MICROPROPAGATION OF *CYMBIDIUM* SPP. BY SOMATIC EMBRYOGENESIS TECHNIQUE

Tran Van Minh[1]

Abstract: *Cymbidium* spp. was regularly produced through micropropagation by protocorm like bodies (PLBs) and multiple shoots; 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 to resolve the above difficulties. The method was to use PLBs as planting materials to produce somatic callus cell and embryos. Results were followed as: PLBs were cut into slices and placed on the medium for callus initiated and used as materials for embryo formation study. A fresh weight of callus were used for the experiment of about 100 mg. The medium for the initiation of embryonic callus was composed of: MS + BA (0.1 mg/l) + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (1mg/l) or 2.4D (1 mg/l) and was proliferated on the medium MS + pepton (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (1 mg/l) or 2.4D (1 mg/l). Somatic cell suspensions were initiated and proliferated on the medium consisting of MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (1 mg/l) + BA (0.1 mg/l). Somatic cell suspensions were differentiated to embryonic cell suspensions on the medium MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with BA (1 mg/l). Embryonic cell suspensions were plating and regeneration on the medium MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (0.1 mg/l) + BA (1 mg/l). Micropropagation of *Cymbidium* spp. via embryogenesis technique was set up to produce 3,800 plantlets per one liter of somatic embryogenesis suspension.

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Introduction

Traditional breeding in orchids nowadays results in the problem that common for micropropagation are tissue cultures that often grow slowly and involve a lot of work to produce seedlings. Large quantities are available at high market prices for seedlings (Mamood, 1993). The somatic embryo propagation system (Evans et al, 1981) addresses the above barriers with the advantages of: rapid multiplication in the form of cells, the embryos are a differentiation with high regeneration coefficient, they are dynamic and also cost effective (Kurata and Kozai, 1992). The initial material in the embryo culture plays a role in ensuring parental traits and maintaining high regeneration rates over a long period of time (Chang and Chang, 1978). The medium for the embryo culture plays an important role in the growth and differentiation of embryonic cells (Arditii and Erent, 1993). Control of differentiation and regeneration of embryos under the influence of growth regulators has become the rule (Huan et al, 2004; Haun and Tanaka, 2004). PLB is a particular embryo that arose in the cultivation of cytokinin-induced orchids (Park et al, 2003). A study on embryo callus growth, embryo culture, and embryo regeneration are the first barriers in somatic embryos (Jaime et al, 2006; Nayak et al, 2002). This paper investigates the rapid multiplication of *Cymbidium* through the somatic embryo technique.

Materials and Methods

Materials: Varieties of *Cymbidium* sp. imported from Australia were used. Sample culture: Shoot tip growth *in vitro* in 20 days. (ii) Young shoots (white shoots) *in vitro* in 20 days of age. (iii) PLB is cut into thin slices.

Base medium for culture is MS (Murashige and Skoog, 1962). Additive: BA (6-benzylaminopurine), TDZ (thidiazzuron), 2iP (2-isopentyl adenine), IBA (β-indol butyric acid), NAA (α-napthalene acetic acid), B1 (5 mg/l), Activated charcoal (1 g/l), Coconut water (30%), sucrose (30 g/l)

Conditions of culture: room temperature 26 $\pm$ 2ºC, RH = 65%, lighting time 10 hours/day, light intensity 33.3 μmol/m^2/s, shake rate 100rpm.

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

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

Results and Discussion

Cultivation and Formation of Callus

Formation of protocorm like body

The sample culture was young leaves. The PLB formation medium: MSC + B1 (5 mg/l) + CW (10%) + pepton (1 g/l) + sucrose (20 g/l) + activated charcoal (1 g/l) with NAA supplement (0, 0.1, 1, 3 mg/l) and TDZ (0, 0.1, 1, 3 mg/l). Results of the study (Table 1): After 45 days of culture. The rate of formation of high PLB (80-90%) supplemented with NAA (0.1 mg/l) + TDZ (3 mg/l) and NAA (1 mg/l) + TDZ (3 mg/l). Growth regulators TDZ play an important role in the generation of PLB embryos. The number of PLB formation was high in NAA (0.1 mg/l) + TDZ (3 mg/l) and NAA (1 mg/l) + TDZ (3 mg/l) with 10-20 PLB/ sample.

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

Medium MS supplemented with (mg/l)		**Formation rate PLB (%)**	**Number of protocorm /sample**
TDZ	**NAA**		
0.0	**0.0**	0	0.0
	0.1	8	1.2
	1.0	6	2.6
	3.0	4	2.2
0.1	**0.0**	8	2.4
	0.1	34	6.8
	1.0	22	4.4
	3.0	14	2.0
1.0	**0.0**	14	4.2
	0.1	64	12.0
	1.0	42	5.4
	3.0	22	4.6
3.0	**0.0**	32	6.4
	0.1	90	20.2
	1.0	82	10.4
	3.0	68	8.0
CV%		14.6	8.6

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		2	0.9
0.5		2	0.9
1.0		28	2.6
2.0		24	1.4
3.0		6	1.0
	0.1	1	0.8
	0.5	1	0.8
	1.0	22	2.2
	2.0	16	1.8
	3.0	4	1.2
CV%		14.2	9.0

Source: Author

Formation of embryogenic callus

PLB has a slow *in vitro* multiplication rate. It has a large size, limited to micropropagation. It was used as raw material for the production of embryogenic callus. The PLB was cut in thin slices and placed on the culture medium to create the callus. The culture medium for callus formation: MS + BA (0.1 mg/l) + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (0.1, 0.5, 1, 2, 3 mg/l), 2.4D (0.1, 0.5, 1, 2, 3 mg/l). Results of the study (Table 2): After 45 days of culture, the culture

medium containing the NAA (1 mg/l) or 2.4D (1 mg/l) stimulated embryogenic callus formation, with the formation rate of callus being low and the diameter of the callus being small. The culture process occured outside, the callus was green; otherwise, in the dark, it was ivory-white color. The ivory-white-color embryonic callus cells were suitable for the liquid multiplication of shaking under diffused light.

Proliferation of embryogenic callus

The embryonic callus from the above study was used as culture material. The weight of tissue to be cultured was 1 g/50ml culture medium. The culture medium proliferated the mass of callus: MS + BA (0.1 mg/l) + peptone (1 g/l) + activated charcoal (1 g/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 (Table 3) show that: a suitable proliferation culture medium was a medium supplemented with NAA (1mg / l) or 2.4D (1 mg/l). The ivory-white somatic cells were cultured in the dark at a rapid rate of proliferation of 4-5 times more than under diffused light after 30 culture days.

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		2,264	2.2
0.5		2,652	2.6
1.0		5,244	5.2
2.0		5,627	5.6
3.0		4,834	4.8
	0.1	1,886	1.8
	0.5	2,466	2.4
	1.0	4,628	4.6
	2.0	3,942	3.9
	3.0	2,886	2.8
CV%		12.6	8.2

Source: Author

Regeneration of embryogenic callus

The embryonic callus from the above study was used as culture material. The mass of tissue injected into the culture was 500mg. The culture medium for regeneration of callus was: MS + peptone (1 g/l) + activated charcoal (1 g/l) + rhizogenic (5 mg/l) supplemented with NAA (0, 0.1, 0.5, 1, 2 mg/l), BA (0, 0.1, 0.5, 1 mg/l). The results of the study (Table 4): After 45 days of culture. The highest regeneration was achieved in culture medium supplemented with BA (0.5 mg/l). The *Cymbidium* shoots grew well *in vitro* and have roots in good growth. When the medium only has NAA, PLB formed vigorously and restricted regeneration. In combination with NAA + BA, low regeneration rates and PLB continued to occur.

Effects of Tissue and Genotype on Regeneration of callus

Effect of tissue culture on regeneration of embryogenic callus

PLB was cut into thin slices and young leaves *in vitro* (10-20 mm) separated shoots were used as culture materials to produce and regenerate the embryonic callus. The culture medium produced the embryonic callus: MS + BA (0.5 mg/l) + peptone (1 g/l). The results of the study showed that (Table 5) the culture medium produced the embryonic callus: MS + BA (0.5 mg/l) + peptone (1 g/l). After 45 days of culturing: Both tissues cultured produced calluses. The culture process was set externally, the embryogenic callus was green; in contrast, in the dark it was ivory-white. The ivory-white embryonic callus cells are suitable for the liquid multiplication of shaking with diffused light. In culture medium, regeneration of embryonic callus: MS + BA (0.5 mg/l) + peptone (1 g/l): After 45 days of culture: callus from both tissues had high shoots, achieving 100% regeneration rate of culture samples.

Effects of genotypes on regeneration of embryogenic callus

PLB culture specimens from apical shoots and hybrid seed were incubated. Cultured embryogenic callus growth medium: MS + BA (0.1 mg/l) + peptone (1 g/l). The results of the study (Table 6)

showed that: After 45 days of culture, both types of PLB were incubated to produce callus. The culture process is set externally, the embryogenic callus was green; in contrast, in the dark it was ivory-white. The ivory-white embryonic callus cells are suitable for the liquid multiplication of shaking with diffused light. The culture medium for regeneration of callus: MS + BA (0.5 mg/l) + peptone (1 g/l) + activated charcoal (1 g/l): After 45 days culture: thin layer of the two samples were cultured to produce high shoots, reach 4-5 shoots/culture and the rate of regeneration of 100% for the samples put into culture.

Table 4: Effects of BA, 2.4D and NAA on regeneration of callus

Medium MS supplemented with		**Regeneration rate (%)**	**Number of shoot/cluster**	**Number of protocorm/cluster**
BA (mg/l)	**NAA (mg/l)**			
0.0	**0.0**	00	-	-
	0.1	10	1.0	3.0
	0.5	6	1.0	3.6
	1.0	4	1.0	4.2
0.1	**0.0**	28	1.8	1.4
	0.1	14	1.4	1.8
	0.5	12	1.0	2.6
	1.0	10	1.0	3.6
0.5	**0.0**	100	4.4	1.2
	0.1	32	3.2	1.4
	0.5	26	2.6	2.2
	1.0	18	1.8	2.6
1.0	**0.0**	72	3.2	1.6
	0.1	24	2.6	1.8
	0.5	18	2.0	3.0
	1.0	10	1.6	3.4
CV%			10.6	8.4

Source: Author

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

Callus origin	**Regeneration rate (%)**	**Number of shoot/cluster**	**Number of protocorm/cluster**
Form thin slice PLB	100	4.6	1.4
From young leaves	100	4.2	1.2

Source: Author

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	4.2	1.4
From PLB hybrid seed	100	4.8	2.6

Source: Author

Formation and Proliferation of Callus Suspension

Formation of embryogenic callus suspension

Callus embryos are used as culture materials to produce a callus suspension. The base liquid medium for callus suspension formation is: MS + BA (0.1 mg/l) + peptone (1 g/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 culture days, 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 culture 2,4D was easy to variate. The appropriate growth regulator in culture was NAA (1 mg/l). The suspension was ivory-white, cells were uniform in shape and size.

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		2,248	2.2
0.5		2,462	2.4
1.0		3,486	3.4
2.0		3,826	3.8
3.0		2,846	2.8
	0.1	1,844	1.8
	0.5	2,024	2.0
	1.0	2,834	2.8
	2.0	2,486	24
	3.0	2,248	2.2
CV%		14.2	10.4

Source: Author

Proliferation of embryogenic callus suspension

The somatic cell suspension after 3 times subcultured were used as culture material. The volume of culture was 20%. The culture medium for proliferation of embryogenic callus suspension was: MS + BA cellulose (0.1 mg/l) + peptone (1 g/l) supplemented with NAA (0.1, 0.5, 1, 2, 3 mg/l). The results showed that (Table 8): After 20 culture days, the suspension of somatic cell was rapidly produced on culture medium supplemented with NAA (1 mg/l), with 8-10 times proliferation in biomass. It was ivory-white, cells were uniform in shape and size. Partial somatic cell suspension activity differentiated into somatic embryos.

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	3,426	3.4
0.5	5,684	5.6
1.0	10,268	10.2
2.0	8,642	8.6
3.0	6,424	6.4
CV%	14.4	10.8

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	0.8×10^4	52.6
2	0.9×10^4	66.8
3	1.1×10^4	80.6
4	1.3×10^4	92.2
5	0.8×10^4	64.6
6	0.7×10^4	56.2
CV%	10.6	15.2

Source: Author

Regeneration of Embryogenic Callus Suspension

Activation induction of somatic embryo

The embryonic callus suspension was used as a culture material. Cell volume of 10 g/50 ml of culture medium induced somatic embryos: MS + peptone (1 g/l) supplemented with BA (1 mg/l). The results of the study show (Table 9): After 45 days of culture, the culture medium of MS + BA (1 mg/l) stimulates somatic embryogenesis. Time of differentiation of embryos increased with time of culture

from 1-4 weeks after culture, then gradually decreased. Embryonic induction > 75% (including spherical, heart shape, torpedo), the appropriate time for regeneration. The cells were white-yellow, large, with concentrated cytoplasms.

Splatting and regeneration of somatic embryos on agar medium

The embryonic cell suspension was used as a culture material. Cell volume of 5 ml/60 ml of culture medium for regeneration of somatic embryo suspension: MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (0.1, 0.5, 1 mg/l) and BA (0.1, 0.5, 1 mg/l). Illumination intensity was 33.3 µmol/m^2/s. Lighting time was 10 hours/day. Results of the study (Table 10): After 30 days of culture, the somatic embryo culture medium supplemented with BA (1 mg/l) + NAA (0.1 mg/l) was suitable for somatic embryo differentiation. Blue somatic embryos were differentiated into PLB; and shoot regeneration was recorded after 60 culture days with 19 shoo/50ml embryonic cell suspension (Table 4). 3,800 buds of *Cymbidium* sp. per one liter of embryonic cell suspension were collected.

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

Culture medium (mg/l)		**Number of regeneration shoots/5 ml of embryonic cell suspension**
BA	**NAA**	
0.1	**0.0**	4
	0.1	10
	0.5	6
	1.0	4
0.5	**0.0**	10
	0.1	14
	0.5	10
	1.0	6
1.0	**0.0**	16
	0.1	19
	0.5	14
	1.0	9
CV%		11

Source: Author

Conclusion

The PLB was cut in thin slices and used as culture materials. The thin slices were cultured on callus formation medium: MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (1 mg/l) or 2.4D (1 mg/l) and proliferation medium: MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (1 mg/l) or 2,4D (1 mg/l). The formation and proliferation of callus suspension on medium: MS + peptone (1 g/l) + activated charcoal (1 g/l) supplemented with NAA (1 mg/l) + BA (0.1 mg/l). The medium of regeneration of embryonic callus: MS + peptone (1 g/l) + activated charcoal (1 g/l) + B1 (5 mg/l) + BA (0.5 mg/l).

The culture medium to produce embryonic callus: MS + BA (0.5 mg/l) + peptone (1 g/l). After 45 days culturing: Both cultured tissue (PLB sliced and young leaf) produced callus. The culture process was set externally, the embryonic callus was green; in contrast, in the dark it was ivory-white. The ivory-white embryonic callus cells were suitable for the liquid multiplication of shaking with diffused light. In culture medium for regeneration of embryonic callus: MS + BA (0.5 mg/l) + peptone (1 g/l): After 45 days of culture: the callus from both tissues produced shoots, reaching the rate of regeneration of 100% for the samples put into culture.

The cultured medium to produce embryonic callus: MS + BA (0.1 mg/l) + pepton (1 g/l) + activated charcoal (1 g/l). After 45 days culturing: Both PLB cultured (from apical shoots and hybrid seeds) produced embryogenic calluses. The ivory-white embryogenic callus cells were suitable for the liquid multiplication of shaking with diffused light. On the culture medium regenerate callus: MS + BA (0.5 mg/l) + peptone (1 g/l): After 45 days of culture: the embryogenic callus from PLB sliced produced high rate shoots, reach 4-5 shoots/culture the rate of regeneration 100% of the samples put into culture.

The somatic suspension formed on culture medium: MS + BA (0.1 mg/l) + peptone (1 g/l) + NAA (1 mg/l) or 2.4D (1 mg/l), after 30 days of culture. However, during prolonged culturing, 2.4D is easy to

variate. The appropriate growth regulator in culture was NAA (1 mg/l). The suspension was ivory-white, and cells were of uniform shape and size. The somatic cell suspension regeneration on medium (0.1 mg/l) + peptone (1 g/l) + NAA (1 mg/l) with biomass increments 10.2 times after 20 days cultured. The suspension was ivory-white, and cells were of uniform shape and size. Partial somatic cell suspension activity differentiates into somatic embryos.

The cell volume of 10 g/50ml culture medium induced embryos: MS + peptone (1 g/l) + BA (1 mg/l) activation of somatic embryo development after 45 days cultured. Time of differentiation of embryos increased with time of culture from 1-4 weeks after culture, then gradually decreased. Embryogenic induction > 75% (including spherical, heart shape, torpedo), the appropriate time for transplant regeneration. Cell volume of 5 ml/60 ml of culture medium to regenerate somatic suspension: MS + peptone (1 g/l) + BA (1 mg/l) + NAA (0.1 mg/l) suitable for differentiation somatic embryos, forming a somatic embryo cell layer, were blue, they were differentiated into PLB; and shoots regenerated after 60 culture days, 3,800 shoots per one liter of embryogenic cell suspension were collected.

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