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Thin Cell Layer Culture of *Phalaenopsis* sp. and *Rhynchostylis* sp.

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Abstract: *Phalaenopsis sp*: The corm of shoot tip from elite shoot 90-day-old was used as material on the medium of MS + BA (1mg/l) + NAA (0.1mg/l) to rise to 2-4 PLB/corm after 90 days cultivation. PLBs were used as materials for in vitro propagation. PLB was cut into thin slices and cultures on the PLB initiation medium MS + peptone (1g/l) + BA (1mg/l) + NAA (0.1mg/l). After 3 months, PLB was initiated on different samples with 11.8 PLB/corm shoot tip sample, 7.4 PLB/leaf sample, and 6.2 PLB/thin slice of PLB. PLB has multiplied on the medium MS + BA (1mg/l) + NAA (0.1mg/l) to rise 11,4 PLB/sample. PLB was regenerated and rooted on the medium MS + NAA (0.1mg/l) with a high rate of 100%. The thin cell layer culture of *Phalaenopsis* sp. was established.

Rhynchostylis sp: Shoot in 60-day-old clone in the pot was used as material for set up in vitro shoots by cultured on the medium MS + 2iP (1mg/l) + IBA (0,5mg/l) to raise 4-6 shoot/sample after 30 days of cultivation. Young shoots were multiplied on the medium MS + BA (1mg/l) + IBA (0.1mg/l) to produce 5-8 shoots/sample. The young shoot released young leaves and corms of shoot tip meristem used as material in thin cell layer culture. The corm shoot tip meristem was cultured on medium MS + BA (1mg/l) + IBA (0,5mg/l) to rise 4.8 shoots/sample; and young leaves were cultured on medium MS + 2iP (1mg/l) + IBA (0,5mg/l) to rise 2.4 PLB/sample and 3.6 shoots/sample. Shoots were multiplied on medium MS + BA (1mg/l) + IBA (0,5mg/l) and PLB was regenerated and rooted on medium MS + IBA (0,5mg/l). The thin cell layer culture of *Rhynchostylis* sp. was established.

Keywords: *Phalaenopsis* sp., *Rhynchostylis* sp. somatic embryogenesis, micropropagation, protocorm-like bodies, bioreactor technique.

I. Introduction

Mineral composition and plant growth regulators play an important role in the growth and development of plants, especially in vitro plants [1]. Mineral nutrient composition in each environment has different nutrient compositions and concentrations. Each plant species is suitable for a certain composition and concentration of mineral nutrients. Plant growth regulators are commonly used in vitro to regulate homeostasis and control morphogenesis, organogenesis in vitro [2]. The balance of growth regulators determines the in vitro culture process [3]. Controlling the composition and concentration of growth regulators determines the direction of cell and tissue differentiation in vitro [4]. The cytoskeleton is a thin piece of tissue that contains several layers of cells. Thin slices of cells are commonly cultured from leaves, stems, and inflorescences [5]. Cells of thin layer culture usually contains many types of somatic cells, interspersed with cells capable of differentiating into buds [6]. Its difficult and low percentage using cell culture technique to regenerate tissues and cells into plantlets [7]. There are leat successful of *Catleya* sp. [8], *Dendrobium* sp. [9], *Rhynchostylis* sp. [10] *Phalaenopsis* sp. [11], *Panax gingseng* [12], *Brassia napus* [13], *Petunia hybrida* and *Nicotina plumbaginifolia* [14], morphogenesis [15], embrygenesis [16] and soot regeneration [17] in TCLs culture. This paper studies the technique of thin cell layer culture on *phalaenopsis* sp. and *rhynchostylis* sp.

II. Materials And Methods

Materials

-The shoots of phalaenopsis were 90-day-old and rhynchostylis were 60-day-old

-The shoot apical corm of phalaenopsis and rhynchostylis was in vitro 20-days-old plantlets

-The young sheath-leaf (5-10mm) 20-day-old in vitro shoots of *phalaenopsis* and *rhynchostylis*.

-The thin-slices in vitro PLB of *phalaenopsis* and *rhynchostylis*

Method

Mineral nutrient medium: MS (Murashige &. Skoog, 1962).

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Added: BA (6-benzyl amino purine), 2iP (2-isopentyl adenine), IBA (β-indol butyric acid), NAA (α-naphthaleneacetic acid), Adenine (10mg/l), B1 (10mg/l), peptone (1g/l), CW coconut water (10%), sucrose (30g/l), activated carbon (1g/l), rhizogen.

Culture conditions: room temperature 26±2°C, RH = 65%, lighting time 10 hours/day, light intensity 33.3µmol/m^2/s in room, 55.5µmol/m^2/s outside course.

The experiment was arranged in the full block with 3 replicates, each time 3 triangle flasks, inoculating 3-5 samples/triangle flask. The data were statistically processed by using MSTATC software.

III. Results and Discussion

Phalaenopsis

Source Material for Thin Cell Layer Culture

Culture sample to create starting material: The 90-day-old shoot apical region was used as the culture material and cultured on medium: MS + peptone (1g/l) + BA (1mg/l) + NAA (0.1mg/l). Research results show that: After 90 days, PLBs generate 4-6 PLBs/culture.

Propagation of raw materials: PLB was used as culture material. PLBs were thinly sliced and cultured on a PLB-generating medium: MS + peptone (1g/l) + BA (5mg/l) + NAA (0.1mg/l). PL. Research results show that: After 3 months of culture, 4-6 PLBs/cultured samples were generated in the first culture. PLBs were regenerated into plantlets on the medium of MS + peptone (1g/l) + NAA (0.1mg/l) after 2 months, with a high regeneration rate of 100%. PLB and plantlets were used as raw materials for further studies.

Culture Samples for Creating Thin Cell Layer Culture Materials

The cultures were (i) thinly sliced apical corms (ii) thinly sliced PLBs (iii) and in vitro young sheath-leaf were used as materials cultured on protocorm induction media: MS + peptone (1g/l) supplemented with BA (1mg/l) + NAA (0.1mg/l). The results showed that (Table 1): After 3 months of culture: PLB arose on all three types of cultured samples. The number of PLBs arising was different with samples: thinly sliced apical corms reached 11.8 PLB/sample, young white sheath-leaves with white sheath was 7.4 PLB/sample, and thinly sliced PLB was 6.2 PLB/sample. However, PLBs arising from the apical corms are suitable for further studies

Protocorms Formation from Thin Cell Layer Culture

Effect of BA and NAA on thin cell layer cultures to protocorm formation: The in vitro shoot growth apical corm was used to culture on medium of MS + peptone (1g/l) supplemented with BA (0.1-1-5mg/l) and NAA (0.1-0.5-1mg/l). Research results showed that (Table 2): After 2 months of culture: PLB appeared more on the culture medium supplemented with a combination of BA (1mg/l) + NAA (0.1mg/l), rising 5.8 PLBs/culture sample. The green PLB was used in propagation

Table 1: Effect of explants on PLBs formation.

Culture medium	Explants	PLBs formation
MS + BA (1mg/l) + NAA (0.1mg/l)	Corm apical bud	11.8a
	Thinly sliced PLB	6.2c
	Young leaf sheath	7.4b
CV(%)		8.2

Table 2: Effect of BA and NAA in TCL cultures on PLBs formation

BA (mg/l)	NAA (mg/l)	PLBs formation
0.1	0.1	2.1d
	0.5	1.2e
	1.0	0.8e
1.0	0.1	5.8a
	0.5	3.2c
	1.0	2.6d
5.0	0.1	4.2b
	0.5	2.4d
	1.0	1.2e
CV(%)		14.6

Protocorm Propagation from Thin Cell Layer

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The cultures were PLBs formation by thin cell layer cultures: PLB thinly slices were cultured on PLB regeneration media: MS + peptone (1g/l) supplemented with BA (0.1- 1-5mg/l) and NAA (0.1-0.5-1mg/l). Research results showed that (Table 3): After 2 months of culture: PLB appeared more on medium of MS + peptone (1g/l) + BA (1mg/l) + NAA (0.1mg/l) with 11.4 PLB/culture sample. The green PLB was used in regeneration.

Table 3: Effect of BA and NAA on TCL cultures to PLBs propagation

BA (mg/l)	NAA (mg/l)	PLBs propagation
0.1	0.1	2.4d
	0.5	1.4e
	1.0	0.8e
1.0	0.1	11.4a
	0.5	4.6c
	1.0	3.2d
5.0	0.1	6.8b
	0.5	2.8c
	1.0	2.1d
CV(%)		14.6

Regeneration, Rooting, and Acclimatization of Shoots from Protocorm

Regeneration of PLBs to shoot: PLBs were cultured for regeneration on medium of MS + peptone (1g/l) supplemented with NAA (0.1-0.5-1mg/l), BA (0.1-0.3-0.5mg/l). Research results showed that (Table 4): After 90 days of culture: PLB on regeneration medium supplemented with a combination of BA (0.1mg/l) + NAA (1mg/l) gave a high rate of shoot regeneration. There was 100% regeneration efficiency. The number of shoots regenerated per PLB was a single shoot.

Table 3: Effect of BA and NAA on PLBs regeneration

BA (mg/l)	NAA (mg/l)	Regeneration rate (%)	Number of shoots /PLB	Schoot height (mm)
0.1	0.1	68c	1	36b
	0.5	85b	1	32b
	1.0	100a	1	40a
0.3	0.1	48d	1	18d
	0.5	56d	1	24c
	1.0	72c	1	28c
0.5	0.1	32e	1	16d
	0.5	47e	1	26c
	1.0	65d	1	32b
CV(%)		14		18

Rooting of plantlets: Shoots regenerated from PLB were cultured on medium of MS + peptone (1g/l) + NAA (0.1mg/l). Research results show that: After 45 days of culture: shoots have high rate of development into plantlets with roots formation.

Acclimatization: In natural light with diffused light, the light intensity is 55.5µmol/m^2/s, acclimatizing time 30 days. The substrate of acclimatization was moss.

Procedure for Regeneration of Tissue Culture Plants from Thin Cell Layer Culture

No.	Content	Time	Culture medium
1	Prepare materials	90	MS + BA (1mg/l) + NAA (0.1mg/l)
2	PLBs formation	60	MS + BA (1mg/l) + NAA (0.1mg/l)
3	TCL culture for PLBs propagation	60	MS + BA (1mg/l) + NAA (0.1mg/l) + peptone (1g/l)
4	Regeneration	90	MS + BA (0.1mg/l) + NAA (1mg/l) + peptone (1g/l)
5	Rooting	45	MS + NAA (0.1mg/l) + peptone (1g/l)
6	Acclimatization	30	Using Moss

Rhynchostylis

Source: Material for Thin Cell Layer Culture

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Cultivation of shoots: young shoots of *rhynchostylis* in pots, with a growth period of 60 days, were used as culture samples. The shoots were cultured on semi-solid agar MS + BA (1mg/l) + IBA (0.5mg/l). Research results showed that new young shoots appeared in day 60 after culture. In vitro shoots appear at the base of the shoot. The average number of shoots formed 4-6 shoots/culture sample.

In vitro propagation: In vitro young shoots of *rhynchostylis*, which have a growth period of 45 days, were used as culture samples. In vitro shoot was cultured on semi-solid agar medium of MS + BA (1mg/l) + IBA (0.1mg/l). Young shoots were defoliated for leaf sheaths and used in vitro cultures. Research results show that young shoots arise quickly on a semi-solid culture medium after 30 days of culture. The number of shoots obtained 5-8 shoots/culture. Young shoots through subculture 6 times were used as raw materials for thin cell layer culture.

Culture Samples for Creating Thin Cell Layer Culture Materials

The effect of the culture sample was the growing apical corm region on the regeneration ability of the thin cells layer: The culture sample was the young shoot after 30 days of a culture that has a bud height of 20mm. The apical corm zone (leaving off the leaves, and the corm at the base of the shoot, called the growing apical corm zone). The apical corms were cultured on induction medium: MS + Adenine (10mg/l) + BA (1mg/l) + IBA (0.5mg/l). Research results show that after 30 days of culture, young shoots appear on the growing apical corm: 4.8 shoots/corm apical growth.

The effect of the culture sample was the young leaf sheath on the ability to regenerate the thin layer cell: The cultured specimen was the young shoot of the growing apical corm region, which was thinly sliced, the young leaves are still white (10-20mm) and the young shoots were used as a culture material. Culture medium MS + Adenine (10mg/l) + 2iP (1mg/l) + IBA (0.5mg/l). The results showed that (Table 5): After 45 days of culture: young shoots and PLBs arose on two types of culture samples. The culture specimen was the young sheath-leaf for PLB regeneration that regenerated 3.6 shoots/explant and 2.4 PLB/cultivation. The culture specimen was a thin slice growth apical corm suitable for research on micropropagation and culture of somatic embryos.

Table 4: Effect of culture samples on shoots and PLBs formation

Culture medium	Culture samples	Number of shoots/sample	Number of PLBs/cluster
BA (1mg/l) + IBA (0.5mg/l)	Growth apical corm	4.8a	-
2iP (1mg/l) + IBA (0.5mg/l)	Young sheath-leaf	3.6b	2.4
CV(%)		12.4	

Shoots and Protocorm Formation from Thin Cell Layer Culture

Effect of BA and 2iP on the culture of the apical corms on shoot regeneration: The culture sample was the growing apical corms cultured on the medium: 1/2 MS + Adenine (10mg/l) + IBA (0.5mg/l) supplemented with BA (0.5-1-2mg/l), 2iP (0.5-1-2mg/l). The results showed that (Table 6): MS + Adenine (10mg/l) + IBA (0.5mg/l) + BA (1mg/l) basal medium for fast shoot growth and many shoots (4.6 shoots/sample) after 30 days of culture. The regerated shoots were separated and transferred to a micropropagation medium.

Table 5: Effect of BA and 2iP on culture samples of growth apical corm region on shoots regeneration

BA (mg/l)	2iP (mg/l)	Number of shoots/sample
0.5		2.9c
1.0		4.6a
2.0		3.8b
	0.5	1.4d
	1.0	2.8c
	2.0	2.2c
CV(%)		7.8

Effect of BA and 2iP on young sheath-leaf culture on shoots and PLBs regeneration: The culture sample was young sheath-leaf cultured on shoot regeneration medium: MS + Adenine (10mg/l) + IBA (0.5mg/l) supplemented with BA (0.5-1-2mg/l), 2iP (0.5-

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1.2mg/l). Research results showed that (Table 7): After 45 days of culture: medium of MS + Adenine basal medium (10mg/l) + IBA (0.5mg/l) + 2iP (1mg/l) + IBA (0,5mg/l) was used for rapid shoot formation (4-6 shoots/sample) and 1-3 PLB/explant after 45 days of culture. The germinated shoots were separated and transferred to a micropropagation medium.

Table 6: Effect of BA and 2iP on culture samples of young sheath-leaf on shoots regeneration and PLBs formation

BA (mg/l)	2iP (mg/l)	Number of shoots/sample	Number of PLBs/cluster
0.5		1.8c	-
1.0		2.6b	-
2.0		2.2b	-
	0.5	2.4b	-
	1.0	5.2a	2.2
	2.0	4.8a	-
CV(%)		1..2	

Shoots Propagation from Thin Cells Layer

Shoot multiplication: Shoots formed through thin cell layer culture were cultured on shoot propagation medium MS + Adenine (10 mg/l) + IBA (0.5 mg/l) supplemented with BA (0.5-1-2 mg). /l), 2iP (0.5-1.2mg/l). Research results showed that (Table 8): After 45 days of culture, shoots proliferated strongly on shoot multiplication medium supplemented with a combination of BA (1mg/l) + IBA (0.5mg/l). The Number of shoots arising was 4-8 shoots/cluster.

Table 8: Effects of BA and 2iP on shoots propagation in vitro

BA (mg/l)	2iP (mg/l)	Number of shoots/sample
0.5		4.4c
1.0		7.8a
2.0		6.2b
	0.5	1.8d
	1.0	3.6c
	2.0	2.4d
CV(%)		15.2

Regeneration, Rooting, and Acclimatization of Shoots from Protocorm

Root culture: young shoots through micropropagation were used as culture material and cultured on medium of MS + IBA (0.5mg/l). Research results show that the roots emerged after 14 days of culture. After 30 days of culture: the number of roots was 3-5 roots/shoot with 20-30mm length, and the shoot height was 20-30mm. The flask of *rhynchostylis* was placed in shaded conditions. After 30 days of pre-acclimatization, the shoots grew strongly, the leaves were dark green, the height of the shoots reached 80mm, the roots developed strongly, and the root diameter reached 0.3-0.8mm. The transplanted plants were then introduced to the nursery.

Acclimatization: In natural light with diffused light, the light intensity was 55.5µmol/m^2/s. acclimatization time 30 days. The substrate of acclimatization was moss.

Tissue Culture Regeneration Process from Thin Cell Layer Culture

No.	Content	Time	Culture medium
1	Prepare materials	30	MS + BA (1mg/l) + IBA (0.5mg/l)
2	Material multiplication	30	MS + BA (1mg/l) + IBA (0.1mg/l)
3	Thin cell layer culture	45	Corm: MS + BA (1mg/l) + IBA (0.5mg/l) Based young leaves: MS + 2iP (1mg/l) + IBA (0.5mg/l)
4	Regeneration	45	MS + BA (1mg/l) + IBA (0.5mg/l)
5	Rooting	30	MS + IBA (0.5mg/l)
6	Acclimatization	30	Using Moss

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VI. Conclusion

Phalaenopsis: The 90-day-old shoot apical region was used as culture material. On the following medium: MS + BA (1mg/l) + NAA (0.1mg/l) induce 2-4 PLB/sample after 90 days of culture. PLBs were regenerated on MS + NAA medium (0.1 mg/l) + peptone (1g/l).

Thin cell layer culture for protocorm induction: After 90 days of culture, the green PLBs arise from the apical corm were suitable for subsequent propagation

PLBs propagation: On the medium of MS + peptone (1g/l) + BA (1mg/l) + NAA (0.1mg/l): PLBs sliced were induced 8-12 PLBs/sample. The green PLB was used in regeneration

Rooting ability: After 45 days of culture: PLB has a high rate of regeneration into complete shoots with 100% regeneration efficiency. The technology to thin cell layer culture of phalaenopsis was developed.

Rhynchostylis: The 60-day-old shoots were cultured on MS basal medium supplemented with 2iP (1mg/l) + IBA (0.5mg/l) for rapid shoot induction and reached 4-6 shoots/sample after 30 days of culture. The germinated shoots were separated and transferred to a propagation medium.

Thin layer cell cultures: After 45 days of culture, the number of shoots generated was 4.8 shoots/sample of apical corm grown on MS + BA (1mg/l) + IBA (0.5mg/l) medium; and produced 2.4 PLBs/sample and 3.6 shoots/slices of young leaves on MS + 2iP (1mg/l) + IBA (0.5mg/l) medium. Regeneration of shoot from thin cell layer was developed

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