Effects of organic nutrients on shoot proliferation and chemical component of in vitro cultured *Dendrobium officinale* Kimura et Migo

5

6Description of the subject. The development of tissue culture technology has enabled the creation of biomass in some species of medicinal plant to accumulate active secondary metabolisms in short periods of time. Plant growth regulators, most of which are harmful to human bodies, have been extensively used as vital agents in the culture processes. However, the abuse of those substances exposes the difficulty in separating their residues from the expected products. In this study, we have chosen to investigate undefined supplements such as coconut water and banana extract as safe alternative regulators in the generation of medicinal products.

7Objectives. Studying the effect of organic additives such as coconut water and banana extract on Protocorm-like bodies (PLBs) proliferation and shoots regeneration. Besides, chemical composition and bioactivities of *Dendrobium officinale* PLBs were also evaluated.

8Methods. *Dendrobium officinale* Kimura et Migo PLBs were subcultured on Murashige and Skoog (MS) medium supplemented with 30 g.L⁻¹ sucrose, 8 g.L⁻¹ agar, and different concentrations (0, 10, 20, 30, 40, and 50 % v/v) of coconut water or banana extract with different concentrations (0, 10, 20, 30, 40, and 50 % v/v). After 8 weeks of culture, data such as fresh weight and number of shoots were recorded. After statistical analysis, the most suitable medium was then chosen for the rapid production of PLBs biomass. Subsequently, the chemical composition of the material was qualitatively analyzed by standard color reactions and the methanol extract was also-tested upon DPPH radical scavenging and inhibition assay for antioxidant and antimicrobial activity assessment respectively.

9Results. The nutrient screening showed that treating 0.3 g fresh PLBs of *Dendrobium officinale* Kimura et Migo in medium containing 20 % coconut water produced the highest PLBs biomass (2.21 g) whereas the similar culturing conditions using 20 % banana extract generated only 1.98 g of biomass. The in vitro culture is a source of biomass. Quantitatively, it is a "production" and not a "creation".
the material. 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 0.8425 mg.mL$^{-1}$. The antimicrobial tests demonstrated stronger inhibitory effects of the methanol extract against Gram negative bacteria than Gram positive bacteria.

**Conclusions.** Coconut water was proven to be a potential alternative nutrient to common unhealthy regulators in the production of the biomass of interest. Particularly, culturing *Dendrobium officinale* Kimura et Migo PLBs in the presence of 20% coconut water produced the biomass with 7.37-fold increase compared to the initial weight and 7.5-fold greater number of shoots compared to the control growing medium. In addition, the composition of the obtained biomass was determined to contain a variety of metabolites including glycosides, flavonoids, steroids, triterpenes, phenolic compounds and saponins. Although a thorough study in identifying individual chemical components of the material was not performed, the DPPH assay was carried out showing a prospective antioxidant activity of the mixture. Furthermore, the methanol extract indicated a potential antimicrobial activity after evaluation against various bacterial strains, and the results indicated potential antimicrobial activity.

**Keywords.** banana extract, coconut water, *Dendrobium officinale*, DPPH assay, phytoconstituents

**1. INTRODUCTION**

*Dendrobium officinale* Kimura et Migo, also known as “Thạch họa tía” or “Thiết bị thác họa” in Vietnamese, belongs to *Orchidaceae* family. This species mostly grows in mountain areas at altitudes ranging from 1000 to 3400 m where the ambient conditions (70% humidity, 12 – 18 °C average temperature, precipitation of 900 – 1500 mm) are of tropical or subtropical climates. In Vietnam, these are abundantly found in the northern midland regions (Do Tat Loi, 2004). In Vietnam, “Thạch họa tía” contains many precious biological substances, amongst which are polysaccharide (23%), alkaloids (0.02 – 0.04%), amino acids (135 mg.g$^{-1}$) and minerals (iron (292 mg.g$^{-1}$), zinc (12 mg.g$^{-1}$), manganese (52 mg.g$^{-1}$), copper (3.6 mg.g$^{-1}$)).
Medicinally, *Dendrobium officinale* Kimura et Migo is used as an additional drug for the treatment of malignant cancers. It improves the health of patients as well as reduces the side-effects of radio- and chemotherapy medical treatments. According to Pharmacopoeia Commission (Pharmacopoeia Commission of PRC, 2010), “Thạch höchstia” is classified as a medicinal plant that increases the cancer resistance and patients life longevity. Moreover, it enhances insulin and reduces glucose, cholesterol and triglyceride contents in blood (Xie et al., 2016). Lin et al. (2011) showed that *Dendrobium officinale* polysaccharides of *Dendrobium officinale* could alleviate the abnormality of aquaporin 5 (AQP-5), the pro-inflammatory cytokines and inhibits apoptosis in the Sjögren’s syndrome of 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 syndrome (SS). In 2015, Yan et al. (2015) assembled 1.35 Gb genome sequences of *D. officinal*. They 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. officinal* 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 in vitro 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⁻¹ coconut water, 10 g.L⁻¹ sucrose, and 6 g.L⁻¹ agar for PLBs or shoot clusters multiplication (Vu Ngoc Lan and Nguyen Thi Ly Anh, 2013). About in the in vitro study of the propagation of *D. officinale* Kimura et Migo (2014), Nguyen Thi Son (2014) successfully identified the suitable culture conditions that were suitable for growing plants from seeds. Specifically, Vacin and Went (VW) medium supplemented with 10 g.L⁻¹ sucrose, 6 g.L⁻¹ agar and 100 mL⁻¹ coconut water was determined as the optimal nutrient environment for the seeding stage while MS medium supplemented
with 100 mL⁻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 proliferation stage.

The rapid development of tissue culture industry has allowed the production creation of biomass in some plant species of medicinal plant to rapidly generate medicinally active metabolites.

The culture processes typically require growth the employment of regulators which usually leave irremovable traces into the expected medicinal products. Such regulators may cause detrimental effects to human when consumed (like??, + reference please). Therefore, seeking for safer alternative nutrients is of great importance. In this study, we chose to utilize undefined additives such as coconut water and banana extract as harmless plant growth regulators in the culture of to grow Dendrobium officinale Kimura et Migo PLBs in vitro. Additionally, the chemical composition and bioactivities including antioxidant and antimicrobial activities of obtained biomass were had to be also evaluated.

2. MATERIAL AND METHODS

2.1. Plant material

Dendrobium officinale Kimura et Migo PLBs were cultured at Plant Cell Technology Department, Institute of Tropical Biology (9/621 Ha Noi avenue, Linh Trung ward, Thu Duc district, Ho Chi Minh city, Vietnam).

2.2. Culture medium

In this study, basal MS medium (Murashige and Skoog, 1962) supplemented with 30 g.L⁻¹ sucrose, 8 g.L⁻¹ agar and organic nutrients at with different concentrations was used in all experiments.

Banana extract used as growth regulator was obtained from fruit of banana fruits of Musa acuminata, “Grand Nain” (could you mention a maturation stage of the fruit?? as reference) (Musa acuminata, “Grand Nain”) were applied to produce banana extract. Banana extract were obtained by cutting banana into thin circular slices and followed by blending these them in the presence of with distilled water in the ratio of 4:1 (w/v).

2.3. Experimental design

Increasing biomass Protocorm like body (PLB) stage
The role of organic nutrients such as 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 containing 30 g L⁻¹ sucrose, 8 g L⁻¹ agar, and supplemented with coconut water and or banana extracts at different concentrations (0, 10, 20, 30, 40, and 50 % v/v). After 8 weeks of culture, the following data were collected: fresh weight and number of shoots. After statistical analysis, the suitable medium was chosen to rapidly proliferate PLBs biomass. This material was used for subsequent experiments.

2.4. Qualitative chemical components of PLB explants

Plant extract preparation

The fresh PLBs of *D. officinale* were dried in the open air and ground into powder form by use of a clean electrical blender. The powders were kept at room temperature until further experiments. Dried PLBs powder (1.0 g) were extracted with 10 ml absolute methanol by maceration for 5 hrs. Extract was filtered and the filtrate was used for phytochemical screening.

Qualitative chemical components

The methanol extract of PLBs powder was analyzed for their chemical components by using standard qualitative chemical procedures (described by Culei, 1982; Harbone, 1984). The color reactions were used to test the presence of common metabolite classes such as glycosides, flavonoids, steroids, triterpenes, phenols and saponins.

2.5. Antioxidant and antimicrobial activity

Solution preparation

PLBs dried powder (5 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.
2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity assay was used to determine the total antioxidant activity of the PLB(s) 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 mg.mL\(^{-1}\)) were used as negative and positive controls, 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).

\[
\text{AA} \% = 100 - \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \right)
\]

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 %.

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-15114781 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\(^6\) 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 two times.

2.6. 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.

3. RESULTS AND DISCUSSION

3.1. PLBs (Protocorm like bodies) biomass growth stage: the effect of organic nutrients on PLBs proliferation and shoot regeneration

In previous researches, demonstrated that coconut water was demonstrated to have influences on the embryo proliferation of Datura stramonium (Huang et al., 2010), the callus induction and proliferation of some Citrus species (Burnet and Ibrahim, 1973), and on 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 contains cytokinins that are known from a long time (Letham, 1974). Therefore, coconut water has been used as a natural source of plant growth regulators. On the other hand, it helps produce/stimulate natural production.

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

The data from Table 1 showed differences between treatments. Fresh weight of PLBs of treatment supplemented with 20% coconut water was the highest one (2.21 g), which expressed 7.37-fold increase compared to the initial weight while PLBs which were cultured on medium supplemented with 20% banana extract had 1.98 g of fresh weight (6.6-fold increase). The lowest fresh weight of PLBs was of the control (0.93 g).

As mentioned earlier, coconut water is a rich supplier of amino acids, organic acids and cytokinins, which facilitate shoot development. However, when coconut water concentration exceeded 20%, fewer fresh weights were collected. On the other hand, the number of shoots increased proportionally to coconut water concentration. In medium supplemented with 20–50% coconut water, the average numbers of shoots (per Flask/initial PLBs explant) 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) where in which MS medium supplemented with 20% coconut water was the most efficient nutrient concentration for the proliferation process of *Drosera tundifolia*. In addition, Nambiar et al. (2012) showed that coconut water was found to be superior to a variety of other organic additives (like ??? Could you add them for comparison purpose) for the proliferation of *Dendrobium Alya Pink* PLBs.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration (%)</th>
<th>Initial fresh weight (g)</th>
<th>PLBs fresh weight (g)</th>
<th>Number of shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.3</td>
<td>0.93c</td>
<td>3.78c</td>
</tr>
<tr>
<td>Banana</td>
<td>10</td>
<td>1.01c</td>
<td>8.11c</td>
<td></td>
</tr>
</tbody>
</table>

Adaptation of the title to the data is required

Without any growth regulator!!!


<table>
<thead>
<tr>
<th></th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
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<tbody>
<tr>
<td>Extract</td>
<td>1.98ab</td>
<td>1.66abc</td>
<td>1.08c</td>
<td>0.94c</td>
<td>1.62abc</td>
<td>2.21a</td>
<td>1.48abc</td>
<td>1.24bc</td>
<td>1.16c</td>
</tr>
<tr>
<td>Coconut</td>
<td>10.22c</td>
<td>9.44c</td>
<td>4.78c</td>
<td>2.44c</td>
<td>29.89b</td>
<td>28.44b</td>
<td>41.11a</td>
<td>30.78b</td>
<td>26.67b</td>
</tr>
<tr>
<td>Water</td>
<td>3.24bc</td>
<td>4.78c</td>
<td>2.44c</td>
<td>1.16c</td>
<td>9.44c</td>
<td>10.22c</td>
<td>4.78c</td>
<td>2.44c</td>
<td>1.16c</td>
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<tr>
<td>NOVA</td>
<td>*</td>
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</tr>
<tr>
<td>CV%</td>
<td>59.01</td>
<td>92.99</td>
<td>59.01</td>
<td>92.99</td>
<td>59.01</td>
<td>92.99</td>
<td>59.01</td>
<td>92.99</td>
<td>59.01</td>
</tr>
</tbody>
</table>

Means in the same column that are followed by different letters are significantly different (p ≤ 0.05) using Duncan’s Multiple Range Test. *: * significantly different with P ≤ 0.05

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 18PLBs. 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 easier than that supplemented with coconut water.

### 3.2. 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. In addition to these compounds, the methanol extract was also inferred to contain flavonoids and phenolic compounds. Preliminary phytochemical screening for organic elements results were shown in table 2. These tests were performed to detect the major chemical groups occurring in the extracts. The components detected are well known to possess medicinal capabilities. Therefore, the presence of these compounds suggests that *D. officinale* could be potentially employed in pharmaceuticals.
Table 2. Results of qualitative test for chemical components

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoside</td>
<td>+*</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>++</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
</tr>
<tr>
<td>Triterpene</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic</td>
<td>++</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
</tr>
</tbody>
</table>

Legend: +, Rare; ++, Abundant

237 The in vitro antibacterial activities of methanol extract of PLB D. officinale which were shown in table 3 indicated that the methanol extract of PLB D. officinale at concentration of 100 mg.mL⁻¹ could inhibit the growth of *Salmonella typhimuricum* and at the concentration of 500 mg.mL⁻¹ could inhibit the growth of all four tested microorganism strains (*Salmonella typhimuricum*, *S. aureus*, *P. aeruginosa*, *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. It is worth mentioning that the extracts of *Dendrobium 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 different human bacterial pathogens which cause various diseases. Some bioactive components such as phenolics, terpenoids and saponins were implicated in plant 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 3. Diameter inhibitory zone (mm) of sample against bacteria strain

<table>
<thead>
<tr>
<th>Test strain</th>
<th>500 mg.mL⁻¹</th>
<th>100 mg.mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhimuricum</em></td>
<td>30.0</td>
<td>18.5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>20.5</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>13.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Antioxidant activity

To investigate the antioxidant activity of PLB₅ *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 (Figure 1) with the IC₅₀ value being 0.8425 mg.mL⁻¹.

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 PLB₅ *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.

![Figure 1. DPPH radical scavenging activity of methanol extract of PLB *D. officinale*](image)
Figure 2. PLBs were cultured on MS supplemented with 20% banana extract (a); 20% coconut water (b);

Antibacterial activity of PLBs methanol extract of PLBs: Salmonella typhimurium (c); Staphylococcus aureus (d); Pseudomonas aeruginosa (e); Bacillus subtilis (f).

4. CONCLUSION

Coconut water was shown to be a potential alternative nutrient to common unhealthy growth regulators in the production of healthy biomass of interest. Particularly, culturing Dendrobium officinale Kimura et Migo PLBs in the presence of 20% coconut water produced the biomass with 12-fold increase compared to the initial weight and 7.5-fold greater number of shoots compared to the control that reached 3 times the initial weight!!!
the control growing medium. In addition, the composition of the obtained biomass was determined to contain a variety of metabolites including glycosides, flavonoids, steroids, triterpenes, phenolic compounds 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.

Bibliography


