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Changes in shoot proliferation and chemical components of *in vitro* cultured *Dendrobium officinale* due to organic additives

T.T. Tuan[1], N.S. Thien[2], H.C. Nguyen[2], D.H. Nguyen[2], L.Q. Loan[1], T.D. Thai[1], N.T.H. Trang[1], N.H. Dung[1], D.D. Giap[1], T.X. Du[1], T.T. Huong[3] and D.H. Truong[2]*

*[1]Institute of Tropical Biology, Vietnam Academy of Science and Technology, 9/621 Ha Noi Avenue. Linh Trung ward, Thu Duc District, Ho Chi Minh City 700000, Vietnam. [2]Faculty of Applied Sciences, Ton Duc Thang University, 19 Nguyen Huu Tho, Tan Phong, District 7, Ho Chi Minh City, Vietnam. [3]Ho Chi Minh City University of Food Industry, 140 Le Trong Tan, Tay Thanh, Tan Phu, Ho Chi Minh City, Vietnam. *E-mail: truongthidieuhien@tdt.edu.vn*

Abstract

Tissue culture has become a promising technique to produce biomass and active secondary metabolites in some species of medicinal plant with in short period of time. However, most of the plant growth regulators utilized as vital agents in the culture process are harmful to human bodies. In this study, *Dendrobium officinale* Kimura *et* Migo PLBs was subcultured on Murashige and Skoog medium supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar, and various concentrations of coconut water or banana extract to investigate the effect of these organic additives on Protocorm-like bodies (PLBs) proliferation and shoot regeneration. Subsequently, the chemical composition of the material was qualitatively analyzed by standard color reactions and the methanolic extract were also tested upon DPPH radical scavenging and inhibition assay for antioxidant and antimicrobial activity assessment, respectively. The nutrient screening showed that treating 0.3 g fresh PLBs of *D. officinale* in medium containing 20 % coconut water produced the highest PLB biomass (2.21 g) whereas the similar culturing using 20 % banana extract generated only 1.98 g PLB biomass. The qualitative chemical tests recognized the presence of various phytoconstituents such as glycosides, flavonoids, steroids, triterpenes, phenolic compounds and saponins. The DPPH assay revealed the antioxidant activity of the methanol extract in a dose dependent manner with the IC_{50} value of 0.84 mg mL^{-1}. In addition, this methanol extract showed a stronger inhibitory activity against Gram negative bacteria than Gram positive bacteria. These finding suggested that coconut water could be a potential alternative nutrient to common unhealthy regulators in the production of the biomass of interest.

Key words: Banana extract, coconut water, *Dendrobium officinale,* DPPH assay, phytoconstituents.

Introduction

Dendrobium officinale Kimura *et* Migo, also known as "Thạch hộc tía" or "Thiết bì thạc hộc" in Vietnamese, belongs to Orchidaceae family. The species grows mostly in mountain areas at the altitude ranging from 1000 to 3400 m where the ambient condition (70 % humidity, 12-18 ^{o}C average temperature, precipitation of 900-1500 mm) is of tropical or subtropical climate (Do, 2004). In Vietnam, it is abundantly found in the northern midland regions (Do, 2004). The plant contains many precious biological substances including polysaccharide (23 %), alkaloids (0.02 – 0.04 %), amino acid (135 mg g^{-1}) and minerals such as iron (292 mg g^{-1}), zinc (12 mg g^{-1}), manganese (52 mg g^{-1}), copper (3.6 mg g^{-1}).

D. officinale Kimura *et* Migo is used as an additional drug for treatment of malignant cancers to improve health of patients as well as to reduce the side-effects of radio- and chemotherapy. According to Pharmacopoeia Commission (Pharmacopoeia Commission of PRC, 2010), *D. officinale* is classified as a medicinal plant that increases the cancer resistance and patient longevity. Moreover, it enhances insulin and reduces glucose, cholesterol, and triglyceride contents in blood (Xie *et al.*, 2016). Lin *et al.* (2011) reported that *D. officinale* polysaccharides could alleviate the abnormality of aquaporin 5 (AQP-5), pro-inflammatory cytokines and inhibit apoptosis in the Sjogren's syndrome mice. The results showed that *D. officinale* polysaccharides (DP) could suppress the progressive lymphocytic infiltration and apoptosis and could balance the chaos of pro-inflammatory cytokines in the submandibular gland. Moreover, DP could also maintain the functional importance of AQP-5 in saliva secretion. The protection of AQP-5 by DP was further supported by an *in vitro* study on salivary gland tumor cell line A-253. This study hence signified the possibility of DP to be a promising plant extract for the therapy of Sjögren's sydrome (SS). Yan *et al.* (2015) assembled 1.35 Gb genome sequences of *D. officinale* and analyzed the biosynthesis pathways of medicinal components of the species and found extensive duplication of *Sps* and *SuSy* genes, which were related to polysaccharide generation. The understanding of *D. officinale* genome assembly enables the access to deciphering larger complex genomes as well as the study of medicinal components formation and the potential genetic breeding of *Dendrobium*.

In Vietnam, several studies have been carried out on *Dendrobium* orchid species. With *Dendrobium nobile* Lindl, the optimal culture media were Knudson C (KC) for protocorm growth and MS supplemented with 100 mL L^{-1} coconut water, 10 g L^{-1} sucrose, and 6 g L^{-1} agar for PLBs or shoot clusters multiplication (Vu and Nguyen, 2013). In the *in vitro* study of the propagation of *D. officinale,* Nguyen *et al.* (2014) successfully identified the culture conditions that were suitable for the plant to grow *in vitro* from seeds. Specifically, Vacin and Went (VW) medium supplemented

with 10 g L^{-1} sucrose, 6 g L^{-1} agar, 100 mL L^{-1} coconut water was determined as the optimal nutrient environment for the seeding stage while MS medium supplemented with 100 mL L^{-1} coconut water, 20 g L^{-1} sucrose, 6 g L^{-1} agar, and 60 g L^{-1} ripe banana was appropriate for shoot clusters micropropagation stage.

The rapid development of tissue culture industry has allowed the production of biomass in some species of medicinal plant to rapidly generate bioactive compounds. The culture processes typically require the employment of regulators which usually leave irremovable traces in the expected medicinal products. Such regulators may cause detrimental effects to human when being consumed. Therefore, questing for safer alternative nutrients is of great importance. In this study, undefined additives such as coconut water and banana extract were used as harmless regulators in the culture of *D. officinale* PLBs. Additionally, the chemical composition and bioactivities including antioxidant and antimicrobial activities of obtained biomass were also evaluated.

Materials and methods

Plant material and medium: *D. officinale* PLBs were obtained from Institute of Tropical Biology (Ho Chi Minh city, Vietnam) and used for cultivation. The basal MS medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar and organic nutrients with different concentrations was used in all experiments.

Fruit of banana (*Musa acuminata,* "Grand Nain") were applied to produce banana extract. Banana extract was obtained by cutting banana into thin circular slices and blending these with distilled water in the ratio of 4:1 (w/v).

The effect of organic nutrients on Protocorm like body (PLB) growth: To investigate the effect of coconut water and banana extract on PLB proliferation and shoot regeneration, PLBs *D. officinale* Kimura *et* Migo (0.3 g) were subcultured on MS medium supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar, and coconut water or banana extract with different concentrations (0, 10, 20, 30, 40, and 50 % v/v). After 8 weeks of cultivation, the fresh weight and the number of shoots were recorded to determine the optimal medium for the proliferation and shoots regeneration of PLB. All measurements were performed in triplicate.

Qualitative chemical components of PLB explants: The fresh PLBs of *D. officinale* were dried in the open air and ground into powder by using a clean electrical blender. The powder (1.0 g) was then extracted with 10 mL absolute methanol by maceration for 5 hrs. The extract was filtered and analyzed for the chemical components by using standard qualitative chemical procedures described by Culei (1982) and Harbone (1984). The color reactions were used to test the presence of common metabolite classes such as glycosides, flavonoids, steroids, triterpenes, phenols and saponins. All measurements were performed in triplicate.

Antioxidant and antimicrobial activity

Solution preparation: PLB dried powder (5.0 g) was extracted three times with 30 mL absolute methanol (Sigma) each time for 24 hrs. The extract was filtered and the filtrate was then evaporated in a rotary evaporator (Heidolph Laborota 4000, Germany) at 40 °C to obtain 1 mL of crude extract. This mixture was used for bioactivity analysis.

Antioxidant activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity assay was used to determine the total antioxidant activity of the PLB crude extract. The extract was initially diluted to concentrations varying from 0.0625 mg mL^{-1} to 1 mg mL^{-1} and assays were performed in a 96-well microplate. The reaction mixture in each well of the 96-well microplate consisted of 100 µL of DPPH solution (300 µM) and 100 µL of the sample. Ethanol and ascorbic acid (2.0 mg mL^{-1}) were used as negative and positive control, respectively. The plate was kept for 30 minutes at 37 °C and the absorbance was immediately recorded at 517 nm on a Bio-Rad Benchmark Plus Microplate Spectrophotometer (USA). The scavenging activity percentage was determined according to Mensor *et al.* (2001).

The radical scavenging activity of the extract was expressed in form of the IC_{50} values defined as the concentration of the sample required to decrease the absorbance at 517 nm by 50 %. All measurements were performed in triplicate.

Antimicrobial activity: The bacteria strains selected for this assay were *Salmonella typhimurium* NRRL B-4420, *Bacillus subtilis* NRRL B-354, *Staphylococcus aureus* NRRL B-313, *Pseudomonas aeruginosa* NRRL B-14781, and *Escherichia coli* NRRL B-409, all of which were maintained at 4 °C in Luria-Bertani (LB) agar slant.

The agar well diffusion method was used for the determination of the antibacterial activity of the methanol extract. The bacterial stocks were incubated for 24 hours at 37 °C in nutrient broth medium and were diluted to the concentration of 10^8 cfu.mL^{-1}. 100 µL suspension of tested bacteria was then spread uniformly on a sterile Mueller Hinton (MH) agar Petri dish. The crude methanol extract was diluted using 5 % DMSO solution to obtain the concentration of 100 mg mL^{-1} and 500 mg mL^{-1}. 100 µL of each extract solution was added to the corresponding well (6 mm diameter holes cut in the agar gel). 100 µL of 5 % DMSO solution was added to the negative control well. The petri dishes were incubated for 24 hours at 37 °C under aerobic conditions. After incubation, confluent bacterial growth was observed by measuring the diameter of the inhibition zones. Each test was performed three times.

Statistical analysis: The data were reported as mean values and statistical analysis was executed using Statgraphics centurion XV software for comparisons. Any *P*-value lower than 0.05 was considered as statistical significance.

Results and discussion

Effect of organic nutrients on PLB proliferation and shoot regeneration: It was reported that coconut water has influence on embryo proliferation of *Datura stramonium* (Huang *et al.,* 2010), callus induction and proliferation of some citrus species (Burnet and Ibrahim, 1973), and the growth of *Deosera rotundifolia* (Donia, 2009). Especially, the effects of coconut water on the growth and germination of some *Dendrobium* sp. were also observed (Niimoto and Sagawa, 1961). According to the nutritional composition analysis by World Health Organization (WHO), coconut water contains proteins, carbohydrates, calcium, iron, and some vitamins such as thiamine, riboflavin, niacin, ascorbic acid as well as amino acids and other organic compounds. Moreover, it also consists of cytokinin plant growth hormones (Letham, 1974). Therefore, coconut water has been

used in culture media as natural source of plant growth regulators. On the other hand, it helps create safe medicinal sources for human health (Reddy *et al.,* 2014).

Along with coconut water, banana extract has also been received increasing attention as a natural culture medium supplement for promoting the growth of *Dendrobium* sp. and some other species. Banana extract contains potassium, phosphorus, magnesium, iron, calcium, starch, carbohydrates, vitamin A, C, B, and cytokinins (Van Staden, 1975). Therefore, in this research, the effect of banana extract and coconut water on PLB proliferation and shoot induction of *D. officinale* Kimura et Migo was tested at different concentrations. After 8 weeks of culture, the results were recorded and statisticaly analyzed (Table 1).

As shown in Table 1, the biomass varied among treatments. The highest biomass (2.21 g, fresh weight) was obtained by culturing PLBs on MS medium supplemented with 20 % coconut water, which expressed 7.37-fold increase in comparison to the initial weight. PLBs cultured on medium supplemented with 20 % banana extract demonstrated a low biomass with the fresh weight of 1.98 g (6.6-fold increase) but higher than the control (0.93 g).

Coconut water is the supplier of amino acids, organic acids and cytokinins, which facilitate shoot development. However, when coconut water concentration exceeded 20 %, less fresh weight was recorded. On the other hand, the number of shoots increased proportional to coconut water concentration. In medium supplemented with 20-50 % coconut water, the average number of shoot were high. In terms of morphology, PLBs, which were cultured on coconut water medium, were green and friable, and hence it would be easy for generated shoots to grow strongly. This result was in accordance with the study of Doina *et al.* (2009) in which MS medium supplemented with 20 % coconut water was the most efficient nutrient for the proliferation process of *Droseraro tundifolia.* In addition, Nambiar *et al.* (2012) showed that coconut water was found to be superior to a variety of other organic additives for the proliferation of *Dendrobium Alya Pink* PLBs.

The influence of banana extract on the growth of *Dendrobium* was observed in several previously published studies. In 1993, Shobhana demonstrated that banana powder supported the growth of *Dendrobium* sp. In the study of using organic compounds in *in vitro* propagation of *Dendrobium*, Aktar *et al.* (2008) showed that banana powder significantly affected the growth of PLBs. Results from Table 1 also showed modulating effect of banana extract on the growth of *Dendrobium officinale* Kimura *et* Migo PLBs though to lesser extent. Unlike treatments with coconut water, fresh weight and number of shoots decreased when culturing in media supplemented with high concentration (30-40 %) of banana extract. Morphologically, PLBs achieved from treatments with 20 % banana extract were tough, non-friable and dark green. This would make the shoot induction treated with banana extract harder than that supplemented with coconut water.

Table 1. The influence of organic nutrients such as coconut water and banana extract on PLB proliferation and shoot regeneration

Treatments	Concentration (%)	Initial fresh weight (g)	PLB fresh weight (g)	Number of shoots
Control	-		0.93c[x]	3.78c
Banana extract	10		1.01c	8.11c
	20		1.98ab	10.22c
	30		1.66abc	9.44c
	40		1.08c	4.78c
	50	0.3	0.94c	2.44c
Coconut water	10		1.62abc	29.89b
	20		2.21a	28.44b
	30		1.48abc	41.11a
	40		1.24bc	30.78b
	50		1.16c	26.67b

[x] Means in the same column that are followed by different letters are significantly different ($P \leq 0.05$) using Duncan's Multiple Range Test.

Quantitative chemical constituents of PLB explants: Specific and color change reactions were used to screen phytochemical components of the methanol extract of the PLB dried powder. The qualitative chemical tests showed the presence of various phytoconstituents such as glycosides, flavonoids, steroids, triterpenes, phenolic compounds and saponins (Table 2). In addition to these compounds, the methanol extract was also inferred to abundantly contain flavonoids and phenolic compounds. These components detected have been known to possess medicinal capacity. Therefore, the presence of these compounds suggests that *D. officinale* could be potentially employed in pharmaceuticals.

Antimicrobial activity: The *in vitro* antibacterial activities of methanol extract of PLB *D. officinale* indicated that the methanol extract of PLB *D. officinale* at concentration of 100 mg mL^{-1} could inhibit the growth of *Salmonela typhimuricum* and at the concentration of 500 mg mL^{-1} could inhibit the growth of all four tested microorganism strains (*Salmonela typhiumricum, S. aureus, P. aeruginosa,* and *B. subtilis*) with the measured inhibitory zones being 30.0 ± 0.8 mm, 12.5 ± 1.4 mm, 20.5 ± 0.5 mm, and 15.5 ± 0.5 mm, respectively (Table 3). It is worth mentioning that the extracts of *D. officinale* PLBs evaluated against gram negative bacteria (*S. typhimuricum* and *P. aeruginosa*) gave larger inhibitory diameters than those tested against gram positive bacteria (*B. subtilis* and *S. aureus*). These results suggested that PLB *D. officinale* could contain antibacterial substances and could be used to control human bacterial pathogens which cause various diseases (Fig. 2). Some bioactive components such as phenolics, terpenoids, and saponins were implicated which could also be the reason for the activity of plants (Wink *et al.*, 2015). Therefore, the results from this work may encourage intensive researches on antimicrobial activities of *D. officinale*.

Table 2. Results of qualitative test for chemical components

Compound	Sample
Glycoside	+[*]
Flavonoid	++
Steroid	+
Triterpene	+
Phenolic	++
Saponin	+

[*]Legend: +, Rare; ++, Abundant

Table 3. Diameter inhibitory zone (mm) of sample against bacteria strain

Test strain	500 mg mL^{-1}	100 mg mL^{-1}
Salmonella typhimuricum	30.0	18.5
Staphylococcus aureus	12.5	-
Pseudomonas aeruginosa	20.5	-
Bacillus subtilis	13.5	-

All values are the mean (n=3).

Antioxidant activity: To investigate the antioxidant activity of PLB *D. officinale*, DPPH assay was performed. The scavenging ability of methanol extract on DPPH free radical was examined in the concentration range of 0.0625 to 1.000 mg mL^{-1}. The results showed that the methanol extract exhibited the antioxidant activity in a dose dependent manner (Fig. 1) with the IC_{50} value being 0.84 mg mL^{-1}.

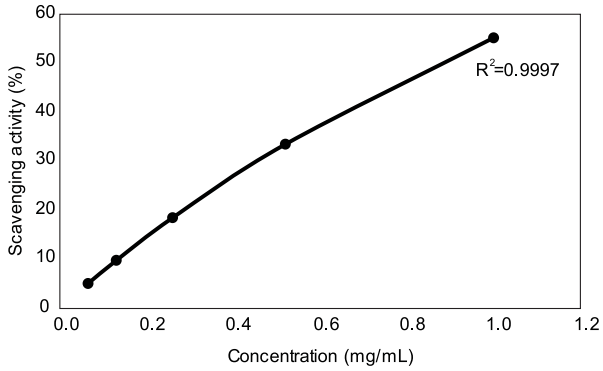

Fig. 1. DPPH radical scavenging activity of methanol extract

The antioxidant activities of plants have been reported to express free radical scavenging activity (Mukherjee *et al.*, 2010). The results from DHHP assay indicated that methanol extract of PLB *D. officinale* contained compounds capable of donating radical hydrogens to trap the oxidant's radicals, which accounts for the antioxidant activity (Olayinka and Anthonay, 2010). In addition, the presence of flavonoids and phenolic compounds implied the prospective antioxidant ability. The study on the mechanism of the observed antioxidant activity is in progress.

In conclusion, coconut water was shown to be a potential alternative nutrient to common unhealthy regulators in the production of the biomass of interest. Particularly, culturing *D. officinale* Kimura *et* Migo PLBs in the presence of 20 % coconut water produced the highest biomass with 7.37-fold increase compared to the initial weight and 7.5-fold greater number of shoots compared to the control medium. In addition, the composition of the obtained biomass was determined to contain a variety of metabolites including glycosides, flavonoids, steroids, triterpenes, phenolic, and saponins. Although a thorough study in identifying individual chemical components of the material was not performed, the DPPH and antimicrobial assay were carried out showing prospective antioxidant and antimicrobial activities of the mixture.

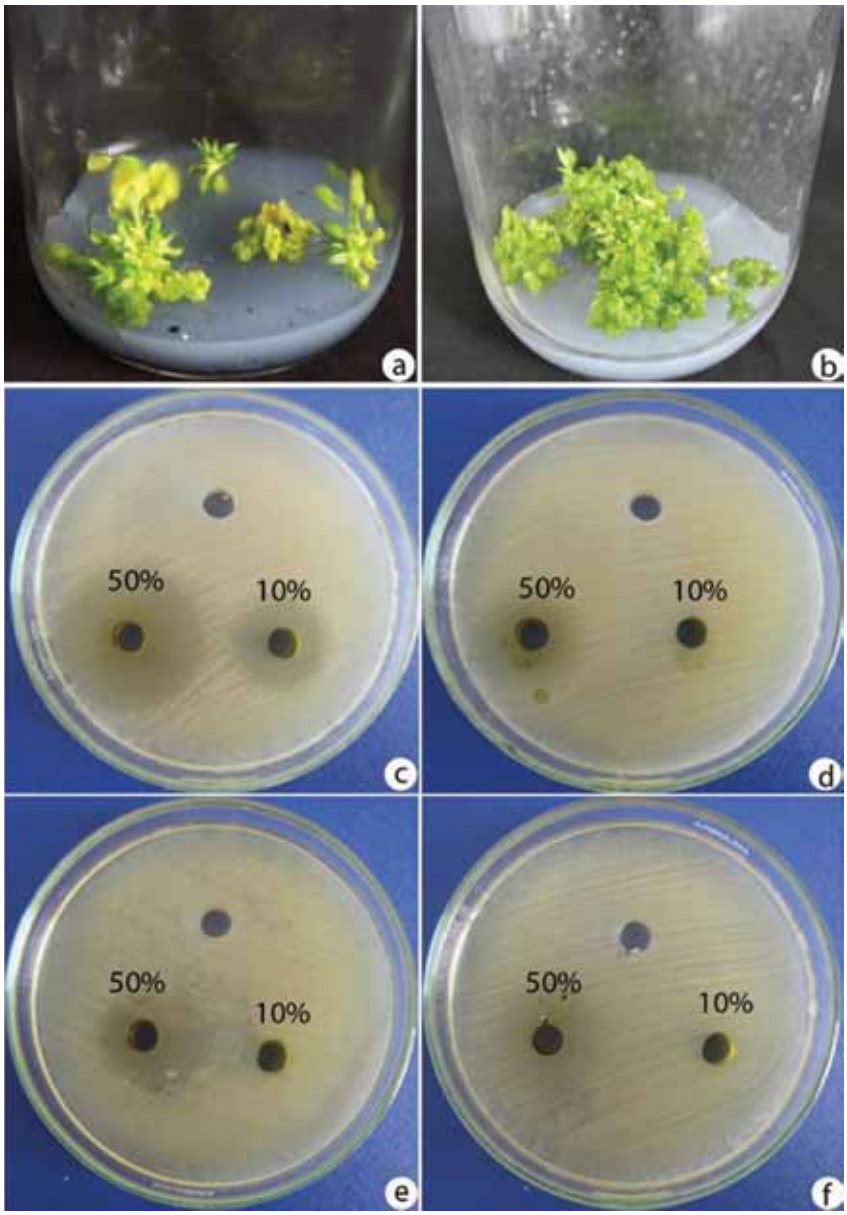

Fig. 2. PLBs were cultured on MS supplemented with 20 % banana extract (a); 20 % coconut water (b); Antibacterial activity of methanol extract of PLBs: (c) *Salmonella typhimuricum*; (d) *Staphylococcus aureus*; (e) *Pseudomonas aeruginosa;* (f) *Bacillus subtilis.*

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