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Stem disc culture: Development of a rapid mass propagation method for *Dendrobium moschatum* (Buch.-Ham.) Swartz – An endangered orchid

The clonal propagation from *Cymbidium* apical shoot meristem by Morel[1] has been the most sensational development which has revolutionized the orchid industry and triggered global expansion of tissue culture for rapid propagation. The technique has been especially important for orchids as their genotypes are highly heterozygous and sexual reproduction is an extremely slow process. Morel estimated that it is possible to obtain more than four million plantlets in a year from a single explant[2]. Among different methods available[3,4], shoot tip and axillary bud cultures are widely used[5]. Regeneration potential of alternative plant parts other than shoot tip and axillary buds like stem, leaf, root and inflorescence have been worked out in some species of orchids[6–17]. Both shoot tips and axillary buds produce protocorm-like bodies (PLBs) which subsequently develop into plantlets. The clonal propagation from shoot meristem culture has proved disadvantageous particularly for endangered orchid taxa because continuous excision of meristematic region may threaten the existence of the mother plant. So, the emphasis has now been shifted towards faster methods by exploring alternative plant parts whereby the mother plant can be saved[17]. Studies on rapid regeneration have been scanty in view of the success reports in few orchid taxa when compared to the large size of the orchid family[5,18].

The present investigation deals with the development of a rapid regeneration method using thin sections of stems from *in vitro* raised seedlings of *Dendrobium moschatum* (Buch.-Ham.) Swartz, an epiphytic endangered orchid taxon[19]. During last two decades extensive work has been done on micropropagation of orchids and vast literature has been accumulated in this area. However, information in this regard in *D. moschatum* (Figure 1 *a*) is entirely lacking. Hence, the present work was undertaken with a view to finding out a suitable method for rapid micropropagation of this orchid taxon.

Shoots of 6–8-week-old *in vitro* raised seedlings of *D. moschatum* were used as explant source in the present study. Shoots were taken out of conical flasks aseptically and the leaves and roots were removed. Transverse sections measuring 1–1.5 mm were prepared by cutting the stem with a sharp, sterile surgical blade. Two different basal media, Vacin and Went[20] and Knudson C (KnC)[21] both in liquid (without agar) and semisolid (with agar) form were tested. A positive and most encouraging result was obtained in KnC medium. Both the media were suitably modified. The modified media were supplemented with coconut milk (CM, 15% v/v), various concentrations of indoleacetic acid (IAA; 0.5, 1.0, 2.0 and 3.0 mg l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5, 1.0, 2.0 and 3.0 mg l^{-1}), and different combinations of a naphthaleneacetic

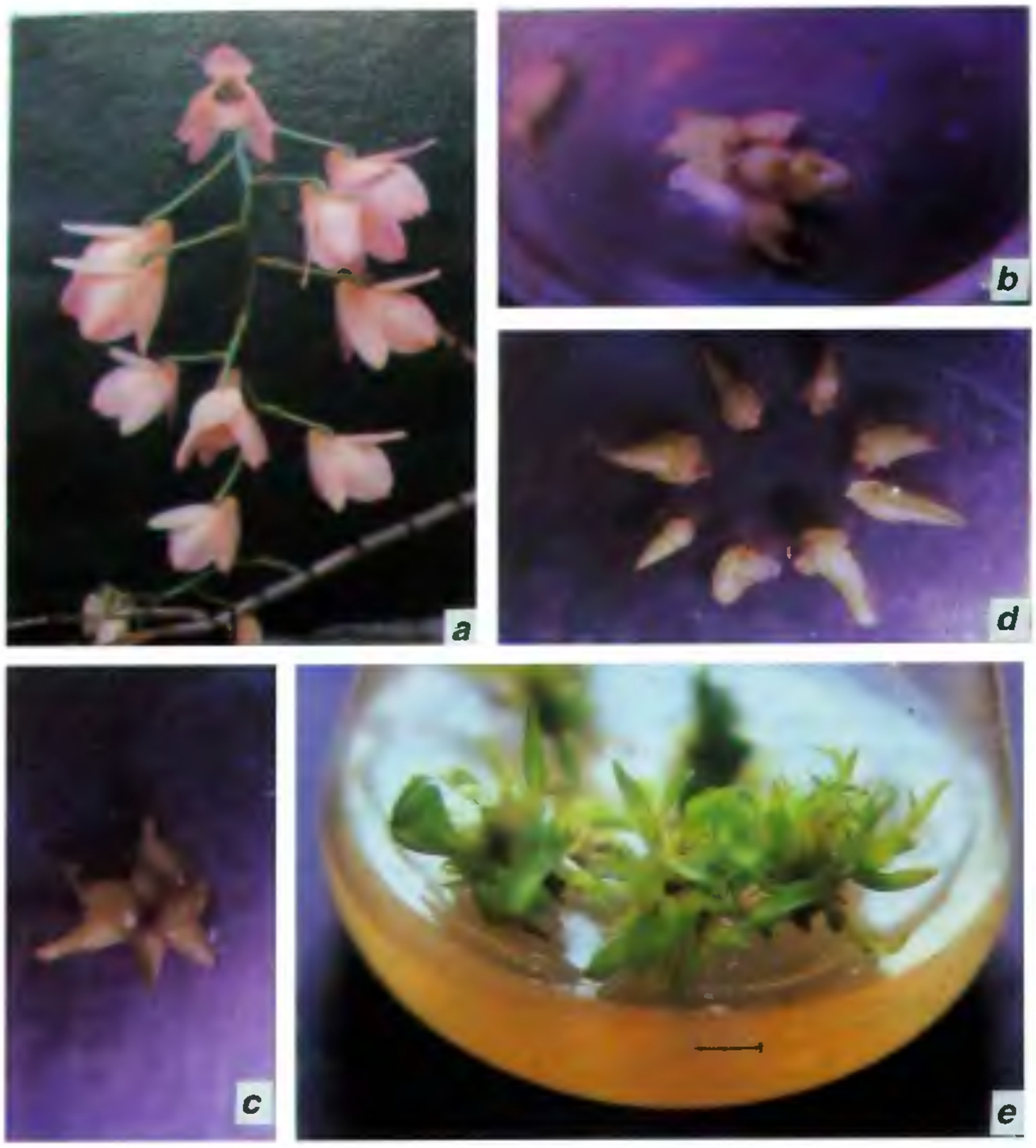

Figure 1. Formation of PLBs in *D. moschatum* and their regeneration into plantlets. The bar represents 10 mm for all figures. *a*, Flowering shoot of naturally occurring *D. moschatum*; *b*, PLBs formed in the conical flasks containing liquid medium; *c*, Cluster of PLBs; *d*, Separated PLBs; and *e*, Plantlets (8-week-old) obtained through regeneration of PLBs.

acid (NAA; 0.5, 1.0, 2.0 and 3.0 mg l^{-1}) and 6-benzyl amino purine (BAP; 0.5, 1.0, 2.0 and 3.0 mg l^{-1}) depending on the objective of the experiment. The pH of the media was adjusted to 5.2. All media either liquid or semisolid were autoclaved at 110 kPa for 20 min at 120°C. Explants cultured on liquid media were continuously agitated on a rotary shaker at 80–120 rpm. Conical flasks were incubated at 25 ± 2°C under 16 h photoperiod from cool white light giving 1000 lux at culture level. Observations were made at regular intervals of 10 days. PLBs produced in liquid culture were subcultured in the semisolid medium for further multiplication. For growth and differentiation, the clumps of PLBs were finally transferred into conical flasks containing semisolid KnC medium enriched with CM (15%), BAP (3 mg l^{-1}) and NAA (2 mg l^{-1}). Subculturing was carried out once every two weeks. All treatments had 5 replicates and were repeated at least thrice. Survival percentage of the explants and the PLB formation per explant were evaluated after 6 weeks following inoculation.

Stem discs cultured both in liquid and semisolid modified KnC media expanded during the middle of the second week. Expansion of the stem disc was the first visible change. PLB (Figure 1 *b*) formation was initiated at the beginning of the third week. 59% and 56% explants survived and 2.0 and 1.6 PLBs on an average were formed from each explant when they were cultured in modified basal (without any organic additives and growth regulators) liquid and semisolid KnC media, respectively. Addition of CM has been found to increase the survival percentage and the PLB formation as well. The survival percentage and PLB formation per explant were calculated as 86 and 3.6 on an average, respectively, when cultured in liquid KnC medium. On the other hand, 81% of explants survived and 3.0 PLBs were formed from a single explant when cultured in semisolid medium. However, higher concentrations of CM more than 15% were not found suitable. Various growth regulators were tried either individually or in combination with CM (15% v/v) supplemented liquid and semisolid KnC media and different responses were noted. 2,4-D at 1 mg l^{-1} concentration showed a significant increase in PLB formation in liquid culture. 7.6 PLBs were produced from a single explant and the survival percentage of the PLBs was as high as 93. But in semisolid medium, the same concentration of 2,4-D yielded only 4.8 PLBs from an explant which was quite low in comparison to the liquid culture. Concentrations of 2,4-D higher than 1 mg l^{-1} were found to induce callusing (Table 1). Addition of IAA at 2 mg l^{-1} concentration with CM supplemented liquid and semisolid KnC media, was found to increase PLB production. 6.4 and 4.8 PLBs were produced from a single explant in liquid and semisolid culture, respectively (Table 1). NAA and BAP when used independently had very little or no effect on PLB formation and survival percentage of the explants. However, their combination resulted in a sharp and significant increase in PLB production. When BAP (3 mg l^{-1}) and NAA (2 mg l^{-1}) were added with KnC medium, 13.6 and 8.4 PLBs on an average were produced in liquid and semisolid medium respectively (Table 2). The survival percentage was also very high. However, concentrations of NAA higher than 2 mg l^{-1} were found inhibitory.

In all the cases the PLBs were green, healthy and in cluster (Figure 1 *c*). The PLBs were separated for subculturing. Separated PLBs (Figure 1 *d*) were subcultured for further multiplication and within 10–12 weeks following subculture, the PLBs developed into healthy plantlets (Figure 1 *e*).

15-week-old plantlets were taken out of the conical flasks and washed thoroughly with distilled water to remove

Table 1. Effect of different concentrations of growth regulators on PLB formation in *D. moschatum*. Data taken 6 weeks following inoculation. All treatments had 5 replicates and were repeated thrice

Hormone	Concentration (mg l^{-1})	Survival percentage ± SE		PLB formatrion per explant ± SE	
		Liquid	Semisolid	Liquid	Semisolid
IAA	0.0	89 ± 1.44	85 ± 2.57	3.0 ± 0.41	2.4 ± 0.40
	0.5	87 ± 1.52	83 ± 2.18	5.0 ± 1.09	3.2 ± 0.67
	1.0	92 ± 1.88	82 ± 2.07	5.8* ± 1.00	4.2* ± 0.58
	2.0	93* ± 2.80	88 ± 1.64	6.4** ± 1.20	4.8** ± 0.37
	3.0	71*** ± 1.34	72** ± 1.70	3.8 ± 0.80	3.2 ± 1.00
NAA	0.0	81 ± 2.03	80 ± 2.00	2.6 ± 0.73	2.6 ± 0.66
	0.5	82 ± 1.20	80 ± 2.07	3.2 ± 0.58	2.0 ± 0.40
	1.0	76 ± 2.19	83 ± 2.03	3.6 ± 0.50	2.8 ± 0.20
	2.0	84 ± 2.47	81 ± 3.00	4.6* ± 0.90	3.0 ± 0.43
	3.0	76 ± 2.80	77 ± 3.50	2.2 ± 0.30	2.0 ± 0.28
2,4-D	0.0	86 ± 2.19	79 ± 3.47	2.8 ± 0.80	3.0 ± 0.30
	0.5	85 ± 3.90	76 ± 2.7	4.8* ± 0.40	2.6 ± 0.60
	1.0	93* ± 2.80	89* ± 2.9	7.6** ± 0.60	4.8 ± 0.70
	2.0	68*** ± 2.08	72 ± 3.2	CD	CD
	3.0	66*** ± 1.58	65 ± 1.40	CD	CD
BAP	0.0	81 ± 1.94	76 ± 2.34	2.4 ± 0.60	2.0 ± 0.20
	0.5	79 ± 2.9	80 ± 1.80	4.0* ± 0.44	2.8 ± 0.40
	1.0	74 ± 2.6	70* ± 4.6	4.2*** ± 1.03	3.0*** ± 0.38
	2.0	83 ± 1.87	81* ± 2.46	3.8 ± 0.80	2.6 ± 0.20
	3.0	84 ± 2.70	81 ± 3.80	4.6** ± 0.50	3.8* ± 0.40

Values followed by asterisks in each hormone treatment within the same column are significantly different from control (no growth regulators; only KnC + CM), using Student's *t* test at *5% level; **1% level and ***0.1% level. CD represents callus development.

Table 2. Effect of NAA–BAP combination on PLB formation in *D. moschatum*. Data taken 6 weeks following inoculation. All treatments had 5 replicates and were repeated thrice

Hormone concentration		Percent of explant survived ± SE		PLB formation per explant ± SE	
NAA	BAP	Liquid	Semisolid	Liquid	Semisolid
0.0	0.0	80 ± 2.75	81 ± 3.07	3.2 ± 0.40	2.8 ± 0.30
0.5	0.5	78 ± 2.40	73* ± 2.34	4.0*** ± 0.58	3.4*** ± 0.50
0.5	1.0	87 ± 2.90	86*** ± 1.04	3.8 ± 0.60	4.0* ± 0.32
0.5	2.0	80 ± 3.19	83 ± 2.67	4.6*** ± 0.80	4.2** ± 0.86
0.5	3.0	89* ± 1.81	86.5 ± 2.09	5.4*** ± 0.90	4.6*** ± 0.60
1.0	0.5	90* ± 1.59	87** ± 0.84	3.4 ± 0.50	2.8 ± 0.37
1.0	1.0	92* ± 1.26	89 ± 1.5	5.0*** ± 1.04	4.2** ± 0.60
1.0	2.0	88 ± 2.16	85 ± 2.88	5.8** ± 0.80	4.6** ± 0.20
1.0	3.0	91** ± 1.30	89 ± 3.50	6.6*** ± 0.92	5.2** ± 0.40
2.0	0.5	87 ± 2.56	89 ± 3.27	3.0 ± 0.40	3.4 ± 0.70
2.0	1.0	91* ± 1.50	86 ± 1.72	7.0** ± 0.60	5.8* ± 0.49
2.0	2.0	93** ± 1.82	87* ± 2.38	9.2*** ± 0.48	6.8** ± 0.80
2.0	3.0	92** ± 4.51	89 ± 3.02	13.6*** ± 0.30	8.4*** ± 0.70
3.0	0.5	79 ± 3.93	83 ± 2.19	4.6* ± 0.59	3.8 ± 0.37
3.0	1.0	80 ± 4.00	77 ± 1.87	3.6 ± 0.40	4.0 ± 0.70
3.0	2.0	83 ± 1.51	80 ± 2.06	5.0*** ± 1.08	5.4 ± 0.80
3.0	3.0	75 ± 2.58	77 ± 3.57	2.4 ± 0.53	1.8* ± 0.60

Values followed by asterisks in each treatment within the same column are significantly different from control (no hormone; only KnC + 15% CM), using Student's *t* test at *5% level; **1% level and ***0.1% level.

f agar particles. Following fun- (Bavastin) treatment they were red to community pots contain- ing a mixture of brick bats, charcoal chips, sand and soil (1:1:1:1). High humidity (about 90%) was maintained around the plantlets for two we tially the plantlets did not sh growth symptom. Most of the

grown roots died. Supplementation of the pot mixture with varying concentrations of IAA revealed that root development was induced at 1 mg l^{-1} level. Better rooting was obtained with NAA (1 mg l^{-1}). Hardened plantlets with 3–4 new roots on transferring to field showed significantly high (48%) survival.

The present study deals with the development of a suitable method for rapid mass propagation of an endangered orchid taxon *D. moschatum* (Buch.-Ham.) Swartz. Results obtained in the present study clearly indicate the efficiency of the protocol. It has recently been discovered that if meristems are cultured in liquid media in shake flasks, PLB production can be greatly increased[22]. The present investigation substantiates this. It further reveals that PLB production per explant has always been higher in agitated liquid culture than in semisolid culture. So, liquid culture is by far more suitable for rapid micropropagation in this taxon. With this thin section culture method more than 1,10,000 plantlets could be produced from a single *in vitro* raised seedling within a year.

CM (15% v/v) has been found to increase the survival percentage of the explants. As CM contains many nutritional and hormonal substances, it is very difficult to make any conclusive inferences for such a high percentage of survival of explants. However, this may be due to the presence of cytokinin and sugar in CM[5].

Significant increase in PLB production has been noted both in liquid and semisolid cultures when auxins like IAA and 2,4-D were used with CM supplemented KnC medium. NAA, on the other hand, had very little effect on PLB production when used individually. The result is the same in case of BAP. But a BAP–NAA combination (BAP 3 mg l^{-1} with NAA 2 mg l^{-1}) resulted in significant increase in PLB production from a single explant. 13.6 PLBs were obtained in the mentioned BAP–NAA combination when added to CM supplemented liquid KnC medium. In semisolid culture, the PLB production per explant was 8.4. The interesting point is that, BAP or NAA when used individually had very little or no effect but their combination produced the best result. In the present study, it has been noted that the number of PLBs is always higher in agitated liquid culture than that in semisolid culture. This may be due to the fact that liquid media provide better aeration and optimum conditions for respiration and salt uptake. Moreover, in liquid culture, the prolifering tissues having increased surface area enable more uptake of nutrients resulting in better growth and differentiation[23].

With the recent developments in tissue culture techniques, emphasis has now been shifted towards exploring the potentials of alternative plant parts as explant source. In the present investigation, stem sections of *in vitro* grown plants were used as explant source. This has two advantages – one is the easy availability of explants without damaging the natural source and the other is the aseptic availability of explants which will help minimize contamination. The agitated liquid culture method has already been proved advantageous for rapid micropropagation[22]. The present study will add a new dimension in this direction. The thin section liquid culture method described in the text is a highly efficient method for rapid micropropagation of at least *D. moschatum* (Buch. Ham.) Swartz.

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