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CLONAL PROPAGATION AND ESTABLISHMENT OF PLANTLETS OF *DENDROBIUM FIMBRIATUM* VAR. *OCULATUM* HOOK. F.

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SUMMARY

Protocorm-like bodies were developed from the apical meristems and axillary buds of *Dendrobium fimbriatum* var. *oculatum* cultured on a new formulated medium supplemented with growth regulators. Maximum number of plbs were formed on the medium supplemented with 10·0 μM of IAA and 0·5 μM of BAP. However, at 2·5 μM each of IAA and BAP, caulogenesis was initiated directly from the apical meristem. A large number of plbs were formed from axillary buds on medium supplemented with 5·0 μM of NAA. NAA (10·0 μM) and BAP (0·5 μM) stimulated the formation of both plbs and small shoots from axillary buds. On being left undisturbed the plbs developed into complete plantlets. These clonally propagated plantlets were hardened and established in the glasshouse under optimum conditions of temperature, light and moisture. The substratum comprising brick and charcoal pieces, vermiculite and a layer of moss on top resulted in 70% survival and healthy growth of plantlets.

KEY WORDS

Dendrobium fimbriatum var. *oculatum*, micropropagation, protocorm-like bodies

ABBREVIATIONS

Plbs, protocorm-like bodies; IAA, indole–3–acetic acid; NAA, α–naphthaleneacetic acid; 2,4–D, 2,4–dichlorophenoxyacetic acid; BAP, 6–benzylaminopurine; KN, kinetin

INTRODUCTION

Clonal propagation of orchids by means of explants is gaining wide importance in the tissue culture industry. It is particularly valuable in perpetuating clones of special merit. Morel (1960) for the first time noted the formation of plbs around the shoot tips of *Cymbidium* cultured *in vitro*. These plbs when cut into small sections and subcultured on fresh nutrient medium, multiplied and, on being left undisturbed, developed into complete plantlets. This work led to the mass propagation of desirable virus-free varieties at a very high rate.

Tissue culture methods for the propagation of orchids were introduced by several workers using various plant parts and a number of media (Bergman, 1972; Wang, 1989). *Cymbidium, Dendrobium, Vanda, Spathoglottis* and allied genera are widely propagated by tissue culture throughout the world (Vajrabhaya, 1977; Oliva *et al.*, 1985). According to Murashige (1978), important orchids except *Paphiopedilum* are clonable *in vitro*. Kusumoto (1979) used the shoot apices of *Cattleya* buds for the production of plbs. Cional propagation of *Phalaenopsis* by means of flower stalk, bud culture and shoot-tip culture has also been reported (Yoneda *et al.*, 1983). Explants from the floral stalk have been used in studies on *in vitro* propagation of orchids (Tanaka and Sakanishi, 1978; Homma and Asahira, 1985; Yoneda, 1986; Momose and Yoneda, 1988). Reports concerning the propagation of orchids through leaf segments have been published (Tanaka, 1987; Vij and Pathak, 1990). Phillip and Nainar (1988) reported the *in vitro* transformation of root meristem to shoot and plantlets in *Vanilla planifolia*. Sanchez (1988) carried out micropropagation of *Cyrtopodium* cf. *punctatum* seedlings, grown asymbiotically *in vitro*, through root tip culture. Yoneda and Momose (1988) reported plbs and plantlets formation by root tip cultures in *Phalaenopsis*. *In vitro* shoot regeneration from root tips of *Mormodes histrio* has also reported been by Holters and Zimmer (1990).

Different tissue culture techniques have been developed to promote the selected clones multiplication in Dendrobiums (Kim *et al.*, 1970; Mosich *et al.*, 1974; Kukulczanka and Wojciechowska, 1983). But these techniques differ from species to species. The explants employed for the tissue culture respond selectively depending on their source, physiological state and nutrient environment. The following investigation deals with tissue culture of *D. fimbriatum* var. *oculatum* for mass propagation of identical genotypes of this orchid.

MATERIALS AND METHODS

Different explant sources such as leaf parts, root tips, axillary buds and apical meristems were obtained from the plants maintained in the Botanical Garden of North-Eastern Hill University, Shillong. About 1–2 cm segments of the explants were cut and cleaned gently scrubbing with a soft brush and mild detergent. These were then washed in running tap water for about 15–20 min and were rinsed with distilled water. The surface sterilization of the explants was done by sodium hypochlorite solution (0·8–1·0% available chlorine) for 15–20 min. The explants were thoroughly rinsed with sterile distilled water to remove the sterilant. Leaf bases, leaf tips and roots tips measuring about 2 mm were cut from the larger sections of the explant sources. The nodal sections containing the axillary buds were also cut into 3–4 mm size after the removal of the leaves, dry sheaths and other external tissues. The apical meristems measuring about 1 mm were excised from the shoot tips aseptically and cultured on different media.

A number of media were tried for the clonal propagation of *D. fimbriatum*. A new medium comprising inorganic and organic nutrients along with the vitamins was developed after trying different combinations of nutrients (data not presented here). The composition of the medium is given in Table 1. The sterilized explant pieces were inoculated on this medium aseptically. The cultures were maintained at 24 ± 2°C with 16 hr illumination of 3000 lux light intensity. The pH of the medium was adjusted to 5·5 prior to autoclaving. The medium was supplemented with different growth regulators both separately and in combination so as to initiate the formation of plbs and shoot buds on the explants. The different combinations of growth regulators tried were 2, 4–D + KN, IAA + KN, NAA + KN, NAA + BAP and IAA + BAP in a range of 0–10·0 μM.

Table 1. Basal medium devised for clonal propagation

Macro and micro nutrients	mg/*l*	Macro and micro nutrients	mg/*l*
NH_4NO_3	825	KNO_3	950
KH_2PO_4	200	H_3BO_3	0·5
KI	0·02	$Na_2MoO_4.2H_2O$	0·025
$CoCl_2.6H_2O$	0·02	$MgSO_4.7H_2O$	275
$MnSO_4.4H_2O$	6·8	$ZnSO_4.7H_2O$	1·0
$CuSO_4.5H_2O$	0·05	$CaCl_2.2H_2O$	88·0
$CaHPO_4$	400·0		
Na_2 EDTA	3·72	$FeSO_4$	2·785
$AlCl_3$	0·03	$NiCl_3.6H_2O$	0·03
Vitamins			
Inositol	100	Thiamine HCl	0·4
Pyridoxine HCl	0·3	Nicotinic acid	0·3
Glycine	2·0		
Addenda			
Agar	9 g	Sucrose	20 g
N/1 HCl	6 ml		

RESULTS

Out of the different explant sources tried, the apical meristems and axillary buds were found to be suitable for clonal propagation of this dendrobe. The apical meristem enlarged and produced the protocorm mass in about 3–4 weeks time in the medium supplemented with IAA and BAP, both separately and in combination (Table 2). IAA at 10·0 μM concentration resulted in the formation of plbs on apical meristem. The maximum number of plbs were formed on the medium supplemented with 10·0 μM of IAA and 0·5 μM of BAP. For caulogenesis from apical meristem, a balanced supply of 2·5 μM each of auxin and cytokinin in combination was found to be effective. The axillary buds responded better to the medium containing NAA and BAP. It took about 4 weeks for the formation of plbs or shoot buds from axillary buds. A large number of plbs formed in the presence of NAA at 5·0 μM in the medium. NAA (10·0 μM) and BAP (0·5 μM) stimulated the formation of plbs and small shoots (Table 3). The plbs were multiplied by further cutting and subculturing on fresh medium. The formation of complete plantlets from apical meristems (Fig. 1) and axillary buds (Fig. 2)

Table 2. Effect of IAA and BAP individually and in combination on the formation of plbs and caulogenesis in the apical meristem

Medium* + Growth regulator	Conc. µM	% response	Nature of response		Remarks
			Plbs	Caulogenesis	
Control	–	–	–	–	No morphogenetic response observed
IAA	0·5	15	–	+	Apical meristem differentiated into a solitary shoot bud
	2·5	10	–	+	1–2 shoot buds emerged from meristem
	5·0	20	–	++	Few shoot buds appeared on surface of the tissue
	10·0	50	+	+	Small green plbs and shoot buds appeared
BAP	0·5	30	–	+	Green protuberances, i.e., shoot buds appeared
	2·5	20	–	–	Tissue enlarged and remained green. No further development occurred
	5·0	15	–	–	No growth noticed
	10·0	15	–	–	Tissue remained green
IAA + BAP	0·5 + 0·5	20	–	–	Tissue remained green
	0·5 + 2·5	60	+	+	Both plbs and shoot buds emerged from meristematic tissue
	0·5 + 5·0	–	–	–	Tissue dried up
	0·5+10·0	–	–	–	Tissue dried up
	2·5 + 0·5	40	–	–	Distal end of explant showed swellings
	2·5 + 2·5	30	–	++	Emerging shoot buds differentiated into complete plantlets
	2·5 + 5·0	20	–	+	Few shoot buds appeared from tissue
	2·5+10·0	–	–	–	Tissue dried up
	5·0+0·5	50	+	–	Very few plbs appeared which turned brown
	5·0 + 2·5	60	–	+	Single shoot emerged, turned yellow subsequently
	5·0+5·0	40	–	+	Shoot buds differentiated but turned brown
	5·0+10·0	–	–	–	Explant dried up
	10·0+0·5	80	++	+	Numerous healthy plbs developed
	10·0+2·5	50	–	+	Poorly developed shoot buds emerged
	10·0+5·0	20	–	+	Solitary shoot developed
	10·0+10·0	–	–	–	Explants dried up

* Basal medium used; – no response; + moderate; ++ appreciable

Table 3. Effect of NAA and BAP individually and in combination on the formation of plbs and caulogenesis in the axillary buds

Medium* Growth Regulator	Conc. µM	% response	Nature of response: plbs	Nature of response: Caulogenesis	Remarks
Control	–	50	–	+	Single solitary shoot developed from axillary bud
NAA	0·5	30	–	+	Bud proliferated into a single shoot
	2·5	60	–	+	2–3 shoots developed
	5·0	70	–	++	Axillary bud enlarged into a swollen mass which developed into small shoots
	10·0	50	++	+	Small shoot buds and plbs formed
BAP	0·5	35	–	–	Bud development into a single shoot took place
	2·5	20	–	–	Signs of shoot initiation observed, no further development
	5·0				Explant remained green. No growth noticed
	10·0	–	–	–	" " " "
NAA+BAP	0·5+0·5	30	–	+	2–3 shoots developed
	2·5+0·5	80	+	+++	A large number of small shoots emerged
	5·0+0·5	60	–	++	Multiple shoots developed
	10·0+0·5	40	+	+	Small shoots and plbs emerged out
	0·5+2·5	20	–	+	2–3 shoots developed
	2·5+2·5	30	–	+	" "
	5·0+2·5	20	–	+	Developing shoots turned yellow subsequently
	10·0+2·5	20	–	+	Poor development of shoots
	0·5+5·0	40	–	+	Development of single shoot
	2·5+5·0	50	–	+	Development of single shoot
	5·0+5·0	40	–	+	Developing shoots turned brown
	10·0+5·0	–	–	–	Explant dried up
	0·5+10·0	10	–	+	Formation of a small and poor plant
	2·5+10·0	–	–	–	Explant dried up
	5·0+10·0		–	–	" "
	10·0+10·0		–	–	" "

*Basal medium used; – no response; + moderate; ++ appreciable; +++ good.

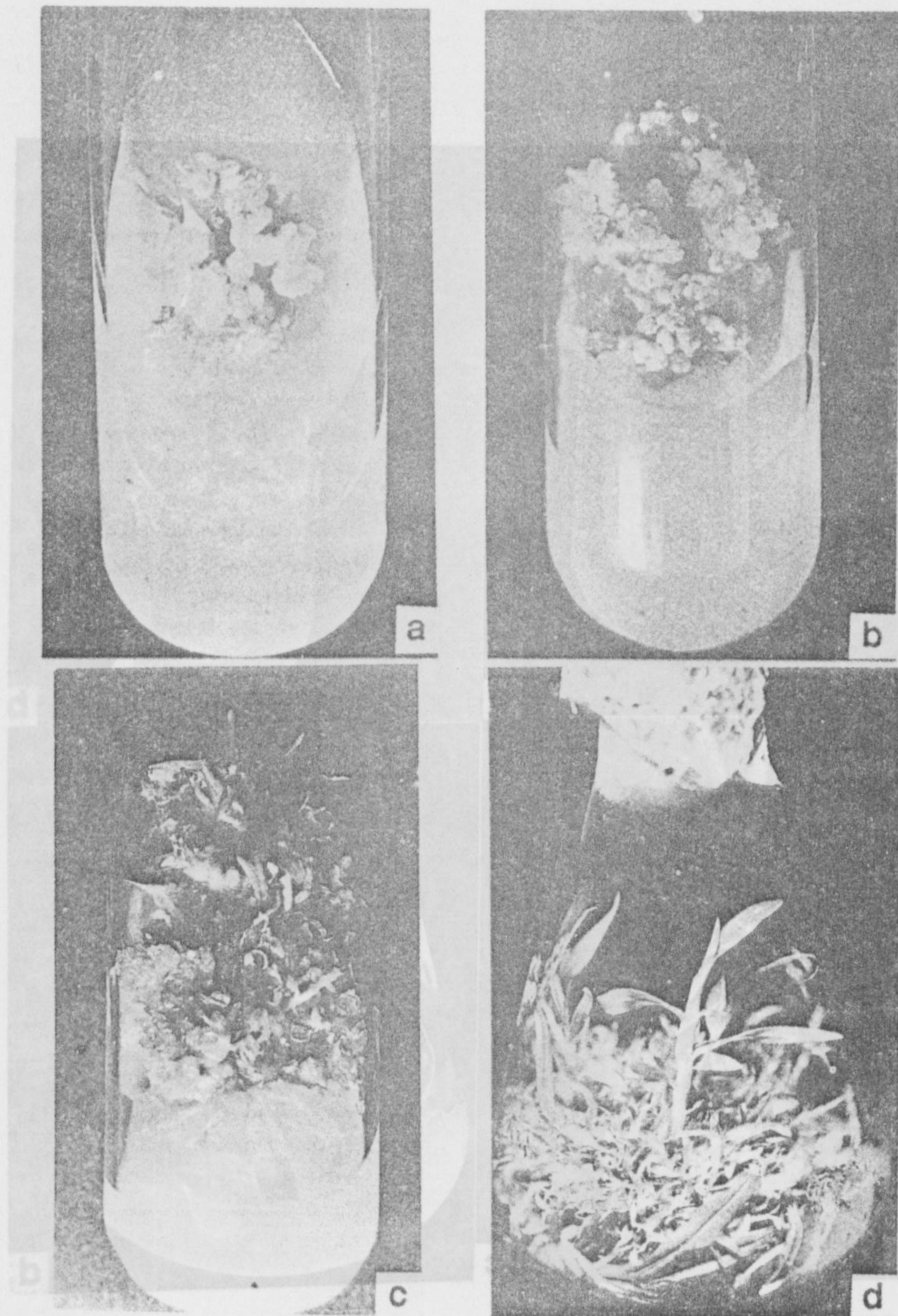

Fig. 1. Development of complete plantlets from apical meristem. (a) Formation of protocorm-like bodies. (b) Multiplication of protocorm-like bodies. (c) Development of shoots. (d) Complete rooted plantlets in culture

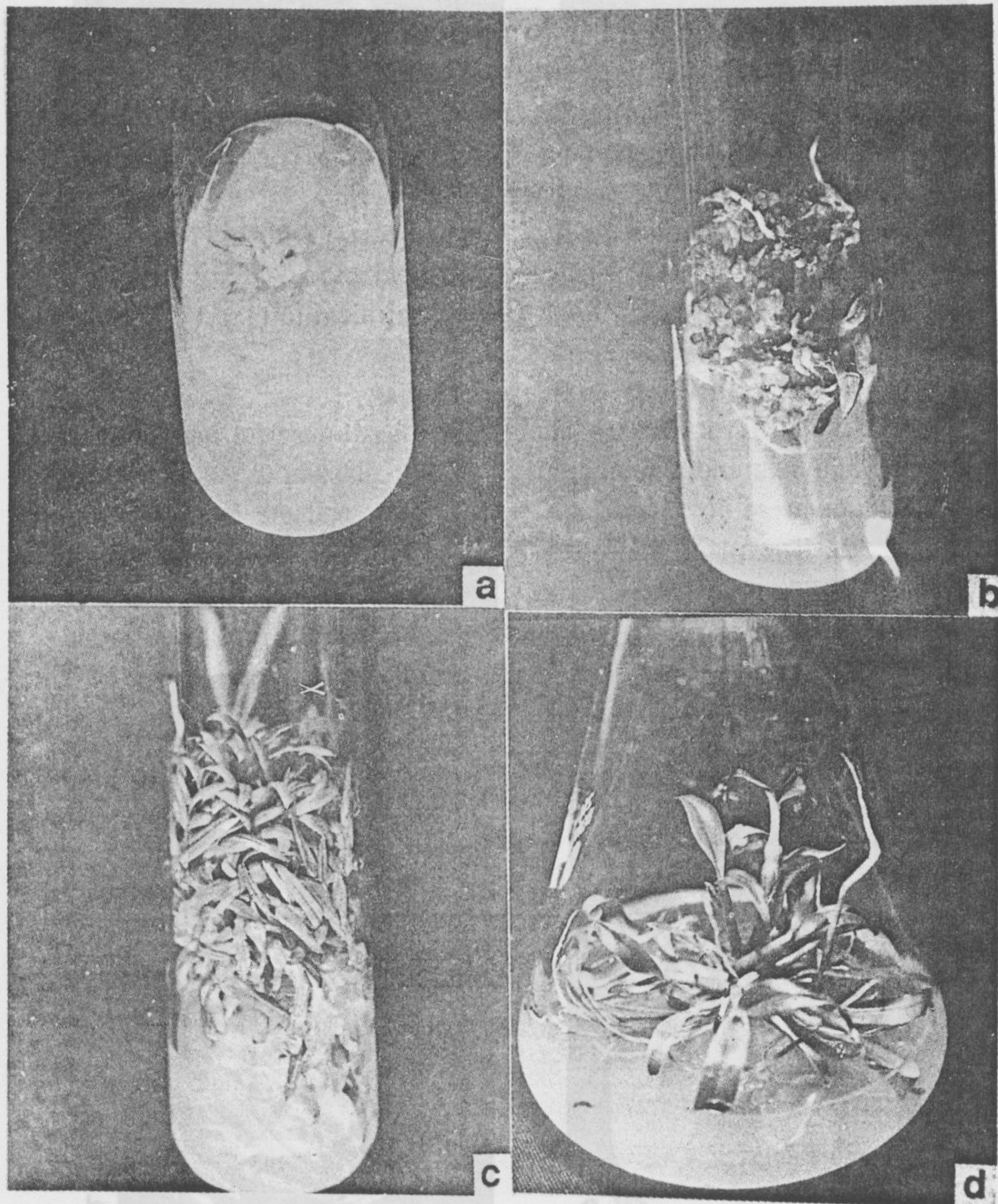

Fig. 2. Development of complete plantlets from axillary bud (a) Initiation of protocorm–like bodies (b) Multiplication and development of protocorm–like bodies. (c) Formation of shoots from protocorm–like bodies. (d) Rooted plantlets in culture.

took place in about 8 weeks' time on leaving the plbs undisturbed. The other combinations of NAA and BAP tried, also stimulated the differentiation of the plantlets from the axillary buds. Conversely, other growth regulators and their combinations tried were not found suitable for plb formation and caulogenesis. The leaf tips dried up in about 2 weeks' time. However, the leaf bases remained green for a longer time. The root tips also remained green for some time and showed slight swelling.

Tiny plantlets measuring 2·5–3·0 cm were transferred to clear clay pots of 10 cm diameter containing different mixture of compost *viz.* :

(i) Charcoal chunks and brick pieces (1 : 1),
(ii) Charcoal chunks, brick pieces and coconut husks (1 : 1 : 1/2),
(iii) Charcoal chunks, brick pieces (1 : 1) and a layer of moss,
(iv) Charcoal chunks, brick pieces, vermiculite (1 : 1 : 1/2) and a layer of moss, and
(v) Charcoal chunks and a layer of moss.

The potted plantlets were kept in the glass-house for hardening. The minimum and maximum temperatures of the glass-house at the time of transplantation were 16°C and 22°C, respectively and the relative humidity was 70–80%. The plantlets were fed with 1/10 MS nutrient salt solution fortnightly and kept in shade in the glass–house. The compost comprising charcoal chunks, brick pieces, vermiculite and a layer of moss was found to be the most suitable for the healthy growth of the plantlets (Table 4). The plantlets were hardened and established in 6–7 weeks' time (Fig. 3).

Table 4. The response of *in vitro* grown plantlets to different potting media

	Substrate used	% Survival	Growth
(i)	Charcoal + brick	10	–
(ii)	Charcoal + brick + coconut husk	15	–
(iii)	Charcoal + brick + moss layer	60	+++
(iv)	Charcoal + brick + vermiculite + moss	70	+++
(v)	Charcoal + brick + vermiculite	60	++
(vi)	Charcoal + moss	45	+

– poor growth; + fair growth; ++ good growth; +++ best growth

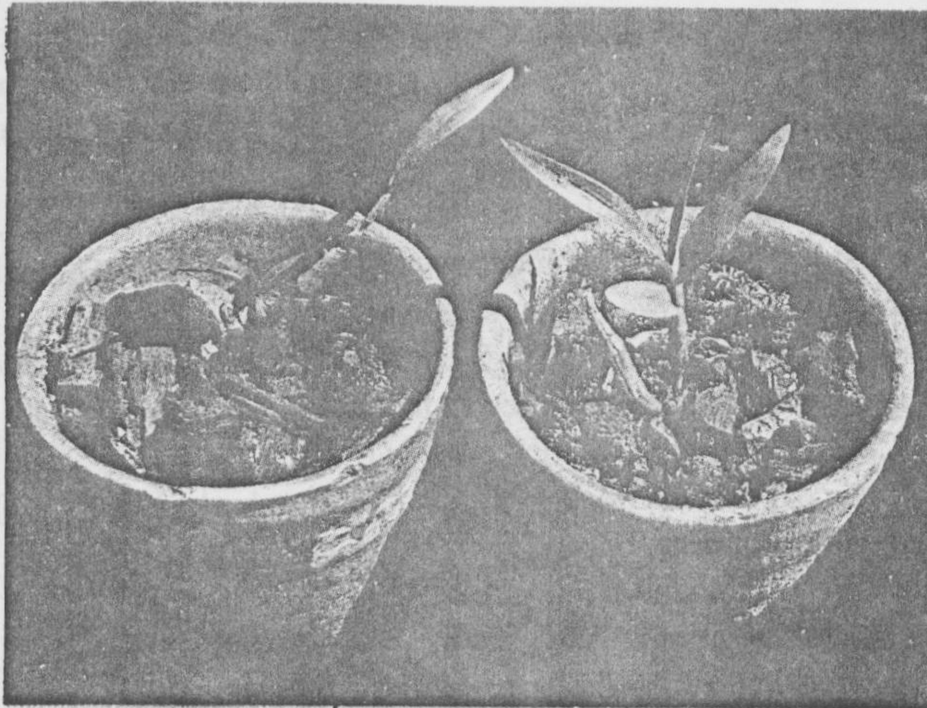

Fig. 3. Complete plantlets transferred to pots in glass-house

DISCUSSION

The causative factors controlling morphogenesis and regeneration from various plant parts are diverse. Nutritional requirements for the optimal growth of the plants *in vitro* vary from species to species. The concentrations and chemical forms of the components vary considerably. Some media contain components of defined chemical nature (salts, vitamins, hormones, amino acids, organic acids, nucleotides, nucleic acids and chelating agents), while the others have complex, not entirely defined ones (peptone, tryptone, casein hydrolysate, yeast and/or potato extract, and green or ripe banana). Considerable variations are reported to exist between media used for species of the same genus or different genera of orchids (Arditti, 1977). In the present study, a new medium was formulated which proved to be highly suitable for micropropagation of *D. fimbriatum.* This medium was composed of nutrient salts and vitamins and was purely synthetic (Table 1). Although Heller (1953) reported that aluminium and nickel were not essential for tissue culture of plants, in the present case the chlorides of these elements were used and found useful. Increased concentration of chloride ions in the medium might have promoted the induction of plbs. The use of hydrochloric acid in the medium was additional and it maintained the required acidity of the medium.

The axillary buds and apical meristems were found to be the suitable explant sources for the formation of plbs. Clonal propagation of

Dendrobium through shoot tips and nodes has been successfully accomplished in some other species (Kim *et al.*, 1970; Mosich *et al.*, 1974). The other explants of *D. fimbriatum* failed to respond in culture. Although Rao (1977) had reported tissue culture of *Dendrobium* through leaf and root explants, the media used were not purely synthetic. The successful propagation through these explants has also been reported in other orchid species (Kraus and Kerbauy, 1987; Sanchez, 1988; Yoneda and Momose, 1988; Kraus and Monteiro, 1989; Holters and Zimmer, 1990). The use of growth regulators in the medium is beneficial and results in different morphogenetic responses. It has been reported that the tissues from diffrent organs or/and parts differ in their growth substance requirements. These differences are mostly marked in respect of the auxin requirements (Audus, 1972). In the present study, the combinations of IAA and BAP, and NAA and BAP resulted in the formation of plbs from apical meristems and axillary buds, respectively, which is consistent with the earlier reports (Ponchet *et al.*, 1985; Sanchez, 1988). Fonnesbech (1972) reported that in low concentration and in combination with auxin, BAP induced the formation of plbs and small shoots from the explants. Similar findings are reported in the present study where the addition of IAA and NAA at higher concentrations brought about the formation of plbs, both when added separately and in combination with BAP. The morphogenetic responses of the explants varied with the concentrations of the growth regulators used. The incorporation of NAA and BAP along with 10% coconut milk in the medium was found to increase the rate of plbs formation in *Phalaenopsis*, where internodal sections of the flower stalk were used as the explant source (Yoneda *et al.*, 1983).

The transfer of plants from the culture vessels to the glass-house conditions requires a careful, stepwise procedure. Healthy plantlets showing vigorous growth in the culture vessels were transferred to the pots. It is a well-established fact that the healthy and vigorously growing seedlings are easier to transplant and these are less susceptible to diseases and mechanical injuries. The transferred plantlets had a healthy and vigorously growing root system which ensured higher establishment and growth. Charcoal chunks, brick pieces, vermiculite and a layer of moss formed the best substratum for the growth of the plantlets as it may have facilitated proper drainage and aeration for root respiration. The layer of moss on top proved to be beneficial due to higher retention of moisture. Direct sunlight was harmful to the transferred plantlets which may be due to the increase in temperature

at the leaf surface. Feeding the plantlets with nutrient salt solutions has been reported to be beneficial for the promotion of orchid plant growth (Mukherjee, 1983). The present procedure described for the hardening and transplanting of the plantlets resulted in 70% survival and healthy growth of the plantlets. The compost used is easily available, porous and convenient for the transfer and establishment of the *in vitro* clonally propagated plantlets.

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