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Research Article

In Vitro Culture of *Phalaenopsis amabilis* (L.) Blume Orchid for Seedling Production with Banana Extract Supplementation and Light Treatment for *Ex Situ* Conservation

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ABSTRACT
In vitro culture is one of the effective cultivation methods for seedling production of orchids that can be used as a powerful tool in the conversation of orchids. The aims of the present study were to (i) investigate the effects of the addition of banana extract (0 g/l, 100 g/l, and 150 g/l) on media (NP media), in two different light regimes (light and dark conditions) on the growth of plantlets of an epiphytic orchid *Phalaenopsis amabilis* in *in vitro* culture. Methods used included (i) subculturing orchid seedlings in treatments media, (ii) measuring leaves and roots chlorophyll content and growth parameters, (iii) anatomical preparation of leaves and roots of the seedlings. The results showed that the best condition for getting greater seedlings of *P. amabilis* plantlets is in media with an addition of 100 g/L banana extract in light condition. The highest amount of chlorophyll in the *P. amabilis* leaves was found in medium with the addition of 100 g/L banana extract medium in light conditions. The thickness of mesophyll and the largest root diameter of *P. amabilis* seedlings were also found in media with the addition of 100 g/L banana extract medium in light condition. In conclusion, the addition of 100gr/L banana extract into basic culture medium will be beneficial for seedlings production of *P. amabilis* with great appearance, for *ex situ* orchid conservation programs.

INTRODUCTION
One of the orchid genera that has high commercial value and is widely traded is the *Phalaenopsis* genus. *Phalaenopsis amabilis* orchid is a popular plant (Theng & Korpenwar 2014) and is also used as a mother plant to generate superior hybrids of *Phalaenopsis* orchids (Semiarti et al. 2007). Overcollection of these orchids from their natural habitat in the forest for cultivation at home or nurseries and for commercialisation (Setiari et al. 2018) causes the population to decrease and even become endangered (Rukmana 2000). Therefore, propagation of orchids to generate many seedlings is required to fulfil marker demand on the *Phalaenopsis* and to reduce overcollection from their natural habitats. *In vitro* is one of cultivation methods for facilitating mass propagation of orchids to produce numerous seedlings as well as one of the programs of *ex-*

situ conservations of orchids (Semiarti et al. 2010).

The nutrient composition in medium is one of the crucial and essential factors in the *in vitro* growth period of orchid seedlings. Several studies reported that the addition of some organic substances in medium culture, such as fruit extract can enhance plantlet growth. The fruit extracts in culture medium contain many nutrients providing hormones and plant growth regulator useful to increase plant growth (Souza et al. 2013). The addition of banana extract (50 g/L) in culture medium has been shown to promote protocorm-like bodies (PLBs) viability (Yulianti et al. 2016)

Musa acuminata x *Musa balbisiana* is one of the Indonesian banana cultivars called "Pisang Raja" that Indonesian people widely consume. Djajanegara (2010) showed that the insertion of 100 g/L banana extract of "Pisang Raja" into medium culture affects the percentage of shoots number, plantlet height, leaves number, and roots number on *P. amabilis* orchids.

Other studies also reported that the addition of 100 g/L banana extract into medium culture affects the percentage of shoots number, plantlet height, leaves number, and roots number on *P. amabilis* orchids (Djajanegara 2010). Furthermore, the addition of 150 g/L banana extract into culture medium can increase the number of roots and growth of *Dendrobium lasianthera* (Utami et. al. 2016). Banana is known to contain high nutrients including sugar, phosphorus, thiamin which can accelerate cell division in root meristems (Pazil 2009; Sallolo et al. 2012; Hapsari & Lestari 2016).

There are some factors affecting plantlet growth in *in vitro* culture including culture media (Qomariyah & Dewanti 2019) and light conditions. Currently, there are no studies of plantlet growth of *P. amabilis* in relation to the addition of banana extract into culture media and light conditions. The present study aimed to investigate the effects of (i) the addition of banana extracts into culture media (ii) light conditions on the growth of *P. amabilis* plantlets. Furthermore, morphological, physiological, and anatomical characteristics of the plantlets were also investigated. This study is a part of programs to support *ex situ* conservation of orchids.

MATERIALS AND METHODS

Plant Materials, Medium Preparation and Culture Conditions

Plant materials used in this study were 18 months-old *P. amabilis* plantlets with ≥ 2 leaves without root generated from seed germination in *in vitro* culture. The plantlets were cultivated in basic medium, New Phalaenopsis (NP) medium with the addition of banana extracts (0 g/l: 100 g/l and 150 g/l) (Arditti 2008), under two light regimes (light and dark conditions) at 25° C. The plantlets were planted in culture bottle with 5 plantlets in each bottle as replication. Every treatment has 2 bottle culture with 10 plantlets in total as replication. Plantlets were sub-cultured every two weeks. The measurements of plantlet growth were carried out by photographing the plantlets using a Canon 800D DSLR *camera* and processed using an Image Raster 3.0.

The Banana extract was made by weighing the peeled banana fruit us-

ing a digital scale, 100 g and 150 g, respectively. The bananas were mashed using a blender with 100 ml of water for 100 g peeled banana and 150 ml of water for 150 g peeled banana. The homogenate obtained was filtered twice, then added to the NP basic medium and homogenized.

Measurement of Chlorophyll Content in Leaves and Roots of *P. amabilis*

The methods used for measuring chlorophyll content are based on Harborne (1998) with modification. Leaves sample was weighed at 0.03 g and then put into a microtube and crushed with a micro-pestle. The crushed leaves were dissolved in 1.5 ml of 80% acetone. After that, the sample was vortexed for a few moments for homogenization. The homogeneous sample was centrifuged at 8000 rpm for 15 minutes. The supernatant was taken to measure the content of chlorophyll a and b and it was performed by determining the absorbance value using spectrophotometer with wavelength of 646 nm and 663 nm.

Anatomical Preparation

The anatomical preparation were prepared by free-hand sectioning method based on Berlyn and Miksche (1976). The samples of *P. amabilis* leaves and roots were taken after eight weeks of treatment with banana extract and light the samples were sliced transversely as thin as possible using a razor blade. The incision of the sample is placed into the water for a while. Subsequently, the sample is placed in an object glass and dripped with water and then covered with cover glass to be observed under a microscope (Nikon, Japan) and optilab (Miconos, Indonesia).

Data Analysis

Quantitative data for plant morphological and physiological analysis were carried out by measuring plant height, leaves length and number, root length and number; and chlorophyll contents. The quantitative data for anatomy were collected by measuring root diameter and mesophyll thickness. The quantitative data were analyzed using SPSS (Statistical Package for the Social Sciences) v.23 which includes analysis of variance (ANOVA). Significant variance between treatments were subsequently tested with Duncan's test or Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The Growth Response of Plantlets

Results showed that treatments of lighting condition and the addition of banana extract in basic medium affect the growth of *P. amabilis* plantlets after eight weeks of subculturing. Leaf length, root length and plant height of *P. amabilis* plantlets in the treatment of the addition of 100 g/L banana extract in NP medium in the dark showed the highest values compared to other treatments (Table 1). Futhermore, leaf number was not significantly different

Table 1. The growth of *P. amabilis* plantlets under treatment of medium and light conditions for 8 weeks culture.

Treatments / Parameters		Average of Leaves Number	Length of Leaves (µm)	Average of Roots Number	Length of Roots (µm)	Plant Height (µm)
Light Treatments (1224 lux)	NP 0	3.9±1.101[a]	6.46±1.821[b]	1.2±0.422[a]	3.24±1.321[a]	16.02±5.096[bc]
	NP 100	3.5±0.849$_a$	6.74±1.296[b]	1.3±0.483[a]	2.93±1.165[a]	16.49±2.963[bc]
	NP 150	3.3±0.823[a]	4.08±0.618[a]	1.3±0.483[a]	2.87±0.769[a]	10.93±1.498[ab]
Dark Treatments (3 lux)	NP 0	3.5±0.971[a]	6.73±1.484[b]	2.1±1.449[b]	6.55±3.892[b]	16.97±2.691[c]
	NP100	3.5±0.849[a]	8.53±2.955[c]	1.2±0.422[a]	6.64±1.830[b]	18.45±4.456[c]
	NP150	3.6±0.966[a]	4.46±1.007[a]	1.0±0.000[a]	3.87±1.948[a]	13.43±4.153[ab]

Note: Data in the same column followed by the same letters are not significantly different by Duncan's test at $p \leq 0.05$. Details: NP 0: control medium; NP 100: NP+100 g/l banana extract; NP 150: NP+150 g/l banana extract

between *P. amabilis seedlings in all treatments*. This might be influenced by internal factors such as genotype and plantlet physiological conditions (Haris & Mercuriani 2018).

Leaf length and plant height of *P. amabilis* plantlet were highest in the treatment of the addition of 100 g/l banana extract (Table 1). This might be due to the auxins and cytokinin contained in banana extract that commonly has effects to promote plant growth, where auxin plays a key role in cell elongation while cytokinin regulates plant cell proliferation and differentiation (Armarego-Marriott et al. 2020). Hasanah et al (2014) reported that banana extract contains 0.00035% IAA (auxin) and 0.00020% cytokinin. Furthermore, the action of auxin and cytokinin was influenced by light, where both hormones were degraded when exposed to light (Manzur et al. 2014). This might explain the higher values of plant height and leaf length in medium NP+100 g/l banana extract in the dark than in light conditions.

Root length of seedlings in medium NP+100 g/l banana extract was also higher in the dark than in the light condition (Table 1), as in light conditions auxin activity decreased as the auxin is degraded in light conditions. However, dark conditions can cause etiolation, and the etiolation process causes the plantlets to have pale green colour leaves leading to skotomorphogenic phenotype (Armarego-Marriott et al. 2020).

Apart from auxin and cytokinin, banana extract also contains thiamine which can stimulate cell division in root meristem to grow faster which affects the length of the roots (Sallolo et al. 2012). Furthermore, potassium (K) plays an important role for root growth by affecting N distribution and controlling the photosynthesis rate by carbohydrate translocation (Xu et al. 2020). The combination between auxin, thiamine, and potassium contained in banana extract that have promoting effects can lead to the increase of plant growth, especially in the dark. In the present study, leaves length, plant height and roots length were the highest in medium NP+100 g/L banana extract in dark conditions. Results of the present study were similar to the results of study by Djajanegara (2010).

The present study also showed that the best medium for root growth is NP 0 basic medium (without the addition of banana extract) (Table 1). The largest number of roots on medium without the addition of banana extract

indicates that the amount of endogenous auxin that is essential for the formation adventitious roots and elongation of root cells of the seedlings, is sufficient. Therefore, the addition of some growth regulator such as auxin contained in banana extract did not have significant effects (Arimarsetiowati & Ardiyani 2012). This is also relevant with the theory stated by Moore (1989) that the use of plant hormones generally must be in accordance with their needs. Adding exogenous hormones that exceed the critical level will interfere with plant metabolism or have no significant effect (Moore 1989).

The present study also showed that the addition of 150 g/l banana extract into medium culture did not significantly affect the growth of *P. amabilis* plantlets, both in dark or light conditions. This might be due to the PGR contained in 150 g/l banana extract exceeded the optimal levels for plantlets growth of *P. amabilis*. In contrast, Utami et al. (2016) reported the addition 0f 150 g/L banana extract of same cultivars improve the number of roots in other orchid species, *Dendrobium lasianthera*. This indicates that the concentration of banana extract gives a different response on different species of orchids that might be related to the amount of endogenous auxin of each species. Each plant requires different levels of PGR and gives a different effect related to the amount of endogenous auxin of each species (Moore 1989).

Although, the growth of the plantlets showed the highest value in the dark conditions, as seen in Figure 1, this condition was not recommended for continuous treatment. The dark condition caused plantlets to have pale green leaves compared to the light treatment, as seen in Figure 1. It proves that the leaves contain lack of chlorophyll and are in line with Figure 2A. Dark conditions are usually applied to stimulate plantlet's root growth (Monteuuis & Bon 2000). When the root starts to appear, culture is placed in normal condition (Monteuuis & Bon 2000) with sufficient light intensity for multiplication phase of *in vitro* culture around 1000-10.000 lux (Yuniardi 2019).

Chlorophyll Content in Leaves and Roots of *P. amabilis*

Results of the present study demonstrated that the highest leaf chlorophyll content was found on light treatment in NP+100 g/L banana extract medium (Figure 2A). This might be related to the essential compounds contained in banana including vitamins, iron, and magnesium which is important for the formation of chlorophyll and avoid chlorosis (McCauley et al. 2009). In addition, potassium (K) levels which are found in banana extract at appropriate concentration can increase leaf chlorophyll levels and facilitate the integrity of the chloroplast ultrastructure (Tränkner et al. 2018). Perceiving light signal in plant affects the uptake and utilize multiple nutrients including macronutrients and micronutrients. Availability of light also activates some cryptochrome that regulates plant development and also influences chlorophyll content in plant. Light also triggers expression of gene that controlling nutrient utilization and plant hormone (Xu et al. 2021).

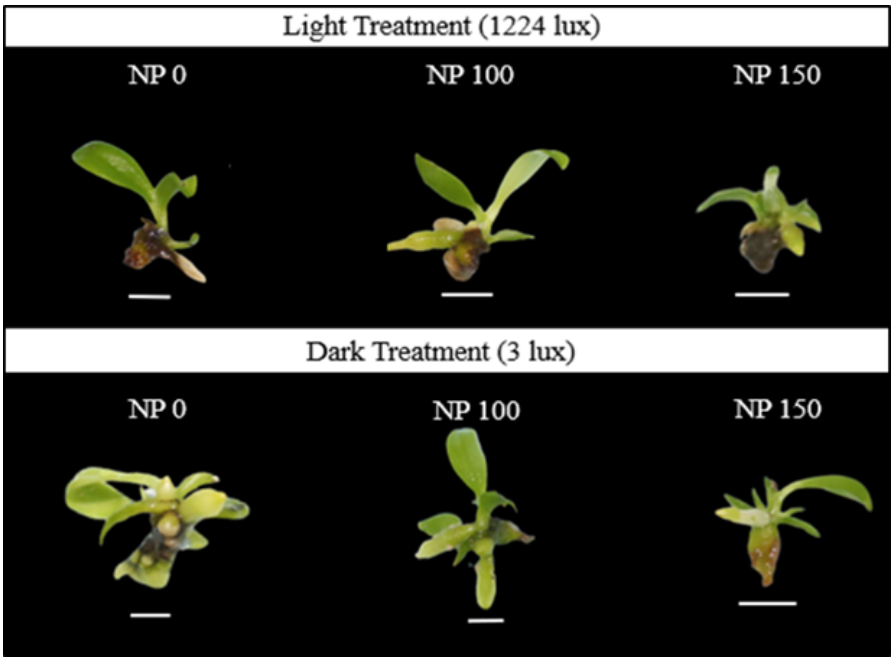

Figure 1. Growth comparison of *Phalaenopsis amabilis* plantlets on NP medium and dark-light treatment after 8 weeks of culture. Details: NP0: control medium; NP 100: NP+100 g/L banana extract; NP 150: NP+150 g/L banana extract. Bars: 0.5 cm

Furthermore, the highest chlorophyll content in the *P. amabilis* roots was found in NP0 basic medium under light treatment (Figure 2B). The nutrients contained in the NP0 medium might be sufficient for the synthesis of chlorophyll in *P. amabilis* roots.

Almost all the light treatment conditions have higher chlorophyll content than the dark treatments. This condition showed that the formation of chlorophyll is strongly influenced by light. Light plays an important role in converting proplastids into normally functioning chloroplasts, while dark conditions cause proplastids to develop into etioplast (Cortleven & Schumulling 2015).

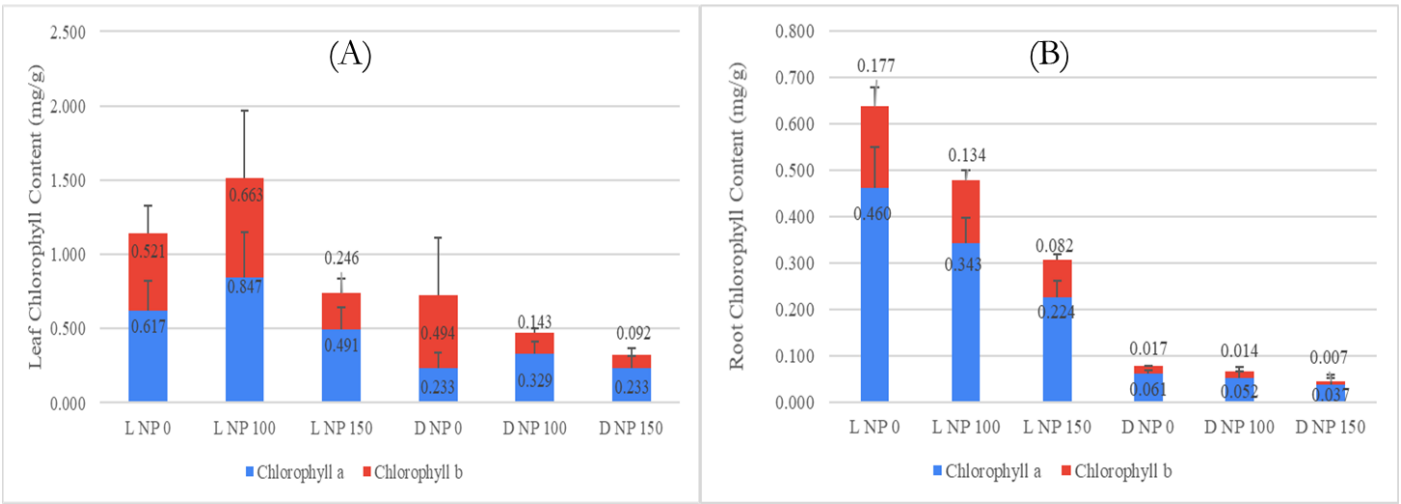

Figure 2. Chlorophyll content in leaves and roots of *Phalaenopsis amabilis*. (A) leaves, (B) roots with various medium treatment and dark-light conditions. Details: D: dark 3 lux; L:light 1224 lux; NP0: control medium; NP 100: NP+100 g/L banana extract; NP 150: NP+150 g/L banana extract.

Anatomical Features of Leaves and Roots of *P. amabilis*

The present study also showed anatomical features of *P. amabilis* leaves and roots. Anatomical features of *P. amabilis* leaves consisted of upper and lower epidermis, stomata, mesophyll, and vascular bundle (xylem and phloem) (Figure 3).

The outermost layer of the leaf is the epidermis which acts as a protective tissue, prevents water loss and gas exchange through epidermis derivatives. The tissue under the epidermis is the mesophyll which is homogeneous with parenchyma cells that are polygonal in shape and contain chloroplasts, in which photosynthesis occurs (Bercu et al. 2011). *P. amabilis* leaves have closed collateral type of vascular bundle, where the xylem located opposite to phloem and fascicular cambium is not present between phloem and xylem (Rindyastuti et al. 2018; Pradhan & Bajracharya 2020). In the *P. amabilis* leaves, the stomata are found on abaxial and adaxial epidermis surface with parallel position (Bercu et al. 2011).

Table 2 shows that the thickest of the mesophyll in *P. amabilis* was found in light treatment (1224 lux) on NP+100 g/L banana extract medium. The type of nutrient availability influenced the quantitative variation and differentiation of leaf tissue in orchid (Silva Junior et al. 2013). Thus, nutrient contained in NP+100 g/L banana extract, especially macronutrients such as N, P, and K helps differentiation of leaf tissue and affect the photosynthesis process. This is also in accordance with previous study by Silva Junior et al. (2013) where nitrogen application to *Laelia purpurata* orchid in appropriate levels will affect significantly on leaf mesophyll thickness. However, higher concentration of N application can cause toxicity, resulting in a reduction in thickness of mesophyll (Silva Junior et al. 2013).

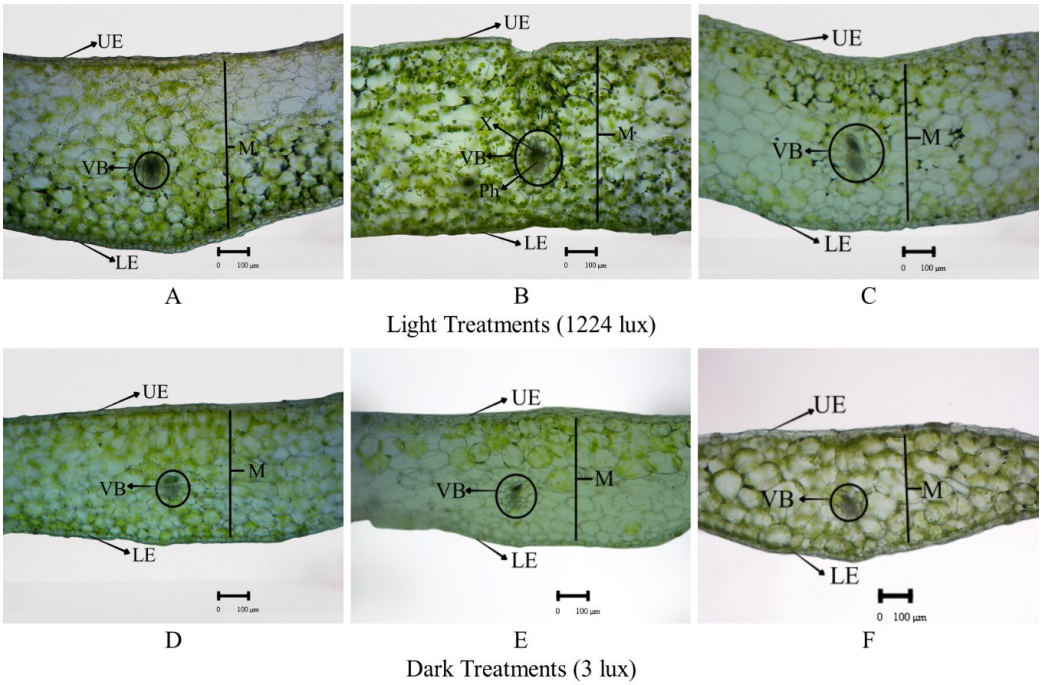

Figure 3. Cross section of leaves of *Phalaenopsis amabilis* orchids. (A) NP0; (B) NP100; (C) NP150; (D) NP0; (E) NP100; (F) NP150. Details: M: Mesophyll; LE: Lower Epidermis; Ph: Phloem; UE: Upper Epidermis; VB: Vascular Bundles; X: Xylem. Bars: 100µm

Table 2. Comparison of roots diameter and mesophyll thickness in *P. amabilis* under different medium and lighting condition.

Treatments / Parameters		Roots Diameter (mm)	Mesophyll Leaves Thickness (µm)
Light (1224 lux)	NP 0	1.64±0.031101^{bc}	471. 25±66.420^{c}
	NP 100	1.795±0.079^{c}	550.65±1.486^{d}
	NP 150	1.65±0.004b^{c}	412.09±24.637^{bc}
Dark (3 lux)	NP 0	1.54±0.019^{bc}	377.45±9.963^{b}
	NP 100	1.37±0.021^{ab}	350. 33±12.684^{ab}
	NP 150	1.19±0.006^{a}	286.97±27.381^{a}

Note: Data in the same column followed by the same letters are not significantly different by Duncan's test at $p \leq 0.05$. Details: NP 0: control medium; NP 100: NP+100 g/L banana extract; NP 150: NP+150 g/L banana extract.

Moreover, light changes the anatomical structure of leaves by affecting the differentiation of parenchymal cell arrangement of mesophyll layer (Zheng & Van Labeke 2017). Leaves with thick mesophyll can effectively reduce transpiration that is beneficial for the growth of *in vitro* culture plantlets after acclimatization (Zheng & Van Labeke 2017). Plants that get sufficient light tend to have a thicker mesophyll layer indicating the high effectiveness of photosynthesis, while plants that get low intensity have a thinner mesophyll layer due to barriers to the differentiation process of mesophyll cells (Paiva et al. 2003).

The present study also demonstrated anatomical features of orchid roots which consisted of epidermis, velamen, exodermis, cortex, endodermis, and vascular bundle. Epidermis as the outermost layer of root anatomy orchids forms a derivative layered, known as velamen which is composed of cellulose with suberin and lignified cells in assorted size (Nurfadilah et al. 2016). Velamen has essential functions in defence, assimilation of water and nutrients, and defence against water loss caused by evaporation (Simpsons 2019). The thickness of the velamen layer depends on light intensity and humidity fluctuations (Moreira et al. 2013).

The outer layer of cortex is exodermis which functions as a control of water and nutrients pathway to the roots, protection against water evaporation, and controlling the entry of mycorrhizae (Beck 2010; Nurfadilah et al. 2016). The cortex is composed by parenchyma cells which have thin wall in various proportions and includes the chloroplast inside (Nurfadilah et al. 2016). The cortex serves as a pathway for water and nutrients entry from external environment to inside cell of the stele and as food reserve (Metusala et al. 2017). The innermost layer of the cortex is the endodermis which is arranged by one layer of cells and the cell wall can be thickened by lignin and suberin substance to prevent backflow in the cortex and prevent water loss (Muthukumar & Shenbagam 2018). Root anatomy of *P. amabilis* has radial vascular bundle type where xylem located alternate with phloem position and forming circular radii or *arch* (Beck 2010).

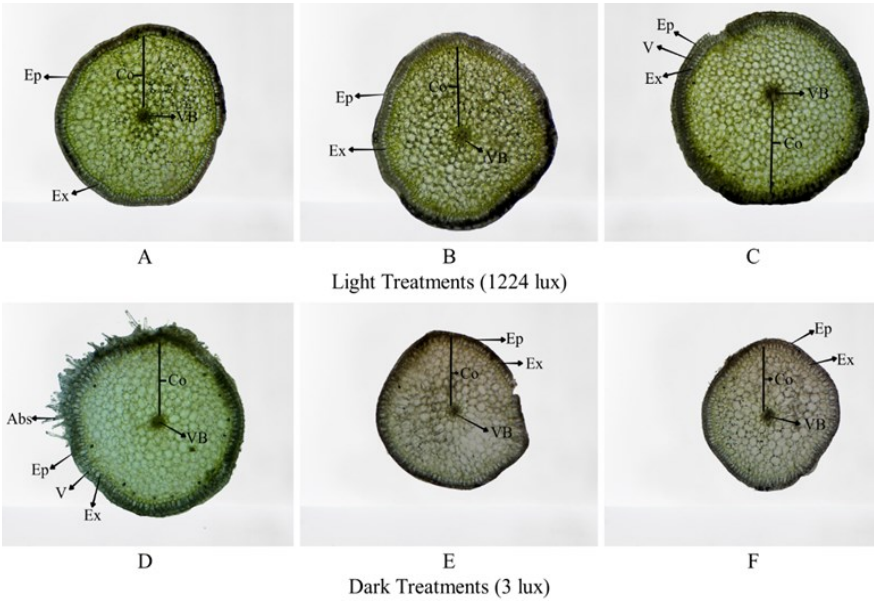

Figure 4. Cross section of roots *Phalaenopsis amabilis* orchids. (A) NP0; (B) NP100; (C) NP150; (D) NP0; (E) NP100; (F) NP150. Details: Abs: Absorbing hair; Ep: Epidermis; V: Velamen; Ex: Exodermis; Co: Cortex; VB: Vascular bundles; Ed: Endodermis; P: Pith; X: Xylem; Ph: Phloem. Bars: 100µm

The present study also showed that the largest root diameter in *P. amabilis* was found in NP+100 g/L banana extract medium in light conditions (Table 2 and Figure 4). It can be concluded that medium type NP+100 g/L banana extract and in light conditions produced better root anatomical characteristics compared to other treatments. Nutrients obtained in 100 g/L banana extract are at sufficient levels and the presence of light is needed for the development of root cells of *P. amabilis*. Light signaling interacts with the root environment, including nutrient acquisition (Joca et al. 2017). Root of dark-grown still develop but have a far thinner diameter than the ones of light-grown seedlings (van Gelderen et al. 2018). That previous research was consistent with the results, where plantlets roots diameter grown in dark conditions had thinner diameter than the light ones.

The largest diameter of roots in NP+100 g/L banana extract might be related to the phosphorous (P) content that has essential functions to support greater root growth (Heydari et al. 2018). According to Heydari et al. (2018) the root diameter decreases significantly under the lack of P. Besides P, potassium (K) also affect root diameter (Filho et al. 2017). The roots diameter increases linearly with the increase of K rate (Filho et al. 2017).

Data of morphological, physiological, and anatomical features showed that *P. amabilis* plantlets grow better in NP+100 g/L of banana extract. Therefore, medium NP+100 g/L banana extract can be recommended for seedling production of *P. amabilis* to produce many seedlings with great performance to fulfill market demand. This work could contribute to reduce exploitation of *P. amabilis* in natural habitats.

CONCLUSION

It can be concluded that NP+100 g/L of banana extract is the best culture

medium for *P. amabilis* plantlets as providing seedlings with high quality. Light treatment at 1224 lux becomes the best condition for *P. amabilis* plantlets growth. The combination of light treatment at 1224 lux and medium NP+100 g/L banana extract is suitable for increasing seedling production of *P. amabilis* by affecting morphology growth, chlorophyll content and anatomy structure. This work could contribute to provide a mean to produce many seedlings for *ex situ* orchid conservation and reduce exploitation of *P. amabilis* in natural habitats.

AUTHORS CONTRIBUTION

DAPA carried out the analysis of growth plantlets, anatomical studies, and chlorophyll content in *P. amabilis*, also drafted the manuscript. ES was responsible for coordinating the implementation of the research and discussion of the research results. All authors read and approved this final manuscript.

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CONFLICT OF INTEREST

The authors declare there is no competing interest.

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