Micropropagation of Mokara Orchid by Temporary Immersion System Technique

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Abstract: **Based young leaves of *Mokara Leuen Berger Gold* were used as cultured materials. Callus was initiated and increase biomass on medium of semisolid and liquid cultures: MS medium supplemented with CW (30%), sucrose (30 g/l), 2.4D (1 mg/l). Callus was used as materials for initiation and biomass propagation on medium: (1) MS medium supplemented with NAA (1 mg/l), B1 (5 mg/l), Adenin sulfate (10 mg/l), peptone (1 g/l), CW (10%), sucrose (30 g/l) (2) MS medium supplemented with 2.4D (1 mg/l), CW (30%), Adenin sulfate (10 mg/l), peptone (1 g/l), CW (10%), sucrose (30 g/l). Semisolid medium for callus cell regeneration and to induce multiple shoots were: MS medium supplemented with BA (0.5 mg/l), B1 (5 mg/l), Adenin sulfate (10 mg/l), peptone (1 g/l), CW (10%), sucrose (20 g/l). Multiple-shoots were propagated on MS medium supplemented with BA (0.5 mg/l), B1 (5 mg/l), CW (10%), sucrose (20 g/l). Propagation of multiple-shoots in TIS on MS medium supplemented with BA (0.5 mg/l), B1 (5 mg/l), CW (10%), sucrose (20 g/l). Interval culture time was optimum for 2 hours floating and 1 minute rising. Plantlets were induced roots on MS medium supplemented with NAA (1 mg/l), B1 (5 mg/l), CW (10%), sucrose (20 g/l). Experiments on callus formation and callus growth of mokara orchids both on semi-solid medium (agar) and liquid medium to create callus suspension, the best medium selected was: MS supplemented with 2.4D (1 mg/l), CW (30%), sucrose (30 g/l)**

Keywords: **Bioreactor temporary immersion system, callus, callus cell suspension, multi-shoots**

I. INTRODUCTION

Orchids are a beautiful flower with high economic value, currently having a strong domestic and export market. Some flower varieties can be grown in the humid tropical conditions of Ho Chi Minh City such as: Dendrobium, Mokara, Phalaenopsis, Cattleyas, Vandaceous, Oncidium... In which, the cut branches of orchids belonging to the Mokara and Dendrobium groups are grown by many households and have a high profit rate despite the high initial investment, especially seed investment.

Mokara orchid group is the main group of varieties in developing area and providing cut orchid products in Ho Chi Minh City. The efficiency of growing cut branches orchids is very high, and the demand for domestic and export markets is also very large. Mokara is a group of flower varieties crossed from the varieties*: Arachnis x Vanda x Ascocentrum*. This group of varieties has similar characteristics to the Vanda group, which is a single species of orchid, with a long cylindrical body that continues to grow taller, without pseudobulbs, long leaves in the shape of a trough or a cylinder growing on either side of the stem. Inflorescence develops from the leaf axils in the center of the stem, inflorescence long with many flowers that are usually unbranched. Flowers medium to large, sepals very large. Flowers have many colors from white, purple, rose red, orange, yellow brown, blue. On the petals often have dots, spots or very beautiful checkered. This group of varieties is very suitable to produce cut branches flowers due to diligent flowering, which can reach 6-8 flowers/year.

However, at present, the price of seedlings is still high, such as Mokara seedlings from 40,000 - 45,000 VND/plant with an average size of 35 - 40cm. If investing in an orchid garden area of at least 1,000m2 in a net house, the average number of seedlings to invest is 4,000 plants, the value of seedlings is up to 160-200 million VND. Not to mention the cost of net house and other necessary materials from 60 to 80 million VND/1,000m2 of net house. The initial cost for Mokara seedlings accounts for 70% of the total cost. Therefore, it is necessary to reduce the cost of seedlings to provide producers and expand the area.

Currently, several private institutions, universities and research institutes have developed new techniques such as photoautotrophs, bioreactors, etc. to propagate orchids, but have not yet applied them (Paek *et. al*., 2005, Park *et. al*., 2000). The Center for Biotechnology in Ho Chi Minh City has also been using the TIS (Temporary Immersion System) bioreactor culture method to increase the efficiency of phalaenopsis orchid propagation, initially obtained good results (Nguyen Quoc Thien, 2009).

The temporary submerged culture system is a system that not only takes advantage of the advantages of liquid culture and culture on agar, but also limits the disadvantages of the two above culture systems to help create a culture medium. aeration, healthy seedlings, high survival rate, reduced labor costs, saving and reducing culture media costs by using less media per explant and not using agar, multiplier is increased many times compared to when propagated on conventional culture systems (Ducus *et. al*.,1993, 2000, Matsumoto *et. al*, 2009, Paek *et. al*., 2005, Park *et. al*., 2000).

For the reasons mentioned above, it was the basis of the research "Research on industrial propagation of some Mokara orchid varieties by intermittent submerged bioreactor technique." aims to investigate the applicability of this culture system in improving the quantity and quality of Mokara orchid seedlings when compared with conventional culture systems to contribute to opening production possibilities with

many good quality seedlings to meet the market demand in Vietnam.

In this study, a temporary submerged culture system used in plant propagation made in Taiwan, plantima was used for basic research and compared with the conventional culture method used by laboratories. Tissue culture in Vietnam such as culture on agar, liquid culture, liquid with shaking in the rapid multiplication of Mokara orchids.

II. MATERIALS AND METHODS

Materials

Mokara species: *Mokara Leuen Berger Gold*.

Callus tissue samples: young leaf sheaths of invitro plant.

Culture conditions

MS culture medium supplemented with coconut water (CW), 2.4D (Dichloropheoxy acetic acid), NAA (α-Naphthalene acetic acid), BA (benzyl adenine), B1 (5 mg/l), Adenin sulfate (10 mg/l), peptone (1 g/l).

Culture conditions: Room temperature 28+2^{o}C, RH=75%, callus formation in the dark, culture suspension and shoot clusters under 1500lux lighting condition.

Methods

Callus formation

Using young leaf sheath, white part and cutting off green part of leaf, transplanted into medium supplemented with 2.4D with concentrations of 0.5-1-2-3-4 mg/liter. Count the percentage of callus formed after 4 weeks of culture.

Callus biomass multiplication

Put a 500 mg callus sample into 60ml of culture medium supplemented with 2.4D with concentrations of 0.5-1-2-3-4 mg/liter. Weigh the callus after 1, 2, 3 weeks.

Suspension culture

Use 2g of fresh callus cultured in 50ml of liquid medium and place on a shaker with a shaking speed of 80rpm. After 7-14-21 days, centrifuge and weigh the biomass.

Growth culture of embryonic callus suspension

The rate of culture suspension is 20% of the culture medium volume. After 7-14-21 days, centrifuge and weigh the biomass.

Regeneration culture on agar

Sixty milliliter of agar is poured into a 300 ml conical flask. Spread 10 ml of the embryonic cell suspension on agar. Survey the regeneration rate after 30 days of culture. The regeneration rate is calculated on the ability to occupy the agar surface area (agar).

Propagation on agar

Sixty milliliter of propagation medium (agar) is poured into a 300 ml conical flask. Regenerating shoots 2-3 shoots/cluster were used for culture of 6 clusters each. Each treatment 6 bottles.

Propagation in semi-submersible bioreactor

Two hundred fifty milliliter regenerating medium (liquid) is poured into the semi-submersible bioreactor. Rhythm breaks for 1 hour (60 minutes) and immersion for 1 minute. Number of cultured samples was 16. Cultured samples of 2-3 shoots/cluster. Count the number of shoots formed after 40 days of culture.

Incubation rhythm in semi-submersible bioreactor

One-two-three hours interval and 1 min immersion. Number of culture samples was 40. Culture samples were 2-3 shoots/cluster. Count the number of shoots formed after 40 days of culture.

Rooting culture

Single shoot with height >2.5 cm. Rhythm breaks 2 hours (60 minutes) and immersion for 1 minute. Culture 100 plants in each pot. Measure the rate of rooting, number of roots and length of roots/plant.

Statistical analysis

Culture in conical flasks: 3 replicates, 3 flasks each time.

Culture in semi-submersible bioreactor: take the average of the number of culture samples.

Data were analyzed by ANOVA using MSTSC software (p=0.05).

III. RESULTS

Table 1: Effect of 2.4D on callus formation

2.4D (mg/l)	Callus formation rate (%)		
	Rate of callus formation	Creating callus on the cutting sample	Creating calluses of the whole sample
0.5	62.5b	61.3b	1.2c
1	78.4a	73.9a	4.5a
2	72.6a	69.4a	3.2a
3	65.4b	63.2b	2.2b
4	60.2c	58.4c	1.8b
CV%	12.2	11.6	10.5

Table 2: Effect of 2.4D on callus biomass multiplication

2.4D (mg/l)	Ability to increase callus biomass (mg)		
	7 days	14 days	21 days
0.5	540c	834c	1262c
1	580a	1652a	3245a
2	592a	1684a	3294a
3	604b	1722b	3316b
4	625b	1782b	3348b
CV%	8.6	10.4	14.2

Table 3: Effect of 2.4D on the process of callus suspension formation

2.4D (mg/l)	Ability to create biomass of callus suspension (mg)		
	7 days	14 days	21 days
0.5	2246c	2864c	3425c
1	2456a	4278a	6874a
2	2514a	4294a	6924a
3	2583b	4328b	6985b
4	2634b	4412b	7087b
CV%	7.6	10.6	12.4

Table 4: Effect of NAA and 2.4D on callus suspension proliferation

Auxin (mg/l)	Ability to increase the callus suspension proliferation (mg)		
	7 days	14 days	21 days
NAA (0.5)	2192b	2786b	3245b
NAA (1)	2892a	5046a	7425a
NAA (2)	2984a	5278a	7206a
2.4D (0.5)	2358b	2896b	3564b
2.4D (1)	2746a	4825a	7258a
2.4D (2)	2842a	4985a	7486a
CV%	9.2	12.2	14.6

Table 5: Effect of BA in embryo regeneration of callus suspension

BA (mg/l)	Embryo regeneration rate (%)		
	7 days	14 days	21 days
0.1	0.5c	32.6b	86.4b
0.5	2.8a	42.6a	100a
1	2.1a	28.2c	62.4c
2	1.4b	26.8c	58.6c
CV%	12.2	13.6	14.6

Table 6: Effect of BA on shoot multiplication on semi-solid medium

BA (mg/l)	Shoots/cluster		
	7 ngày	14 ngày	21 ngày
0,1	2,4b	4,2b	6,5b
0,5	2,6a	5,8a	12,6a
1	2.,4a	3,8c	7,2b
2	2,2c	3,6c	6,8b
CV%	9,4	10,6	12,2

Table 7. Propagation of propagule in bioreactor temporary immersion system

Order of shoot clusters	Number of shoots/clusters	Shoot height (cm)	Diameter shoot cluster (cm)
1	16	3.0	3.0
2	12	1.8	2.6
3	19	3.0	2.8
4	18	1.8	2.2
5	11	2.2	1.6
6	14	2.4	1.6
7	16	3.0	1.3
8	20	2.6	2.0
9	19	2.9	2.8
10	14	1.8	2.2
11	15	3.1	1.6
12	13	2.0	1.0
13	15	1.6	1.0
14	12	1.6	1.0
15	13	2.0	3.0
16	17	2.0	2.0
Average	15.2	2.3	1.9

Table 8: Culture rhythms in the bioreactor temporary immersion system

Interval (hour)	Number of shoots/clusters	Height shoots (cm)	Diameter shoots cluster (cm)	Number of dead shoots/clusters
1	10.6b	1.8a	1.8a	3.2
2	14.8a	2.6a	2.1a	0.0
3	8.2c	2.2a	1.6a	2.2
CV%	12.2	8.6	16.4	-

Table 9: Effect of NAA on rooting ability in bioreactor temporary immersion system

NAA (mg/l)	Root formation		
	Rooting rate (%)	Number of roots/shoots	Root length (cm)
0.1	68b	2.2b	1.8b
0.5	72b	2.8b	2.2b
1	100a	3.2a	2.5a
2	100a	3.2a	2.4a
CV%	14.2	10.4	12.2

IV. DISCUSSION

Callus formation

The white part of the young leaf sheath was inoculated into the treatments of different media at the concentration of 2.4D. After 4 weeks of culture to count the number of calli generated (Table 1).

Callus biomass multiplication

Five hundred milligram callus was inoculated into 60ml of agar medium with different concentrations of 2.4D. After 1, 2, 3 weeks of culture (Table 2). In the treatment of 1 mg/liter of 2.4D, the medium had better callus-forming biomass. Significant difference compared with treatments of 0.5-3-4 mg/l of 2.4D medium, not significant difference compared with treatment of 2 mg/liter of medium, but for more even and firm callus for later regeneration. The best callus formation medium MS supplemented with 2.4D (1 mg/l), CW (30%), sucrose (30g/l)

Culture to create suspensions

Two grams of callus cultured into 50 ml of medium with different concentrations of 2.4D. After 7, 14, 21 days of culture was centrifuged and weighed. Treatments 1 and 2 mg/l 2.4D were significantly different from the other treatments (Table 3). The treatment of 2 mg/l 2.4D of biomass medium had higher growth than the treatment of 1 mg/l of 2.4D medium. However, this treatment gave more soft tissue and was not good for regeneration. The best callus suspension medium MS supplemented with CW (30%), sucrose (30g/l), 2.4D (1 mg/l).

Suspension culture proliferation

Testing the concentration of 2.4D and NAA with different concentrations on the medium to grow the callus mass. After 7, 14, 21 days of culture, both media supplemented with 1 mg/l 2.4D and NAA had high biomass growth index and better callus quality than other concentrations (Table 4). The culture medium to increase the suspension biomass: (1) MS supplemented with NAA (1 mg/l), B1 (5 mg/l), Adenin sulfate (10 mg/l), peptone (1 g/l), CW (10%), sucrose (30g/l), (2) MS supplemented with 2.4D (1 mg/l), CW (30%), Adenin sulfate (10 mg/l), peptone (1 g/l), CW (10%), sucrose (30 g/l)

Embryo regeneration on agar

Callus regeneration test on agar medium, spread 10 ml of the above cultured suspension into 60 ml of medium with different concentrations of BA. After 7, 14, 21 days of culture, the embryos were count on the counting chamber. The regeneration rate after 7, 14, 21 days of culture on medium supplemented with BA (Table 5). The callus suspension in the treatment of 0.5 mg/l BA medium was the best. At higher concentrations regeneration was slower due to the increased number of embryos. Embryo regeneration culture medium (on semi-solid medium – agar): MS supplemented with BA (0.5 mg/l), B1 (5 mg/l), Adenin sulfate (10 mg/l), peptone (1 g/l), CW (10%), sucrose (20 g/l).

Propagation on agar

Shooting coefficient on agar with different concentrations of BA showed that at higher concentration of BA than 0.5 mg/l. The shoot growth was slow because shoot clusters tend to form embryos. The growth of shoot clusters on medium supplemented with 0.5 mg/l BA was the best (Table 6) and significantly different from other treatments. MS supplemented with BA (0.5 mg/l), B1 (5 mg/l), CW (10%), sucrose (20 g/l).

Propagation on bioreactor temporary immersion system

Trials of culturing shoot cluster in bioreactor temporary immersion system, separating shoots from 2-3 individuals and transplanting them into flasks. After 40 days, the results were shown in Table 7

Culture rhythms in the bioreactor temporary immersion system (TIS)

To find out the best time and interval for the growth of Mokara orchids. The experiment was arranged at different intervals. After 40 days of culture, interval culture time for the development of Mokara orchids was optimum for 2 hours floating and 1 minute rising (Table 8).

Root formation

Root growth of Mokara orchid cultured in bioreactor temporary immersion system with different concentrations of NAA. Measured roots after 40 days of culture: Investigation of the rooting of Mokara orchids in bioreactor temporary immersion system on the medium supplemented with NAA with different concentrations (Table 9). At an additional concentration of 1 mg/liter of NAA, the medium was best, significantly different from other concentrations. Root growth medium in semi-submersible bioreactor MS supplemented with NAA (1 mg/l), B1 (5 mg/l), CW (10%), sucrose (20 g/l).

V. CONCLUSION

A flush out system for Mokara micropropagation via manipulation of temporary immersion system techniques was set up. Experiments on callus formation and callus growth of Mokara orchids both on semi-solid medium (agar) and liquid medium to create callus suspension, the best medium selected was MS + CW (30%) + sucrose (30 g/l) + 2.4D (1 mg/l). Following the stage of cell suspension cultures; Regenerating the callus cell suspension to create shoots on semi-solid media; Propagating shoots and Rooting in the bioreactor temporary immersion system

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