RAPID MICROPROPAGATION OF VU NU ORCHID (*ONCIDIUM* SP.) BY USING TISSUE CULTURE TECHNIQUE

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Abstract: The high demand of *Oncidium* orchids leads us to find out efficient methods of propagating them. However, the propagation rate of traditional methods are low in nature and a hybrid seed is not genetically stable. Thus, plant cell biotechnology is examined as the most effective way to resolve the barrier of elite clone production. Shoot clusters were cultured on MS medium supplemented with 2,4-D (1 mg/l) for callus induction (76.19%) before induced callus was favoured for PLBs regeneration (98 PLBs/callus cluster) on MS medium supplemented with NAA (0.75 mg/l); the combination of BA (0.5 mg/l) and NAA (0.5 mg/l) was favoured for PLBs regeneration (28.18 PLBs/shoot cluster) from shoots cultivation. The PLBs (79.21 PLBs/PLB cluster) were then proliferated on MS medium supplemented with NAA (1 mg/l) and BA (1 mg/l) for shoot regeneration (12.42 shoots/PLBs cluster). Multiple-shoots were divided to 3-4 shoots/cluster for micropropagation on the MS medium supplemented with the combination of BA (0.25 mg/l) and NAA (0.25 mg/l) to reach 11.66 shoots/cluster. Shoots were finally separated to single-shoot for rooting on the MS medium supplemented with NAA (0.75 mg/l). A scheme for *Oncidium* micropropagation using PLBs culture techniques was set up.

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Introduction

Orchidaceae orchids include many of the flowers spices used in the cut flower industry. Vu Nu (*Oncidium*) orchid is a fragrance that is popular in the world market in the form of flower pots and cut branches (Arditti, 2008). Low natural shoots and hybrid shoots do not guarantee parental lineage. The technique of tissue culture has been successfully applied in the conservation and development of rare orchids, cut flowers, condiment and medicinal plants (Arditti, 2008).

Vu Nu orchid culture has yielded the groundwork for the construction of micropropagation technology, such as the shoot tip, root tip, inflorescence culture of *O. Sweet Sugar* (Chen and Chang, 2000b) culture of young leaf producing direct and callus-formed PLBs (Chen and Chang, 2000a), proliferation of PLBs of *O. Gower Ramsey* (Kusumoto et al, 1998), regeneration of PLBs of *O. Sharry Baby* OM8 (Li et al, 2005) and *O. Sweet Sugar* (Hong et al, 2008).

Natural regeneration of Vu Nu is low about 1-3 shoots/cluster in one year. The culture of PLB occurs primarily in monocots such as orchids, which are directly regenerated from tissue, pollen, and orchid cells cultured in vitro. PLBs were proliferated and developed into complete plants in vitro (Arditti, 2008). By this method, a large number of plantlets of the same genetic origin can be obtained in a short time. The study of callus production, PLBs production, PLBs formed shoot regeneration and rapid multiplication of shoot need to be developed in order to rapidly multiplying *Oncidium* sp. orchids in vitro.

Materials and Methods

Vu Nu orchids (*Oncidium* sp.) in Vietnam are used as research materials for conservation and development purposes. The cultured samples are in vitro shoots of Vu Nu orchid. The research was conducted in the Plant Biotechnology Laboratory, International University.

The Murashige-Skoog nutrient medium (Murashige & Skoog, 1962) was added with BA (6-benzylaminopurin), NAA (α -naphthalenacetic acid), 2,4-dichloro-phenoxyacetic acid, sucrose (20 g/l), Coconut water (10%), agar (Hai Phong).

The culture medium was adjusted to pH 5.8 before autoclaving at 121 ° C and 1 atm for 40 minutes. The culture condition was set up with room temperature at 26 ± 2 ° C, illumination intensity in 22.2 $\mu\text{mol}/\text{m}^2/\text{s}$ for 12 hours per day, relative humidity RH = 65%.

The experiment was set up in randomly complete block (RCB), with 3 replications, each with 3 Erlenmeyer flask (300 ml), each with 5 explants. Experimental data were recorded after 90 days of

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culture and ANOVA was analyzed together with Turkey HSD ($p = 0.05$) with SPSS, version 16 (<http://www-01.ibm.com/>). Software/analytics/spss/).

Experiment Design*

Experiment 1: Callus culture of Vu Nu orchid: MS medium was supplemented with 2,4-D in 6 treatments from C1 to C6 (0, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/l). The cultured explants were shoot clusters (3-5 buds/cluster). The culture time is 90 days. Observed indicators: the rate of callus formation (%) and diameter of callus are recorded (mm). The best callus in one of these six treatments will be used as a material for experiment 2.

Experiment 2: Effect of NAA on PLB formation from callus: The PLB production from the best callus in one of the six treatments of experiment 1. MS medium was supplemented with NAA in 5 treatments from A1 to A6 (0, 0.1, 0.25, 0.5, 0.75, 1 mg/l). Cultured explants were callus (5 clusters of calli/Erlen). The observed indicator was PLBs/callus. The best PLBs in one of the five treatments will be used as material experiment 4.

Experiment 3: Effect of NAA and BA on PLB formation from bud cluster: MS medium was supplemented with BA in 6 treatments from G1 to G6 (0, 0.1, 0.25, 0.5 mg/l) and NAA (0, 0.5, 1 mg/l). The cultured explants were shoot cluster (3-5 buds/cluster). Observed indicator: number of PLBs/shoot cluster. PLBs in one of six treatments will be used as a material for experiment 4.

Experiment 4: Effect of NAA on proliferation of PLB biomass: MS medium was supplemented with NAA in 5 treatments from E1 to E5 (0.10, 0.25, 0.50, 0.75, 1 mg/l). The cultured explants were PLB clusters (3-5 PLB/cluster). The observed indicator is PLB number/cluster. PLBs in one of five treatments will be used as materials for Experiment 5.

Experiment 5: Effect of BA on shoot regeneration from PLBs: PLBs were formed from treatment E5 used as culture material. MS medium was supplemented with NAA in 5 treatments from B1 to B5 (0, 0.1, 0.25, 0.5, 0.75, 1 mg/l). The cultured explants were PLB clusters (3-5 PLB/cluster). The observed indicator was the number of shoots/cluster.

Experiment 6: The combined effect of BA and NAA on bud proliferation: The PLBs was formed from treatment E5 used as the culture material. MS medium was supplemented with BA (0, 0.1, 0.25, 0.5 mg/l) and NAA (0, 0.1, 0.25 mg/l) in six treatments ranging from D1 to D6. The cultured explants were shoot clusters (3-4 buds/cluster). The observed indicator was the number of shoots/cluster.

Experiment 7: Effect of NAA on root formation: MS medium was supplemented with NAA in six treatments from F1 to F6 (0, 0.1, 0.25, 0.5, 0.75, 1 mg/l). The culture explants were single shoots (> 30 cm). Observed indicators were the number of roots/shoots and root length.

Results and Discussion

Culture of Callus Formation of Vu Nu Orchid

Callus formation was carried out on MS medium containing different concentrations of 2,4-D. The effect of 2,4-D on callus formation is shown in Table 1. Callus is formed at the base of the Vu Nu orchid bud after 90 days of culture. The incidence of callus formation varies depending on the different concentrations of 2,4-D added to the culture medium. Observations show that there are three kinds of callus: (i) cream color, fragile, and lumps; (ii) light blue, and sticky; (iii) blue and sticky.

On MS medium without 2,4-D supplement (control), callus formation did not occur. On the MS medium supplemented with 2,4-D, shoot clusters produced callus-formed response significantly. The highest incidence of callus formation was 76.19% recorded in treatment A5 supplemented with 1 mg/l 2,4-D and two kinds of callus formed were (ii) light green and (iii) blue, compact with 5.57 mm in cluster size. In A2 treatment supplemented with 0.25 mg/l 2,4-D, the diameter of the callus reached the maximum size of 8.02 mm/cluster.

Wu et al. (2004) investigated the effects of two groups of auxins and cytokinins on embryo formation from calluses obtained from the root sample, the authors noted that there was no PLB on the MS medium without 2,4-D, but they still induced callus from this medium. Our results are similar to the rate of callus formation of 76.19% on MS medium supplemented with 1 mg/l 2,4-D. Jheng et al. (2006) added with 2,4-D in the lower concentration to culture medium affected cell proliferation, which was similar to that obtained with low concentrations of 2,4-D in this study. Experimentally on

callus formation, high concentrations of 2,4-D should be not used as it was the cause of mutant formation (Arditti, 2008) and the callus appeared to mutate in long-term culture (Jheng et al, 2006).

Table 1: Effect of 2,4-D on callus formation

Treatment	2,4-D (mg/l)	Callus formation rate (%)*	Diameter of callus (mm/cluster)*	Morphology of callus
A0	0,00	0,00 ± 0,00b	0,00 ± 0,00b	No callus formation
A1	0,10	47,62 ± 20,75ab	3,48 ± 0,63ab	Cream, fragile, and lumps
A2	0,25	42,86 ± 16,49ab	8,02 ± 2,52a	Light green, and sticky
A3	0,50	54,76 ± 16,67ab	5,14 ± 2,21ab	Light green, and sticky
A4	0,75	23,81 ± 12,60ab	1,38 ± 0,70ab	Green and sticky
A5	1,00	76,19 ± 4,76a	5,57 ± 1,86ab	Cream, fragile, and lumps mixed with - light blue, and sticky
A6	2,00	33,33 ± 4,76ab	2,05 ± 0,40ab	Light green, and sticky

Source: Author

Effect of NAA on PLB Formation from Callus

Study on PLBs formation by callus culture on MS medium supplemented with NAA was performed. In Table 2, NAA was not added to medium showed no PLB was formed. The NAA in the higher concentration favoured the greater number of PLBs produced. Addition of 0.5 mg/l and 0.75 mg/l NAA into MS medium was for formation of 67.25-98 PLB/clusters. The MS medium supplemented with 0.75 mg/l NAA was suitable for the embryo PLB culture.

Chen and Chang (2000b) reported on MS medium supplemented with appropriate concentrations of NAA and TDZ will speed up the formation of PLBs. In this study, we tested the influence of NAA on the formation and growth of PLB biomass. The results recorded that supplemented with NAA in culture medium at a concentration of 0.1-1 mg/l had a significant effect. In a few other studies by other scientists, auxin and cytokinin have also been incorporated (Juliana et al., 2010).

Table 2: Effect of NAA on PLB formation from callus tissue

Treatment	NAA (mg/l)	Number of PLB/cluster*
B0	0,00	0,00 ± 0,00d
B1	0,10	26,83 ± 9,74c
B2	0,25	28,86 ± 5,60c
B3	0,50	67,25 ± 1,76b
B4	0,75	98,00 ± 3,79a
B5	1,00	5,00 ± 5,00cd

Source: Author

Effect of NAA and BA on PLB Formation from Shoots

On the MS medium, the addition of a combination of NAA and BA concentrations has had some effects. After 90 days of culture, all treatments had PLB formation, except the control medium without NAA plus BA with no PLB formation. Most of the cultured shoots produced PLB and the highest number of PLB/ bud cluster were recorded in treatment C3 (with the addition of 0.5 mg/l NAA and 0.5 mg/l BA) for the formation of 28.18 PLB/ shoot cluster, PLBs are blue and well-developed. Table 3 shows that the NAA and BA in MS culture medium significantly influenced production and morphology of PLBs. When combined with NAA (1 mg/l) and BA (0.1-0.5 mg/l), the PLBs formed with a low rate. MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA was suitable for the production of PLBs.

Rahman et al. (2005) recorded a combination of 1 mg/l BA and 0.05 mg/l NAA obtained the highest rate of Vu Nu PLB formation (90%). Juliana et al. (2010) also recorded a combination of 0.25 µM NAA and 13.5 µM TDZ for up to 80% of the PLB formation. In the results of this study, 28,18

PLB/shoots per cluster were also obtained on MS supplemented with NAA (0.50 mg/l) and BA (0.50 mg/l). Arditti (2008) concludes the PLBs from callus and shoots have both the nature and structure of cells capable of producing the same embryos, so PLBs from callus and shoots are used as raw material for proliferation culture research.

Table 3: Effect of NAA and BA on PLB formation

	Treatment	NAA (mg/l)	BA (mg/l)	Number of PLB/ shoot cluster*
	C0	0,00	0,00	0,00 ± 0,00d
	C1	0,50	0,10	9,65 ± 1,07c
	C2	0,50	0,25	5,81 ± 1,78cd
	C3	0,50	0,50	28,18 ± 1,62a
	C4	1,00	0,10	19,42 ± 3,11b
	C5	1,00	0,25	10,33 ± 1,56c
	C6	1,00	0,50	9,42 ± 1,47c

Source: Author

Effect of NAA on PLB Proliferation

The objective of this study was to accelerate the multiplication of PLBs to provide the material for subsequent experiments. Results showed that NAA was added to the appropriate MS culture medium for embryos PLB. Treatments supplemented with NAA had a good response during the proliferation process. After 90 days of culture, MS medium supplemented with 1 mg/l NAA (treatment D5) produced the highest PLB with 79.21.

Table 4: Effect of NAA on PLB proliferation

	Treatment	NAA (mg/l)	Number of PLB/sample *
	D0	0,00	33,53 ± 0,47d
	D1	0,10	41,62 ± 1,20cd
	D2	0,25	38,14 ± 3,36cd
	D3	0,50	55,00 ± 1,15b
	D4	0,75	43,67 ± 1,07c
	D5	1,00	79,21 ± 1,62a

Source: Author

Effect of BA on Shoot Regeneration from PLBs

Cytokinins play an important role in the reproduction of shoots. The culture medium of MS with different BA concentrations achieved effective regeneration (Table 5). On medium MS supplemented with 1 mg/l, BA yielded highly with 12.42 shoots/cluster after 90 days of culture. Shoots are green and have strong vigor with a height of 3-5 cm and 6-9 leaves.

Table 5: Effect of BA on shoot regeneration from PLBs

	Treatment	BA (mg/l)	Number of shoot/sample*	Morphology
	E0	0,00	4,43 ± 0,30b	No more new shoots
	E1	0,10	11,48 ± 1,31a	The green and the buds are uneven
	E2	0,25	9,83 ± 0,49a	Green and bud uniform Green and some dominant shoots
	E3	0,50	9,50 ± 0,29a	Green and bud uniform
	E4	0,75	10,92 ± 0,65a	Green and bud uneven
	E5	1,00	12,42 ± 0,60a	Green and bud uniform Green and some dominant shoots

Source: Author

The Combined Effect of BA and NAA on Shoot Proliferation

The research of shoot proliferation in vitro of Vu Nu is an important determinant of multiplication. Amongst the MS medium with BA and NAA after 90 days of culture, the supplemented treatment with 0.25 mg/l BA and 0.25 mg/l NAA gave best results with 11.66 shoots/cluster (Table 6).

Table 6: The effects of BA and NAA on shoot proliferation				
Treatment	BA (mg/l)	NAA (mg/l)	Number of shoot/sample*	Morphology
F0	0,00	0,00	4,43 ± 0,30d	No new shoots
F1	0,10	0,10	11,19 ± 0,17ab	Green and shoot uniform
F2	0,25	0,10	6,50 ± 1,28bcd	Blue and some dominant shoots
F3	0,50	0,10	10,50 ± 1,26abc	Green and bud uniform
F4	0,10	0,25	9,33 ± 0,70abcd	Green and bud uniform Green and some dominant shoots
F5	0,25	0,25	11,66 ± 1,74a	Dark green and uniform shoot
F6	0,50	0,25	6,10 ± 0,86cd	Blue and some dominant shoots

Source: Author

Effect of NAA on Rooting

MS medium was supplemented with NAA at different concentrations to study root shoots in vitro. The results of Table 7 show that there is no difference in rooting ability in the treatments. The addition of 0.75 mg/l NAA to the MS medium resulted in the highest number of roots with 2.64 roots/shoot and 1 mg/l NAA for the longest root length with 12.58 mm.

The experimental results achieved was similar to Rahman et al. (2005). The highest number of roots at 1.5 mg/l NAA of 3.2 roots and the longest root length of 25 mm on the culture medium with supplementation of 1 mg/l NAA.

Table 7: Effect of NAA on rooting				
Treatment	NAA (mg/l)	Number of root/sample*	Root length (mm)*	
G0	0,00	0,00 ± 0,00b	0,00 ± 0,00b	
G1	0,10	1,97 ± 0,35a	2,25 ± 0,14b	
G2	0,25	2,23 ± 0,23a	9,35 ± 0,85a	
G3	0,50	1,47 ± 0,37ab	2,85 ± 0,15b	
G4	0,75	2,64 ± 0,59a	10,11 ± 1,49a	
G5	1,00	1,83 ± 0,33a	12,58 ± 1,56a	

Source: Author

Conclusion

The culture of the shoot cluster on MS medium supplemented with 1 mg/l 2,4-D gave the highest incidence of formed callus with 76.19%. The PLB culture from callus on medium supplemented with 0.75 mg/l NAA gave PLB as much as 98 PLB/callus.

The culture of the shoot cluster on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA for direct generation of PLBs was 28.18 PLB/shoot cluster.

PLB proliferation was cultured on MS medium supplemented with 1 mg/l NAA reached 79.21 PLB/shoots.

On MS medium supplemented with 1 mg/l BA for shoot regeneration with 12.42 shoot/PLB cluster. Shoots were rapidly multiplied on medium supplemented with 0.25 mg/l BA and 0.25 mg/l NAA reached 11.66 shoots/cluster. These shoots are 3-5 cm tall and have 6-9 leaves. On the MS medium supplemented with 0.75 mg/l NAA, root formation was stimulated with a maximum number of roots of 2.64 roots and supplemented at 1 mg/l NAA for the longest root length of 12.58 mm.

From the callus or the shoot cluster, we are capable of forming PLBs. The ability of the cell culture to proliferate is faster than PLBs and the ability to regenerate PLB high or low depends on the cultivar, so it is necessary to select appropriate culture techniques for each species in breeding. The process of rapid multiplication of Vu Nu orchids in vitro has been elaborated using the technique of tissue culture: from shoot cluster → callus → PLBs → PLBs multiplication → regenerate complete plants.

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