MICROPROPAGATION OF *PHALAENOPSIS* SPP. BY SOMATIC EMBRYOGENESIS TECHNIQUE

Minh Van Tran[1]

Abstract: *Phalaenopsis* spp. was regularly produced through micropropagation by protocorm like bodies (PLBs); micropropagation takes a lot of labor, and has high cost of seedlings, energy and material. The purpose of this paper was to study the new technique of using in vitro embryogenesis culturing for microprogation. The method involved using protocorm like bodies as planting materials. PLBs were cut into slices and placed on the medium for callus initiation. The callus was initiated on the medium MS + BA (0.1 mg/l) supplemented with NAA (1 mg/l) or 2,4D (1 mg/l) and was proliferated on the medium MS + BA (0.1 mg/l) supplemented with NAA (1 mg/l). Somatic cell suspensions were initiated and proliferated on the medium MS + BA (0.1 mg/l) supplemented with NAA (0.5, 1 mg/l). Somatic cell suspensions were differentiated to embryonic cell suspensions on the MS medium supplemented with NAA (0.1 mg/l) + BA (0.5 mg/l). Embryonic cell suspensions were plated and regenerated on the medium: 1/2MS supplemented with NAA (0.1 mg/l) + BA (0.5 mg/l). Micropropagation of *Phalaenopsis* sp. via the embryogenesis technique was set up to produce 5,800 plantlets per one liter of somatic embryogenesis suspension.

UDC Classification: 57.01; **DOI:** http://dx.doi.org/10.12955/cbup.v6.1313

Keywords: Callus, protocorm like bodies (PLBs), micropropagation, cell suspension, embrygenesis

Introduction

Traditional breeders that breed Phalaenopsis nowadays have a common problem that microbiological laboratories often face, that is that tissue culture often grows slowly, and it involves a lot of work to produce seedlings with at a high volume (Mamood, 1993). The somatic embryo propagation system solves the above problems with the advantages of: rapid multiplication in the form of cells, cloned embryos are a differentiation with a high regeneration coefficient, lower labor costs and price (Aitken-Christie et al, 1994). The initial material in cloned culture has the role of ensuring parental traits and maintaining a high rate of regeneration over long periods of time in orchids (Lin et al, 2000). The appropriate somatic embryo culture medium plays an important role in the growth and differentiation of embryogenic cells (Arditii & Ersnt, 2004) and controls the differentiation and regeneration of embryos under the influence of the growth regulator (Ishii et al, 1998 & Huan et al, 2004). PLB is a specialized clonal embryo that develops in cultured orchids (Young et al, 2000) that are highly cytokinin-affected such as BA, 2iP, and TDZ (Park et al, 2002). Studies on embryo callus growth, somatic embryo culture, and somatic embryo regeneration are the first barriers in somatic embryo development (Wu et al, 2004). This article studies the rapid multiplication of *Phalaenopsis* by somatic embryo technology.

Materials and Methods

Materials: *Phalaenopsis* spp. was imported from Singapore. Culture sample: (i) Shoot tip of corm growth in vitro. (ii) young shoots in white shoots in vitro. (iii) PLB was cut into thin slices.

The base medium is cultured in MS (Murashige and Skoog, 1962). Added to the medium: BA (6-benzylaminopurine), 2iP (2-isopentyl adenine), TDZ (thidiazuron), IBA (β- indol butyric acid), NAA (adrenaline), pantone Coconut water (30%), B1 (5 mg/l), sucrose (30 g/l). Conditions of culture: room temperature 26 $\pm$ 2°C, RH = 65%, lighting time 10 hours/day, light intensity 33.3 μmol/m^2/s, shake rate 100rpm.

Experimental Design: Randomly arranged block of 3 replicates, each with 3 replications. Erlenmeyer flask (containing 60 ml semi-solid medium or 50 ml liquid medium). Initial cell culture biomass 10 g/100ml suspension of media. Data was analyzed with MSTATC software (t = 0.05).

Results and Discussion

Cultivation and Formation of Callus

Formation of protocorm like body

Culture sample was young leaf tissue. PLB culture medium: MS supplemented with NAA (0, 0.1, 1 mg/l) and TDZ (0, 0.1, 1, 3 mg/l). Results of the study (Table 1): After 45 days of culture. The high embryogenic rate (80-90%) on PLB culture medium supplemented with NAA (0.1 mg/l) + TDZ (3

[1] Tran Van Minh, International University, Vietnam National University Ho Chi Minh City, Viet Nam, drminh.ptntd@yahoo.com

mg/l) and NAA (1 mg/l) + TDZ (3 mg/l). It was shown that TDZ plays an important role in the generation of PLB embryos. The number of embryos highly produced in the medium that was NAA combination (0.1 mg/l) + TDZ (3 mg/l) and NAA (1 mg/l) + TDZ (3 mg/l) with 8-12 PLB embryos/sample.

Table 1: Effect of TDZ and NAA on PLB embryo formation

Medium MS supplemented withed (mg/l)		PLB formation rate (%)	Number of protocorm/sample
TDZ	**NAA**		
0.0	**0.0**	-	-
	0.1	-	-
	1.0	-	-
	3.0	-	-
0.1	**0.0**		-
	0.1	2.6	1.2
	1.0	1.4	1.0
	3.0	0.6	0.8
1.0	**0.0**	-	-
	0.1	6.2	1.4
	1.0	4.8	2.4
	3.0	2.2	1.8
3.0	**0.0**	-	-
	0.1	48	12.2
	1.0	36	8.6
	3.0	22	6.2
CV%		18,6	16.4

Source: Author

Table 2: Effects of 2,4D and NAA on callus formation

Medium MS + BA (0,1 mg/l) supplemented with		Formation rate of callus (%)	Callus diameter (mm)
2.4D (mg/l)	**NAA (mg/l)**		
0.1		38	2.4
0.5		52	3.8
1.0		98	7.4
2.0		82	6.4
3.0		76	5.2
	0.1	36	2.2
	0.5	54	3.6
	1.0	96	6.2
	2.0	78	5.8
	3.0	62	4.6
CV%		12	14.8

Source: Author

Formation of embryonic callus

PLB embryos have a slower rate of multiplication in vitro due to the larger size of the PLB, which is limited to micropropagation. PLB was used as a material for embryonic callus growth. PLB was sliced thin and placed on the culture medium to induce the embryogenic callus: MS + BA (0.1 mg/l) supplemented with NAA (0.1, 0.5, 1, 3 mg/l) , 2.4D (0.1, 0.5, 1, 2 mg/l). Results of the study (Table 2): After 45 days of culture. Culture medium supplemented with NAA (1 mg/l) or 2.4D (1 mg/l) stimulated embryo callus growth (Table 2). The culture process was light, the embryonic callus was blue, in contrast, in diffused light, the embryonic callus was ivory-white. The cells of the embryonic callus which were ivory-white were suitable for liquid mass multiplication of shaking under diffused light. Green-celled callus growing embryos were cultured to increase biomass on semi-submerged bioreactors with illumination.

Proliferation of embryonic callus

The embryonic callus from the above study was used as culture material. The amount of callus applied to culture was 1 g/callus cluster. The culture medium increased the cell biomass of the callus: MS + BA (0.1 mg/l) supplemented with NAA (0.1, 0.5, 1, 2, 3 mg/l), 2.4D (0.1, 0.5, 1, 2, 3 mg/l). The results of the study show that (Table 3): The suitable growth culture medium increases the embryo callus with NAA supplementation (1 mg/l) or 2.4D (1 mg/l). In order to avoid somatic cell deformation, the cells were cultured for a prolonged time, NAA supplementation (1 mg/l) was appropriate. The ivory-white somatic cells were cultured in the dark, with rapid growth rates of 10-12 folds after 45 days of culture.

Table 3: Effect of 2,4D and NAA on callus proliferation

Medium MS + BA (0.1mg/l) supplemented with		Biomass after 45 days of culture (mg/cluster)	Biomass growth index
2.4D (mg/l)	**NAA (mg/l)**		
0.1		1,859	1.8
0.5		3,168	3.1
1.0		6,208	6.2
2.0		5,258	5.2
3.0		4,278	4.2
	0.1	1,954	1.9
	0.5	3,206	3.2
	1.0	5,462	5.4
	2.0	5,161	5.1
	3.0	4,058	4.0
CV%		9.4	12.2

Source: Author

Table 4: Effects of BA and NAA on regeneration of callus

Medium MS supplemneted with		Regeneration rate (%)	Number of shoot/cluster	Number of protocorm/cluster
BA (mg/l)	**NAA (mg/l)**			
0.0	**0.0**	18	0.4	1.0
	0.1	48	2.4	3.2
	0.3	32	1.2	3.8
	0.5	22	0.8	4.2
0.1	**0.0**	32	1.0	1.2
	0.1	72	3.6	3.8
	0.3	60	1.8	4.0
	0.5	46	1.0	4.2
0.3	**0.0**	48	1.0	1.6
	0.1	88	4.2	2.6
	0.3	76	2.8	2.2
	0.5	62	1.2	1.8
0.5	**0.0**	64	1.2	1.0
	0.1	98	5.6	1.4
	0.3	82	3.2	1.8
	0.5	78	1.8	2.2
CV%			1.6	8.2

Source: Author

Regeneration of embryonic callus

The embryonic callus from the above study was used as culture material. The culture medium for regeneration of the callus was: 1/2MS supplemented with NAA (0, 0.1, 0.3, 0.5 mg/l), BA (0, 0.1, 0.3, 0,5 mg/l). The results of the study showed that (Table 4): After 2 months of culture: In the medium of regeneration of callus, supplemented with NAA (0.1 mg/l) + BA (0.5 mg/l) for high shoot regeneration. A high yield of 100% sample for regeneration. The number of shoots was 3-5 buds/cluster. The differentiated embryonic callus to PLB was small in size and these PLBs continued to regenerate directly *in vitro* bud formation.

Effects of Tissue and Genotype on Regeneration of callus

Effect of tissue culture on regeneration of embryonic callus

The PLB was cut into thin slices and young leaves *in vitro* (10-20 mm) with separated shoots were used as culture materials to produce and regenerate the embryogenic callus. The culture medium produced the embryogenic callus was: MS + BA (0.1 mg/l) + NAA (1 mg/l). Results (Table 5): After 45 days of culture: regeneration of callus: MS + BA (0.5 mg/l) + NAA (0.1 mg/l) a callus appeared form both tissue types. The culture process was light, the embryonic callus was blue, in contrast, in diffused light, the embryonic callus was ivory-white. The ivory-white cells of the embryonic callus were suitable for liquid mass multiplication in shaking with diffused light. After 2 months of culture: Callus embryos from PLB were thin and young leaves produced shoots. A high yield of 100% sample for PLB formation. The number of shoots was 5.8-5.2 shoots/cluster.

Table 5: Effect of tissue origin on regeneration of embryonic callus

Callus origin	Regeneration rate (%)	Number of shoot/cluster	Number of protocorm/cluster
Form thin slice PLB	100	5.8	1.6
From young leaves	100	5.2	1.2

Source: Author

Effects of genotypes on regeneration of embryonic callus

PLB culture specimens from apical shoots and hybrid seeds were incubated. The cultured embryonic callus growth medium was: MS + BA (0.1 mg/l) + NAA (1 mg/l). The results of the study showed that (Table 6): In culture medium: MS + NAA (0.1 mg/l) + BA (0.5 mg/l): After 45 days, two phalaenopsis lines were capable of producing high rate of embryonic callus. The culture process was light, the embryonic callus was blue, in contrast, in diffused light, the embryonic callus was ivory-white. The ivory-white cells of embryonic callus were suitable for liquid mass multiplication in shaking with diffused light. After 2 months of culture: Callus embryos from PLB were thin and young leaves produced shoots. Number of shoots was 5.6-4.8 shoots/cluster.

Table 6: Effect of genotypes on regeneration of embryogenic callus

Callus origin	Regeneration rate (%)	Number of shoot/cluster	Number of protocorm/cluster
From PLB cultured apical shoot	100	5.6	1.8
From PLB hybrid seed	100	4.8	2.2

Source: Author

Formation and Proliferation of Callus Suspension

Formation of embryonic callus suspension

The somatic cells obtained from study 3 were used as culture material for the suspension production. The initial culture biomass was 1g/50ml of culture medium. The culture medium for somatic cell suspension was: MS + BA (0.1 mg/l) supplemented with NAA (0.1, 0.5, 1, 3 mg/l), 2.4D (0.1, 0.5, 1, 2 mg/l). The results showed that (Table 7): After 30 days of culture the somatic cell suspension was formed on a suspension culture supplemented with NAA (1 mg/l) or 2.4D (1 mg/l). However, during prolonged culturing, 2.4D was easy to produce variation. So, the appropriate growth regulator in culture was NAA (1 mg/l). The suspension had ivory-white cells which were uniform in shape and size.

Proliferation of embryonic callus suspension

The somatic cell suspension obtained from the above study was used as the suspension culture material. The initial culture biomass was 1 g/50ml of culture medium. The culture medium increased somatic cell suspension biomass: MS + BA (0.1 mg/l) supplemented with NAA (0.1, 0.5, 1, 2, 3 mg/l). Results of the study (Table 8): After 20 days of culture. The somatic cell suspension was produced on a suspension culture medium supplemented with NAA (0.5, 1 mg/l) a rapid growth rate was observed with a 10-12 fold increase in biomass. The suspension had ivory-white cells which were uniform in shape and size. Partial somatic cell suspension activity differentiates into somatic embryos.

Table 7: Effect of 2,4D and NAA on formation of callus suspension

Medium MS + BA (0,1 mg/l) supplemented with		Biomass after 30 days of culture (mg/50ml)	Biomass growth index
2.4D (mg/l)	**NAA (mg/l)**		
0.1		1,244	1.2
0.5		1,632	1.6
1.0		3,264	3.2
2.0		2,646	2.6
3.0		2,262	2.2
	0.1	1,458	1.4
	0.5	1,608	1.6
	1.0	2,742	2.7
	2.0	2,625	2.6
	3.0	2,048	2.0
CV%		11.8	10.6

Source: Author

Table 8: Effect of NAA on proliferation of embryogenic callus suspension

Medium MS + BA (0.1 mg/l) supplemented with NAA (mg/l)	Biomass after 30 days of culture (mg/50ml)	Biomass growth index
0.1	5,434	5.4
0.5	10,862	10.8
1.0	12,452	12.4
2.0	8,244	8.2
3.0	6,432	6.4
CV%	12.4	9.6

Source: Author

Table 9: Effect of culture time on induction efficiency of embryo induction

Culture time (week)	Activated cell density (CFU/ml) after 45 days	Activation efficiency (%) compared to initial density
1	0.7×10^4	50.0
2	0.8×10^4	58.3
3	1.1×10^4	72.6
4	1.2×10^4	78.4
5	0.7×10^4	52.8
6	0.6×10^4	44.6
CV%	14.4	10.2

Source: Author

Regeneration of Embryonic Callus Suspension

Activation induction of somatic embryo

The embryonic callus suspension from study 8 was used as a suspension culture material. The initial culture biomass used was 1 g/50 ml of culture medium. The somatic embryo suspension was produced in the medium: MS supplemented with NAA (0.1 mg/l) + BA (0.5 mg/l). Results of the study (Table 9): After 6 weeks of culture, the culture medium supplemented with BA (0.5 mg/l) + NAA (0.1 mg/l) stimulated somatic embryogenesis. Growth performance of embryos increased with time of culture from 3-4 weeks after culture and decreased after 4 weeks of culture. Embryonic induction > 75% (including spherical, heart shape, torpedo), the appropriate time for transplant. The cells were white-yellow cells, large, and with concentrated cytoplasms.

Spreading and regeneration of somatic embryos on agar medium

The embryonic cell suspension was used as a culture material. A cell volume of 5 ml/60 ml of culture medium was used for the regeneration of the somatic embryo suspension: MS supplemented with NAA (0.1, 0.5, 1 mg/l) and BA (0, 0.1, 0.5, 1 mg/l) . Results of the study (Table 10): After 30 days of culture, the somatic cell culture supplemented with NAA (0.1 mg/l) + BA (1 mg/l) was suitable for the somatic embryogenesis homogenization, resulting in a somatic embryonic cell layer with green,

somatic embryos differentiated into small PLB, and which regenerated after 60 days of culture, received 5,800 shoots of orchid per 1 liter of cell suspension.

Table 10: Effect of growth regulator on regeneration of somatic embryo suspension

Culture medium		Number of regeneration shoots/5 ml of embryonic cell suspension
BA (mg/l)	**NAA (mg/l)**	
0.1	**0.0**	4
	0.1	12
	0.5	8
	1.0	6
0.5	**0.0**	10
	0.1	18
	0.5	12
	1.0	8
1.0	**0.0**	16
	0.1	29
	0.5	22
	1.0	18
CV%		10.4

Source: Author

Conclusion

Slices of protocorm like bodies (PLBs) were used as raw materials for cultures. The callus produced on MS + BA (0.1 mg/l) supplemented with NAA (1 mg/l) or 2.4D (1 mg/l) and was cultured in medium on MS + BA (0.1 mg/l) + NAA (1 mg/l). In the regenerative medium, MS + NAA (0.1 mg/l) + BA (0.5 mg/l) gave a high complete regeneration rate of 3-5 shoots/cluster. The differentiated embryonated callus to PLB was small in size and these PLBs continued to regenerate directly in vitro bud formation.

The culture sample consisted of thin sliced PLB and a young leaf (0.5 cm^2). The callus was grown on a medium: MS + BA (0.1 mg/l) + NAA (1 mg/l). The callus was incubated in regeneration culture medium, after 50 days of culture on medium: MS + BA (0.5 mg / l) + NAA (0.1mg / l) it showed two tissues types that produced calluses. The culture process was light, the embryonic callus was blue, in contrast, in diffused light, the embryonic callus was ivory-white. After 2 months of culture: Callus embryos from PLB slices and young leaves had white shoots. The number of shoots was 5.8-5.2 shoots/cluster.

PLB culture specimens from apical shoots and hybrid seeds were incubated. The callus grew on culture medium: MS + BA (0.1 mg/l) + NAA (1 mg/l). The callus weight of 1 g/50ml was cultured on a culture medium for regenerating calluses: MS + NAA (0.1 mg/l) + BA (0.5 mg/l): After 45 days culture: Both cell lines showed an ability to generate embryo callus. The cells of embryonic calluses were ivory-white and suitable for liquid mass multiplication in shaking with diffused light. After 2 months of culture, calluses from PLB slices of two genotypes were regenerated. The number of shoots was 5.6-4.8 shoots/cluster.

The results of the study showed that a somatic cell suspension was formed on suspension medium MS + BA (0.1 mg/l) + NAA (1 mg/l) or 2.4D (1 mg/l)). However, when growing the culture for a longer period of time, 2.4D is easily to produces variation, so the appropriate growth regulator in culture was NAA (1 mg/l). The somatic suspension was formed on culture medium MS + BA (0.1 mg/l) + NAA (1 mg/l) after 20 days of rapid growth, with a biomass increase of 12.4. The suspension was ivory-white and cells were uniform in shape and size. Partial somatic cell suspension activity differentiates into somatic embryos.

The cell suspension was produced and proliferated on medium MS + BA (0.1 mg/l) + NAA (0.5, 1 mg/l). The cell suspension was differentiated embryos on medium MS + BA (0.5 mg/l) + NAA (0.5 mg/l). The embryo cell suspensions were grown and regenerated on semi-solid medium: 1/2MS + BA (0.5 mg/l) + NAA (0.1 mg/l). The results of the study have established a fast-growing system for *Phalaenopsis* using the somatic embryogenesis technology, which collected 5800 shoots of *Phalaenopsis* per 1 liter of embryo cell suspension.

References

Aitken-Christie, J., Kozai, T. & Smith, M. A. L. (1994). Automation and environmental control in plant tissue culture. Kluwer

Arditii, J. & Ersnt, R. (1993). Micropropagation of orchids. Wiley

Huan, L. T. V., Takamura, T. & Tanaka, M. (2004). Callus formation and plant regeneration from callus through somatic embryo structure of *Cymbidium* orchid. Plant Science; 166:1443-1449.

Ishii, Y., Takamura, T., Goi, M. & Tanaka, M. (1998). Callus induction and somatic embryogeneis in *Phalaenopsis*. Plant Cell Report;17:446-450.

Lin, Y. H., Chang, C. & Chang, W. C. (2000). Plant regeneration from callus culture of a *Paphiopedillum* hybrid. Plant Cell, Tissue and Organ Culture;62:21-25.

Mamood, M. (1993). Application of plant in vitro technology. Proceeding, 16-18 Nov 1993, Univ. of Malaysia, Malaysia.

Murashige, T. & Skoog, R. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum; 15:431-497.

Park, S. Y., Yeung, E. C. & Chakrabarty, D. (2002). An efficient direct induction of PLB from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. Plant Cell Report;21:46-51

Wu, I.F., Chen, J.T. & Chang, E.C. (2004). Effects of auxin and cytokinins on embryo formation from root derived callus of *Oncidium.* Plant Cell, Tissue and Organ Culture;77:107-109.

Young, P.S., Murthy, H.N. & Paek, K.Y. (2000). Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. Plant Cell, Tissue and Organ Culture;63.