RAPID IN VITRO PROPAGATION OF NATIVE *DENDROBIUM CHRYSOTOXUM*

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Abstract: *The rapid micropropagation of Dendrobium chrysotoxum by applying in vitro tissue culture has the following results: The VW containing 20g/l sucrose and 8g/l agar was the most suitable medium for seed germination. The appropriate medium for rapid protocorm multiplication and shoot formation was the basic MS supplemented with 20g/l sucrose and 8g/l agar. The basic MS containing 20g/l sucrose, 8g/l agar, TDZ 4μM and NAA 1μM was the most optimum medium for shoot propagation from seedlings and gave the best shoot quality. NAA had little effect on generating roots of Dendrobium chrysotoxum in vitro.*

Keywords: *Dendrobium chrysotoxum, TDZ, NAA, protocorm, shoot.*

1. Introduction

The family Orchidaceae is one of the largest families in the world with more than 35,000 species scattering throughout the earth [1], in which *Dendrobiums* include about 1400 species [5]. *Dendrobium chrysotoxum*, known as the "Gold Orchid", is the popular and favorite species due to their large, beautiful yellow flowers with sweet smell. *Dendrobiums* are normally reproduced asesxually by forming offshoots at a very slow rate in natural conditions [9]. The sexual reproduction of these orchids is difficult because their seeds are minute and have no endosperm (without nutrients). Consequently, they need symbiotic fungi in order to germinate. Therefore, tissue culture is the only method that can be used to produce large numbers of seedlings at low cost to meet the market demand and minimize the exploitation of wild orchids. There are many published studies about propagation of *Dendrobiums in vitro* such as *D. candidum* [11], *D. fimbriatum* [10], *D. nobile* [8], *D. tosaense* [6] and *D. densiflorum* [7]. However *in vitro* propagation of *Dendrobium chrysotoxum* is rarely mentioned [11]. The present study was carried out to establish an efficient *in vitro* propagation protocol for native *Dendrobium chrysotoxum* collected in Thanh Hoa province, Vietnam.

2. Materials and methods

2.1. Plant materials

7- month unripe green capsules of *Dendrobium chrysotoxum* were collected in the nature in Thanh Hoa province, Vietnam and used as explants to initiate culture.

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The surface of capsules was sterilized by totally dipping in 96% Ethanol for 5 minutes, then passing over the alcohol light. Each capsule was split open longtitudinally by using sterile scalpel to scoop out numerous minute exalbuminous seeds and diluted in 120ml of sterile distilled water. Seed suspension was spread on the surface of different medium.


Figure 1. *Dendrobium chrysotoxum and 7- month unripe green capsules*

2.2. Research methods

The experiments were carried out in the tissue culture laboratory. Samples were incubated in a culture room at 25±2°C for 10 hour photoperiod provided by white fluorescent light of 2,300 lux intensity. The pH of medium was adjusted to 5.8 prior to sterilizing at 121°C at 1atm for 20 minutes. The experiments were arranged according to the normal methods of tissue culture, following the Randomized Complete Block Design with 3 replicates/ treatment (10 Erlenmayer flasks/ replicate).

Seeds were sowed on three types of media: Vacin and Went 1949 (VW), KnudsonC 1965 (KC), Murashige-Skoog 1962 (MS) to determine the suitable medium for seed germination and protocorm generation (1st Experiment). Good protocorms inducted from the 1st Experiment (Ex) were transplanted into different media (MS, VW and KC) to study the effect of these media on the multiplying ability of protocorms (2nd Experiment). Six- week good seedlings inducted from optimum medium of the 2nd Ex were transplanted on the base medium (the optimum medium resulted from the 2nd Ex) supplemented with TDZ *(Thidiazuron or 1-phenyl-3-(1,2,3 thiadiazol-5-yl) urea)* ranging from 0-6 µM and NAA ranged from 0-3 µM to evaluate the effects of TDZ and NAA on growth and development of *Dendrobium chrysotoxum*'s shoots and seedlings. Measures for monitoring and evaluating the results were carried out according to normal methods of tissue culture. Data obtained from the experiments was analysed by using Microsoft Excel and 5.0 IRRISTAT software.

3. Results and discussion

3.1. Effect of medium on seed germination, protocorm generation and seedling formation of Dendrobium chrysotoxum

Seeds germinated on all three types of media. When sowing seeds on VW, seeds germinated earliest (1.43 weeks) at 100% success rate and the highest percentage of protocorm and seedling emergence rate (100 % and 90% respectively). On MS medium, seeds germinated 4 days later than those on VW with low percentage of seedlings (only about 30% of 50% germinated seeds).

The results of this experiment were consistent with the statement of Kauth *et al* (2008) about the effect of ammonium salt on germination of orchid seeds [4]. Ammonium salt is essential for germination of orchid seeds. However ammonium salts inhibit germination of some orchid species seeds such as *Dactylorhiza incarnata*, *Vanda tricolor*. Our experiment showed that the germination initiation and germination percentage of the seeds decreased significantly with the increase of the ammonium concentrations (Ammonium concentration on VW, MS and KC is 7.57 mM, 10.31 mM and 13.82 mM respectively).

Although 30% of the orchid seeds germinated into the dark green color sample on KC medium, the sample was not able to form protocorms and was completely dead after 10 weeks of culture (Fig 2). In conclusion, the suitable medium for seed germination of *Dendrobium chrysotoxum* is VW medium.

Table 1. *Effect of medium on seed germination, protocorm generation and seedling formation*

Trea-ment	Media	Initiation of germi-nation (Weeks)	Seed germi-nation percen-tage (%)	Develop-ment of protocorm (Weeks)	Protocorm percentage (%)	Initiation of the 1st two leaves (Weeks)	Seedling percen-tage (%)
1	MS	2,0	30,25 (+)	7,14	50,0 (++)	14,20	30,50 (++)
2	VW	1,43	100 (+++)	6,0	100 (+++)	12,13	90,25 (+++)
3	KC	3,14	30,22 (+++)	_	0	_	

Note: "_" not initiation; "+" Bad quality, light green; "++" Medium quality, green; "+++" Good quality, dark green.

Figure 2. *Effect of media on seed germination, protocorm generation and seedling formation (from the left to the right MS, KC và VW)*

3.2. Effect of culture medium on protocorm multiplication and shoot initiation

The 8-week protocorms (from sowing) obtained on VW from the 1st Ex were transferred to different media including MS, VW and KC to determine the effect of medium on protocorm multiplication. The results obtained after 4 weeks are presented in Table 2.

Table 2. *Effect of media on protocorm multiplication and shoot initiation 4 weeks after the first transplantation*

Treatment	Medium	Percentage of samples formed protocorm (%)	Protocorm multiplication	Shoot percentage (%)
1	MS	100	+++ Dark green	97,13
2	VW	100	++ Light green	50,35
3	KC	100	+ Light green	0

Note: "+" Slow multiplication; "++" Medium multiplication; "+++"Fast multiplication

The transplanted protocorms to three media were capable of producing new protocorms. On MS, protocorms had the highest multiplication and shoot emergence with 97.13%. Protocorms and shoots on MS were very good and had a dark green color. On VW, the percentage of shoot emergence was only 50.35% with the light green color. The protocorms initially created new protocorms with very slow rates and stopped completely after two weeks of culturing on KC. These protocorms were not able to form shoot buds (Fig 3).

The results showed that protocorms and shoots occurred respectively on all media. Shoot buds only initiated as the formation of new protocorms has slowed or stopped. VW was the optimal medium for seed germination but was not the suitable medium for protocorm propagation. MS was the suitable medium for rapid protocorm multiplication and budding of *Dendrobium chrysotoxum*. KC medium was not recommended for this orchid species.

Figure 3. *Effect of culture medium on protocorm multiplication and shoot initiation after 4 weeks of the first transplantation (from the left to the right MS, KC and VW)*

3.3. Effect of TDZ and NAA on the shoot initiation and the development of seedlings

Many authors concluded that the *in vitro* differentiation of plant organs is the interaction of auxin and cytokinin group. High incidence of auxin/cytokinin will stimulate rooting. On the other hand, low incidence will promote the differentiation of shoots [3]. The results of the study on the effect of TDZ and NAA on shoot initiation and development of seedlings are presented in Table 3.

Table 3. *Effect of TDZ and NAA on shoot initiation and the development of seedlings after 4-week culturing of seedlings taken from the 2nd transplantation (*)*

Treatment	Concentration (µM)		Number of leaves/ explant	Number of roots/ explant	Shoot initiation percentage (%)	Coefficient of shoot multiplication (times)
	TDZ	NAA				
CT1(Control)	0	0	2,17 **a**	2,25 **a**	0,0	1,00 **a**
CT2	2	0	5,00 **b**	0,67 **b**	80,3	2,10 **b**
CT3	4	0	5,14 **b**	0,24 **c**	88,25	2,29 **c**
CT4	6	0	4,15 **c**	0,00 **d**	75,52	1,93 **d**
CT5	0	1	4,08 **c**	0,83 **e**	17,34	1,58 **e**
CT6	2	1	5,13 **b**	0,73 **be**	83,02	2,17 **bc**
CT7	4	1	5,92 **d**	0,00 **d**	100	2,42 **c**
CT8	6	1	4,46 **e**	0,00 **d**	83,23	2,0 **bd**
CT9	0	3	3,72 **f**	1,11 **f**	33,06	1,5 **e**
CT10	2	3	4,54 **e**	0,89 **e**	86,25	2,11 **b**
CT11	4	3	5,54 **g**	0,79 **be**	88,12	2,29 **c**
CT12	6	3	4,75 **e**	0,65 **b**	79,41	2,1 **b**
LSD $_{0,05}$			0,224	0,157		0,146

Note: Means within collumn followed by different letters are significantly different (p= 0.05) based on LSD0,05. (*) 1st transplantation was carried out after 8 weeks since sowing. 2nd transplantation was carried out after 6 weeks since the 1st transplantation.

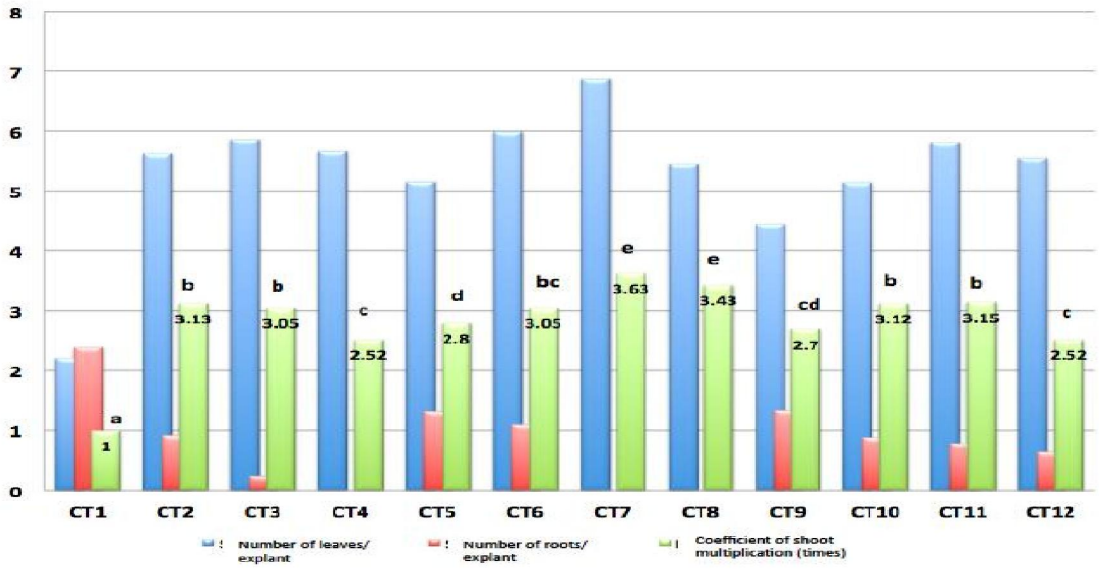

Figure 4. *Effect of TDZ and NAA on shoot initiation and the development of seedlings after 8- week culturing of seedlings taken from the 2nd transplantation*

3.3.1. Effect of TDZ and NAA on shoot initiation

Shoot multiplication coefficient of *Dendrobium chrysotoxum* after 4-week culturing was the highest at TDZ 4µM in combination with 0 or 1 or 3µM NAA. After 8 weeks of culturing, shoot multiplication was the highest at TDZ 4µM + NAA 1µM and TDZ 6µM + NAA 1µM. Thus on the TDZ 4µM + NAA 1µM medium, seedlings had stabler and better shoot multiplication than those on the other media. In the control treatment, all seedlings were not able to produce buds and had morphological variations that were significantly different from those of the other treatments. After 3 weeks of culturing, seedlings on this medium had pseudobulbs. This was the reason that these plants were less likely to produce buds than plants in other treatments (Fig.5).

3.3.2. Effect of TDZ and NAA on rooting

D. chrysotoxum plants rooted best on the medium without growth regulator, followed by those on NAA 3µM supplemented medium (Fig.5). Plants in other media had lower rooting ability. Especially high shoot multiplication plants had no roots (Fig.5).

According to Gantait *et al* (2009) [2], different auxins have different effects on root formation of orchids. This effect is probably due to the affinity of auxin receptors when involved in root formation. For *D. chrysotoxum*, Gantait claimed that IBA has much greater positive effect on root formation than other auxins including NAA. This conclusion was also drawn from the experiments of Sreckumar *et al* (2000) about *Hermidesmus indicus* [13]. Our results were consistent with previous research that NAA had little effect on the rooting ability of *D. chrysotoxum*. Therefore, the most suitable medium for rooting was the one without TDZ as well as NAA.

3.3.3. Effect of TDZ and NAA on leaf initiation

In treatments with TDZ 4µM + NAA 1µM as well as TDZ 4µM + NAA 3µM, the plants had the highest number of leaves compared to those in other treatments. This can be easily explained because these plants had the highest shoot multiplication coefficient which resulted in stronger leaf initiation (Fig 5). Plants in Control had the least number of leaves (2.17 leaves /tree in average) (Fig.5).

Figure 5. *4- week seedlings from seedlings obtained from 2nd transplantation on medium with TDZ 4µM + NAA 1µM; Control; and TDZ 0.0µM + NAA 3.0µM (from the left to the right)*

4. Conclusion

The VW contained 20g/l sucrose and 8g/l agar was the most suitable medium for seed germination *in vitro*.

The appropriate medium for rapid protocorm multiplication and shoot formation was the basic MS supplemented with 20g/l sucrose and 8g/l agar.

The basic MS contained 20g/l sucrose, 8g/l agar, TDZ 4μM and NAA 1μM was the most optimum medium for shoot propagation from seedlings and gave the best shoot quality.

NAA had little effect on generating roots of *Dendrobium chrysotoxum* in *in vitro* condition.

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