In vitro culture of *Dendrobium lineale* Rolfe orchid for plant breeding and propagation

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Abstract. *D.lineale* is included in the CITES appendix II due to *D. lineale* has great potential as a cut flower and the parent of hybrid orchids. *In vitro* culture can be used for mass propagation of orchids for commercial breeding as well as conservation. Basic culture medium with the addition of organic substances can increase the growth of plantlets. The objective of this research was to determine the best condition for *in vitro* culture of *D. lineale* orchids for producing a mass number of plantlets. Methods used include planting orchid seedlings in various variations of culture media, plantlet subcultures, observation of the anatomy of leaves and roots, as well as the measurement of chlorophyll content. This research uses ‘pisang raja’ as organic material in the culture medium. The highest chlorophyll content in the leaves was 1.58 mg/g, while the highest chlorophyll content in the roots was 0.34 mg/g. Both were found in medium NP+100 g/L bananas in light conditions (1317 lux). The anatomical structure of the leaves shows that the mesophyll is thicker in plants placed under lighting 1317 lux because it shows good effectiveness in photosynthesis. The anatomy of the orchid roots showed differences in diameter in each treatment because the addition of bananas to the medium stimulated rapid and abundant root formation, but the size was smaller. Based on these results, it can be concluded that the addition of 100 gr/L bananas as organic substances in *in vitro* medium culture increases the growth rate of *D. lineale* orchids, which will be beneficial for conservation efforts.

1. Introduction

*D.lineale* is an orchid that must be considered for its existence in nature. Based on CITES [1], this orchid is included in appendix II, so that its trade needs to be considered to prevent overexploitation. The *D. lineale* orchid has great potential as a cut flower and is the parent of orchid hybrids [2]. Therefore, *D. lineale* has a high potential for extinction, so a fast orchid breeding method that produces lots of seeds is needed. However, orchid seeds don’t have an endosperm so it is difficult to survive when they germinate. Orchid plant growth is also known to take a long time [3].

Orchid habitat conditions are already depleted and even damaged, causing ex situ conservation to be a solution to maintain an orchid species. *In vitro* culture is a very useful method for mass propagation of orchids which can maintain the status of orchids so that they do not become extinct [4]. *In vitro* culture techniques are very helpful in the cultivation and conservation of rare orchids because they are able to suppress the exploitation of orchids in nature [6][7].

Souza et. al. [8] reported that the use of fruit extracts in culture medium was able to replace hormones and plant growth regulators, thus enabling the production of orchids to be more economical. This will support the cultivation of orchids and support conservation efforts for rare orchids. According to Utami
et al. [9], the addition of 150 g/L banana extract was able to induce more roots in *D. lasianthera*. Meanwhile, according to Djajanegara [10], the addition of 100 g/L banana and 100 ml/L coconut water can increase shoot and leaf growth optimally. Therefore, this study was conducted to increase the production of orchid seedlings *in vitro* through the subculture of *D. lineale* using New Phalaenopsis (NP) medium with the addition of ‘pisang raja’ banana extract of 100 g/L and 150 g/L to see the growth of shoots and roots of the plant previously had stagnant growth.

2. Materials and methods

2.1. Materials

This research was conducted at the Laboratory of Biotechnology, Laboratory of Plant Structure and Development, and the Joint Research Facility Laboratory of the Faculty of Biology, Gadjah Mada University from January 2021 – May 2021. The plant used in this study was the 2-year-old *D. lineale* orchid plantlets.

2.2. Subcultures

Plantlets were grown in NP medium with the addition of 100 g/L and 150 g/L ‘pisang raja’ banana extracts. Plantlets were subcultured every 2 weeks and placed in dark (2 lux) and light (1317 lux) conditions. Plantlets were captured using a camera Canon D900 and then measured using an image raster 3.0.

2.3. Chlorophyll measurements

Calculation of chlorophyll content following the method of Harborne [11] with modification. Samples of leaves taken as much as 0.3 g were put into a tube and then crushed using a micropestle. 1.5 ml acetone 80% was added to the crushed sample then the sample was mixed with a vortex. Samples were centrifuged at 8000 rpm for 15 minutes. The supernatant was taken to measure the absorbance of chlorophyll using a spectrophotometer at 663 nm and 646 nm wavelengths.

2.4. Anatomical preparation

Anatomical preparations used in this study were free hand sections or fresh preparations. The selected sample was inserted into a slice of carrot so that its position was clamped by the carrot to make cutting easier. The sample was then sliced thinly using a razor blade. The thinly sliced sample was placed in a glass object and dripped with aquadest, covered with a cover glass. Samples were directly observed under a microscope and captured using optilab 3.0.

2.5. Data analysis

The quantitative physiological methods were obtained from measurements of chlorophyll content, as well as growth measurements which included number and size of leaves, number and size of roots, and plant height. Quantitative data were processed using Microsoft Excel and analyzed using SPSS which included ANOVA and Duncan's test if there was a significant difference.

3. Result and Discussion

The results showed that lighting and the addition of banana extract in basic medium was able to increase the growth of *D. lineale* orchid plantlets after 8 weeks of subculturing. The treatments in this study included planting orchids on NP0 medium, NP+100 g/L, and NP+150 g/L ‘pisang raja’ banana extract. The three types of media are also placed in the incubation room with temperature 25°C, treatment light condition with intensity 1317 lux and dark conditions with intensity 2 lux.
Figure 1. The growth of *D. lineale* plantlet for 8 weeks subculture. Scale bar: 5 mm. Each subculture, it is known that there is an increase in the number and size of leaves, number and size of roots, and plantlet height.

Table 1. The growth of *D. lineale* plantlets various media and light conditions for 8 weeks culture

| Parameters          | Treatments                                      | Light (1317 lux) | Dark (2 lux) | sig. |
|---------------------|-------------------------------------------------|------------------|--------------|------|
|                     |                                                 | NP0              | NP+100g/L    | NP+150g/L | NP+100g/L | NP+150g/L |
|                     |                                                 | Banana 'pisang raja' | Banana 'pisang raja' | NP0    | Banana 'pisang raja' | Banana 'pisang raja' |
| Leaves Length (mm)  |                                                 | 11.17±2.78a      | 8.89±2.37a   | 8.52±2.21a | a        | 10.136±2.58 | 10.58±2.30 | 0.146 |
| Number of Leaves    |                                                 | 5.80±1.32ab      | 4.60±1.27a   | 5.10±0.99ab | 5.40±1.51ab | 6.10±1.37b | 5.00±0.94a | 0.103 |
| Root Length (mm)    |                                                 | 3.840±1.93a      | 2.77±1.03a   | 3.72±1.87a | 3.311±2.71a | 3.026±1.84a | 3.96±2.09a | 0.713 |
| Number of Root      |                                                 | 2.10±0.74b       | b            | 2.60±1.58bc | 1.00±0.94a | 1.80±1.32ab | 3.00±1.05c | 0.005 |
| Plant Height (mm)   |                                                 | 16.34±2.89b      | b            | 15.14±2.55b | 17.47±2.56b | 16.55±4.93b | 9.52±2.09a | 0     |

*The letter behind each number indicates the significance of <0.05 on the ANOVA Test.

The number of roots and plant height had a significance value of less than 0.05 so that the two parameters gave significant differences in each treatment. The most significant difference in the number of roots was found in light medium NP 0, dark medium NP 0 and NP150. The highest number of roots was found in plantlets which were planted in the medium with 150 g/L 'pisang raja' banana extract in dark condition (2 lux). As for plant height, NP+150 g/L dark medium gave very significant results compared to the other five mediums.

This can happen because bananas ‘pisang raja’ as the best organic additive to *in vitro* medium [12] contain thiamine, glucose, carbohydrate, auxin hormones, and gibberellins. Thiamin is a type of vitamin B1 that is able to stimulate cell division in the root so that the roots can grow faster. Vitamins in general are also necessary for the growth of cells that are still actively dividing because they act as catalysts in metabolism [13][14]. High carbohydrates and sugars can help facilitate plant metabolism and can increase cell growth and differentiation [15][16]. In dark conditions, the plant will be etiolated, which is a condition where plants grow faster but are weak, stems are not sturdy, leaves are small, and are pale in color [5]. Dark conditions also stimulate auxin activity [17] so that it can induce the emergence of more roots and plants to grow faster. Auxin will work optimally in dark conditions, but will be degraded in light conditions.
Figure 2. Chlorophyll content in leaves and root of D. lineale orchid. LNP0 = Light NP0; LNP100= Light NP+100 g/L banana extract; LNP150= Light NP+150 g/L banana extract; DNP0= Dark NP0; DNP100= Dark NP+100 g/L banana extract; DNP150 = Dark NP+150 g/L banana extract. Light : 1317 lux, dark: 2 lux.

Based on Figure 2, it can be seen that the chlorophyll in the leaves is generally higher than the chlorophyll in the roots of orchids. Medium NP0+100 g/L ‘pisang raja’ banana extract became the medium with the highest chlorophyll content, both in the dark and light treatments. However, medium NP150 g/L ‘pisang raja’ banana extract actually reduced the chlorophyll content in the leaves and roots of the orchid. This is because the concentration of bananas added is too much so that it reduces the quality of plant growth. This is due to the presence of organic substances in bananas that supply vitamins, nitrogen, iron, and magnesium so that they can help the formation of chlorophyll levels and prevent chlorosis. Nitrogen is needed by plants to produce proteins, nucleic acids, and chlorophyll. Magnesium is a central molecule in chlorophyll and plays an important role as a cofactor in ATP synthesis. Iron has an important role in respiration and photosynthetic reactions. Although not part of the chlorophyll molecule, iron can help the formation of chlorophyll and play a role in electron transfer [22][23].

The results of the spectrophotometer showed that there was chlorophyll in the roots of the orchid, this is a characteristic of orchids root. Chlorophyll has a very important role in the energy conversion and biosynthesis of several essential primary and secondary metabolites, and light is a very essential condition for the formation of chlorophyll. Chlorophyll is present in chloroplasts that develop from proplastids which are present in immature plant meristem cells. If the leaves grow in a dark place, the proplastids will develop into etioplasts. When dark growing seedlings are exposed to light, chloroplasts are formed. After chlorophyll a and b are formed, chloroplasts are fully functional and can photosynthesize [18]. Therefore, based on this study (Figure 2.), chlorophyll levels in plants placed in the dark (2 lux) were lower than in plants exposed to light (1317 lux). However, several studies have shown that the highest chlorophyll levels are when the plant is not fully exposed to light and too high an intensity. This shows that each plant has a maximum tolerance limit to light conditions [19][20].

Anatomically, orchid leaves consist of epidermis, stomata, mesophyll, bundle sheath, xylem, and phloem. The epidermis, which has varying cell shape, has an important role in ecophysiology, that is protecting the mesophyll from radiation and high heat from sunlight. Thicker layers of the epidermis can reflect more heat and are sometimes able to store water [23][24]. Mesophyll is a tissue composed of thin-walled parenchyma cells with an oval or polygonal shape. Mesophyll tissue in orchids does not undergo differentiation [23]. Mesophyll tissue contains chlorophyll which plays a vital role in the process of photosynthesis. The thickness of the mesophyll layer supports the formation of succulent leaves which allow for more water storage so that it helps the photosynthesis process, especially in dry environmental conditions [24]. The vascular bundles on the leaves of D. lineale are arranged in one row. The vascular bundle is surrounded by bundle sheath consisting of sclerenchyma cells. The vascular bundle type in D. lineale leaves is a closed collateral type, where the xylem and phloem are located side by side but there is no cambium between them [23][24][25]. Stomata on orchid leaves are found on the
adaxial and abaxial epidermis, but are more commonly found on the abaxial. The position of the stomata with the epidermis parallel.

![Figure 3. Comparison of the anatomy of D. lineale orchid leaves in various treatments. A: Light NP0, B: Light NP 100, C: Light NP 150, D: Dark NP 0, E: Dark NP 100, and F: Dark NP 150. Ep: epidermis, M: Mesophyll, VB: Vascular Bundles, X: xylem, P: phloem, BS: bundle sheath, and St: stomata. Scale bar: 50µm. Light: 1317 lux, dark: 3 lux.](image)

Based on Figure 3, it’s known that the mesophyll of orchid leaves is thicker in plants placed under lighting (1317 lux). This is because chlorophyll is located in the mesophyll, so the thickness of the mesophyll shows good effectiveness on chlorophyll because it plays a role in photosynthesis. Meanwhile, in plant leaves that are placed in a dark place, photosynthesis does not take place, thus inhibiting the formation and role of chlorophyll as indicated by a thinner mesophyll layer.

Orchid root structure (Figure 4.) from outside to inside includes epidermis, velamen, exodermis, cortex, bundle sheath, xylem, and phloem. The epidermis is the outermost layer of the anatomy of orchid roots, the epidermis forms a derivative in the form of a layered epidermis called velamen, composed of cellulose with various proportions of lignin and suberin [26]. Velamen functions in protection, absorption of water and minerals, and prevents water loss due to evaporation [27]. In vitro plants live with sufficient water and nutrient availability.

Orchid root cortex also found the presence of chlorophyll. The cortex serves as a pathway for the entry of water and nutrients from outside the plant to the stele [28]. The vascular bundle is a transport system in plants consisting of xylem and phloem. Xylem functions to transport water and nutrients from the soil, while phloem functions to transport photosynthetic products from leaves [26]. Xylem consists of dead cells with thick walls. While the phloem consists of living cells and has a thin wall. The vascular bundle is surrounded by endodermal
tissue consisting of sclerenchyma cells [27]. The vascular bundle on the roots of orchids is of the radial type, i.e. the location of the xylem and phloem alternately forming circular radii [25].

Figure 4. Comparison of the anatomy of *D. lineale* orchid roots in various treatments. A: Light NP0, B: Light NP 100, C: Light NP 150, D: Dark NP 0, E: Dark NP 100, and F: Dark NP 150. Ep: Epidermis, V: velamen, Ex: Exodermis, Co: Cortex, Ed: endodermis, VB: Vascular bundle, X: xylem, Ph: Phloem, and P: Pith. Scale bar : 100 µm.

The anatomy of *D. lineale* orchid roots in each treatment shows that there was a difference in the diameter of the roots. It is known that the highest root diameter is found in the NP0 medium. The addition of 150 g/L banana ‘pisang raja’ to the medium stimulated root formation. This causes rapid and abundant root growth, but is smaller in size compared to the other two types of medium. These results are in accordance with the research conducted by Utami et. al. [9] where the addition of 150 g/L bananas was able to induce an increase in the number of roots. The use of banana organic matter as an addition to the basic *in vitro* medium was able to increase the growth of *D. lineale* orchids. This is expected to help increase the production of superior *D. lineale* orchid seeds to meet market demand and orchid cultivation efforts for conservation. If the needs of orchids in the market can be met, then the exploitation of orchids from nature can also be reduced. In addition, the return of cultivated orchids to the wild is also important to maintain the existence of these orchids in their natural habitat.

4. Conclusions
The best medium for the growth of *D. lineale* orchid plantlets *in vitro* is NP medium with addition of 100 mg/L banana. The best conditions for the growth of *D. lineale* orchid plantlets *in vitro* are lighting (1317 lux) using continuous white light. The addition of bananas affects root diameter, and lighting affects leaf mesophyll thickness.
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