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Effect of light and media composition on growth and stomatal density of *Phalaenopsis amboinensis*

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Abstract. *Nugraha MA, Puspitaningtyas DM, Haryanto. 2025. Effect of light and media composition on growth and stomatal density of* Phalaenopsis amboinensis. *Nusantara Bioscience 17: 243-252. Phalaenopsis amboinensis* J.J.Sm. is an endangered orchid species endemic to Indonesia, valued for both its conservation significance and horticultural potential. However, limited natural populations and slow propagation rates hinder sustainable conservation efforts. This study investigated the combined effects of culture media composition and light conditions on the *in vitro* growth, chlorophyll content, and stomatal density of *P. amboinensis* plantlets. A factorial experiment was conducted using nine media formulations under light and dark conditions. Significant interactions between media and light treatments were observed across all measured parameters ($p < 0.05$). The KC EO2 medium, containing 2 g/L KC, 40 g/L potato (*Solanum tuberosum* L.) extract, 2 g/L peptone, and MS vitamins under light, produced the most vigorous vegetative growth (5.40 ± 2.50 leaves, 1.44 ± 0.31 cm leaf length, 2.00 ± 0.82 roots, and 1.89 ± 0.45 cm root length). In contrast, the KC EO3 medium with 2 g/L KC, 150 g/L mung bean sprout (*Vigna radiata* (L.) R.Wilczek) extract, and 150 mL/L young coconut water (*Cocos nucifera* L.) under light yielded the highest chlorophyll a content (0.695 ± 0.113 mg/g fresh weight) and stomatal density (77.57 ± 2.72 stomata/mm²). Control plantlets cultured without organic supplements under dark conditions showed poor growth and negligible chlorophyll accumulation. These findings demonstrate that organic-enriched media, especially when combined with light exposure, significantly enhance physiological and morphological development *in vitro*. The optimized culture conditions offer a practical and scalable protocol for *ex situ* conservation and commercial propagation of *P. amboinensis,* supporting the preservation and sustainable use of this rare orchid species.

Keywords: Chlorophyll, *ex situ* conservation, *in vitro* culture, *Phalaenopsis amboinensis*, stomata, tissue culture media

INTRODUCTION

The Orchidaceae family is estimated to comprise over 28,000 species across 760 genera, making it one of the two largest angiosperm families worldwide and accounting for approximately 10% of the global floriculture trade value in potted plants and cut flowers (Singh 2020; Wei et al. 2025). The remarkable diversity in flower morphology, coloration, and vase life establishes orchids as high-value ornamental commodities and subjects for ecophysiological research, particularly due to their unique survival strategies, including epiphytism and morphophysiological adaptations to low light conditions in tropical forests (Zhang et al. 2018). Epiphytic orchids exhibit various structural modifications, such as velamen, aerial roots, and specialized stomata, that enable survival in water and light-limited environments.

Phalaenopsis amboinensis J.J.Sm., endemic to Moluccas Island and Sulawesi, is listed as a protected species under Indonesian Government Regulation No. 7/1999. Its population continues to decline due to illegal harvesting and limitations of conventional propagation methods, necessitating urgent conservation and mass propagation efforts (Machmudi et al. 2019; Utami and Haryanto 2019). Beyond ecological value, *P. amboinensis* holds genetic significance as a donor of yellow pigmentation in modern *Phalaenopsis* Blume breeding programs, particularly for developing fragrant yellow cultivars demanded by global markets (Sevilleno et al. 2023). Conservation of this species is crucial not only for preserving local biodiversity but also for maintaining valuable genetic resources for sustainable horticulture industries.

In vitro tissue culture enables large-scale vegetative propagation under controlled conditions, shortens production cycles, and provides pathogen-free plantlets (Sawardekar et al. 2023; Guo et al. 2024). Medium efficacy depends not only on macro and micronutrients but can be enhanced with natural organic supplements like young coconut water (*Cocos nucifera* L.), mung bean sprout (*Vigna radiata* (L.) R.Wilczek) extract, and tomato (*Solanum lycopersicum* L.) extract as growth stimulants (George et al. 2008; Park et al. 2011; Apriliyani and Wahidah 2021). For instance, supplementation with 100 g L^{-1} banana extract significantly improved *Phalaenopsis amabilis* (L.) Blume seedling performance *in vitro* (Arum and Semiarti 2023), demonstrating the potential of natural organic compounds as growth enhancers. These organic compounds contain phytohormones (auxins and cytokinins), vitamins, and sugars to promote cell division and tissue differentiation.

Light serves dual roles as both a photosynthetic energy source and a morphogenic signal. Spectrum, intensity, and photoperiod influence leaf development, chlorophyll synthesis, hormonal activity, and stomatal density. In certain orchids, specific red, blue LED combinations enhance protocorm-like body formation, while initial dark treatment accelerates root initiation (Cavallaro et al. 2022; Naderi et al. 2023). Previous studies on *Phalaenopsis* hybrids showed that 55% shading yielded peak photosynthetic pigment content and modified stomatal guard cell dimensions (Fauziah et al. 2022). Excessive light intensity may cause oxidative stress in young tissues, while insufficient light can inhibit chlorophyll synthesis and vegetative growth.

Although numerous studies have examined media or light factors separately, systematic investigations of media-light interactions specifically in *P. amboinensis* remain scarce, with current reports limited to germination optimization and protoplast isolation (Machmudi et al. 2019; Utami and Haryanto 2019). Therefore, an experimental approach combining both factors is essential for a comprehensive understanding of *P. amboinensis* morphophysiological responses.

Chlorophyll content and stomatal density in plantlet leaves serve as indicators of photosynthetic adaptation and gas exchange efficiency. Shading variations alter pigment levels and stomatal guard cell dimensions in *Phalaenopsis* hybrid plantlets (Fauziah et al. 2022), while other studies demonstrate increased stomatal density in coconut fiber media (Jyothsna and Srivastava 2023) with positive correlations to chlorophyll content and leaf physiological performance (Asyary et al. 2024). Integrative evaluation of these leaf physiological and anatomical responses can also serve as parameters for selecting superior genotypes adapted to specific growth conditions.

This study evaluates the combined effects of culture media with light and dark conditions on *P. amboinensis* plantlet growth, chlorophyll content, and stomatal density will addressing critical knowledge gaps and establishing a foundation for developing efficient propagation protocols supporting both conservation and premium planting material production. The research outcomes are expected to benefit not only *ex situ* conservation but also the development of orchid agribusiness at national and international scales.

MATERIALS AND METHODS

Media preparation

This study used *P. amboinensis* plantlets derived from *in vitro* seed germination, previously sub-cultured for four months on Knudson C (KC) medium. The plantlets selected for the experiment were 0.6-1.0 cm in length, possessed two leaves, and had not yet developed roots. A total of nine culture media with different organic supplement compositions were prepared (Table 1). All media contained 7.5 g/L agar, 1 g/L activated charcoal, and 15-20 g/L glucose. The organic components included young coconut water (*C. nucifera*), tomato (*S. lycopersicum*), potato (*Solanum tuberosum* L.), mung bean sprouts (*V. radiata*), peptone, MS vitamins (myo-inositol, nicotinic acid, pyridoxine-HCl, thiamine-HCl, glycine), and naphthaleneacetic acid (NAA). The media pH was adjusted to 5.7 before autoclave at 121°C for 20 minutes.

Experimental design

The experiment employed a completely randomized factorial design with two factors: (i) nine types of culture media; and (ii) two illumination conditions: 16 hours of light at 4000 lux and continuous dark. The dark treatment was achieved by placing culture bottles in a tightly sealed rack within the same growth chamber to ensure complete light exclusion. Each treatment combination consisted of five culture bottles, with each bottle containing two plantlets (n = 10 plantlets per treatment combination), resulting in a total of 180 plantlets. Cultures were maintained in a growth chamber at 25°C for 18 weeks.

Table 1. Recapitulation of media for the growth and physiological development of *Phalaenopsis amboinensis* orchid

Basal medium	**Composition**
WMO	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L
VW	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, VW media 1.67 g/L
VW EO	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, VW media 1.67 g/L tomato (*Solanum lycopersicum*) 100 g/L, mung bean sprouts (*Vigna radiata*) 100 g/L, coconut water (*Cocos nucifera*) 150 mL/L, NAA 5 ppm, thiamin 1 ppm
GM	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, fertiliser Grow More 32-10-10 0,5g/L
GM EO	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, fertilizer Grow More 32-10-10 0,5g/L potato (*Solanum tuberosum* L.) 40 g/L, peptone 2 g/L, KH2PO4 0.125 g/L, MS vitamins (Myo inositol 100 mg/L, nicotinacid, pyridoxine-HCL 0.5 mg/L, Thiamin HCl 0.4 mg/L, glicine 2 mg/L)
KC	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, KC medium 2 g/L
KC EO 1	Agar 7.5 g, sugar 20 g/L, charcoal 1 g/L, KC media 2 g/L, tomato (*Solanum lycopersicum*) 100 g/L, mung bean sprouts (*Vigna radiata*) 100 g/L, young coconut water (*Cocos nucifera*) 150 mL/L.
KC EO 2	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, KC medium 2 g/L, potato (*Solanum tuberosum*) 40 g/L, peptone 2 g/L, MS vitamins (Myo inositol 100 mg/L, nicotinacid, pyridoxine-HCL 0.5 mg/L, Thiamin HCl 0.4 mg/L, glicine 2 mg/L).
KC EO 3	Agar 7.5 g/L, sugar 20 g/L, charcoal 1 g/L, KC media 2 g/L, mung bean sprouts (*Vigna radiata*) 150 g/L, young coconut water (*Cocos nucifera*) 150 mL/L.

Note: EO: Enriched with organic supplements; WMO: Without basal medium and organic supplements. VW: Vacin & Went; KC: Knudson C; GM: Grow More

Growth measurement

Plantlet development was monitored every two weeks for a total of 18 weeks. Morphological data collected included the number of leaves, leaf length (cm), number of roots, and root length (cm). Measurements were made using a digital caliper, and the mean of each replicate was recorded. At the end of the culture period, samples were further analyzed for chlorophyll content and stomatal density.

Pigment analysis

Chlorophyll a and b contents were determined following a modified method from Mackinney (1941). Approximately 0.1 g of fresh leaf tissue was ground using a mortar and pestle in 5 mL of 96% ethanol. The extract was transferred into microtubes, vortexed briefly, and centrifuged at 8000 rpm for 15 minutes. Absorbance readings were taken at 665 nm and 649 nm using a Shimadzu UV-2600 spectrophotometer. Chlorophyll concentrations were calculated using the following formulas:

$$\text{Chlorophyll a (mg/g FW)} = 13.36 \times A665 - 5.19 \times A649$$

$$\text{Chlorophyll b (mg/g FW)} = 27.43 \times A649 - 8.12 \times A665$$

The results were expressed in milligrams of chlorophyll per gram of fresh weight (mg/g Fresh Weight).

Stomata observation

Stomatal density was measured on the abaxial surface of the second fully expanded leaf. A 1 × 2 cm strip of transparent adhesive tape was applied to the leaf surface, and the adaxial side was gently scraped using a sterile razor blade until the underlying tissue became translucent. The tape was then mounted on a microscope slide and observed under an Olympus U-TV0.5XC-3 light microscope at 400× magnification. Stomata were counted per microscopic field of view and converted into units of stomata per mm².

Data analysis

All quantitative data, including growth parameters, chlorophyll content, and stomatal density, were subjected to normality and homogeneity tests. When necessary, data were transformed to meet parametric assumptions. A two-way Analysis of Variance (ANOVA) was conducted at a 5% significance level (α = 0.05) to evaluate the effects of media type and light condition, followed by Duncan's Multiple Range Test (DMRT) for post hoc comparison among treatment means. All statistical analyses were performed using SPSS version 21.

RESULTS AND DISCUSSION

Media and dark-light interaction on vegetative growth

The number of leaves

The analysis of variance results showed a significant interaction between media type and lighting conditions on the leaf count of *P. amboinensis* plantlets. Based on Table 4 and Figure 1, the KC EO 2 treatment under light conditions produced the highest leaf number (5.40 ± 2.50), which was statistically significantly different from most other treatment combinations. Conversely, the lowest leaf number was recorded in the VW EO treatment under dark conditions, with an average value of 2.40 ± 0.70 leaves. The KC EO 2 medium exhibited consistent leaf growth from week 6 to week 18. In contrast, plantlets in the VW EO treatment showed slow and stagnant leaf growth throughout the observation period. These findings confirm that the presence of complex organic compounds in the medium, along with bright lighting, plays a crucial role in stimulating the growth of vegetative leaf organs *in vitro*.

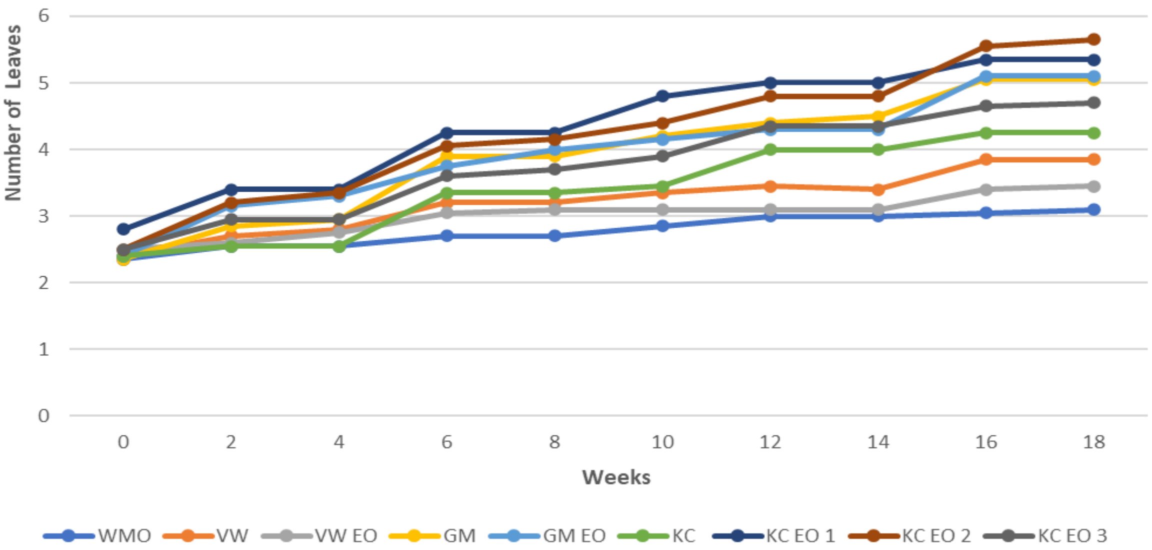

Figure 1. Leaf number of *Phalaenopsis amboinensis* under different culture media and light conditions over 18 weeks. Each data point represents the mean number of leaves recorded at two-week intervals from week 0 to week 18. Among all treatments, KC EO 2 medium under light conditions appeared to promote the highest leaf production throughout the culture period, as shown in the graph

Leaf length

The KC EO 2 treatment under light exposure produced the highest leaf length at 1.44 ± 0.31 cm, significantly different from most other treatments. The VW EO under dark conditions resulted in the lowest value at 0.49 ± 0.09 cm, indicating that the absence of lighting simultaneously significantly inhibited leaf elongation. Visual differences in leaf length were also observed in Figure 2, where plantlets in KC EO 2 medium (a) appeared to have longer leaves compared to those in VW EO medium (b). The leaf growth pattern generally accelerated from week 12 to week 18, particularly under light conditions. These results support the role of lighting and organic compounds in triggering the biosynthesis of growth hormones such as auxin and cytokinin, as well as cell expansion processes that promote leaf elongation during *in vitro* culture.

The number of roots

The highest average root number was found in the KC EO 2 light treatment with bright lighting at 2.00 ± 0.82, significantly different from most other treatments. Among all combinations, the VW EO under dark conditions produced the lowest average root number at 0.20 ± 0.63, indicating that complex organic compounds in the medium were ineffective without light support. Meanwhile, other treatments, such as KC EO 1 under light conditions, produced 2.40 ± 0.84 roots, and GM EO under dark conditions produced 2.10 ± 0.99 roots. They showed relatively high values but were not consistently superior across all replicates. The root growth pattern became evident from week 6, with significant increases between weeks 12 and 18, indicating that the active rooting phase occurred during the mid to late culture period.

Root length

The KC EO 2 under light conditions produced the highest root length in *P. amboinensis in vitro*, at 1.89 ± 0.45 cm, and was statistically significantly different from all treatments (Table 4). The relatively high results were statistically different from KC EO 2. VW EO in dark conditions produced the shortest root length at 0.10 ± 0.32 cm, indicating significant inhibition of root system growth. Generally, treatments with lighting tended to increase root length compared to dark conditions on the same medium, suggesting that light plays a crucial role not only in leaf formation but also in root elongation processes through the activation of physiological and hormonal pathways.

Chlorophyll content

Across all treatments, chlorophyll a content was consistently higher than chlorophyll b, indicating the dominant role of chlorophyll a in the photosynthetic reaction center. The best treatments were observed in the media KC EO 3 and KC EO 2 under light conditions, which produced chlorophyll a level of 0.695 ± 0.113 mg·g^{-1} and 0.641 ± 0.089 mg·g^{-1}, respectively. These two treatments were also the only ones showing detectable chlorophyll b content, albeit low, ranging from 0.000–0.002 mg·g^{-1}, which was still higher than all other treatments, where chlorophyll b was almost entirely zero. In contrast, the lowest chlorophyll content was recorded in the WMO under light conditions as control media under dark conditions, with chlorophyll a level of only 0.044 ± 0.001 mg·g^{-1} and no detectable chlorophyll b (Table 2).

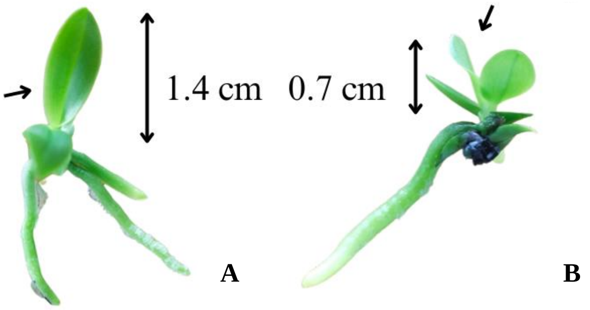

Figure 2. Comparison of leaf length in *Phalaenopsis amboinensis* planlets grown under different culture media: A. KC EO 2, showing longer leaves (1.4 cm); B. VW, showing shorter leaves (0.7 cm)

Table 2. Interaction of media modification and light-dark conditions on chlorophyll content of *Phalaenopsis amboinensis* orchid

Treatments		**Chlorophyll content**	
Conditions	**Media**	**Chlorophyll a (mg/g FW)**	**Chlorophyll b (mg/g FW)**
Light	KC EO 3	0.695 ± 0.113^{g}	0.000 ± 0.001^{a}
Dark	WMO	0.044 ± 0.001^{a}	0.000 ± 0.000^{a}

Note: Mean ± Standard Error (SE) followed by the same letter in the same column are not significantly different at the 5% level ($p \leq 0.05$) of the Duncan MRT. Only the highest and lowest values for each parameter are shown; the full dataset is provided in Table 3

Table 3. Effect of media modification and light and dark conditions on chlorophyll content of *Phalaenopsis amboinensis* orchid

Treatment		**Chlorophyll content**	
		Chlorophyll a (mg/g FW)	**Chlorophyll b (mg/g FW)**
Light	WMO	0.029 ± 0.001^{a}	0.000 ± 0.000^{a}
	VW	0.396 ± 0.012^{d}	0.000 ± 0.001^{a}
	VW EO	0.478 ± 0.049^{e}	0.000 ± 0.001^{a}
	GM	0.559 ± 0.024^{f}	0.001 ± 0.000^{a}
	GM EO	0.463 ± 0.004^{e}	0.000 ± 0.000^{a}
	KC	0.266 ± 0.011^{c}	0.000 ± 0.000^{a}
	KC EO 1	0.501 ± 0.024^{ef}	0.000 ± 0.001^{a}
	KC EO 2	0.641 ± 0.089^{g}	0.002 ± 0.003^{b}
	KC EO 3	0.695 ± 0.113^{g}	0.000 ± 0.001^{a}
Dark	WMO	0.044 ± 0.001^{a}	0.000 ± 0.000^{a}
	VW	0.062 ± 0.001^{a}	0.000 ± 0.000^{a}
	VW EO	0.083 ± 0.013^{ab}	0.000 ± 0.000^{a}
	GM	0.081 ± 0.002^{ab}	0.000 ± 0.000^{a}
	GM EO	0.057 ± 0.000^{a}	0.000 ± 0.000^{a}
	KC	0.065 ± 0.000^{ab}	0.000 ± 0.000^{a}
	KC EO 1	0.134 ± 0.012^{b}	0.000 ± 0.000^{a}
	KC EO 2	0.058 ± 0.005^{a}	0.000 ± 0.000^{a}
	KC EO 3	0.073 ± 0.000^{ab}	0.000 ± 0.000^{a}

Note: Numbers followed by the same letter in the same column are not significantly different at the 5% level ($P \leq 0.05$) of the Duncan MRT

Table 4. Effects of media modification and light-dark conditions on the growth of *Phalaenopsis amboinensis* orchid

Treatment		Parameters			
		Number of leaves (per plantlet)	Leaf length (cm)	Number of roots (unit)	Root length (cm)
Light	KC EO 2	5.40±2.50^{e}	1.44±0.31^{efgh}	2.00±0.82^{bc}	1.89±0.45^{c}
Dark	VW EO	2.40±0.70^{a}	0.49±0.09^{a}	0.20±0.63^{a}	0.10±0.32^{a}

Note: Mean ± Standard Error (SE) followed by the same letter in the same column are not significantly different at the 5% level ($p \leq 0.05$) of the Duncan MRT. Only the highest and lowest values for each parameter are shown; the full dataset is provided in Table 5

Table 5. Effects of media modification and light-dark conditions on the growth of *Phalaenopsis amboinensis* orchid

Treatment		Parameters			
		Number of leaves (Unit)	Leaf length (cm)	Number of roots (unit)	Root length (cm)
Light	WMO	3.10±1.20^{ab}	0.48±0.18^{a}	1.00±0.47^{ab}	1.63±1.39^{bc}
	VW	3.30±0.67^{abc}	0.67±0.35^{ab}	1.00±0.94^{ab}	1.04±0.93^{b}
	VW EO	4.60±2.01^{bcde}	0.74±0.20^{abc}	1.50±1.18^{bc}	1.11±0.66^{b}
	GM	5.00±3.94^{cde}	0.91±0.28^{bcd}	1.80±2.15^{bc}	1.01±1.00^{b}
	GM EO	5.30±1.49^{de}	1.05±0.35^{bcde}	1.90±0.74^{bc}	1.39±0.61^{bc}
	KC	3.60±0.84^{abcd}	0.98±0.34^{bcd}	1.80±1.48^{ab}	1.23±1.03^{bc}
	KC EO 1	4.60±0.84^{bcde}	1.02±0.26^{bcd}	2.40±0.84^{c}	1.39±0.42^{bc}
	KC EO 2	5.40±2.50^{e}	1.44±0.31^{efgh}	2.00±0.82^{bc}	1.89±0.45^{c}
	KC EO 3	5.30±2.31^{de}	1.11±0.59^{cdef}	2.00±1.56^{bc}	0.96±0.60^{b}
Dark	WMO	3.10±0.74^{ab}	0.89±0.43^{bcd}	1.10±0.32^{ab}	1.52±0.70^{bc}
	VW	4.40±1.17^{bcde}	1.49±0.71^{fgh}	2.00±0.94^{bc}	1.47±0.45^{bc}
	VW EO	2.40±0.70^{a}	0.49±0.09^{a}	0.20±0.63^{a}	0.10±0.32^{a}
	GM	5.10±0.88^{de}	1.23±0.46^{defg}	1.70±0.67^{bc}	1.50±0.40^{bc}
	GM EO	4.90±0.99^{cde}	1.60±0.37^{ghi}	2.10±0.99^{bc}	1.24±0.49^{bc}
	KC	4.90±1.20^{cde}	1.29±0.60^{defg}	1.60±1.35^{bc}	0.90±0.56^{b}
	KC EO 1	4.80±1.69^{bcde}	1.72±0.36^{hi}	1.40±0.52^{bc}	1.51±0.36^{bc}
	KC EO 2	5.90±2.13^{e}	1.62±0.33^{ghi}	2.10±1.37^{bc}	1.30±0.33^{bc}
	KC EO 3	5.40±1.17^{e}	1.96±0.65^{i}	1.60±0.52^{bc}	1.52±0.62^{bc}

Note: Numbers followed by the same letter in the same column are not significantly different at the 5% level ($P \leq 0.05$) of the Duncan MRT

Table 6. Effect of media modification and light and dark conditions on the stomatal density of *Phalaenopsis amboinensis* orchid

Treatments		Stomatal Density (stomata/mm^2)
Conditions	Media	
Light	KC EO 3	77.57 ± 2.72^{c}
Dark	WMO	39.38 ± 1.66^{b}

Note: Mean ± Standard Error (SE) followed by the same letter in the same column are not significantly different at the 5% level ($p \leq 0.05$) of the Duncan MRT. Only the highest and lowest values for each parameter are shown; the full dataset is provided in Table 7

Stomatal density

The KC EO 3 and KC EO 2 under light conditions produced the highest stomatal density, at 77.57 ± 2.72 stomata/mm^2 and 76.38 ± 3.73 stomata/mm^2 (Table 6), respectively. Meanwhile, the lowest stomatal density was recorded in the WMO medium under dark conditions, at 39.38 ± 1.66 stomata/mm^2. In general, all light treatments showed an increase in stomatal density compared to dark conditions. Media with organic compounds under light conditions consistently produced stomatal density values >75 stomata/mm^2. This suggests that lighting stimulates stomatal development pathways through the activation of gene transcription, such as SPCH and the EPFL9 peptide, while organic compounds support optimal epidermal differentiation. In contrast, dark conditions inhibited this process, even in the same media. This pattern reinforces the synergistic role of light and nutrient composition in regulating the formation of anatomical structures critical for gas exchange and photosynthetic efficiency.

Table 7. Effect of media modification and light and dark conditions on stomatal density of *Phalaenopsis amboinensis* orchid

Parameters		Stomatal density
Conditions	Media	
Light	WMO	46.60 ± 3.28^{bc}
	VW	71.09 ± 2.69^{d}
	VW EO	77.48 ± 2.84^{e}
	GM	71.72 ± 1.41^{d}
	GM EO	77.12 ± 3.20^{e}
	KC	77.02 ± 3.28^{e}
	KC EO 1	75.20 ± 2.72^{e}
	KC EO 2	76.38 ± 3.73^{e}
	KC EO 3	77.57 ± 2.72^{e}
Dark	WMO	39.38 ± 1.66^{a}
	VW	45.96 ± 2.67^{bc}
	VW EO	44.95 ± 2.21^{b}
	GM	45.96 ± 1.50^{bc}
	GM EO	45.59 ± 1.97^{b}
	KC	45.23 ± 3.21^{b}
	KC EO 1	47.24 ± 3.05^{bc}
	KC EO 2	48.70 ± 2.90^{c}
	KC EO 3	45.59 ± 2.14^{b}

Note: Numbers followed by the same letter in the same column are not significantly different at the 5% level ($p \leq 0.05$) of the Duncan MRT

Discussion

Interaction of culture media in light and dark conditions on leaf number

In general, plantlets cultured under light conditions exhibited a higher leaf number compared to those in dark conditions on the same medium. This indicates that consistent lighting plays a crucial role in supporting shoot meristem activity and vegetative organ formation. Light acts as a primary environmental signal, triggering photomorphogenesis pathways through the activation of receptors such as phytochromes and cryptochromes (Dobisova et al. 2017). These pathways regulate the expression of various leaf development genes, including those involved in photosynthesis, chloroplast formation, and the regulation of growth hormones like auxin and cytokinin (Dobisova et al. 2017; Huq 2018). The presence of light also enables plantlets to perform limited photosynthesis, depending on chlorophyll and sugar availability, thus generating the energy and metabolites needed for leaf formation and elongation (Cavallaro et al. 2022; Saeedi et al. 2023).

On the other hand, media composition also significantly contributed to the increase in leaf number. The KC EO 2 medium under light treatment contains organic compounds such as coconut water (*C. nucifera*) and peptone, which showed the highest number of leaves. Coconut water (*C. nucifera*) is known to contain various bioactive substances, including natural cytokinin, amino acids, and phenolic compounds, which can enhance meristem activity and promote the formation of new shoots and leaves (Werner et al. 2003; Chickarmane et al. 2012). Cytokinin plays a role in stimulating cell division and tissue differentiation by regulating specific gene expression (Wu et al. 2021). Peptone, as an organic nitrogen source, supports growth by providing free amino acids required for protein biosynthesis (Fouda et al. 2024).

Previous studies on *Phalaenopsis* genus have shown that optimal lighting can enhance leaf size and number. Lee et al. (2017) reported that young *Phalaenopsis* exposed to warm white LED lighting produced longer and wider leaves compared to those grown under standard fluorescent lamps. Additionally, a study by Hamdeni et al. (2022) on *Dendrobium* orchids demonstrated that adding organic compounds such as coconut water, tomato juice, and banana to Knudson C medium significantly increased shoot and axillary leaf formation, highlighting the critical role of natural substances in supporting the vegetative phase.

The interaction between lighting and media composition appears to be synergistic. Light supports the activation of metabolic processes and basic photosynthesis, while organic compounds like coconut water (*C. nucifera*) and peptone enhance hormonal responses and provide essential nutrients. This combination creates an optimal environmental condition for leaf development. Thus, the increased leaf number under light treatment reflects the importance of an integrated approach in developing *in vitro* vegetative regeneration protocols, particularly for endemic species like *P. amboinensis*, which holds high conservation and commercial value.

Interaction of culture media in light and dark conditions on leaf length

Leaf length growth results from cell division and expansion activities in mesophyll tissue, which are heavily influenced by hormonal, media, and lighting factors. The medium under light conditions, as the most effective treatment, probably provided optimal physiological conditions through the synergy of organic supplements and lighting. Coconut water (*C. nucifera*) in this medium contains natural cytokinin and trace amounts of auxin, supporting initial cell division and tissue differentiation (Lazim et al. 2015). Cytokinins are known to enhance plant responses to light by increasing the sensitivity of photoreceptors such as phytochromes and cryptochromes (Prerostova et al. 2021), thereby optimizing leaf cell expansion under light exposure.

In addition to coconut water (*C. nucifera*), the presence of peptone as an organic nitrogen source supports protein synthesis required for cell expansion and elongation (Hwang et al. 2024). The combination of coconut water (*C. nucifera*) and fruit homogenates has been shown to enhance *in vitro* orchid growth, as reported by Utami and Hariyanto (2019), where VW medium supplemented with 15% coconut water (*C. nucifera*) and banana produced the longest leaves, 62.1 mm in *P. amboinensis*.

Light plays a crucial role in activating photomorphogenesis, including the differentiation of proplastids into active chloroplasts and enhancing photosynthetic activity. This process generates ATP and carbon assimilates needed for cell expansion (Dubreuil et al. 2018). Additionally, light stimulates the expression of growth-regulating genes such as HY5 and GLK1 and mediates gibberellin hormone pathways that induce the production of cell wall-loosening enzymes like expansins and XET (Sprangers et al. 2020). For example, in *Arabidopsis thaliana* (L.) Heynh., gibberellin treatment significantly increased expansin gene expression, accelerating leaf elongation.

The synergistic effect of light and hormones is also demonstrated in studies on *Phalaenopsis* orchids, where white LED lighting significantly increased leaf area and length (Utami and Hariyanto 2019; Hwang et al. 2024). It can be concluded that lighting is not merely a trigger for photosynthesis but also a molecular regulator that amplifies the effects of growth hormones in *in vitro* culture.

Dark cultures, in contrast, produced inconsistent growth responses. The absence of light inhibited chlorophyll synthesis and photosynthesis, reducing energy production and causing uneven hormone distribution. This is reflected in the high variability of leaf length. The high standard deviation in dark conditions indicates disruptions in metabolism and hormonal regulation (Dubreuil et al. 2018).

Overall, the leaf length growth of *P. amboinensis* is influenced by a complex interaction between media composition and the microenvironment. Media rich in organic compounds provide essential hormones and nutrients, while light activates photosynthetic processes and genetic pathways related to cell expansion. The combination of these factors creates ideal physiological conditions: coconut water (*C. nucifera*) supplies cytokinin and auxin (Lazim et al. 2015), peptone provides organic nitrogen for protein synthesis, and light activates chloroplasts and gibberellin expansion pathways. These findings are supported by previous studies recommending the use of organic-rich media and optimal lighting for *in vitro* orchid growth (Shekarriz et al. 2014; Utami and Hariyanto 2019; Hwang et al. 2024).

Interaction of culture media in light and dark conditions on root number

Roots are critical organs for nutrient uptake and acclimatization of *in vitro-derived* plants, making root number and quality key parameters in evaluating propagation success. Coconut water (*C. nucifera*) provides natural hormones such as auxin and thiamine, which promote root development (Djajanegara 2020), while peptone contains amino acids like tryptophan, a precursor for indole-3-acetic acid (IAA) biosynthesis, the primary hormone stimulating lateral and adventitious root formation (Setiowati and Rahmah 2023). Auxin acts through the activation of ARF-Aux/IAA signaling pathways, such as ARF7, which induces LAX3 gene expression, facilitating increased IAA transport to root primordia and stimulating new root tissue initiation (Overvoorde et al. 2010; Cavallari et al. 2021).

In addition to hormonal factors, lighting also plays a significant role in supporting root growth, despite roots not being primary photosynthetic organs. Lighting during the early culture phase enhances partial photosynthetic activity in the plantlet's photosynthetic parts, generating carbohydrates and energy to support cell division and root tissue expansion (Pospíšilová et al. 1999; Gálvez et al. 2020; Deng et al. 2024).

Light also indirectly contributes to hormonal regulation by enhancing general metabolism and phytohormone synthesis, including auxin. In contrast, dark cultures inhibit photosynthate accumulation and overall metabolic activity, negatively impacting root initiation, as observed in dark treatments with low average root numbers and high standard deviations. These findings align with a study by Xu et al. (2019), which showed that a combination of red-blue light increased root length and activity in *Phalaenopsis* orchids.

The increased root number in the KC EO 2 under light treatment results from the synergy between hormonal and organic nutrient content in the medium and the environmental stimulation provided by bright lighting, which enhances physiological root responses. The strategy of combining organic-rich media with optimal lighting proves crucial for improving root regeneration efficiency in *Phalaenopsis in vitro* propagation (Chugh et al. 2009; Tantasawat et al. 2015; Xu et al. 2019). Therefore, this approach is suitable for tissue culture protocols for orchid species that are difficult to propagate conventionally.

Interaction of culture media in light and dark conditions on root length

Root length is a key indicator of the root system's capacity to penetrate substrates, absorb water and nutrients, and support vegetative plant growth. Coconut water (*C. nucifera*) contains natural phytohormones such as auxin, gibberellin, and cytokinin, with auxin playing a critical role in root elongation by activating cell wall-loosening enzymes like expansion and peroxidases. This mechanism aligns with the acid-growth theory, where auxin enhances H^+-ATPase activity, leading to cell wall acidification that activates expansins to loosen cellulose microfibrils, enabling faster cell elongation (Majda and Robert 2018). Gibberellins in coconut water (*C. nucifera*) also enhance root apical meristem activity, accelerating cell division and elongation (Shtin et al. 2022).

Meanwhile, peptone provides organic nitrogen in the form of hydrolyzed proteins, amino acids, and peptides readily absorbed by plants. Tryptophan in peptone serves as a precursor for endogenous auxin biosynthesis (Setiowati and Rahmah 2023), and other amino acids play essential roles in enzyme and structural cell wall protein synthesis. Previous studies have shown that peptone supplementation in KC medium accelerates germination, PLB formation, and orchid seedling growth even without external hormone addition (Kanjilal and Datta 2000; Nhut et al. 2008; Mondal et al. 2014; Setiari et al. 2016).

In addition to media under light treatment, supported root growth by enhancing photosynthetic activity in the plantlet's green parts, producing carbohydrates like sucrose that are transported to roots as energy and carbon sources for cell elongation (van Gelderen et al. 2018). Research indicates that inhibiting photosynthesis in dark conditions drastically reduces root growth, but this can be mitigated by adding sucrose to the medium (van Gelderen et al. 2018). Light also improves nitrogen use efficiency and the plant's C/N ratio, which are known to play critical roles in root development.

Specific light spectra, such as red-blue LED combinations, have been shown to significantly enhance *Phalaenopsis* root length and activity (Ren et al. 2016). Therefore, the success of the medium under light conditions reflects the synergy between a medium providing natural hormones and organic nutrients and optimal light intensity for metabolism and growth. These results align with studies on *Phalaenopsis* 'Bahia Blanca,' which showed increased root length in media supplemented with coconut water (*C. nucifera*) (Abbaszadeh et al. 2018), and reports that organic supplements like coconut water (*C. nucifera*) and peptone enhance root and PLB growth (Tantasawat et al. 2015; Setiari et al. 2016).

Interaction of culture media in light and dark conditions on chlorophyll a and b

Light is the primary factor in regulating chlorophyll biosynthesis, as it activates the expression of photosynthetic genes such as HEMA1, PORA, CAO, and the transcription factor GLK1, which regulate chloroplast biogenesis and chlorophyll pigment synthesis (Liu et al. 2020). The activity of the LPOR enzyme, which converts protochlorophyllide to chlorophyllide, is highly light-dependent, and in dark conditions, precursor accumulation without conversion leads to stagnation in chlorophyll synthesis. A study by Vendrame et al. (2022) showed that using white LEDs with moderate intensity can increase chlorophyll content in orchid cultures, while excessively high light intensity risks causing photoinhibition and pigment degradation (Streit et al. 2005).

In addition to light, the inclusion of coconut water (*C. nucifera*) and peptone significantly contributed to increased chlorophyll content. Coconut water (*C. nucifera*) contains natural cytokinins, sugars, vitamins, and amino acids that support cell division and chloroplast formation. At the same time, peptone provides organic carbon and nitrogen needed for pigment biosynthesis (Vendrame et al. 2022).

The combination of nutrients and phytohormones supports chlorophyll biosynthesis pathways, resulting in organic-rich media consistently showing higher chlorophyll content compared to control media without supplements. Adaptively, the chlorophyll a/b ratio can vary depending on light conditions. In low-light environments, the expression of chlorophyllide a oxygenase (CAO) increases, converting more chlorophyll a to chlorophyll b to broaden the green-blue light absorption spectrum (Liu et al. 2020).

However, in light conditions, as in this study, the a/b ratio remains high because chlorophyll a remains dominant, and its biosynthesis is supported by light and phytohormones. Overall, these data demonstrate that bright light and organic-rich media composition synergistically enhance chlorophyll a and b biosynthesis and accumulation, supporting the effectiveness of the photosynthetic system in *P. amboinensis* plantlets during *in vitro* culture.

These findings reinforce the understanding that successful vegetative regeneration in orchid culture depends not only on basic media but also critically on lighting and the presence of organic supplements that support complex physiological processes at the cellular and molecular levels.

Interaction of culture media in light and dark conditions on stomata

The results confirm that bright light is the dominant external factor stimulating stomatal formation. Light exposure activates molecular pathways for stomatal lineage differentiation, including increased expression of transcription factors like SPEECHLESS (SPCH) and the production of EPFL9 signaling peptides by mesophyll cells, which promote guard cell formation (Wang et al. 2021; Zhou et al. 2024).

In dark conditions, these pathways are suppressed due to the activation of the COP1 ligase, which degrades SPCH and ICE1 proteins, drastically reducing stomatal formation. In contrast to lighting, media composition also exerts a strong physiological influence on stomatal formation. Media with high organic compounds like coconut water (*C. nucifera*) and peptone, rich in micronutrients, vitamins, and natural growth hormones such as auxin and cytokinin, provide optimal signals and metabolic resources for stomatal formation (Aishwarya et al. 2022). These hormones are known to enhance epidermal cell division and differentiation, including guard cells of stomata (Ando et al. 2024). Peptone, as an organic nitrogen source in the form of amino acids and peptides, supports the synthesis of structural proteins and enzymes involved in leaf morphogenesis and tissue development regulation (Lu and Xiao 2024). At the same time, low-nutrient media like WMO (without basal media and organic compounds) do not adequately support efficient epidermal differentiation.

Physiologically, stomata play a central role in gas exchange, CO_2 and O_2, and leaf transpiration. High stomatal density, as observed in basal media and under light treatments, enables increased CO_2 uptake and supports greater photosynthetic activity, consistent with the high chlorophyll content recorded in these treatments (Bertolino et al. 2019; Lawson and Vialet-Chabrand 2019). Plants with high stomatal density have an adaptive advantage in bright light and nutrient-sufficient conditions, as they can enhance water use efficiency through stomatal aperture regulation (Chua and Lau 2024).

However, this can also lead to increased water loss through transpiration if stomatal closure mechanisms are inefficient. Therefore, in addition to the number, the functionality and responsiveness of stomata to environmental changes are critical. During acclimatization, the transition from *in vitro* to *ex vitro*, plantlets with optimal stomatal numbers and functional stomata have a higher chance of survival and robust growth. Stomatal responses to light and humidity fluctuations help regulate CO_2 uptake and water evaporation in a balanced manner (Bertolino et al. 2019; Lawson and Vialet-Chabrand 2019).

Conversely, plantlets with excessively low or high stomatal density without efficient regulation risk physiological stress when transferred to external environments. Thus, the highest stomatal density in the basal media and under light treatment indicates that the combination of bright light and organic-rich media not only supports morphological stomatal formation but also enhances the physiological readiness of *P. amboinensis* plantlets for *ex vitro* conditions, making it an ideal treatment for orchid propagation and conservation *in vitro*.

In conclusion, the combination of KC EO 2 medium with 16-hour light exposure proved to be the most effective treatment for enhancing the *in vitro* growth of *P. amboinensis*. This treatment significantly increased leaf number (5.40 ± 2.50), leaf length (1.44 ± 0.31 cm), root number (2.40 ± 0.84), root length (1.89 ± 0.45 cm). However, no significant differences were observed between KC EO 3 and KC EO 2 media under light conditions in terms of chlorophyll a content (0.695 ± 0.113 mg/g FW and 0.641 ± 0.089 mg/g FW, respectively), and stomatal density (77.57 ± 2.72 stomata/mm^2 and 76.38 ± 3.73 stomata/mm^2, respectively). KC EO2 and KC EO3 are effective media, with KC EO2 being more suitable for growth enhancement and KC EO3 for improving physiological quality. These results confirm a strong interaction between organic-enriched media and light in promoting plantlet morphogenesis and physiological activity. KC EO 3 and KC EO 2 media are therefore recommended as a standardized protocol for the efficient and cost-effective propagation of *P. amboinensis*. Its application can support *ex situ* conservation efforts, reduce the need for wild harvesting, and offer a practical solution for commercial orchid nurseries seeking high-quality planting material. This approach contributes to both biodiversity preservation and sustainable horticultural development.

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