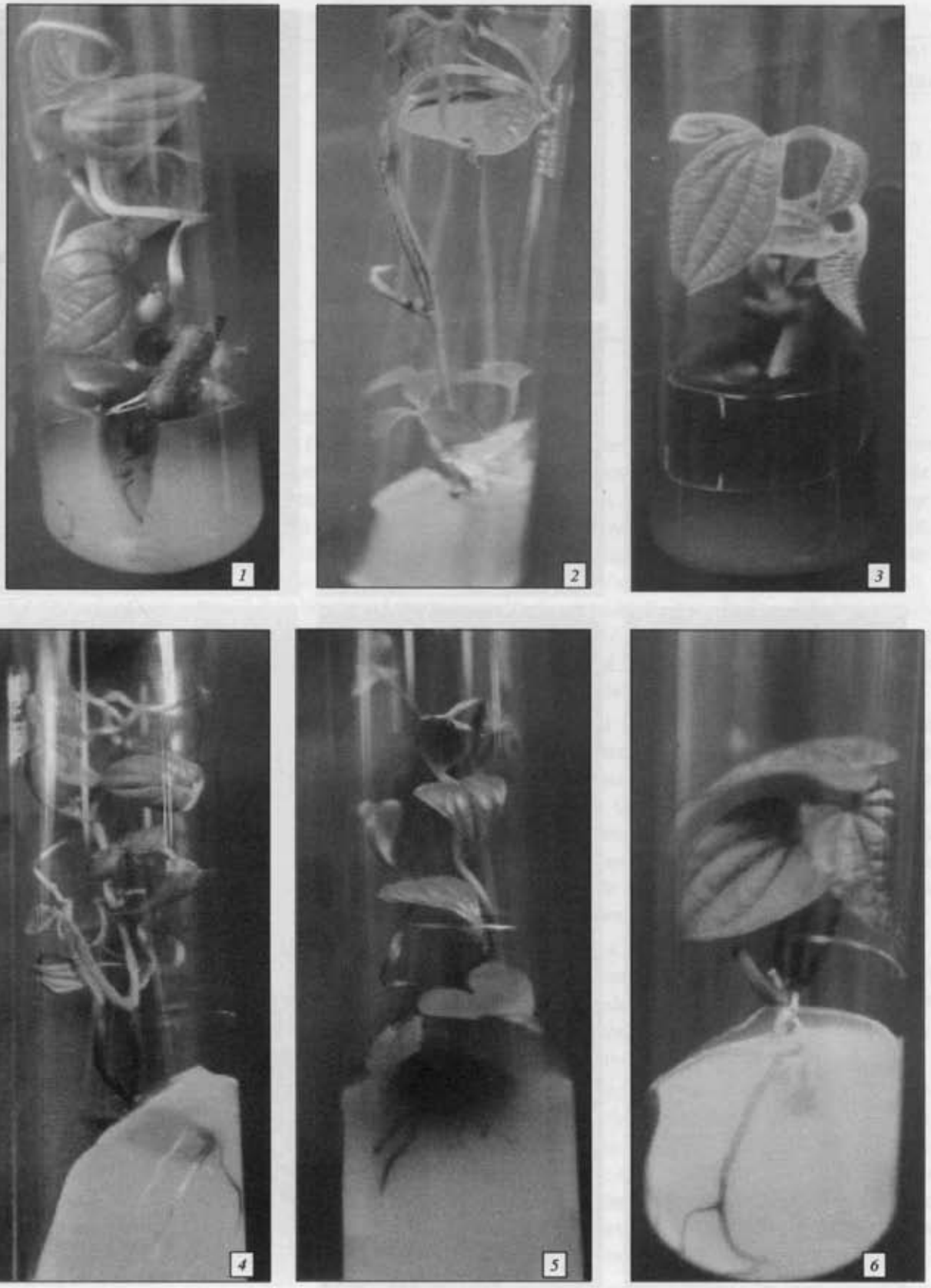

Plate 2

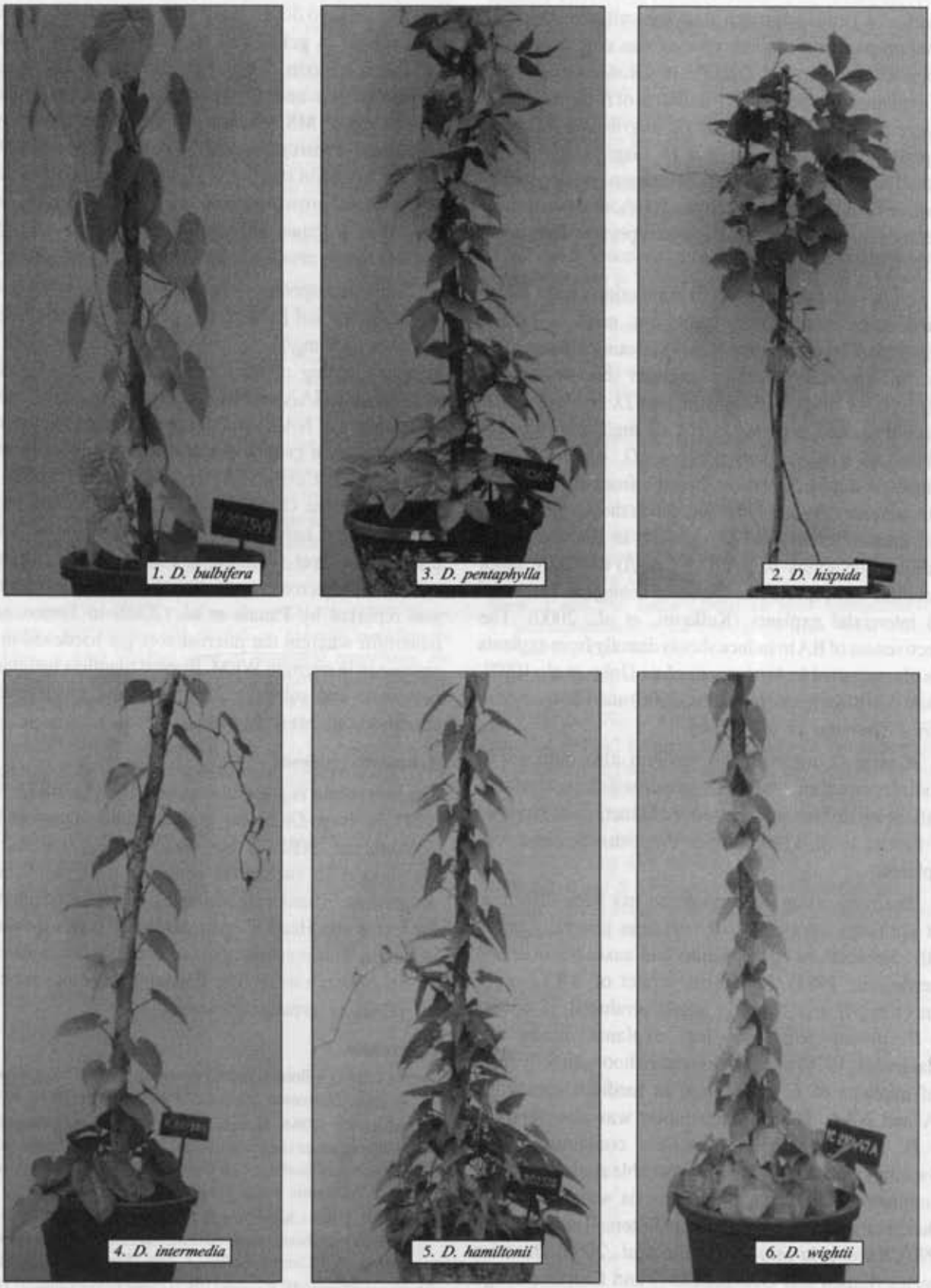
1. D. bulbifera
3. D. pentaphylla
2. D. hispida
4. D. intermedia
5. D. hamiltonii
6. D. wightii

Plate 3

organs as a better approach than the callus method for clonal propagation of plant species was emphasized by Bhojwani and Razdan (1983). In *Dioscorea* species, leaf explants responded well in cultures of *D. pentaphylla, D. hamiltonii, D. wightii* and *D. intermedia* followed by petiole in *D. bulbifera* and *D. hispida*. The stem, root and bulbil explants did not give *de novo* organogenesis. Superiority of leaf explants for greater shoot regeneration potential is in accordance with earlier reports by Kohmura, *et al.* (1995) in *D. opposita*.

Cytokinins are effective in promoting direct shoot initiation. Of the various cytokinins used to induce adventitious bud sprouting from various explants, BA was effective in producing maximum shoots from the leaf explants of *D. pentaphylla* and *D. wightii*. In *D. pentaphylla*, leaf explants in BA (2 mg/l) developed 3 bulbils and 8 shoot buds while in *D. wightii*, BA at 4 mg/l produced 3 shoots directly from the explant. Kohmura *et al.* (1995) could induce direct shoot regeneration from immature leaves of *D. opposita* in BA containing medium. The ability of BA (0.5-5 mg/l) to induce shoot formation was reported in *Withania somnifera* from leaf and internodal explants (Kulkarni, *et al.*, 2000). The effectiveness of BA to induce shoots directly from explants was also reported in *Naragamia alata* (John *et al.*, 1997), *Vanda coerulea* (Seeni and Latha, 2000) and *Clerodendron inerme* (Baburaj *et al.*, 2000).

Kinetin (2 mg/l) in the medium also induced 16 shoots from leaf explants of *D. hamiltonii*. Similar pattern of shoot bud initiation in presence of kinetin was reported by Pereira *et al.* (1995) from *Maytenus ilicifolia* root explants.

The application of a cytokinin has been effective, but optimum rates of shoot initiation generally occur with combinations of cytokinins and auxin (George and Sherrington, 1984). Synergistic effect of BA (3 mgl), Kin (1 mg/l) and NAA (1 mg/l) produced 12 shoots in *D. pentaphylla* from leaf explants. Sinha and Chaturvedi (1979) could also induce shoots directly from leaf explants of *D. floribunda* in medium containing BA and NAA. Direct regeneration was also reported in *D. rotundata* in MS medium containing NAA (Nwachukwu, *et al.*, 1996). A favourable auxin-cytokinin combination for direct organogenesis was reported in other plants like *Camelina sativa* (Tattersall and Milliam, 1999), *Kaemferia galanga* (Shirin *et al.*, 2000), *Brassica* species (Eapen and George, 1997) and *Curcuma longa* (Salvi *et al.*, 2000).

The *de novo* developed microshoots of the 5 species were rooted in gelled full strength basal MS medium or with the auxin NAA. Microshoots of the species *D. pentaphylla* and *D. intermedia* developed healthy roots in basal MS medium itself without any growth regulators. In *Jatropha curcas*, Sujatha and Mukta (1996) could also obtain rooting in regenerated shoots in basal MS without growth regulators. Similar results were noticed in *Solanum viarum* (Chand and Chand, 1995) and *Maranta arundinacea* (Manjula *et al.*, 1999).

The other species such as *D. bulbifera, D. hispida, D. hamiltonii* and *D. wightii* produced roots in presence of NAA (0.5 mg/l). Of the different auxins used for inducing rooting of micro shoots, NAA was found to be superior to IAA and IBA in producing healthy roots. The efficacy of NAA in inducing roots from microshoots was reported in genera like *Manihot esculenta* (Mussio *et al.*, 1998), *Curcuma longa* (Salvi *et al.*, 2000) and *Vanda coerulea* (Seeni and Latha, 2000). The rooted plantlets were hardened in liquid MS medium having 2% sucrose over filter paper bridges. Such results of reduction in sucrose concentration enhancing hardening was reported by Panaia *et al.* (2000) in *Symonanthus bancroftii* wherein the microshoots got hardened in 2% sucrose in ½ strength WPM. Rooted plantlets maintained in soilrite and subsequently transferred to greenhouse conditions showed 85-94% survival.

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