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Analysis effect of shade level on the physiological and anatomical characteristics of hybrid *Phalaenopsis* orchid at the acclimatization stage

Abstract. Production of hybrid *Phalaenopsis* seedlings is generally applied by *in vitro* culture techniques. The final stage of *in vitro* culture is acclimatization. The acclimatization stage is crucial because the plantlets must adapt to the *ex-vitro* environment. Shade supports plantlet growth during the early stages of acclimatization. This study aims to determine the effect of shade level on the physiological and anatomical characteristics of the *Phalaenopsis* hybrid orchid at the acclimatization stage. The research used *Phalaenopsis* plantlet hybrid and shading level. Plantlets were shaded at 40%, 55%, and 70%. Parameters measured included: photosynthetic pigment content, number and area of leaves, number and length of roots, stomata density, size of stomata and fresh weight. This study used a completely randomized design with one factor and ten replications. Data were analyzed using the ANOVA test and LSD test at a significance level of 5% ($p<0.05$). The results showed the highest photosynthetic pigment content in the 55% shade, and there was a difference in the size of the stomatal guard cells between *ex vitro* and *in vitro* leaves at 40% shade. Shade level did not affect leaf growth, roots, fresh weight and stomata density. However, it affected photosynthetic pigment content and size of guard cells in the *ex vitro* leaves of *Phalaenopsis* hybrid orchids. The most optimal growth of the *Phalaenopsis* hybrid was at 55% shade.

Keywords: Acclimatization · Ex vitro leaves · Shade

Analisis efek tingkat naungan terhadap karakteristik fisiologis dan anatomis anggrek *Phalaenopsis* hibrid pada tahap aklimatisasi

Sari. Produksi bibit *Phalaenopsis* hibrid umumnya dilakukan dengan teknik kultur *in vitro*. Tahap akhir dari kultur *in vitro* adalah aklimatisasi. Aklimatisasi merupakan tahap paling penting karena planlet harus beradaptasi di lingkungan *ex vitro*. Naungan menunjang pertumbuhan planlet selama tahap awal aklimatisasi. Penelitian ini bertujuan untuk mengetahui pengaruh tingkat naungan terhadap karakteristik fisiologis dan anatomis anggrek *Phalaenopsis* hibrid pada tahap aklimatisasi. Penelitian menggunakan planlet *Phalaenopsis* hibrid dan tingkat naungan. Planlet diberi paranet 40%, 55%, dan 70%. Parameter yang diukur meliputi: kandungan pigmen fotosintesis, jumlah dan luas daun, jumlah dan panjang akar, densitas stomata, ukuran stomata dan berat segar. Penelitian ini menggunakan rancangan acak lengkap dengan satu faktor dan 10 ulangan. Data dianalisis menggunakan Uji ANOVA dan Uji LSD pada taraf signifikasi 5% ($p< 0,05$). Hasil penelitian menunjukkan bahwa kandungan pigmen fotosintesis tertinggi terdapat pada naungan 55% danterdapat perbedaan ukuran sel penutup stomata antara daun *ex vitro* dan *in vitro* pada naungan 40%. Tingkat naungan tidak mempengaruhi pertumbuhan daun, akar, berat segar serta densitas stomata, namun mempengaruhi kandungan pigmen fotosintesis serta ukuran sel penutup stomata daun *ex vitro* pada anggrek *Phalaenopsis* hibrid. Pertumbuhan *Phalaenopsis* hibrid yang paling optimal berada pada naungan 55%.

Kata kunci: Aklimatisasi · Daun *ex vitro* · Naungan

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Introduction

Orchid plants are ornamental plants from the Orchidaceae family with attractive flower shapes, sizes, and colours. Based on this, orchid plants can provide excellent market prospects, thereby increasing cultivators' interest in producing new hybrid orchids (Yasmin *et al*., 2018). *Phalaenopsis* hybrids are the result from plant breeding activities, so they have superior characteristics to their parents, including more diverse flower colours, larger sizes and less wilting (Kartana, 2017). In 2018, orchid production in Indonesia increased and ranked fourth in the cut flower production category (Badan Pusat Statistik, 2018). Orchid production applies *in vitro* culture techniques, which are capable to produce large quantities of seed quickly. *In vitro* culture systems can minimize stress on plants, so that plants develop quickly, but these plants have abnormal structures and functions. The application of exogenous sugar can grow plantlets that appear normal, but the photosynthetic apparatus is not active, causing a low waxy coating on the leaves and abnormal stomata function so that plants are unable to control water loss due to evaporation. Because the *ex vitro* environment is not ideal and is not able to support the growth of plants that have various variants of structure and function, improvements need to be induced so that the redistribution of inorganic nutrients from roots and photosynthetic products from leaves can proceed normally to enhance viability (Adiputra, 2012). Shade supports the adaptation of plantlets to an *ex vitro* environment with wild microclimate fluctuations. In addition, shade regulates incoming light's intensity so that the plantlets' temperature and humidity are maintained (Sudartini *et al*., 2020).

In spermatophyte plants, photosynthetic pigments such as chlorophyll a, chlorophyll b, and carotenoids play a role in capturing light, so the amount of photosynthetic pigment content can affect the process of capturing light and photosynthesis. Photosynthesis produces glucose and water for use as plant growth materials. Therefore, the range of photosynthetic pigments is used to determine the physiological characteristics of plants that are influenced by the environment and the ability to adapt to different light intensities (Chen *et al*., 2011).

Different light intensity affects the anatomical characteristics of a plant, especially stomata. Stomata opening is influenced by high light intensity, causing stomatal density to increase. Stomata density affects the amount of CO_2 fixed during the photosynthesis process, thus affecting plant metabolic processes (Yuniarachma *et al*., 2019). Plants at low light intensity have a small number of stomata and large size, causing low stomata density. Plant metabolism is disturbed when there is a lack of light, such as a decrease in the rate of photosynthesis and carbohydrate synthesis (Sihotang, 2017).

Research on shade during the acclimatization stage has been reported, among others, on gaharu (Mulyono, 2014), dragon fruit (Basri *et al*., 2013), and *Phalaenopsis* sp. (Yasmin *et al*., 2018). However, there has been no research, especially on *Phalaenopsis* hybrid orchids during the acclimatization stage, so further studies are needed by looking at plantlets' physiological and anatomical characteristics at the shade level.

Materials and Method

Hybrid *Phalaenopsis* was used as research material. The research was conducted in March - June 2021 at the greenhouse located in Sikebrok Hamlet, Beji Village, East Ungaran and the Laboratory of Biological Structure and Plant Function, Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang.

In the first step, coconut fibers were washed with running water and soaked with a 2 g L^{-1} solution of dithane M-45 for 15 minutes. Next, the plantlets were washed with running tap water to remove agar from the roots, soaked with 2 g L^{-1} solution of dithane M-45 for 15 minutes, and then transplanted into a hollow plastic box (20x10 cm) and placed in shaded containers. There were three treatments: shade 40% (720 lux), 55% (510 lux), 70% (380 lux) and application of control based on cultivators using shade range 50%-80%. Plantlets were watered twice a day in the morning and evening. In addition, plantlets were given 1 g L^{-1} vitamin B1 and 2 g L^{-1} of fertilizer once a week using a spray. Research parameters included photosynthetic pigment content, leaf growth (number and area of leaves), root growth (number and length of roots), stomata density and stomata guard cell size.

At the end of the study, three leaves with the same green colour were taken from each

treatment with according color chart to be used as three samples of the chlorophyll test. Then, 0.1 g of the middle leaf was crushed using a mortar and pestle, dissolved with 10 ml of acetone, filtered into a plastic tube, and covered with aluminium foil. The chlorophyll solution was put into a cuvette and analyzed using a UV-VIS spectrophotometer with wavelengths 646, 663, and 470. The pigment content of chlorophyll and carotenoids was calculated based on (Kharkongor and Ramanujam, 2021):

Chlorophyll a = 12.21 (A663) – 2.81 (A646)
Chlorophyll b = 20.13 (A646) – 5.03 (A663)
Total Chlorophyll = Ca + Cb
Carotenoids = (1000 A470 – 3.27 Ca – 104 Cb) / 198
Pigment Content = (C × V) / BS

Abbreviation:

A663, A646, A470	: Absorbance value at wavelength
Ca	: Chlorophyll a
Cb	: Chlorophyll b
C	: Pigment content in $\mu g.ml^{-1}$
V	: Volume of solution (ml)
BS	: Fresh weight of sample (g)

The growth of leaves and roots was observed once a month during acclimatization. The length of the leaves and roots was measured using ropes and a ruler. The length of the leaves and roots were averaged at the end of the study.

The density and size of stomatal guard cells were observed at the end of the study. Each shade was taken three types of leaves such as leaf A, leaf B, and leaf C. Leaf A was a small leaf size when in vitro and grow large when *ex vitro* (A1, A2, A3), leaf B was the size of a large leaf when *in vitro* and the size of the leaves increased large during *ex vitro* (B1, B2, B3), leaf C was a leaf that grow fully during *ex vitro* (C1, C2, C3). The replica method was used to make stomata preparations (Fauziah *et al*., 2019) . The first stage was the leaf cleaned of dust, then the abaxial part of the leaf was smeared with clear nail polish and dried for 5-10 minutes. The part that has been spread was covered with clear tape and then peeled off. After that, it was glued to the glass object and labelled identity. Stomata density was observed three times for each preparation. As for the size of the stomata, ten stomata were taken for each leaf type to measure the guard cells. Observations used a Olympus binocular microscope with 10x magnification and optilab advance. Measurements used a raster image application with units of division = 100 micrometres and a field of view of 0.69 mm^2.

The stomata calculation formula:

$$\text{The Density of stomata} = \frac{\text{Number of stomata}}{\text{Unit area of field of view}}$$

Fresh weight was observed at the beginning and end of the study. At the end of the study, the shoots and roots were separated and then weighed to determine the translocation of photosynthetic results in plantlets.

The study used experimental methods and a one-factor, completely randomized design. Shade levels of 40% (720 lux), 55% (510 lux), and 70% (380 lux) were used as treatments. Each treatment was carried out with ten replications. Data were analyzed using an ANOVA test with a significance level of 5% to determine the effect of shade levels during the acclimatization stage. If there was a significant effect, continue with the LSD test to determine the difference between the three shade levels during the acclimatization stage. The data were analyzed using the SPSS 22.0 application.

Result and Discussion

Photosynthetic Pigment Content. Based on the results of the ANOVA test, the pigment content of chlorophyll a, total chlorophyll, and carotenoids showed a significant effect ($p<0.05$) (Table 1). The highest chlorophyll a, total chlorophyll, and carotenoid content appeared in the 55% shade and decreased in the 40% and 70% shade. In contrast, the chlorophyll b content (Table. 1) did not show a significant effect ($p>0.05$) in three levels of shade.

Chlorophyll a, total chlorophyll and carotenoid pigments decreased at 70% shading (Table 1), which was caused by inefficient light capture because plants were not yet adapted. Generally, plants adapted by increasing chlorophyll pigments in low light conditions (Liu *et al*., 2016). Low shading had a short wavelength of light that could inhibit light capture. Chlorophyll biosynthesis occured when the chlorophyll pigment absorbs light 628-630

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nm (Fanindi *et al*., 2010). Research by Fanindi *et al*. (2010) in line with this study, found that *Calopogonium mucunoides* decreased the content of chlorophyll a and total chlorophyll at 40% light intensity.

Chlorophyll a, total chlorophyll and carotenoid pigments also decreased at 40% shading (Table 1), caused by the plantlets which were still too young, so they were susceptible. Increased light exposure could damage the pigment and structure of the thylakoid membrane (Akmalia, 2017), decreased electron transport (Pires *et al*., 2012), and accelerated the degradation of chlorophyll (Ayu *et al*., 2016). The loss of Mg in the chlorophyll chain could easily degrade the chlorophyll into its derivative molecules, such as pheophytin (Hermawan *et al*., 2011). According to Budiyanto *et al*. (2008), high light exposure triggered the presence of free radical compounds such as O_2^- produced from photooxidation reactions, causing the leaves, which were initially green, to slowly turn yellow and eventually die. Generally, photoprotection occured by carotenoid pigments under conditions of high light exposure (Chen *et al*., 2011), but this was not the case in this study.

The highest pigment content of chlorophyll a, total chlorophyll, and carotenoid appeared at 55% shading (Table 1), caused by plantlets ability to maximize light capture in shaded conditions. The increase in chlorophyll pigment affected light absorption and electron transport used in photosystems I and II (Sirait, 2008). The maximum light capture increased the electron excitation so that the photosynthesis process becomes optimized (Martins *et al*., 2014). The increase in the rate of photosynthesis affected the photosynthate produced so that it could support growth in the vegetative phase (Tini *et al*., 2019). Research by Supriyono *et al*. (2017) revealed that in arrowroot, the highest content of chlorophyll a and chlorophyll b pigments occurred at 50% shade. Khusni *et al*. (2018) also reported that red spinach leaves at 50% shade were greenest due to the presence of chlorophyll pigment.

Leaf Growth. The number of leaves and leaf area had no significant effect (p>0.05) between the three levels of shade at the end of the study (Figure 2), caused by the *Phalaenopsis* hybrids which were still in a period of slow vegetative growth. (Handini *et al*., 2016) stated that the *Phalaenopsis* orchid had a long juvenile phase and relatively slow growth. Priyadi and Hendriyani (2016) also reported that *Bulbophyllum echinolabium*, *Dendrobium fimbriatum*, *Dendrobium spectabile* orchids had slow growth, with an increase of 1-2 leaves per 4 months. In addition, each shade level had a slight difference in temperature and humidity, so it still supports leaf growth. The same thing happened in the research of Sudartini *et al*. (2020) on *Dendrobium* orchids, that shade had no significant effect on the number of leaves because the environment had a very controlled microclimate, which is in a greenhouse under a paranet. Thus, the absorption process can balance the transpiration process.

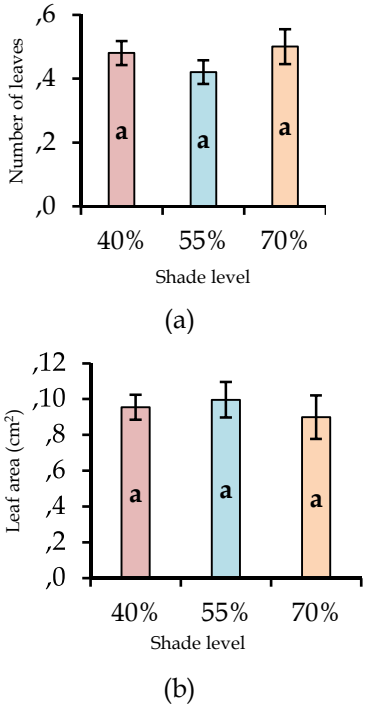


Figure 1. Growth of hybrid *Phalaenopsis* leaves at the end of the study. a). The Number of leaves, and b). Leaf area. The same letter on the histogram shows no significant influence (p>0.05) on the ANOVA test. The number value indicates the mean value of ± SE (n=5)

In the trend of leaf number and area, there was an increasing pattern at all shade levels during the acclimatization period. The 55% shading produced the least number of leaves and the widest leaves. The opposite way appeared in the 70% shade (Figure 3). Plants showed different adaptations to each shade level. The highest photosynthetic pigment content appeared at 55% shade, so leaf widening was necessary in order to maximize the light capture in shaded conditions (Mathur *et al*.,

2018). Shao *et al*. (2014) stated that plants must absorb sufficient light for photosynthesis at low light intensity. If light deficiency appeared, there was an insufficient ATP for carbon fixation and carbohydrate biosynthesis, which resulted in a decreased growth. The same thing happened at 70%, the low light received by the plantlets caused a decrease in the photosynthetic pigment content.

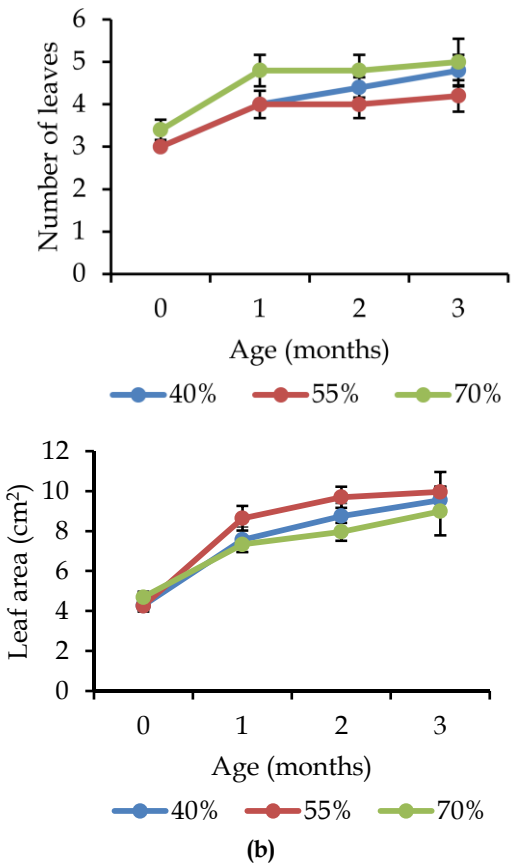
Root Growth. The level of shade had no significant effect (p>0.05) on the number of roots in the ANOVA test. However, there was a significant difference (p<0.05) in the root length of the 40% shade against the 55% shade and 70% on the LSD test (Fig. 4). The high light intensity caused an inhibited root length growth at 40% shade, which caused an increase in transpiration rate, and the absorption process was not optimal. According to Filipović (2020), the lack of water caused an inhibited root growth because there was no flow of nutrients. Edreira and Otegui (2012) stated that high light intensity with no sufficient water availability caused drought stress so that it has an impact on plant productivity. This study was in line with (Lee *et al*., 2017), in *Phalaenopsis* and *Doritaenopsis*, high light intensity inhibited water absorption and root growth. Kircher and Schopfer (2012) also reported that root growth induction depends on the photosynthesis process results. The translocation of sucrose to the roots was the energy for root growth, which did not occur in this study due to a decrease in photosynthetic pigment at 40% shade (Table 1), thus inhibiting sucrose translocation for root growth.

Table 1. The photosynthetic pigment content of hybrid *Phalaenopsis* leaves at the end of the study.

Treatment	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Caroten
40%	311.23[a]	172.18[a]	483.41[a]	75.6
55%	422.59[b]	218.1[a]	640.6[a]	99.8
70%	349.97[a]	185.95[a]	535.91[a]	87.8

The same letter showed no significant (P>0.05) on the ANOVA test. Different letters showed significant differences (P<0.05) in the LSD test. The number value indicates the mean value of ± SE (n=3)

In the trend of root number and length, there was an increasing pattern at all shade levels during the study. The lowest root number and length occurred in the 40% shade (Figure 4). It was suspected that in high light conditions, there was an increase in the rate of transpiration and evaporation of water in the media which disrupted the vegetative growth. Conversely, in low light conditions, the medium became moist, and then the roots could develop to store water, but too much water could increase the leaching of nutrients or asphyxiation of root (Filipović, 2020). Pires *et al*. (2012) reported that low light intensity causes an increase in water use efficiency in *Miltonia avescens* and *Miltonia spectabilis* orchids so that the roots could store water for the photosynthesis process. (Dewir *et al*., 2015) also reported that 30% light intensity resulted in the highest elongation of the main root of *Cattleya* compared to 60% and 90% light intensity. In this study, the lack of light exposure at 55% and 70% shade, so plantlets must maximize the storage by increasing the root growth to optimize the photosynthesis process.



(b)

Figure 2. The growth of hybrid *Phalaenopsis* leaves at different shade levels during the study. a). The Number of leaves, and b). Leaf area. The Bar line indicates the mean ± SE (n=5)

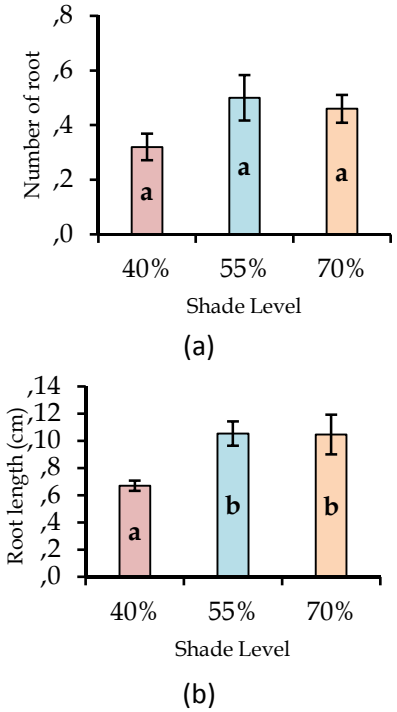


Figure 3. Growth of hybrid *Phalaenopsis* roots at the end of the study. a) Number of roots, and b). Root length. The same letter on the histogram shows no significant influence (p>0.05) on the ANOVA test. The number value indicates the mean value of ± SE (n=5)

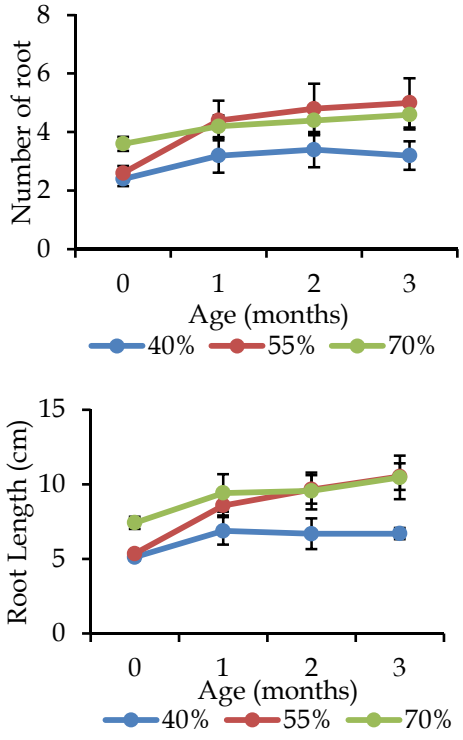


Figure 4. The growth of hybrid *Phalaenopsis* roots at different shade levels during the study. a) Number of roots, and b). Root length. Bar line indicates the mean ± SE (n=5)

Density of Stomata. During acclimatization, there were 3 types of leaf growth (Figure 5). In this study, the level of shade had no significant effect (p>0.05) on the stomatal density of various leaf types (Table 2) which was caused by the plantlets that had not yet been adapted (Table 2). Sihotang (2017) reported that plantlets could adjust to environmental changes by forming stomata. The high light intensity increased the number of stomata. However, in this study, it was assumed that 40% of the shade had not reached high light intensity, so it did not increase the number of stomata. The ambient temperature also affects the stomata density. The ambient temperature in all treatments was almost the same, so the stomatal density was not significantly different. In this study, the maximum temperature at 40% shade is 29°C. Nikmah *et al.* (2017) reported that the temperature was still optimal for *Phalaenopsis* growth, so it was suspected that the CO_2 fixation to 40% shade still supported the photosynthesis process. The stomatal density that was not significantly different was also caused by the shaded and pollutant-free greenhouse environment, so there was no increase or decrease in the stomatal density. Humami *et al.* (2020) reported that the greenhouse environment was surrounded by large trees and it is free of pollution which maintains the microclimate conditions.

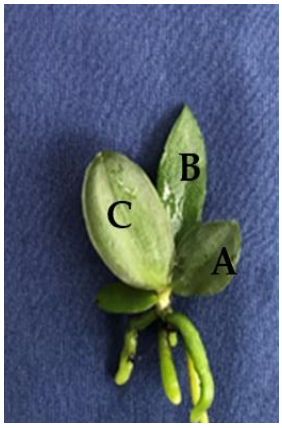


Figure 5. Leaf growth type during the study. a) The leaf that has a small size when *in vitro* and increases in size during *ex vitro*, b). The leaf that is already large *in vitro* and gets bigger during *ex vitro*, and c). The leaf that grows fully during *ex vitro*.

Size of Stomata. The stomata length and the width of leaves A and B at each shade level had no significant differences (p>0.05). However, leaf C at 40% shade was significantly different (p<0.05) against 55% and 70% shade (Table 3), which

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suggests that the stomata on leaves A and B have not functioned optimally. According to Garvita and Wawangningrum (2020), the stomata of leaves *in vitro* were less functional, and the guard cells of leaves A and B were smaller than those of C leaves, thus affecting the opening of the stomata (Figure 6). Priyadi and Hendriyani (2016) reported that *Bulbophyllum echinolabium ex vitro* leaf had a higher number and size of the stomata than *in vitro* leaf, up to five times. The differences in the character of stomata affected the leaf growth. In this study, leaf C had a fast growth (Figure 7), so it was necessary to open the stomata in order to the CO_2 absorption as photosynthetic material to support their growth. However, leaves A and B had a slow growth. Leaf A and B were *in vitro* leaves so that a new leaf covered the leaf position (*ex vitro*), so it was suspected that the stomata did not respond to light. (Shin *et al*., 2014) reported that *Doritaenopsis* had larger *ex vitro* leaves, so the *in vitro* leaves were shaded. *In vitro* leaves also had less functional stomata, few leaf cuticles (Garvita and Wawangningrum, 2020) and thin mesophyll (Zhang *et al*., 2017) These factors inhibited the growth of leaves A and B in this study. Leaves A and B had the slowest growth rate than leaves C (Figure 7).

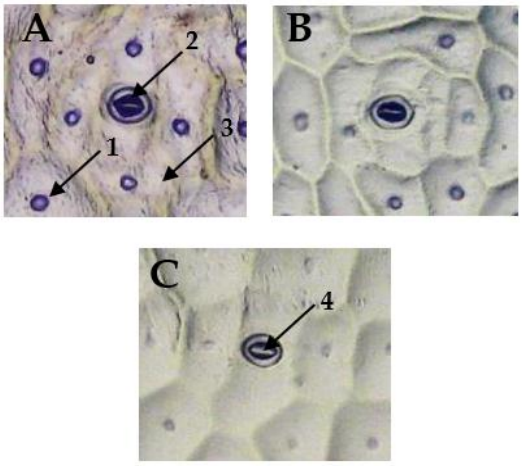

Figure 6. Stomata at a shade level of 40% at the end of the study. A). Porus leaf A closes, B). Porus leaf B closes. C). Porus leaf C opens. Description: 1). cell nucleus, 2). Guard cells, 3). The Epidermis, 4). Porus

Table 2. The density of leaf stomata at different shade levels and leaf types during the study

Treatment	Leaf A	Leaf B	Leaf C
40%	0.1380[a]	0.1602[a]	0.1454[a]
55%	0.1018[a]	0.1356[a]	0.1549[a]
70%	0.1502[a]	0.1648[a]	0.1503[a]

The same letter indicates no significant influence (p>0.05) on the ANOVA test. The number value indicates the mean value of ± SE (n =3)

Table 3. The size of the leaf stomata at different shade levels and leaf types during the study

Treatment	Stomata Length (µm)			Stomata Width (µm)		
	Leaf A	Leaf B	Leaf C	Leaf A	Leaf B	Leaf C
40%	236.1[a]	241.4[a]	260.56[b]	177.48[a]	185.97[a]	201.96[b]
55%	215.31[a]	244.7[a]	233.20[a]	164.23[a]	182.14[a]	166.44[a]
70%	236.64[a]	250.55[a]	234.65[a]	164.55[a]	196.23[a]	182.2[a]

The same letter indicates no significant influence (p>0.05) on the ANOVA test. The number value indicates the mean value of ± SE (n = 10)

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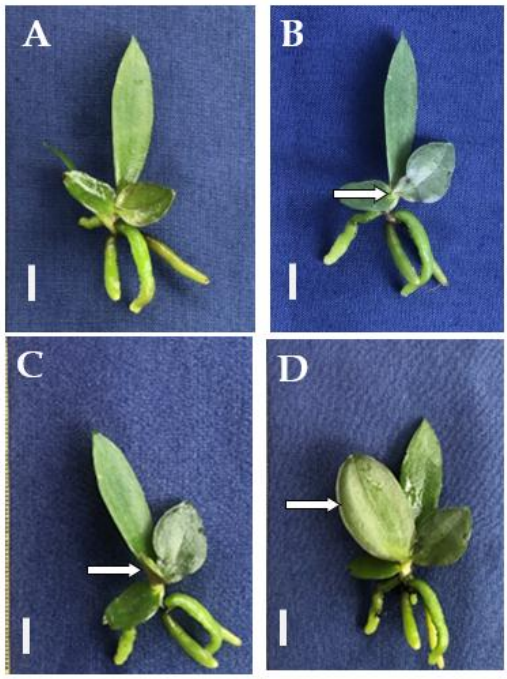

Figure 7. *Ex vitro* leaf growth at a shade rate of 40% during the acclimatization stage. A) Early growth, B). 1st Month, C). 2nd Month, D). 3rd Month. Arrows show *ex vitro* leaves that are undergoing growth

Fresh Weight. The fresh weight of plantlets in each shade showed no significant effect ($p>0.05$) at the end of the study (Figure 8). However, there was an increase compared to the fresh weight at the beginning of the study. Fresh weight was the accumulation of photosynthate and water content (Wu and Lin, 2013). The sunlight, water, and nutrients affected the photosynthate and water content (Febriyono *et al*., 2017). In this study, the growth of leaves and roots did not differ significantly, so it did not affect the fresh weight produced. Based on the results of the fresh weight, it could be interpreted that leaf growth, root growth, and stomata character still supported the development of plantlet photoautotrophs at different light intensities. Shin *et al*. (2014), reported that the ability of photoautotrophs in hybrid *Doritaenopsis* orchids affected the plantlet survival during acclimatization. This was in line with this study, during the acclimatization period, each shade level had a 100% survival rate. da Silva *et al*. (2017) reported that the root system and stomata function in *Dendrobium* plantlets support survival during the acclimatization period. Cha-um *et al*. (2010), reported that on *Phalaenopsis* plantlets, the role of stomata affected the rate of photosynthesis so that plantlets could adapt to *ex vitro* conditions.

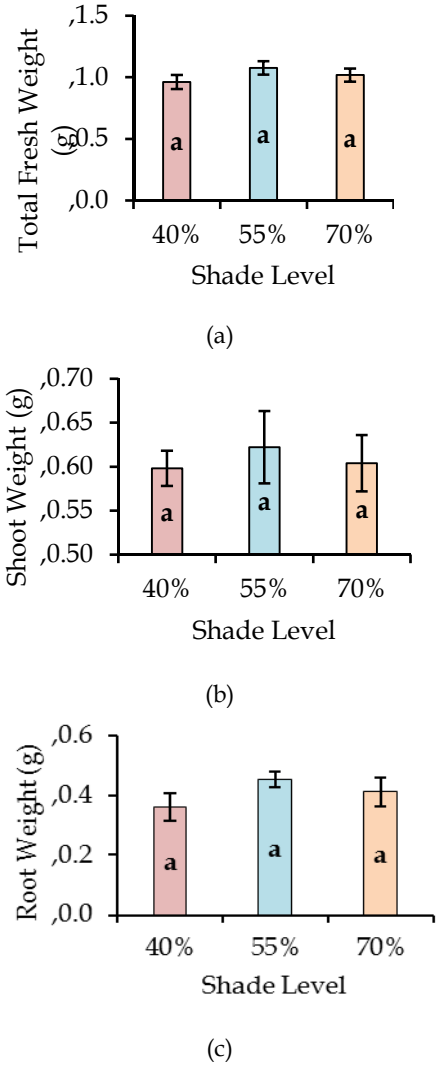

Figure 8. The fresh weight of hybrid *Phalaenopsis* at the end of the study. a). Total fresh weight, b). The weight of the title, and c). The weight of the roots. The same letter indicates an insignificant influence ($p>0.05$) on the ANOVA test. The number value indicates the mean value of ± SE (n=5)

Conclusion

Based on the results of the study, it could be concluded as follows:

1. The level of shade did not affect leaf growth, roots, fresh weight and stomata density. However, it affected the content of photosynthetic pigments as well as the size of stomata in *ex vitro* leaf types in hybrid *Phalaenopsis* orchids.

2. Shade level of 55% resulted in the most optimal growth of *Phalaenopsis* hybrid plantlets.

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