MICROPROPAGATION OF *RHYNCHOSTYLIS GIGANTEA* ORCHID BY SOMATIC EMBRYOGENIC CULTURES

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Abstract: *Rhynchostylis gigantea* is a wild orchid species commonly grown. Flowering at the traditional Tet holiday, the flower season always blooms in December of the lunar calendar. The flower has a faint aroma, so it is called Nghinh spring and is a kind of orchid with a national soul and national essence. *Rhynchostylis gigantea* is found in many areas in Vietnam as it is distributed in the South Central Highlands, especially the areas bordering Laos and Cambodia at low altitudes <600m. *Rhynchostylis gigantea* is a type of heat-resistant orchid, with suitable temperatures for orchid being from 26-30°C. *Rhynchostylis gigantea* species have many different colors such as red, white, shrimp brick color, blue and pink. The single-member tree does not assume pseudobulbs, grows vertically, has many pneumatophores and grows straight from the stem. *Rhynchostylis gigantea* grows slowly and regenerates with seeds. Due to high genetic diversity, it is difficult to maintain the parent's characteristics. Applying techniques of somatic embryo culture in conservation and development of the *Rhynchostylis gigantea* tree to meet the increasing demand for seedlings is urgent.

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Introduction

Orchidaceae make up about 10% of angiosperm species. An orchid is a large species, with diversified shape, size, and color, adapted to a large ecosystem, and is important in the flower industry with 20,000-30,000 different species (Griesbach, 2003).

Cell culture has been used to rapidly multiply orchid species that are suitable for different culture conditions. The protocorm-like body (PLB) is the first embryogenic organism. *Rhynchostylis gigantea* orchid is a single shoot species that are commonly found in Southeast Asia (Vietnam, Laos, Thailand). The propagation of *Rhynchostylis gigantea* orchid using protocorm can be done through culturing shoots, *Rhynchostylis gigantean* young leaf*, Rhynchostylis retusa* root, or PLB regeneration directly from thin cell layer culture (Le et al, 1999).

Somatic embryos are the appropriate system for rapid multiplication of rare orchids (Li et al, 2009). There have been few reports of direct and indirect embryogenesis in orchids such as Vu Nu (Chen, et al, 1999; Chen et al, 2002; Chenet al, 2004), *cymbidiums* (Cheng et al, 1998), *phaleanospsis* (Ishii et al, 1998). *Rhynchostylis retusa* (Thomas et al, 2007) and *Rhynchostylis gigantea* (Lindl) with purple violet spots (Li et al, 2009) were successfully regenerated somatic embryos. The study of callus culture, embryogenesis, and embryo regeneration are the first barriers in somatic embryo culture (Jain et al, 1995). In this paper, rapid multiplication of *Rhynchostylis gigantea* orchids was studied through somatic embryogenic culture.

Materials and Methods

Plant materials: the *Rhynchostylis gigantea* forest of Vietnam is a red blood color. Sample culture: (i) young leaf of shoots in vitro 20-day-old. (ii) PLB thin slices.

Culture medium: The MS (Murashige et al, 1962) medium added: BA (6-benzylaminopurine), 2,4-D (2,4-dichlorophenoxy acetic acid), TDZ (thidiazuron), kinetin (6-furfurylaminopurine) NAA (α-napthalene acetic acid), coconut water (10%), sucrose (30 g/l), active charcoal (1 g/l).

Culture condition: Room temperature 26 + 2°C, humidity 65%, light intensity 22.2 $\mu mol/m^2/s$, lighting time 10 hours/day, sterile culture at 121°C (1 at) for 20 minutes, shaking speed 100 rpm.

Experiment design: with random 3 block replicates, each replicate of 3 flashes (containing 60 ml of semi-solid medium or 50 ml of liquid medium). The initial cell biomass was cultured to form a 10g/100ml suspension of medium. Data were analyzed using MSTATC software ($p = 0.05$).

Formation and Regeneration of Callus Culture

Effect of macromineral medium on PLB formation: Rhynchostylis gigantea shoots of 20-day-old were used as culture materials. The young leaf explant was cultured on the macromineral medium for PLB formation: MS, VW, 1/2M, supplemented with NAA (0.1 mg/l) and TDZ (0.1 mg/l). Cultivation time 30 days.

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Effect of TDZ and NAA on PLB formation: The MS medium supplemented with NAA (0.1, 0.5 mg/l) and TDZ (0.1, 0.5 mg/l). Cultivation time 30 days.

Callus formation: The PLBs were cut into slices that were cultured on the MS medium supplemented with coconut water (10%) and IAA (0.1, 0.5, 1, 2 mg/l). Cultivation time 30 days.

Callus proliferation: Callus from the above study was used in the study of proliferation. Callus mass was initially introduced into culture of 500 mg/sample. The culture medium for callus proliferation was the MS medium supplement with 2,4-D (0.1, 0.2, 0.5, 0.7 mg/l) and kinetin (0.1, 0.5, 1, 2 mg/l)). Cultivation time 45 days.

Callus regeneration: The callus from the above study, used as a regenerative material. The mass of callus was introduced into culture was 500 mg/sample. The culture regeneration medium of callus was the MS medium supplemented with NAA (0, 0.1, 0.5 mg/l), BA (0.1, 0.5, 1 mg/l), TDZ (0.1, 0.5, 1 mg/l). Cultivation time 45 days.

Formation and Proliferation of Callus Suspension

Formation of callus suspension: The callus was used as a culture material for callus suspension. The weight of callus transferred to liquid culture was 500 mg/50 ml. The culture medium for callus suspension: The MS medium supplemented with 2,4-D (0.5 mg/l), NAA (1 mg/l), BA (1 mg/l) and kinetin (1 mg/l). Cultivation time 20 days.

Callus suspension proliferation: The sixth of cell subculture was used as a culture material. The mass callus put into culture medium was 1 g/50ml. The medium for callus suspension proliferation was the MS medium supplemented with NAA and kinetin. Cultivation time 20 days.

Regeneration of Callus Suspension

Induction of somatic embryo: The sixth of cell suspension subculture was used as a culture material. The weight of cell introduced into culture medium was 10 g/50ml. The somatic embryo induction on the MS medium supplemented with BA (0, 0.1, 0.3, 0.5 mg/l). Cultivation time 45 days.

Somatic embryo transfer on agar: The somatic embryo suspension in the above experiment was used as the culture material. The volume of cell spread on solid medium was 5 ml/60ml. The culture medium for somatic embryo was the MS medium supplemented with NAA (0.1, 0.5 mg/l), BA (0.1, 0.5, 1 mg/l). Cultivation time 45 days.

Results and Discussion

Formation and Regeneration of Callus

Effect of Macromineral on PLB Formation

On the MS medium supplemented with NAA (0.1 mg/l) and TDZ (0.1 mg/l) PLB production of 78% was stimulated. PLB formed in clusters and had two different PLB forms, round to light blue and round white with an acorn with high regeneration rates (PLB 4.6). With a ½ MS mineral medium, the PLB has a large rounded shape and low bud regeneration (Table 1). The MS mineral medium was used for the following studies.

Effect of TDZ And NAA on PLB Formation

All treatments had high PLB formation rates. Young leaves were cultured as PLB formation on the MS medium, TDZ (0.1 mg/l), having a PLB production rate of 100% and reaching of 8.6 PLB/sample. However, the majority of PLBs were in blue form, only in the TDZ treatment (0.1 mg/l) appeared another form of small round white eggs of fish. This was the PLB type for high rate of regeneration (Table 2).

Callus Formation

The slices of PLB were used for culture. A callus appeared on the MS medium supplemented with IAA (1 mg/l), reaching a callus diameter of 3.2 mm (Table 3). The PLB appears less on the surface of the callus after 45-50 days of culture. The callus was isolated and cultured on a callus proliferation medium. Because the PLB is an embryo-like, the regenerative method of orchids was cultured in vitro. Therefore, the callus is derived from embryogenic cells.

Callus Proliferation

Rapid callus growth on the semi-solid MS medium supplemented with kinetin (0.5 mg/l) and 2,4-D (0.5 mg/l) yielded biomass of 2.377 mg/block, which has a proliferation coefficient of 4.6 (Table 4).

The callus is ivory white, with enlarged nucleus, cytoplasmic dense structure of pre-embryogenic cells.

Table 1: Effect of macro-mineral medium on PLB formation

Macro-mineral medium	PLB formation rate (%)	Number of PLB/sample
MS	78	4.6
1/2 MS	72	2.4
Vaccine-Went	46	3.2
CV (%)	12	8.4

Source: Author

Table 2: Effect of growth regulator on PLB formation

Growth regulator	PLB formation rate (%)	Number of PLB/sample
NAA (0.1 mg/l)	100	3.2
TDZ (0.1 mg/l)	100	8.6
NAA (0.1 mg/l) + TDZ (0.1 mg/l)	60	4.2
NAA (0.5 mg/l) + TDZ (0.1 mg/l)	74	78
NAA (0.1 mg/l) + TDZ (0.5 mg/l)	80	3.6
CV (%)	14	10.4

Source: Author

Table 3: Effect of IAA on callus formation

IAA (mg/l)	Callus formation rate (%)	Callus diameter (mm)
Control	-	-
IAA (0.1 mg/l)	-	-
IAA (0.5 mg/l)	18	2.2
IAA (1.0 mg/l)	74	2.8
IAA (1.0 mg/l)	100	3.2
IAA (3.0 mg/l)	68	3.0
CV (%)	10.6	9.2

Source: Author

Table 4: Effects of kinetin and 2,4-D on callus proliferation

2,4-D (mg/l)	Kinetin (mg/l)	Callus biomass (mg)	Proliferation rate
0.0	-	512	1.0
0.1	-	652	1.2
0.3	-	924	1.8
0.5	-	1.642	3.6
0.7	-	726	1.4
-	0.1	946	1.8
-	0.5	1.346	2.6
-	1.0	1.684	3.2
-	2.0	1.168	2.2
0.5	0.5	2.145	4.2
0.5	1.0	2.377	4.6
0.5	2.0	1.958	3.8
CV (%)		10.4	8.2

Source: Author

Callus Regeneration

The MS medium supplemented with NAA (0.1 mg/l) and BA (0.5 mg/l) had a 96% bud regeneration yield and 3.6 shoots/cluster (Table 5). The unshaped portion of the cell is green, forming a small PLB. The PLB derived callus has high regeneration efficiency, bud formation and PLB during regeneration. The results of the research were used as a basis for the regeneration of embryogenic callus suspension.

Formation and Proliferation of Callus Suspension

Formation of Callus Suspension

The suspension of mass after 30 days of culture with the MS medium supplemented with 2,4-D (0.5 mg/l) and kinetin (1 mg/l), resulted in a biomass of 2.186 mg/cluster and the coefficient of

proliferation was 4.2 (Table 6). The cell of suspension was relatively homogeneous. The callus in suspension differentiation did not follow certain rules when NAA or BA was added to the MS medium. It expressed in various cellular forms, with multiple cell clusters occurring, and a lower coefficient of proliferation compared to the MS medium supplemented with 2,4-D (0.5 mg/l) and kinetin (1 mg/l). The addition of kinetin and 2,4-D combinations was suitable for the culture of callus suspension compared to BA combined with NAA or 2,4-D.

Table 5: Effect of NAA and BA on regeneration of callus

NAA (mg/l)	BA (mg/l)	Callus regeneration rate (%)	Shoot/cluster
0.0	-	-	-
0.1	-	-	1.8
0.5	-	-	2.6
-	0.1	68	
-	0.5	78	
-	1.0	72	
0.1	0.1	72	2.2
0.1	0.5	96	3.6
0.1	1.0	64	2.8
0.5	0.1	58	2.4
0.5	0.5	42	2.0
0.5	1.0	36	1.8
CV (%)		14	8.2

Source: Author

Proliferation of Callus Suspension

The MS medium supplemented with 2,4-D (0.5 mg/l) and kinetin (1 mg/l) stimulated proliferation of callus suspension, a biomass of 8.256 mg/cluster and the coefficient of proliferation was 8.2 (Table 7). Cells had homogeneous, fast-growing, less clustered cells, with pre-embryogenic cell structure. Addition of kinetin and 2,4-D to the MS medium was appropriate to stimulate the proliferation of suspension.

Table 6: Effect of growth regulators on callus suspension culture

Growth regulators	Callus biomass (mg)	Coefficient of proliferation
2,4-D (0.5 mg/l) + kinetin (1 mg/l)	2.186	4.2
2,4-D (0.5 mg/l) + BA (1 mg/l)	542	1.4
NAA (1 mg/l) + kinetin (1 mg/l)	1.106	2.2
NAA (1 mg/l) + BA (1 mg/l)	744	1.5
CV (%)	10.6	10.2

Source: Author

Table 7: Effect of 2,4-D and kinetin on proliferation of callus suspension

Growth regulators (mg/l)	Callus biomass (mg)	Coefficient of proliferation
2,4-D (0.5 mg/l) + kinetin (0.5 mg/l)	7.642	7.6
2,4-D (0.5 mg/l) + kinetin (1 mg/l)	8.256	8.2
2,4-D (0.5 mg/l) + kinetin (2 mg/l)	6.284	6.2
CV (%)	10.4	8.2

Source: Author

Regeneration of Callus Suspension

Induction of Somatic Embryo Culture

In the culture of cell suspension, the cell suspension is stimulated for regenerated induction in a liquid medium or on a semi-solid medium prior to the culture of regeneration. The process of inducted-stimulation requires the addition of cytokinins and the reduction of auxins. TDZ does not stimulate embryogenesis (Quang, 2011). The suitable medium for induction of embryogenesis was MS supplemented with BA (0.3 mg/l). The embryonic cells in culture medium above were formed with active densities (1.3×10^4 cells/ml) and their efficiency of induction was 82% (Table 8). The rate of biomass proliferation decreases rapidly, whereas the efficiency of embryo-formed activation increases rapidly. The cell suspension differentiated completely to embryo after 45 days of culture.

Table 8: Effect of BA on induction of embryogenesis

BA (mg/l)	Densities of activated-cells (CFU/ml)	Efficiency of activation (%)
0	0.4 x 10^4	26
0.1	1.1 x 10^4	72
0.3	1.3 x 10^4	82
0.5	1.0 x 10^4	68
CV (%)	12.8	10.8

Source: Author

Regeneration of Somatic Embryo on Agar Medium

The MS medium supplemented with NAA (0.1 mg/l) and BA (0.5 mg/l) was suitable for regeneration of callus. Shoot regeneration was healthy, with no variability, with 96% regeneration rate, shoots of 22 shoots/5ml somatic cell suspension (Table 9). Harvest was 4,400 shoots per liter of cell suspension.

Table 9: Effect of NAA and BA on regeneration of callus

NAA (mg/l)	BA (mg/l)	Callus regeneration rate (%)	Number of shoot/5ml embryo cell suspension
-	0.1	66	14
-	0.5	74	18
-	1.0	68	16
0.1	0.1	70	18
0.1	0.5	96	22
0.1	1.0	66	15
0.5	0.1	52	12
0.5	0.5	38	10
0.5	1.0	30	7
CV (%)		12	

Source: Author

Conclusion

Formation and regeneration of callus: Young leaves were cultured to form PLB on the MS medium, TDZ (0.1 mg/l). The slices of PLB was cultured on the MS medium, IAA (0.1 mg/l) for callus formation, callus proliferation on the MS medium, 2,4-D (0.5 mg/l), kinetin (1 mg/l), and regeneration on the MS medium, NAA (0.1 mg/l), BA (0.5 mg/l) yielded high bud regeneration. The non-regeneration portion of the cell turns blue, forming small particles of the PLB.

Formation and proliferation of callus suspension: The cell suspension formation after 10 days cultured on the MS medium supplemented with 2,4-D (0.5 mg/l) and kinetin (1 mg/l), resulted in a coefficient of biomass proliferation of 4.2. Cells that produce suspension did not follow certain rules; appeared as many different cell shapes, and many clusters of cells. After 30 days of culture, the cells had a uniform shape; cells proliferated faster and there was less clumping. There was a part of the callus that differentiates into embryonic cells.

Regeneration of embryogenic callus suspension: The callus suspension was induced to embryo on the MS medium, BA (0.3 mg/l) and regenerated on the MS medium, NAA (0.1 mg/l), BA (0.5 mg/l), a regeneration rate of 96% was achieved and shoots reached 22 shoots/5ml of somatic embryo suspension. A system of rapid micropropagation of *Rhynchostylis gigantea* was established using the somatic embryogenesis technique, which yielded 4,400 shoots per liter of cell suspension.

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References

Chen, J.T., Chang, C. & Chang, W.C. (1999). Direct somatic embryogenesis on leaf explants of *Oncidium* Gower Ramsey and subsequent plant regeneration. Plant Cell Report; 19(2):143-149. https://doi.org/10.1007/s002990050724

Chen, J.T. & Chang, W.C. (2002). Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in *Oncidium* 'Gower Ramse'. Plant Cell Tissue Organ Culture: 69(1):41-44. https://doi.org/10.1023/A:1015004912408

Chen, J.T. & Chang, W.C. (2004). TIBA affects the induction of direct somatic embryogenesis from leaf explants of *Oncidium*. Plant Cell Tissue Organ Culture; 79(3):315-320. https://doi.org/10.1007/s11240-004-4613-5

Cheng, C. & Chang, W.C. (1998). Plant regeneration from callus culture of C*ymbidium ensifolium* var Misericors. Plant Cell Report; 17(6-7): 251-255. https://doi.org/10.1007/s002990050387

Griesbach, R.J. (2003). Orchids emerge as major world floral crop. Chronica Horticulturae; 43:6–12. ISBN: 90 6605 876 5

Ishii, Y., Takamura, T., Goi. M., Tanaka, M. 1998. Callus induction and somatic embryogenesis of *Phalaenopsis*. Plant Cell Report*,* 17(6-7):446-450. https://doi.org/10.1007/s002990050423

Jain, S. M., Gupta, P. K. & Newton, R.J. (1995). Somatic embryogenesis in woody plants. Springer, Dordrecht. DOI 10.1007/978-94-011-0491-3

Le, B.V., Hang, P.N.T, Anh, H.L.T. & Van, T.T. (1999). High frequency shoot regeneration from *Rhynchostylis gigantean* (*Orchidaceae*) using thin cell layers. Plant Growth Regulation; 28:179-185. https://doi.org/10.1023/A:1006210100775

Li, Z.L. & Xu, L. (2009). In vitro propagation of white-flower mutant of *Rhynchostylis gigantea* (Lindl.) Rid through immature seed-derived protocorm-like bodies. Journal of Horticulture and Forestry; 1(6):093-097. http://www.academicjournals.org/jhf

Murashige, T. & Skoog, R. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum; 15:431-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Quang, N.T. (2011). Microropagation of *Rhynchostylis gigantea* with bioreactor technology. Master thesis on Biotechnology 2011, International University.

Thomas, T.D. & Michael, A. (2007). High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume, an exquisite orchid. Plant Biotechnology Report; 1:243–249. https://doi.org/10.1007/s11816-007-0038-z