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Effects of culture medium composition on shoot multiplication and biomass production by *in vitro* culture of *Dendrobium officinale* (Kimura et Migo)

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Abstract

Dendrobium officinale (Kimura et Migo) is one of the most valuable and rare medicinal orchids exhausted in nature. The production of artificial plant biomass in this orchid is really necessary to prevent this risk. This study aimed to evaluate the effect of growth regulators (BAP, NAA, and GA_3) on *in vitro* biomass production of *Dendrobium officinale*. Experiments were arranged in a completely randomized design (CRD) with three replicates. The results showed that MS medium supplemented with sucrose 30 gL^{-1}, agar 6 gL^{-1}, BAP gL^{-1}, and NAA 0,5 gL^{-1} was the most suitable medium for *in vitro* biomass production. *In vitro* biomass of orchids cultured on the medium contained chemical components commonly found in wild *dendrobium* such as: Ca, Mg, protein, polysaccharides, and alkaloids. The success of this study is the basis for *in vitro* biomass production in order to supply materials for the medicine and food industries.

Keywords: *Dendrobium officinale;* MS; Growth regulator; *In vitro* biomass; Polysaccharide; Alkaloid

1. Introduction

Dendrobium officinate Kimura et. Migo (DO), which is used as an herbal and ornamental plant, belongs to the Orchidaceae family. It is a prized herbal folk medicine in various Asian countries [1]. The DO is widely used in traditional Chinese medicine (Editorial Board of China Pharmacopoeia Committee, 2020) [2]. In Chinese traditional medicine, the DO has preeminent functions including benefiting the stomach, increasing body fruids, and boosting immunity [3]. It is reported that the herb contained about 190 compounds of which polysaccharides and alkaloids are the main chemical compositions having potent antioxidant effects and high efficiency in the treatment of diabetes, heart-related diseases, and cancer [4,5,6,7].

The DO distributes in several countries around the world, such as China, the United States, Japan, and Australia. In Vietnam, DO exhibits a distributionin the northern midland regions of Vietnam, including HoaBinh, LaoCai, HaGiang, QuangNinh, and CaoBang provinces, and is exploited by mountainous ethnicpeople to make medicine or sold to Chinese business men[8]. In Vietnam the artificial cultivation technology of DO has been conducted in mountainous provinces. However, the existing cultivation resources of DO are mixed, which results in the low yields and uneven product quality. Aditionally the unsound evaluation system make to greatly affecting the practical and reasonable development and utilization of DO.

In recent years, *in vitro* cell biomass production technology is an effective solution to obtain active ingredients from many precious medicinal species, such as *Panax vietnamensis* [9], *Panax ginseng* [10], *Anoectochilus setaceus*[11], etc. However, *in vitro* biomass production of *D. officinale* is still limited and achieved certain results in micropropagation

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and cultivation underimproved natural forest conditions. Thus, the study aimed to investigate effects of culture mediacomposition on *in vitro* biomass production of *Dendrobium officinale.*

2. Material and method

2.1. Research on *in vitro* biomass production of *Dendrobium officinale*

- *Plant material and medium culture:* DO shoots regenerated from the protocorm like bodies after 40-day old cultures were excised into singles, with each single shoot used as an explant. Six explants were cultured in one vessel and five vessels were used for each treatment. MS medium containing sucrose 30 gL^{-1} and agar 0.65 gL^{-1} was used as a culture medium. Jars of 250 ml (UM culture bottle, as one, Japan) with plastic caps containing 30ml of medium were used for culture vessels. The pH of the medium was adjusted to 5.6-5.8 by using NaOH 1N or HCl 1N before autoclaving at 121 ^oC for 18 min.
- *Determine effect of growth regulator concentration on shoot and biomass proliferation:* Explants were cultured on MS medium[12] supplemented with 6.5 gL^{-1} agar, 10% coconut milk, 30 gL^{-1} sucrose (MS*) and different concentrations of growth regulators in each treatment. Two experiments were conducted to investigate the effect of growth regulator concentrations on shoot and *in vitro* biomass proliferation. In the first experiment, DO shoots were subcultured on MS* supplemented with 6-benzylaminopurine (BAP: 0.0, 0.5, 1.0, 1.5, and 2.0 mgL^{-1}). In the second experiment, MS* supplemented with BAP of 1,0 mgL^{-1} and different concentrations of 1-naphthaleneacetic acid (NAA: 0.0, 0.5, 1.0, 1.5, and 2.0 mgL^{-1})–was investigated for shoot multiplication and biomassproduction. The experiments were replicated five times. Each treatment consists of six explants per replicate.
- Culture conditions and statistical analysis: Cultures were incubated at 25 ± 2^0C with 16 h/8 h of day/night under a cool white led light deliveringapproximately 45 µmol m^{-2} s^{-1}. The research was carried out in the Laboratory of Cell Biotechnology, Institute of Life Sciences, Thai Nguyen University, Vietnam, from February 2021 to February 2022.
- All experiments were arranged in completely randomized designs. Data were analyzed by using one-way ANOVA for shoot proliferation and when F-test showed significant treatment results, separation of treatment means was determined by using Duncan's multiple range test (DMRT) at $p < 0.05$.

2.2. Determination of DO biomass content

In vitro biomass of the DO after harvesting from the most suitable medium (MS* + BAP of 1.0 mg L^{-1} + NAA of 0.5 mg L^{-1}) was removed PB and mini-shoot (less than 0,5 mm), washed by distilled water, and dried by freeze-drying (-40 ^oC) for 24h. The moisture of herbal samples is 10%. The 50-mesh sieved dry powder wasseparately extracted with methanol at a concentration of 60% (vol/vol) at 50 ^oC for 90 minutes by ultrasonic, and then was filtered. The filtrates were combined and concentrated in a vacuum evaporator at 45 ^oC. The dehydrated fractionation was weighted to calculate yield, then dissolved in DMSO to a ragulator concentration and the contentsoftotal minerals, magnesium, calcium, protein, polysaccharide, and alkaloid were measured.

Total minerals were determined by the gravimetric method, metals (Ca, Mn) were determined on the AAS system, and protein was analyzed by the Kjeldahl method. The polysaccharide content was determined according to the phenol-sulfuric acid method[13]. The monosaccharides from hydrolysis reaction of polysaccharides is used for reacting with phenol in an acidic medium to produce a yellow-gold color. The absorbance was measured at 490 nm. Total polysaccharide contents were calculated from a calibration curve using various concentration of glucose (% of dry materials) The alkaloid content was determined according to the method based on the reaction of alkaloid with bromocresol green, forming a yellow colored product[14].

3. Results and discussion

3.1. The effect of growth regulatiors to shoot and biomass proliferation

3.1.1. The effect of BAP concentration in MS medium on shoot multiplication and in vitro biomass of Dendrobium officinale

Supplementing BAP (6-Benzylaminopurin) into MS* media was advantageous and promoted both shoot multiplication and biomass production (Table 1). MS* medium without BAP resulted in poor multiplication rate, change in shoot length andnumber of the leaves per shoot and shoot fresh weight(1.56 folds, 1.97 mm, and 3.67 leaves, respectively). Incorporation of BAPinto MS* medium increased the shoot multiplication rate (from 2.05 to 3.11 folds), change in shoot length (from 2.17 to 3.79 mm) and change in number of the shoot leaves (from 3.89 to 5.56 leaves).

Table 1 The effect of BAP concentrations on shoot multiplication and *in vitro* biomass of DO (after 30 days of culture)

BAP concetrations (mg L^{-1})	Multiplication rate (folds)	Change in shoot length (mm)	Change in no. of leaves per shoot (mm)	Change in explant fresh weight (gram)
0.0	1.56^{c}	1.97^{d}	3.67^{e}	0.43^{c}
0.5	2.37^{b}	2.62^{b}	4.22^{c}	0.66^{b}
1.0	3.11^{a}	3.79^{a}	5.56^{a}	0.80^{a}
1.5	2.26^{b}	2.50^{bc}	4.64^{b}	0.62^{b}
2.0	2.05^{c}	2.17^{cd}	3.89^{d}	0.51^{c}
P	*<0.05*	*<0.05*	*<0.05*	*<0.05*
CV %	*4.54*	*6.84*	*2.06*	*7.16*

a-cvalues with different superscripts were significantly different at P <0.05

Like the shoot multiplication, the change in explant fresh weight in the experimental treatments was also affected by the concentration of BAP (*P<0.05; CV%=7.16*). The highest change in explant fresh weight (0.8 gram/explant) was obtained in MS* medium supplemented with BAP 1.0 mg L^{-1}. The lower change in explant fresh weight was 0.66 gram/explant and 0.62 gram/explant in MS* media supplemented BAP 0.5 mg L^{-1} and 1.5 mg L^{-1}, respectively. The change in explant fresh weight was) the lowest in MS* medium without BAP (0.43 gram/explant) and MS* medium supplemented with BAP 2.0 mg L^{-1} (0.51 gram/explants).

3.1.2. The effect of NAA concentrations in combination BAP 1.0 mg L-1 in MS medium on shoot multiplication and invitro biomass of Dendrobium officinale

Table 2 The effect of NAA concentrations in combination BAP 1.0 mg L-1 in MS medium on shoot multiplication and in vitro biomass of DO (after 30 days of culture)

Growth concentrations (mg L^{-1})		Multiplication rate (folds)	Change in mean shoot length (mm)	Change in mean number of leaves per shoot (mm)	Change in shoot fresh weight (gram)
BAP	NAA				
1.0	0.0	3.06^{b}	3.78^{b}	5.63^{b}	0.80^{c}
1.0	0.5	3.55^{a}	3.93^{a}	6.04^{a}	1.05^{a}
1.0	1.0	2.94^{b}	3.69^{b}	5.22^{c}	0.85^{b}
1.0	1.5	2.77^{c}	3.48^{c}	4.94^{d}	0.84^{c}
1.0	2.0	2.66^{c}	3.48^{c}	4.70^{e}	0.82^{c}
P		*<0.05*	*<0.05*	*<0.05*	*<0.05*
CV %		*2.48*	*1.44*	*2.23*	*2.23*

$^{a-c}$values with different superscripts were significantly different at P <0.05

Flowing to the first test for BAP suitable concentration in shoot and biomass proliferation, the combinations of different NAA concentrations and BAP 1 mgL^{-1} were supplemented in MS* medium to determine influence of the growth regulators on shoot multiplication and *in vitro* biomass of DO. The results were showed in Table 2.

The supplementing of different NAA concentrationsinto MS* media + BAP 1 mg L^{-1}affected shoot multiplication, mean shoot length, mean leave number per shoot, and shoot fresh weight in DO tissue culture (*P<0.05*). MS* medium supplemented with BAP 1 mg L^{-1} and NAA 0,5mg L^{-1}achieved the best multiplication rate (3.55 folds), change in mean shoot length (3,93 mm) , mean number of leaves per shoot (6.04 mm),and shoot fresh weight (1.05 g) compared with other treatment media. In culture mediasupplemented with NAA of 1.0 mg L^{-1}, 1.5 mg L^{-1}, and 2.0 mg L^{-1}, the results of multiplication rate, change in mean shoot length, mean no. of leaves per shoot, and shoot fresh weight were lower than in the treatment medium (incorporation of NAA) (Table 2).

Krikorian (1982) claimed that the success of *in vitro* propagation is directly influenced by the kind and concentration of the used growth regulators [15]. The commonly used growth regulators for micropropagation orchids belong to the auxin and cytokinin groups.In Dendrobium micropropagation, Asghar *et al.* (2011) claimed that BAP 2 mg L^{-1} alone produced the maximum number of shoots in *Dendrobium mobile* [16], While Suntibala and Rajkumar (2009) reported that combination of 2 mg L^{-1} BAP and 1 mg L^{-1} NAA bringed the best effective for shoot multiplication in *Dendrobium transparent* [17]. In this study, the response of the shoot multiplication and *in vitro* biomass production in DO to combinations of cytokinins (BAP 1 mg L^{-1}) and auxins (NAA 0.5 mg L^{-1}) was better compared to the effects of cytokinins (BAP) only. Thus, the differences observed in shoot growth enhancement may be the related response of the genotypes of Dendrobium used to PGR [18].

The results reveal that the addition of 0.5 mg L^{-1} NAA to MS* + BAP 1.0 mg L^{-1} has the effective shoot growth enhancement in DO biomass. However, using too high concentrations of NAA (1-2 mg/l) caused a decrease in multiplication rate, change in mean shoot length, the mean no. of leaves per shoot, and shoot fresh weight.In agreementwith using of low NAA concentration, Pant and Thapa (2012) observed that 0.5 mg L^{-1}NAA supplemented MS + BAP 1,5 ml/L to be the most effective for the shoot multiplication in *Dendrobium primulinum* Lindl[19]. However, the best effective shoot multiplication in *Dendrobium transparent* was reported in supplementing 1 mg L^{-1} NAA into ½ MS + 2 mg L^{-1} BAP. This shows that shoot and biomass proliferation depend on genotype, individual and combined growth regulators (auxin and cytokinin).

3.2. Chemical parameter of biomass of *in vitro Dendrobium officinale*

There were many reports for effects of the growth regulators on shoot multiplication in dendrobium micropropagation [20,21,22]. In this study, beside studying the effects of growth regulators on shoot multiplication and biomass, the authors also evaluated the chemical composition in DO in vitro biomass to make a judgment in the extraction of DO biomass for medicinal and food purposes.The in vitro biomass productions of *Dendrobium officinale* were analyzed to determine the main chemical components and the results are shown in Table 3.

Table 3 The concentration of total mineral, some micronutrient, and chemical components in *in vitro Dendrobium officinale*

Chemical parameter	Units	content
Total mineral	%	9.80 ± 0.06
Mg	%	0.67± 0.02
Ca	%	1.16 ± 0.02
Protein	%	8.54 ± 0.03
Polysaccharides	g/100 g dry materials	23.35 ± 0.04
Alkaloid	mg/100 g dry materials	125.80 ± 1.46

The results shown that the total minerals, Mg, and Ca have values of 9.80%, 0.67% and 1.16%, respectively. Besides, the contents of protein, polysaccharide and alkaloid in in vitro biomass of *Dendrobium officinale* have also positive results: 8.54%, 23.35 g/100g of dry materials, 125.8 mg/100g of dry materials, respectively.

The results of the initial analysis of the chemical composition of the in vitro biomass produced by tissue culture technology showed that *Dendrobium officinale*has important chemical components including calcium, magnesium, protein, polysaccharide, and alkaloid, which is similar to *Dendrobium officinale* growing in the wild. Specially, the previous works reported that polysaccharides have immunomodulatory, antioxidant, and nerve-protective effects[23,24,25,26] and alkaloids have anti-tumor and benefits for alzheimer's disease[27,28,29]. The contents of these components in the wild *Dendrobium officinale*are similar to in vitro culture. The present results proved that it is possible to use the tissue culture method to produce biomass of *Dendrobium officinale* to extract chemical components for the production of health care foods and medicines.

4. Conclusion

The combination of BAP 1 mgL^{-1} and 0.5 mgL^{-1} NAA added to the MS + Sucrose 30gL^{-1}+ Agar 6gram L^{-1} medium is the most suitable for in vitro accumulation of DO biomass. On this culture medium, the shoot multiplication rate of shoot

was 3.54 folds, and the average weight of shoots was 1.05 g. DO biomass produced in vitro on this medium contains total mineral contents, calcium, magnesium, proteins, polysaccharides, and alkaloids with values of 9.80%, 0.67%, 1.16%, 8.54%, 23.35 g/100 g of dry materials, and 125.8 mg/100 g of dry materials, respectively.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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