Industrial Propagation of Rhynchostylis Sp. By Bioreactor Technique

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Abstract: **Rhyncostylis sp. propagation by multi-shoot system induction from meristem culture in invitro. It take more labor, energy, large area, and high cost. Plant cell technology is effective way for micropropagation in bioreactor. Protocorm like bodies were used as planting materials. Somatic embryo callus were initiated on medium MS + IAA (1.0 mg/l). Somatic cell suspension were cultured for initiation and for proliferation on medium MS + 2.4D (0.5 mg/l) + kinetin (1 mg/l). The volume of somatic cell suspension for bioreactor cultivation was 20%. Somatic embryo suspension were cultured in bioreactor for initiation and proliferation on the medium MS + NAA (0,5 mg/l) + 2.4D (1 mg/l). Embryogenic suspension was stimulated on the medium MS + NAA (0.3 mg/l) + BA (0.3 mg/l). In vitro shoots of Rhynchostylis were plating and regeneration on the medium MS + NAA (0,1 mg/l) + BA (0,5 mg/l). Plantlets were enhanced growth and development in immersion-bioreactor cultivation by sinking/rising floated 1min/4hrs. Temperature, light intensity and stirring in stirring-bioreactor cultivation were favoured at 26±2ºC, 11,1-22,2 µmol/m^2/s, and 30 rpm. Micropropagation of Rhynchostylis sp. by bioreactor technique was set up to produce 6,800 plantlets per one liter of somatic embryogenesis suspension.**

Keywords: **Rhynchostylis sp., protocorm like bodies, embryogenic callus, somatic embryogenesis suspension**

I. INTRODUCTION

Traditional micropropagation [1] on orchids currently leads to a problem that micropropagation laboratories often face, which is that tissue culture plants often grow slowly, are very labor-intensive, and costly. it takes a long time to produce seedlings in large quantities when marketed at a high cost of seedlings. The embryo cloning system [2] solves the above barrier with the following advantages: rapid multiplication in the form of cells, the cloned embryo is a differentiated organism with high regeneration coefficient and low cost. labor costs and lower costs [3]. In somatic embryo technology, liquid culture is the basic technique performed on shakers or bioreactors [4,5] with the aim of increasing biomass, inducing homogenous somatic embryogenesis and leading to high efficiency somatic embryo regeneration [6,7]. Bioreactor techniques have been studied and applied to micropropagation in order to reduce the cost of tissue culture products [3]. Culture materials in micropropagation by bioreactor technology such as embryogenic callus cells, clonal embryonic cells, protocorm, bud clusters [7]. And there are many types of bioreactors used for micropropagation such as airlift bubble column-bioreactor, airlift bubble balloon-bioreactor, propeller tank-bioreactor, and semi-bioreactor. contemporary bioreactor [7]. Each type of bioreactor has different features, depending on the physiological properties of the cultured plants, aiming to increase biomass rapidly and enhance growth [5]. Physical and chemical factors are important factors affecting cell proliferation and cell regeneration [7,8]. There have been many successes on orchids micropropagation [9], but there are few articles recorded by using bioreactor. This paper studies the rapid multiplication of Rhynchostylis orchids by bioreactor technology.

II. MATERIALS AND METHODS

2.1 Materials

Varieties: Vietnamese Red Orchid (Rhynchostylis sp.).

Culture specimen: young leaf sheath, PLB.

2.2 Method

The culture mineral nutrient medium is MS [10].

Added: BA (6-benzylaminopurine), TDZ (thidiazuron), NAA (α-naphthalene acetic acid), 2.4D (2,4-dichlorophenoxy acetic acid), adenine (10 mg/l), peptone (1 g/l), CW coconut water (10%), activated carbon (1 g/l).

Culture conditions: room temperature 26±2oC, RH = 65%, lighting time 10 hours/day, light intensity 11.1-33.3 µmol/m2/s, shaking speed 100 rpm and speed impeller in bioreactor 30 rpm.

Experimental design: randomized complete block design, 3 replicates, 3 conical flasks each time (containing 60 ml of semi-solid medium or 50 ml of liquid medium). The volume of callus cells put into culture 10g/100ml of liquid medium generates suspension. The volume of cell fluid was cultured 20% in liquid with shaking and bioreactor 3-5 liters. The volume of cell plating was 5ml/60ml of semi-solid medium. Data were analyzed using MSTATC software (t=0.05).

III. RESULTS

Table 1. Effect of culture samples on protocorm generation

Culture medium	Culture sample	Number of shoots/sample	Number of PLB /sample
MS + BA (1 mg/l) + IBA (0.5 mg/l)	Corm shoot tip	4.8a	-
MS + 2iP (1 mg/l) + IBA (0.5 mg/l)	Young leaf sheath	3.6b	2.4
CV(%)		12.4	

Table 2. Effect of culture state on protocorm generation

Culture medium	Agar medium	Liquid medium	Bioreactor medium
MS + 2iP (1 mg/l) + IBA (0.5 mg/l)	2.6	4.8	5.9

Table 3. Effect of BA and 2iP on protocorm . regeneration

BA (mg/l)	2iP (mg/l)	Number of shoot/sample
0.5		3.6c
1.0		5.8a
2.0		4.4b
	0.5	3.2d
	1.0	3.4c
	2.0	3.7c
CV(%)		10.2

Table 4. Effect of BA and 2iP on protocorm regeneration

Growth regulator	**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

Table 5. Selection of fast-growing callus lines through culture cycles

Year 1 (2007) (mg/cluster)	Cycle 1	Cycle 2	Cycle 3	Cycle 4
	127	141	158	172
	Cycle 5	Cycle 6	Cycle 7	Cycle 8
	182	199	211	231
Year 2 (2008) (mg/cluster)	Cycle 9	Cycle 10	Cycle 11	Cycle 12
	254	268	282	311
	Cycle 13	Cycle 14	Cycle 15	Cycle 16
	342	370	388	394

Table 6. Effect of culture medium on the generation of embryonic callus suspension in bioreactor

Culture medium	NAA (mg/l)	2.4D (mg/l)	Growth coefficient (%)
MS	0.1	0.1	1.6
		0.5	2.4
		1.0	2.6
		2.0	2.1
	0.5	0.1	2.2
		0.5	2.8
		1.0	3.0
		2.0	2.8
	1.0	0.1	1.4
		0.5	1.8
		1.0	2.2
		2.0	1.9
CV%			11.6

Table 7. Effect of culture medium on proliferation of embryonic callus suspension in bioreactor

Culture medium	**NAA (mg/l)**	**2.4D (mg/l)**	**Growth coefficient (%)**
MS	0.1	0.1	6.2
		0.5	9.3
		1.0	10.1
		2.0	8.2
	0.5	0.1	8.5
		0.5	10.9
		1.0	11.8
		2.0	10.9
	1.0	0.1	5.4
		0.5	7.0
		1.0	8.5
		2.0	7.4
CV%			12.4

Table 8. Effect of light on cell suspension proliferation and PLB

Culture medium	Culture sample	11.1 µmol/m^2/s	33.3 µmol/m^2/s
MS + NAA (0.5 mg/l) + 2.4D (1 mg/l)	Cell suspension (after 30 days of culture)	12.5	15.8
MS + 2iP (1 mg/l) + IBA (0.5 mg/l)	PLB (after 60 days of culture)	8.3	12.2

Table 9. Effect of temperature on cell suspension proliferation and PLB

Culture medium	Culture sample	26±2ºC	30±2ºC
MS + NAA (0.5 mg/l) + 2.4D (1 mg/l)	Cell suspension (after 30 days of culture)	12.8	9.0
MS + 2iP (1 mg/l) + IBA (0.5 mg/l)	PLB (after 60 days of culture)	9.6	5.4

Table 10. Effect of stirrer speed on cell suspension proliferation and PLB

Culture medium	**Culture medium**	**30rpm**	**60rpm**
MS + NAA (0.5 mg/l) + 2.4D (1 mg/l)	Cell suspension (after 30 days of culture)	12.9	8.2
MS + 2iP (1 mg/l) + IBA (0.5 mg/l)	PLB (after 60 days of culture)	10.4	8.6

Table 11. Effect of culture medium on induction activation of embryo suspension in bioreactor

Culture medium	NAA (mg/l)	BA (mg/l)	Activation efficiency (%)
MS	0.1	0.1	46
		0.3	64
		0.5	56
	0.3	0.1	68
		0.3	80
		0.5	76
	0.5	0.1	36
		0.3	56
		0.5	48
CV%			14

Table 12. Effect of culture medium on regeneration of embryo suspension in bioreactor

Culture medium	NAA (mg/l)	BA (mg/l)	Number of shoots /5ml of embryo suspension
MS	0.1	0.1	14
		0.5	34
		1.0	32
	0.5	0.1	10
		0.5	22
		1.0	18
CV%			12

Table 13: Effect of culture rhythm on protocorm nucleus and shoot development

Rhythm		Protocorm		Single shoot
Floating (hour)	Sinking (minutes)	PLB	Number of shoot	Leaf length (mm)
1	1	1.4b	1.6b	15b
1	2	1.0c	1.4b	14b
2	1	1.4b	1.8b	16b
2	2	1.2c	1.6b	16b
3	1	1.8a	2.2a	18b
3	2	1.6a	2.0c	16b
4	1	2.2a	2.6a	24a
4	2	1.8a	2.2a	20a
5	1	1.8a	2.4a	20a
5	2	1.4b	2.0b	18b
6	1	1.6b	1.8b	16b
6	2	1.0c	1.6c	14b
Control (agar)		2.0a	2.2a	20a
Test f (0.05)		*	*	*

IV. DISCUSSION

Cultivation and Regeneration of PLB

Cultivation to Produce PLB

The culture sample was the growing apical corm zone: The culture specimen was the young shoot after 30 days of culture. There was a bud height of 20 mm, corm region of the apical bud. The apical corms were cultured on PLB growth medium: MS + Adenine (10 mg/l) supplemented with BA (1 mg/l) + IBA (0.5 mg/l). The results showed that (Table 1): After 30 days of culture, young shoots appeared on the growing apical corm, 4.8 shoots/corm apical growth.

Culture specimens are young leaf sheaths: The culture samples were the growing apical corms which was sliced thinly, and the young leaf sheaths are still white (5-10 mm). MS + Adenine (10 mg/l) culture medium supplemented with 2iP (1 mg/l) + IBA (0.5 mg/l). Research results showed that (Table 1): On MS + 2iP (1 mg/l) + IBA (0.5 mg/l) culture medium, after 45 days of culture: young shoots and PLBs arose on cultured samples. Transplant was young leaf sheath. The culture samples were young leaf sheaths for PLB generation for 3.6 shoots/sample and 2.4 PLBs/cultivars. Culture specimen was a thin-slice apical corm suitable for shoot development.

Rapid Multiplication of PLB on Agar, Liquid and Bioreactor Media

PLBs were thinly sliced and cultured on MS + 2iP (1 mg/l) + IBA (0.5 mg/l) medium, in 3 states of agar, liquid and bioreactor. The results showed that (Table 2): After 45 days of culture, the PLBs generated on agar, liquid and bioreactor were 2.6-4.8-5.9 PLBs/sample. The ability to generate PLB was relatively specific compared to other cultured orchids as a custom

PLB Regeneration

Culture samples are PLB (2-3 PLBs/cluster) cultured on: MS + Adenine (10 mg/l) + IBA (0.5 mg/l) supplemented with BA (0.5-1-2 mg/ l), 2iP (0.5-1-2 mg/l). The research results showed that (Table 3): MS + Adenine (10 mg/l) + BA (1 mg/l) + IBA (0.5 mg/l) basal medium for fast shoot growth: MS + Adenine (10 mg/l) + BA (1 mg/l) + IBA (0.5 mg/l) for rapid shoot growth and many shoots (5.8 shoots/sample) after 30 days of culture. The germinated shoots were separated and transferred to micropropagation medium.

Cultivation of Starting Material
Embryonic Callus Culture

In vitro young shoots were used as culture material, young leaf sheaths were cut into 0.5 cm^2 pieces, placed on MS + CW embryogenic callus cell growth medium (10%) supplemented with IAA (0.1-0.5-1-2 mg/l). The results showed that (Table 4): After 30 days of culture, callus appeared on the culture samples, on MS medium supplemented with IAA (1.0 mg/l). PLB appeared less on the callus surface after 45-50 days of

culture. Callus was separated and cultured on callus proliferation medium after 30 days of culture.

Selective Culture of Fast-Growing Embryogenic Callus Cell Lines

Callus obtained from the above experiment was cultured with selected cell lines on agar. The criterion was to select a cluster of fast-growing callus. The line selection cycle is 45 days, and then repeats. The sampling rate was 20% for each selection. Each cluster of callus was selected, inoculated on agar for subsequent selection with clone numbering. The number of line selections in 2 years was 16 cycles. The mass of cultured callus clusters was 100 mg/cluster. Selected medium MS + IAA (1 mg/l) + CW (10%). Culture samples were placed under diffused light of 22.2 µmol/m^2/s. The results showed that (Table 5): The volume of proliferating cells increased with each cycle of selection. At cycle 7 there was a mass of 211 mg/cluster with a proliferation factor of 2.11. The highest was 370 mg/cluster with a proliferation coefficient of 3.70 in cycle 14 and not much increase in the following cycles. Cyclic callus cells were used as raw materials for bioreactor studies

Cultivation and Proliferation of Cell Suspensions in Bioreactor

Cultivation and Proliferation of Embryonic Callus Suspensions in Bioreactor

Selected callus was used as culture material. Callus suspension cultures were performed on a shaker with a shaking speed of 100 rpm. The mass of cells put into culture was 10g/100ml of medium. The culture medium generated the MS embryonic callus suspension supplemented with NAA (0.1-0.5-1 mg/l) and 2.4D (0.1-0.5-1-2 mg/l). Research results show that (Table 6): After 30 days of culture, the suitable culture medium is MS + NAA (0.5 mg/l) + 2.4D (1 mg/l) to generate suspension, which has proliferation coefficient was 3.0. The suspension was even and has few clumps of cells.

Proliferative Culture of Embryonic Callus Suspension in Bioreactor

The callus suspension from the above experiment was used as the mother fluid and cultured in a bioreactor of 3 liters, with a culture volume of 1 liter, with a parenting rate of 10%, and a stirring speed of 30 rpm. The culture medium in the bioreactor proliferates the MS + BA embryonic callus suspension (0.1 mg/l) supplemented with NAA (0.1-0.5-1 mg/l) and 2.4D (0.1-0.5-1-2 mg/l). Research results show that (Table 7): After 20 days of culture, the appropriate culture medium was MS + NAA (0.5 mg/l) + 2.4D (1 mg/l) cells that proliferate slowly in the slow growth stage. The first week, and proliferate rapidly in the 2-3 week, with little clustering. The suspension was smooth, with the color of coffee and milk. NAA+2.4D was a growth regulator suitable for culture of callus suspension proliferative culture. There was a part of embryonic callus that differentiate into somatic embryonic cells. There was a biomass growth factor of 11.8 times.

Effect of Physical Conditions on Cell Suspension Culture in Bioreactor

Effect of Light (11.1-33.3 mol/m^2/s) on Proliferation of Embryonic Cells and PLB in Stirrer Bioreactor Culture

The culture sample was the embryonic callus suspension: 20% culture volume in bioreactor with culture temperature 26$\pm$2ºC and stirring speed 30 rpm. The culture medium proliferates the suspension of embryonic callus MS + NAA (0.5 mg/l) + 2.4D (1 mg/l). Research results show that (Table 8): After 30 days of culture: cells proliferate rapidly, with little clustering. There was a part of embryonic callus cells that differentiate into somatic embryonic cells. Growth rate was 12.5-15.8 times, no difference at two different light intensities.

Culture sample was PLB pseudo-embryotype: the culture temperature was 26$\pm$2ºC and stirring speed 30 rpm. PLB was cut into thin slices (1 mm thick) and cultured on bioreactor liquid medium to create PLB: MS + 2iP (1 mg/l) + IBA (0.5 mg/l). Research results show that (Table 8): After 60 days of culture: the embryo formation rate was high (80-90%) in the PLB culture medium supplemented with the combination and with 8.3-12.2 PLB embryos/culture at both light intensities.

Effect of Temperature (26-30$\pm$2ºC) on Proliferation of Embryonic Cells and PLB in Stirrer Bioreactor Culture

The culture sample was the embryonic callus suspension: 20% culture volume in bioreactor, illumination intensity 11.1 µmol/m^2/s and stirring speed 30rpm. The culture medium proliferates the suspension of embryonic callus MS + NAA (0.5 mg/l) + 2.4D (1 mg/l). Research results show that (table 9): After 30 days of culture: cells proliferate rapidly, with little clustering. There was a part of embryonic callus cells that differentiate into somatic embryonic cells. The proliferation rate of embryonic callus was 12.8 times at 26$\pm$2ºC compared to that at 30$\pm$2ºC. The suitable temperature for embryonic callus proliferation culture was 26$\pm$2ºC.

Culture sample was PLB pseudo-embryotype: Light intensity 11.1 µmol/m^2/s, stirring speed 30 rpm. PLB was cut into thin slices and cultured on bioreactor liquid medium to create PLB: MS + 2iP (1 mg/l) + IBA (0.5 mg/l). Research results show that (Table 9): After 60 days of culture: the embryo formation rate was high (80-90%) in the culture medium, generating 9.6 PLBs/culture at 26$\pm$2ºC compared with 5.4 PLBs/culturing at 30$\pm$2ºC.

Effect of Propeller Stirring Speed (30-60rpm) on Proliferation of Embryonic Cells and PLB in Stirrer Bioreactor Culture

The culture sample was the embryonic callus suspension: 20% culture volume in bioreactor, illumination intensity 11.1 µmol/m^2/s and culture temperature 26$\pm$2ºC. The culture medium proliferates the suspension of embryonic callus MS + NAA (0.5 mg/l) + 2.4D (1 mg/l). Research results show that (Table 10): After 30 days of culture: cells proliferate rapidly, with little clustering. There was a part of embryonic callus that differentiate into somatic embryonic cells. The proliferation rate was 12.9 at 30 rpm stirring compared to 8.2

at 60 rpm. The appropriate stirring speed for embryonic callus proliferation cultures was 30rpm.

Culture sample was PLB pseudo-embryotype: light intensity 11.1 µmol/m^2/s, culture temperature 26±2ºC. PLB was cut into thin slices and cultured on bioreactor liquid medium to create PLB: MS + 2iP (1 mg/l) + IBA (0.5 mg/l). Research results show that (Table 10): After 60 days of culture: the embryo formation rate was high (80-90%) in the PLB culture medium supplemented with the combination and with 10.4 PLB embryos/culture at a stirring speed of 30 rpm compared to 8.6 PLBs/culture at 60 rpm.

Cell Suspension Regeneration Culture in Bioreactor
Somatic Embryogenesis Induction Culture in Bioreactor

The 6th transfected cell suspension was used as the culture material. The mass of cells put into culture was 10g/100ml of medium. Activated culture medium for MS embryogenesis was supplemented with NAA (0.1-0.3-0.5 mg/l), BA (0.1-0.3-0.5 mg/l) and TDZ (0-0.1-0.5-1 mg/l). Research results show that (Table 11): After 45 days of culture: TDZ does not stimulate embryogenesis, cells were degraded and the death rate was high. The suitable medium for embryogenesis was MS + NAA (0.3 mg/l) + BA (0.3 mg/l) medium. Differentiated callus form embryos in culture, with an activation efficiency of 80% compared to the original cell density in culture. The proliferation rate decreased rapidly, whereas the activation efficiency increased rapidly. The suspension completely differentiated the embryos after 45 days of culture.

Plating Culture and Regeneration of Somatic Embryonic Cells on Agar

The 6th sub-cultured callus suspension was used as the culture material. Spread volume 5ml/60ml semi-solid medium. Spread culture medium of MS embryo suspension supplemented with NAA (0.1-0.5-1 mg/l mg/l), BA (0.1-0.5-1 mg/l), TDZ (0.1- 0.5-1 mg/l). Research results show that (Table 12): After 45 days of culture: TDZ inhibits the process of callus generation, cells are highly necrotic. The culture medium MS + NAA (0.1 mg/l) + BA (0.5 mg/l) was suitable for scar tissue regeneration. The buds regenerated healthy, no mutations appeared. The 6,800 shoots were collected per liter of embryonic cell suspension.

Shoot Growth and PLB in Temporary Immersion System (TIS) Bioreactor

Effect of Culture Rhythm on Protocorm Nucleus and Shoot Development

The results showed that (Table 13), a 4-hour floating rhythm and 1-minute immersion were suitable for protocorm culture and shoot regeneration. Compared with the control, the ability to generate protocorm on agar medium was better than semi-submersible culture (2.2 protocorm compared with 2 semi-submerged cultures) and vice versa, the ability to regenerate shoots cultured in semi-submersible was good. than the agar culture (2.6 shoots regenerated versus 2.2 agar culture). Single shoots that thrived in temporary immersion culture reached 24 mm leaf length compared to 20 mm grown on agar.

V. CONCLUSION

Cultivation and regeneration of PLB: PLBs were cultured arising from apical corms, or young leaf sheaths. PLBs were thinly sliced and rapidly multiplication. The generation of PLB/culture was 2.6-4.8-5.9.

Cultivation of starting material: Callus appeared on culture samples, PLB appeared less on callus surface after 45-50 days of culture on select medium MS + IAA (1 mg/l) + CW (10%). The highest was 370 mg/cluster and the proliferation coefficient was 3.70 in cycle 14 and the following cycles did not increase much. Cyclic callus cells were used as raw materials for bioreactor studies

Cultivation and proliferation of cell suspensions in bioreactor: On suitable culture medium, MS + NAA (0.5 mg/l) + 2.4D (1 mg/l) produced cell suspension with a proliferation coefficient of 3.0. The cell suspension is uniformly smooth and has few clumps of cells. There is a part of embryonic callus cells that differentiate into somatic embryonic cells. Having a biomass growth factor of 11.8 times

Effect of physical conditions on cell suspension in bioreactor: Cell suspensions proliferated rapidly under culture conditions with light intensity of 11.1 µmol/m2/s, temperature of 26±2oC and agitator speed of 30 rpm achieved cell suspension proliferation coefficient 12.5-12.8-12.9

Cell suspension regeneration in bioreactor: embryogenesis is differentiated from callus on MS + NAA (0.3 mg/l) + BA (0.3 mg/l) medium after 45 days culture, with an activation efficiency of 80% compared with the original cell density inoculated

Culture medium MS + NAA (0.1 mg/l) + BA (0.5 mg/l) is suitable for embryogenic callus regeneration after 45 days of culture. The buds regenerated healthy, no mutations appeared. Collect 6,800 shoots per liter of embryonic cell suspension.

Shoot growth and PLB in TIS bioreactor: The 4-hour float and 1-minute immersion rhythms are suitable for protocorm culture, shoot regeneration, and shoot growth. The PPM at a concentration of 2º/oo is suitable for liquid culture to limit the infection rate leading to shoot matter

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