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IN VITRO PROPAGATION OF *DENDROBIUM* AND *PHALAENOPSIS* THROUGH TISSUE CULTURE FOR CONSERVATION

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ABSTRACT

The studies were focused on developing an efficient and effective propagation protocol for orchid species from genera *Dendrobioum* and *Phalaenopsis* through tissue culture. The Materials used were explants from adventive shoot tip, floral stalk buds and PLBs derived from seeds. The results indicated growth and development of adventive shoot tip explants of *D*endrobium: a high survival percentage for explant with green color was shown by *D. racianum*, followed by *D. laxiflorum, D. pseudo-conantum, D. strebloceras, D. lineale, and D. veratrifolium.* However, plantlets regeneration occurred only on *D. pseudoconantum, and D. strebloceras*. Explant regeneration from seed derived protocorm-like bodies on *D. spectabile* occurred 40 days after inoculation transfer and subculture. High survival percentage of explant from floral stalk shoot was shown by *P. amabilis*. There were several plantlets surviving in acclimatisation. Explant regeneration from seed derived from protocorm-like bodies on *P. hieroglypha* occurred 40 days after inoculation and subculture. It was suggested that for *ex situ* conservation on certain species of *Dendrobium* and *Phalaenopsis* in the category of rare germplasms, tissue culture could be applied effectively and efficiently by using explant from adventive shoot tip, floral stalk buds and seed derived protocorm-like body explant for vegetative seed multiplication.

Keywords: orchid, conservation, species, in vitro culture

INTRODUCTION

Germplasms is a very valuable asset as raw materials in any orchid breeding programs. For example, orchids, such as *Dendrobium* and *Phalaenopsis,* which contain some species that are close to extinction, urgently require conservation. Indonesia with its climate and tropical rainforest is an ideal habitat for many orchid species. At present the existence of orchid germplasms in their natural habitat is at risk because of illegal selling, logging, and natural disaster. Their population is also drastically declining because of a lower rate of propagation in nature and overexploitation. One of the means for *ex situ* conservation is by propagation through *in vitro* culture.

Cloning technique by tissue culture resulted in vegetative propagation in mass number and the offspring genetically similar to the parental plant. It has made a possible choice for *ex situ* conservation in orchid. Many types of explants such as shoot tips (Sagawa and Kunisaki 1982; Malabadi *et al.,* 2004; Malabadi *et al.,* 2005), floral stalks (Lim-Ho and Lee, 1987; Young *et.al*, 2000), protocorm-like bodies (Ishii *et al.,* 1998; Lee and Lee. 2003; Huan *et al.,* 2004), seed derived protocorm-like bodies (Chen and Chang, 2004) and protocorm-like bodies derived from mature seeds (Shimura and Koda, 2004) have been used as explants in tissue culture to produce plantlets.

Some *Dendrobium* and *Phalaenopsis* species were chosen to be employed in this study in order to investigate suitable explants material that is good for *in vitro* propagation. By using this method, valuable plants can be conserved and exploited.

MATERIALS AND METHODS

Plant materials used in this experiment were adventive shoot tips, floral stalk buds and seed-derived protocorm-like bodies (plb) from eight species of *Dendrobium* and four species

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Phalaenopsis. *Dendrobium* species used were from different sections: section *Spatulata* (*Ceratobium*) *i.e. Dendrobium stratiotes and D. lasianthera*; section *Eleuteroglossum* i.e *D. canaliculatum*; section *Latourea* i.e. *D. spectabile .* Furthermore, some species from genus *Phalaenopsis* i.e. *Phalaenopsis amabilis, P.. amboinensis, P. hieroglypha* and *P. tetraspis* were used.

Explants from adventive shoot tips of *Dendrobium* and floral stalk buds from *Phalaenopsis* were sterilized in the surface by using Clorox and Teepol. Each explant was placed on medium in 140 ml-volume culture bottle containing 30 ml solid medium. The media were VW, VW+0.5 mg l^{-1}BA, ½ MS and ½ MS+0.5 mg l^{-1}BA. Two months after cultured, explants were cut into pieces and subcultured into fresh medium containing similar medium with the addition of 2 mg l^{-1} BA and 0.5 mg l^{-1}NAA. It was a descriptive experiment and because of insufficient number of explants, there was no replication used.

Another type of explant used in this experiment was four-month old PLBs derived from seeds. These seed-derived protocorm-like bodies (plbs) were longitudinally bisected. Ten explants were placed on medium as previously described. Regenerated plbs were subcultured into fresh medium containing similar medium with the addition of NAA. Cultures were maintained at temperature $25^{o} \pm 2^{o}$ C, light intenstity of 30-40 µ mol m^{-2} s^{-1} and photoperiods of 16 hours in light and 8 hours in dark.

Observation was made, percentage of explant contamination, colour of explants, days of callus or plb initiation, percentage and number of explant development, and number of regenerated plantlets were recorded.

RESULTS AND DISCUSSION

Dendrobium Adventive Shoot Tip Explant

Results of experiments of *in vitro* plant regeneration of *Dendrobium* explants showed that a high percentage of shoot explants with green colour was obtained from *D. racianum* followed by *D. lineale, D. pseudoconantum, D. strebloceras, D. laxiflorum* and *D. veratrifolium.* Percentage of contamination on explants ranged from 0 to 100% (Table 1.) In this experiment, shoot explant with green colour for eight species of *Dendrobium* ranged from 0 to 100 %. From descriptive observation, it was shown that after cultured on medium, some explants of *D. pseudoconantum* turned into yellow and the lowest part of the adventive shoot tip explants of *D. veratrifolium* turned into white. It could be because these explants contained older tissues that made the growth and development (regeneration) slow and became yellow and white in colour. While explants of *D. laxiflorum, D. pseudoconan-tum* and *D. strebloceras* turned into brown, and explants of *D. pseudoconantum* and *D. strebloceras* turned into black. Explants with wide surface cut after several days in culture became brown or black in colour, and could not develope any further. Browning could also be a result of phenolic compound production (Figure. 1.).

Explants with green colour could develop further although not all could grow into new shoot. After 40 days of first subculture, green adventive shoot tip explants *of D. pseudoconantum* formed callus on medium containing ½ MS+BA+NAA. While *D. strebloceras* formed shoot on medium containing ½ MS+ BA+NAA, plb and callus+plb on medium containing VW+BA+NAA. Of eight *Dendrobium* species, only *D. pseudoco-nantum* and *D. strebloceras* were able to regenerate and continue to grow into plantlets (Figure 2.).

Table 1. The growth and development of adventive shoot tip explants of *Dendrobium* species on *in vitro* culture

Species	Media	Number of explants	Contamination (%)	Explant (%) with criterium of colour				
				Green	Yellow	Brown	Black	White
D. laxiflorum	VW	1	0	100	-	-	-	-
	VW+BA	1	0	100	-	-	-	-
	½ MS	1	0	100	-	-	-	-
	1/2MS+BA	2	0	100	-	100	-	-
D.pseudoconantum	VW	2	0	100	100	-	-	-
	VW+BA	2	0	100	100	-	-	-
	½ MS	2	0	100	-	-	100	-
	1/2MS+BA	3	0	100	100	100	-	-
D. canaliculatum	VW	1	100	-	-	-	-	-
	VW+BA	1	100	-	-	-	-	-
	½ MS	1	100	-	-	-	-	-
	1/2MS+BA	2	100	-	-	-	-	-
D. strebloceras	VW	2	50	100	-	-	-	-
	VW+BA	5	40	100	-	100	100	-
	½ MS	4	50	100	-	100	-	-
	1/2MS+BA	4	50	100	-	100	-	-
D. sp. Maluku	VW	3	100	-	-	-	-	-
	VW+BA	2	100	-	-	-	-	-
	½ MS	2	100	-	-	-	-	-
	1/2MS+BA	3	100	-	-	-	-	-
D. lineale	VW	2	50	100	-	-	-	-
	VW+BA	2	50	100	-	-	-	-
	½ MS	2	50	100	-	-	-	-
	1/2MS+BA	2	50	100	-	-	-	-
D. veratrifolium	VW	1	0	-	-	-	-	100
	VW+BA	2	50	-	-	-	-	100
	½ MS	1	0	100	-	-	-	-
	1/2MS+BA	2	50	100	-	-	-	-
D. racianum	VW	1	0	100	-	-	-	-
	VW+BA	1	0	100	-	-	-	-
	½ MS	1	0	100	-	-	-	-
	1/2MS+BA	1	0	100	-	-	-	-

The results of experiment, from which part of the method showed that number of plantlets of *D. pseudoconantum* regenerated on VW and VW+BA+NAA that survived in acclimatisation was 75 and 2, respectively. All plantlets regenerated on ½ MS and on ½ MS+BA+NAA died. While on *D. strebloceras* was 11 on VW, 26 on VW+BA+NAA, 11 on ½ MS, and 11 on ½ MS+BA+NAA (Table 2). The number of explant regeneration from seed derived protocorm-like bodies on *D. spectabile* was 22 calli and 10 plb on VW; 44 shoot on VW+BA; 44 plb and 44 shoot on ½ MS; 10 calli and 11 shoot on ½ MS+BA (Table 3.).

Table 2. Plantlet regeneration of adventive shoot tip explants of *Dendrobium* species on *in vitro* culture 12 months after the first subculture

Species	Media	Number of regenerated plantlets
D. pseudoconantum	VW	75
	VW+BA+NAA	2
	½ MS	0
	1/2MS+BA+NAA	0
D. strebloceras	VW	11
	VW+BA+NAA	26
	½ MS	11
	1/2MS+BA+NAA	11

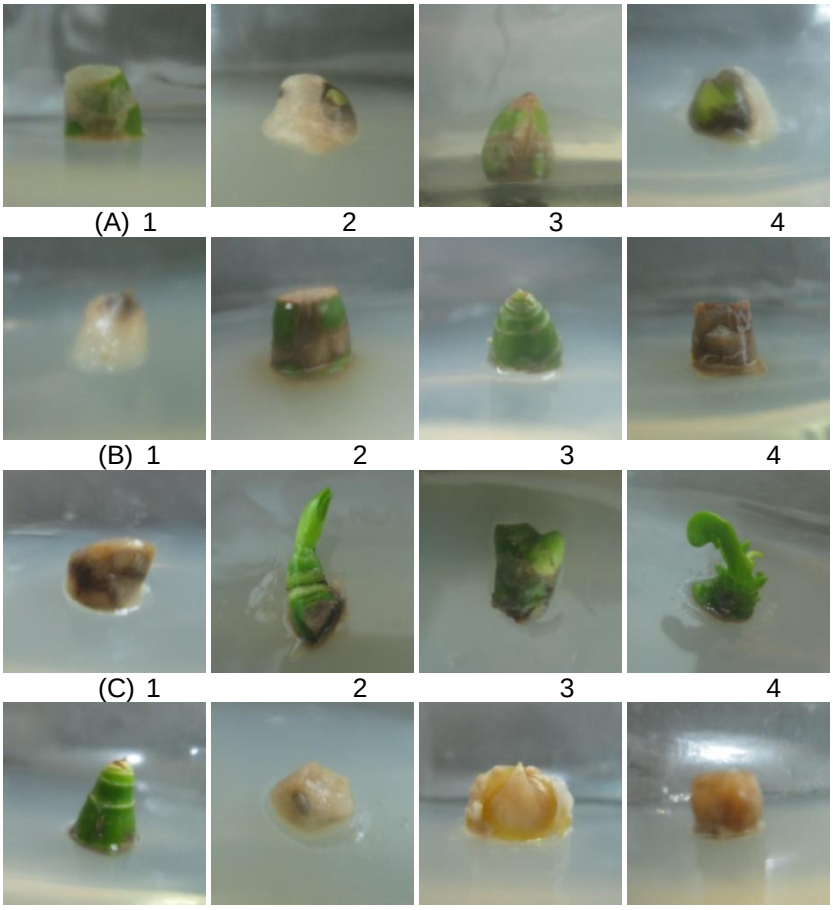

Figure 1. The growth and development of *Dendrobium* adventive shoot tip explant at 40 days after inoculation (A) *D. lineale*, (B) *D. pseudoconanthum*, (C) *D. strebloceras*, (D) *D. veratrifolium*, on media (1)VW, (2) VW + BA, (3) ½ MS, (4) ½ MS + BA

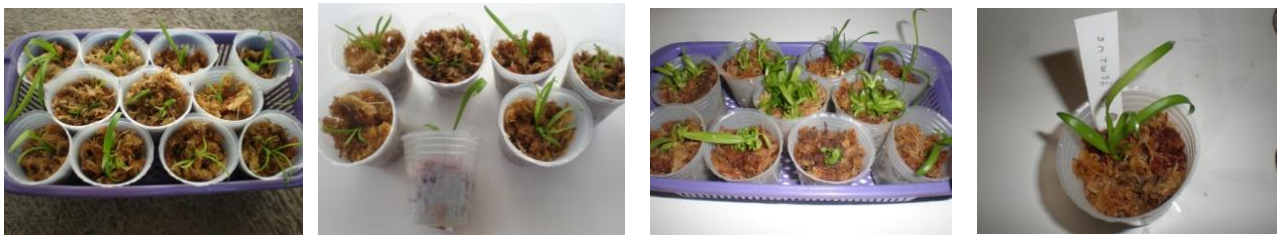

Figure 2. (A) *D. strebloceras* and (B) *D. pseudoconanthum* plantlets after acclimatisation from adventive shoot tip explant from media (1) ½ MS (2) VW (3) VW (4) VW+BA+NAA

Table 3 Explant regeneration of seed derived protocorm-like bodies explants of *Dendrobium* species on *in vitro* culture 40 days after inoculation

Species	Media	Number of explant	Contamination (%)	Number of explant regeneration			Number per explant	
				Callus	PLB	Shoot	PLB	Shoot
D. spectabile	VW	100	0	2.2	10	0	26	0
	VW+BA	100	10	0	0	44	0	4
	½ MS	100	10	0	44	44	7	7
	1/2MS+BA	100	10	10	0	11	0	1
D. lasianthera	VW	50	100	0	0	0	-	-
	VW+BA	50	100	0	0	0	-	-
	½ MS	50	40	0	0	0	-	-
	1/2MS+BA	50	20	0	0	0	-	-
D. ascipilanense	VW	130	76.92	0	0	0	-	-
	VW+BA	80	50.00	0	0	0	-	-
	½ MS	90	55.56	0	0	0	-	-
	1/2MS+BA	50	0	0	0	0	-	-

Although the existence of plant growth hormone was essential for callus induction, callus differentiation took place on ½ MS medium without addition of plant growth hormone. Plb regeneration process from callus and its germination did not depend on exogenous plant growth hormone (Ishii *et al.,* 1998; Roy and Banarjee, 2003; Zhao, *et.al*,, 2008). It differed from embryonic callus of many species which needed the addition of specific plant growth hormone for somatic embryogenesis (Huan *et al.,* 2004; Luo *et al.,* 1999; Chengalrayan *et al.,* 2001). In callus induction, syntesis system of endogenous hormone could be triggered and the rate of hormone raised allowing the cells to proliferate and differentiate on medium without exogenous plant growth hormone (Smith and Krikorian, 1990).

***Dendrobium* Seed-Derived Protocorm Like Bodies Explant**

Table 3 shows that contamination was on plb explant *D. ascipilense* (0-76.92%) and *D. lasianthera* (20-100%).

Meanwhile, on *D.spectabile*, the contamination was 0-10%. Explant regeneration of seed - derived plb of *D. spectabile* 40 days after cultured had formed callus, plb, and shoot (Figure. 3)

The number of explants regenerated after 40 days of inoculation from seed- derived protocorm like-body of *D. spectabile* was 22 calli and 10 plbs on medium VW; 44 shoots on VW+BA; 44 plbs and 44 shoots on ½ MS and 10 calli and 11 shoots on medium 1/2MS+BA. Some other plbs explants had not shown any further development (Table 3). As on *D. lasianthera* and *D. ascipilanense,* there was no significant development on day 40 after inoculation. The number per explant of plbs and shoots of *D. spectabile* after subculture was : plbs 26 on VW; 3 on VW+BA, 40 on ½ MS and 47 on ½ MS+BA; and shoots 38 on VW, 7 on VW+BA, 11 on ½ MS , 47 on ½ MS+BA (Table 4). However, none survived in acclimatisation.

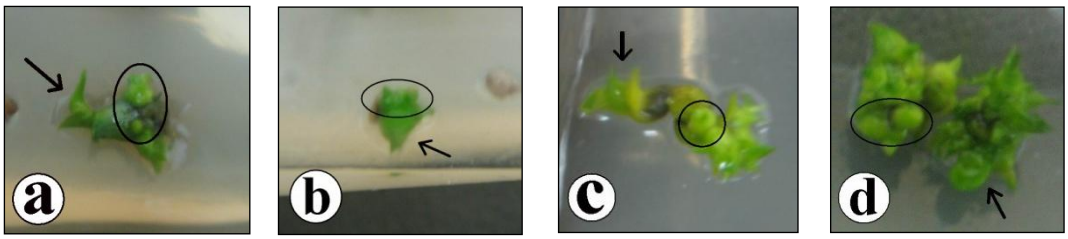

Figure 3. Regeneration of *D. spectabile* seed - derived protocorm-like bodies explant after subcultured on media (a) VW (b) VW+BA (c) ½ MS (d) ½ MS+BA

Table 4. Explant regeneration of seed derived protocorm-like bodies explants of *Dendrobium* species on *in vitro* culture 40 days after subcultured

Species	Media	Explant regeneration after subcultured								
		Days of initiation		Percentage of development (%)					Number per explant	
		PLB	Shoot	Callus	PLB	Shoot	Callus +PLB	Shoot+ PLB	PLB	Shoot
D. spectabile	VW	14	9	44.40	33.30	33.30	22.20	33.30	26	38
	VW+BA+NAA	14	10	0.00	0.00	33.30	0.00	11.10	3	7
	½ MS	13	9	22.20	55.60	11.10	0.00	44.40	40	11
	½MS+BA+NAA	12	3	11.10	44.44	33.30	7.78	22.20	47	47

In many plant species, callus plays an important part in the *in vitro* plant regeneration. In several orchid species, callus has also been induced successfully. Although at first orchid tissue culture did not focus on callus induction because the rate of growth was low and necrotic in the culture ((Zhao *et.al*, 2008). But recently, many lines having been produced on several orchid species were from callus (Lee and Lee, 2003; Lu, 2004). Actually, in order to obtain the same plant material, most of the time, callus was to be avoided since some characteristics might have changed. In this case, it will be better to obtain protocorm-like bodies.

Those calli succeded to grow into plantlet via indirect protocorm like bodies for mass production. Callus induction from protocorm segment was enhanced by growth hormone like BA. It was reported that BA succeeded in inducing callus on *D. fimbriatum* (Roy and Banarjee, 2003), and *D. candidum* (Zhao *et.al*, 2008). Plant regeneration from callus culture on orchid is

Dendrobium spectabile needs warm condition in an open medium. Its growth is specific and it flowers only under specific environmental condition. The condition during usually through middle phase protocorm-like body.

On *D. candidum*, different development of granules globular callus came from inside or outside callus that formed cells with solid cytoplasm and little vacuoles (Zhao, *et al.,* 2008). It was the characteristic of embryonic cells (Eady *et al.,* 1998; Li *et al.,* 2001; Nikam *et al.,* 2003). Those granules could develop into plbs and the plbs in the suitable condition would develop into plantlets. While the other plbs could proliferate further and formed secondary plbs, this was a common characteristic of many orchid species (Wimber, 1963; Arditti and Ernst, 1993). acclimatisation might not meet its requirement, which might explain the death of plantlets at acclimatitation.

Phalaenopsis Floral Stalk Buds Explant

Data show that *P. amabilis* had a high percentage of survival of explants from floral stalk buds (Figure. 4). Table 5 shows that at first culture, contamination percentage ranged from 0 to 100%, while explant life percentage ranged from 0 to 75%.

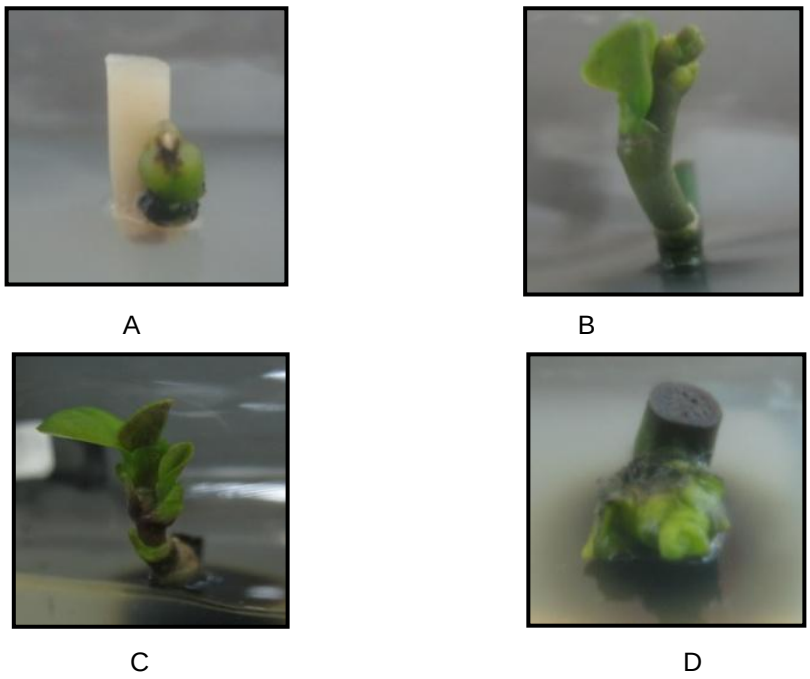

Figure 4. *P. amabilis* floral stalk bud explant regeneration at 60 days after inoculation on media (A) 1/2 MS (B) ½ MS+BA+NAA (C) VW (D) VW+BA+NAA

Table 5. Plant regeneration of *Phalaenopsis* explants from flower stalk buds on *in vitro* culture

	Cultured					**Subcultured**	
Species	**Media**	**Number of explant**	**Days after inoculation**	**Contamination (%)**	**Explant survival (%)**	**Media**	**Number of plantlets (10 months after first subcultured)**
P. amabilis	VW	3	4	0	50	VW	5
	VW+BA	3	4	0	100	VW+ BA+NAA	25
	½ MS	3	4	0	50	½ MS	7
	½MS+BA	4	4	0	75.	½MS+BA+ NAA	10
P.amboinen-sis	VW	1	3	0	50	VW	-
	VW+BA	1	3	0	50	VW+BA+ NAA	-
	½ MS	1	3	0	50	½ MS	-
	½MS+BA	2	3	0	50	½MS+BA+ NAA	-
P. tetraspis	VW	2	3	50	50	VW	
	VW+BA	2	3	100	0	VW+BA+ NAA	-
	½ MS	2	3	50	50	½ MS	-
	½MS+BA	2	3	50	50	½ MS+BA+ NAA	-

(A)

(B)

Figure 5. *P. amabilis* plant from floral stalk bud explant after acclimatisation from media (a) VW (b) VW+BA+NAA

Table 6. Shoot initiation and length of *Phalaenopsis* explants from floral stalk buds on *in vitro* culture

Species	Media	Cultured		Subcultured	
		Shoot initiation (days after inoculation)	**Shoot length 60 days after inoculation (cm)**	**Shoot initiation (days after subcultured)**	**Shoot length 30 days after subcultured (cm)**
P. amabilis	VW	3.	0.97	-	-
	VW+BA+NAA	7	0.47	4	1.30
	½ MS	5	0.20	-	-
	1/2MS+BA	5	0.78	9	0.54
P. amboinensis	VW	-	-	-	-
	VW+BA+NAA	-	-	-	-
	½ MS	3	0.20	0	0
	1/2MS+BA+NAA	-	-	-	-
P. tetraspis	VW	-	-	-	-
	VW+BA+NAA	-	-	-	-
	½ MS	1	0.10	0	0
	1/2MS+BA+NAA	2	0.40	0	0

Remarks: - =explant death; 0= explant alive but no development

Data of acclimatisation shows that number of plantlets regenerating on VW, VW+BA+NAA, ½ MS, and ½ MS+BA+NAA which survived during acclimatisation was 5, 25 , 7, and 10, respectively (Figure. 5).

The mean of percentage of survival of explants of *P. amboinensis* and *P. tetraspis* was around 50%. *Phalaenopsis tetraspis* and *P. amboinensis* are *Phalaenopsis* species with a short stalk, while *P. amabilis*is had a long stalk.

Technically, there were different difficulties in preparing explant from those two types of floral stalk buds. It was more difficult to prepare explant from a type of short flower stalk. Besides, it also grows slowly on in vitro culture and none grew any further.

There was shoot initiation on *P. amabilis, P. amboinensis* and *P. tetraspis* at first culture. At subculture, shoot initiation took place only on *P. amabilis*. Thirty days after subculture, the shoot length was 1.30 cm on VW+BA+NAA and 0.54 cm on ½ MS+BA+NAA. There was no further growth on *P. amboinensis* and *P. tetraspis.*

***Phalaenopsis* Seed - Derived Protocorm- like Bodies Explant**

Table 7 shows that there was good growth and development of explant from seed - derived protocorm like-bodies 40 days after inoculation. The percentage of life explants of *P. hieroglypha* was 100% and explants regenerating into plbs were 12.22 % on 1/2MS+BA and 7.78% on VW+BA.

It had been reported that callus could be formed from seed - derived protocorm with a frequency of 50% (Lu, 2004), from plbs segment 53% (Huan *et.al*, 2004), shoot tips 66.70% (Roy and Banarjee, 2003), and root tips 25% (Chen and Chang, 2000). After several subculturing, the number of callus would replicate three to five times in a month and the average number of protocorm-like bodies was 90.7 per callus culture (Lu, 2004), 134 per 0.01 g fresh weight callus 134 per 0.01 g (Huan *et al.,* 2004), 32.5 per mass callus (Roy and Banarjee, 2003), and 29.1 per 9 mm2 mass callus (Chen and Chang, 2000).

In this experiment, the percentage of the plb formation from explants of *P. hieroglypha* was 8.89% on VW, 1.11% on VW+BA, 6.67% on ½ MS and 3.33% on ½ MS+BA (Figure.6). After subculturing, the percentage of plbs formation was 5% plbs and 0.56% for the shoot formation (Figure. 7).

Table 7. Explant regeneration of *Phalaenopsis* seed - derived protocorm-like bodies 40 days after cultured

Species	Media	Days of initiation			Exlant regeneration (%)					Number per eksplant	
		PLB	Shoot	Callus	PLB	Shoot	Callus+ PLB	Shoot +PLB	Callus+ PLB	PLB	Shoot
P. hieroglypha	VW	12	0	0	8.89	0	0	0	0	1.41	0
	VW+BA	14	0	0	1.11	0	0	0	0	1.22	0
	½ MS	17	0	0	6.67	0	0	0	0	1.11	0
	1/2MS+BA	15	0	0	3.33	0	0	0	0	0.11	0

A **B** **C** **D**

Figure 6. Regeneration of seed - derived protocorm-like bodies explant *P. hieroglypha* formed plbs at 40 days after inoculation on media (A ½ MS (B) ½ MS+BA (C) VW (D) VW+BA

Table 8. Explant regeneration of *Phalaenopsis* explant protocorm-like bodies after first subcultured

Species	First subcultured				
	Days of plb initiation	Percentage of plb from explant (%)	Days of shoot initiation	Percentage of shoot from explant (%)	Number of plb per explant
P. hieroglypha	15	5.00	2	0.56	1.02

Figure 7. *P. hieroglypha* seed-derived protocorm-like bodies explant formed shoots on medium ½ MS, 40 days after subculture

The percentage of plb formation was 8.89% on VW, 1.11% on VW+BA, 6.67% on ½ MS and 3.33% on ½ MS+BA. At the subcultured, the percentage of plb formation was 5% and the percentage of shoot formation was 0.56%.

Table 8 shows that after subcultured, explants of protocorm-like bodies of *Phalae-nopsis* formed plb. However, there was no plantlets of *P.hieroglypha* surviving during acclimatisation. The explant development was slow and the size was too small to be able to develop into normal plantlets.

In this experiment only plbs formed from seed-derived protocorm-like bodies of *P. hiero-glypha* and there was no plantlet surviving during acclimatitation. The regeneration process was slow and the plantlets size was small. It could be due to the genotypes or the consentration of growth hormone used in this experiment. However, further studies are required.

CONCLUSIONS AND SUGGESTIONS

CONCLUSIONS

There were two *Dendrobium* species that continued to regenerate from adventive shoot tip explant. The number of plantlets of *D. pseudoconantum* regenerating on VW, VW + BA that survived during acclimatisation was 75 and 2 respectively. No plantlets regenerating on ½ MS and ½ MS+BA of survived during acclimatisation. The number plantlets of *D. strebloceras* regenerating on VW was 11.26 on VW+BA, 11 on ½ MS, and 11 on ½ MS+BA. These planlets survived during acclimatisation. Explant regeneration from seed - derived protocorm-like bodies was only performed by *D. spectabile.* The number of plbs per explant of *D. spectabile* after subcultured on VW; VW+BA; ½ MS; ½ MS+BA was 26, 3, 40, and 47. Shoot formation on VW, VW+BA, ½ MS and ½ MS + BA was 38, 7, 11, and 47 respectively.

Explant regeneration from floral stalk buds only *P. amabilis.* The percentage of survival plantlets of *P. amabilis* in acclimatisation was 62.5% on VW, 83.33% on VW+BA+NAA, 77.77% on ½ MS and 83.33% on ½ MS+BA+NAA. Survival percentage of explant from protocorm-like bodies on *P. hieroglypha* was 100%. The percentage of plb formation on VW; VW+BA, ½ MS, and ½ MS+BA was 8.89%,; 1.11%; 6.67% and 3.33%. Formation of plbs and shoot was 5% and 0.56% respectively.

SUGGESTIONS

It was suggested that for *ex situ* conservation on certain species of dendrobium and phalaenopsis in the category of rare germplasms, tissue culture could be applied by using explant from adventive shoot tip, floral stalk buds and seed- derived protocorm-like body explant for vegetative seed multiplication. Each species needed specific treatment and environmental condition in acclimatisation. Initial explants used should be in a great number to minimize the risk of explant survival during the in vitro culture and acclimatisation.

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