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Analysis of Chlorophyll *Phalaenopsis amabilis* (L.) Bl. Results of the Resistance to *Fusarium oxysporum* and Drought Stress

Endang Nurcahyani[1], Sumardi[1], Hardoko Insan Qudus[2], Asma Palupi[1], Sholekhah[1]

[1]*Master in Biology Study Program, Faculty of Mathematics and Natural Sciences, University of Lampung, Lampung, Indonesia*
[2]*Master in Chemistry Study Program, Faculty of Mathematics and Natural Sciences, University of Lampung, Lampung, Indonesia*
Corresponding Author: Endang Nurcahyani

Abstract: *Phalaenopsis amabilis* (L.) Bl. *is a native orchid from Indonesia and one of Indonesia's national flowers, included in the list of endangered species. P. amabilis is also one of the orchid plants that is in high demand by various community groups, but P. amabilis production in Indonesia is still lagging behind other countries such as Thailand, Taiwan, Singapore and Australia.The obstacle faced in the growth of the orchid of the month is fusarium wilt caused by Fusarium oxysporum (Fo) which until now has not been effectively overcome. Aside from disease, inadequate water availability is a problem for orchid farmers.Drought stress in plants can result in slow increase in leaf area and affect stomata or photosynthesis in leaves and at mild to moderate levels can reduce plant productivity. The use of high yielding varieties that are resistant to Fo and drought with high yields is an important alternative for disease control and drought stress and does not cause negative impacts.The purpose of this study was to determine the specific expression character of P. amabilis leaflets resistant to fusaric acid (FA) and drought stress resistant in vitro including levels of chlorophyll a, chlorophyll b, and total chlorophyll. This study uses P. amabilis orchid plantlet with 5 levels of fusaric acid concentration, namely 0 ppm, 10 ppm, 20 ppm, 30 ppm, and 40 ppm, and consists of two factors, namely factor A: PEG 6000 concentration consisting of 3 treatments, namely 0% (A1), 5% (A2) and 10% (A3) and factor B: atonic solution consisting of 3 levels of treatment namely 0 mL / L (B1), 2 mL / L (B2) and 3 mL / L (B3).The results showed that the highest chlorophyll a, chlorophyll b, and total chlorophyll content of P. amabilis leaflets were at 40 ppm AF concentration and the lowest at 0 mL / L and PEG 6000 atonic treatment combinations with a concentration of 10%.*

Keywords: *drought stress, Fusarium oxysporum (Fo), Phalaenopsis amabilis* (L.) Bl.

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I. Introduction

Orchidaceae is a family of very large flowering plants, with at least 20,000 species and 735 genera scattered throughout the world, especially in the equatorial region. The most popular type of orchid on the market is *Phalaenopsis amabilis* or known as the moon orchid (Rodica *et al*., 2011). The moon orchid is one of Indonesia's national flowers established by Presidential Decree No. 4/1993, as Puspa Pesona, in addition to jasmine (*Jasminum sambac* L.) as the nation's puspa, and giant padma flowers (*Rafflesia arnoldii* R. Br.) as a rare puspa (Puspitaningtyas and Mursidawati, 2010). Promising economic value makes moon orchids much hunted in nature that threatens their sustainability, so that the conservation status of moon orchids based on IUCN is endangered (Stephan *et al*., 2018).

The obstacle faced in the cultivation of orchids is a disruption in the form of a disease that can make plants damaged and die. Several phalaenopsis fungal diseases have been reported in Taiwan, including diseases caused by *Fusarium oxysporum* (*Fo*), *F. solani*, and *F. proliferatum* (Chung *et.al*., 2011). *Fo* causes fusarium wilt which interferes with the growth of orchids (Djatnika, 2012). In the United States, fusarium wilt can cause crop death and decrease production by more than 50% and control with fungicides has not been able to overcome the disease (Wedge and Elmer, 2008).

Beside from disease, inadequate water availability is a problem for orchid farmers. Drought occurs almost every year, so that it can become a major barrier to plant growth caused by the level of drought. Drought stress in plants can result in slow increase in leaf area and photosynthesis, and can reduce plant productivity (Nio *et al*., 2006).

Efficient, effective and safe ways to control disease, including using resistant varieties. The use of high yielding varieties that are resistant to fo and drought is an important alternative for disease control and drought

stress and does not cause negative impacts (Nurcahyani *et al*., 2016a; Nurcahyani *et al*., *2016*b; Nurcahyani *et al*., 2017 ; Azhari *et al*., 2018; Rosyalina *et al*., 2018; Nurcahyani *et al*., 2019). The development of *Fo*-resistant plantlet varieties can be carried out among others by the in vitro selection method which is culturing explants in the form of tissue or organs on a medium containing selective fusaric acid concentration (Nurcahyani *et al*., 2016a; Nurcahyani *et al*., 2016b; Nurcahyani *et al*., 2014 ; Nurcahyani *et al*., 2017; Nurcahyani *et al*., 2019), while research into the development of drought-resistant stress plantlet was carried out by inducing Polyethylene Glycol (PEG) with a molecular weight of more than 4000 in the in vitro selection medium (Azhari *et al*., 2018; Rosyalina *et al*., 2018).

Fusaric acid (FA) is a metabolite produced by several fungal species of the genus fusarium. FA chemically called 5-n-butylpicolinic acid. This acid can be toxic (concentrations of more than 10^{-5} M), so that it inhibits growth and regeneration of the culture, but at non-toxic concentrations (below 10^{-6} M) it helps to induce phytoalexin synthesis, a form of plant response to inhibit pathogenic activity (Bouizgarne *et.al*., 2006). The in vitro selection approach is reported to have produced resistant varieties in vanilla plantlet (Nurcahyani *et al*., 2012), *Arabidopsis thaliana* (Bouizgarne *et al*., 2006), and *Dendrobium sonia* (Dehgahi *et.al*., 2015).

Polyethylene Glycol is a chemical compound containing ethylene oxide sub unit matrix activity that is able to reduce osmotic potential by binding to water molecules using hydrogen bonds. Giving of PEG to plantlet aims to produce drought stress conditions due to reduced availability of water in plants (Rahayu *et al*., 2005). In vitro selection has been investigated in producing plants resistant to drought stress including hybrid rice plants using concentrations of PEG 5%, 10%, 15%, 20% and 25% (Afa *et al*., 2012); peanut at 10% PEG concentration (Adisyahputra *et al*., 2004); tomatoes with concentrations of PEG 5%, 10%, 15% 20% (Harahap *et al*., 2013); keprok batu 55 orange plantlet (*Citrus reticulata* Blanco var. *crenatifolia*) on atonic combinations 1mL / L, PEG 3% (Azhari *et al*., 2018) and pontianak citrus plantlet (*Citrus Nobilis* Lour. Var. *microcarpa* Hassk.) in atonic combinations 3 mL / L and 4% PEG by in vitro(Rosyalina *et al*., 2018).

Chlorophyll is a pigment that plays an important role in the process of photosynthesis, consisting of chlorophyll A and chlorophyll B as supplementary pigments (Gross, 1991). There are three main functions of chlorophyll, namely utilizing solar energy, triggering the fixation of CO_2 into carbohydrates and providing an energetic basis for the ecosystem as a whole. This study aims to determine the specific expression of *P. amabilis* plantlet which resistant on *Fo* and drought by in vitro including levels of chlorophyll A, chlorophyll B, and total chlorophyll.

II. Material and Methods

The tools used in this study are*autoclave*, *Laminar Air Flow Cabinet* (LAF), spectrophotometer, a 250 ml culture bottle, 100 ml and 500 ml volume measuring cups, 10 cm diameter petri dishes, aluminum foil, tweezers, scalpels, scalpel blades, tip pipettes, micropipets, test tubes, test tube racks, hot plates, Ohaus analytical scales, petridish, tissue, paper label and camera. The materials used in this study were the *Phalaenopsis amabilis* (L.) Bl. obtained from the Borobudur Orchid Garden in Magelang, pure fusaric acid produced by *Sigma Chemical Co*. {*Fusaric acid (5-butylpicolinic acid*) from Giberellafujikuroi}, 70% alcohol, sucrose, Hydrochloric Acid (HCl), Potassium Hydroxide (KOH), distilled water., Polyethylene Glycol (PEG 6000) (0%, 5%, 10%) , atonic solution (0 mL / L, 2 mL / L, 3 mL / L), and the Vacin and Went medium.

Procedure

The medium used is Vacin and Went (VW), the medium is sterilized for 15 minutes. The sterilized VW medium is then added fusaric acid (FA) with a concentration of 0 ppm (control), 10 ppm, 20 ppm, 30 ppm, and 40 ppm for selection of disease resistance.The explants used were sterile plantlets. Planlets from culture bottles were removed with sterile scalpels and one by one placed on a 10 cm diameter petri dish, then plantlets were planted in each culture bottle containing the specified treatment medium. Each concentration was done 3 times and each repetition consisted of 2 *P. amabilis* explants in each culture bottle.

For selection of VW medium drought stress resistance added Polyethylene Glycol (PEG 6000) with concentrations of 0%, 5%, and 10%. Atonic stock solutions are first dissolved with distilled water at certain concentrations of 0 mL/L, 2 mL/L, and 3mL/L. *P. amabilis* roots are soaked with an atonic solution first for one minute.Then the explants were planted in each culture bottle.Each concentration was done 3 times and each repetition consisted of 2 *P. amabilis* explants in each culture bottle.

The material used in the analysis of chlorophyll content is *P. amabilis* plantlet leaves which have been induced by AF, as well as induced atonic solutions and selected with PEG 6000. Chlorophyll analysis on resistance to Fo using the Harbourne method (1987) and using the Miazek method (2002) on drought stress resistance.*P. amabilis* plantlet leaves which were identical to 0.1 g were crushed with mortar (pestle), then added 10 mL 80% acetone. Then, the solution was filtered with Whatmann No.1 paper, and put in a flakon and then tightly closed. Sample solution and standard solution (80% acetone) were taken as much as 1 mL, and put in a cuvette.

Absorption readings by UV spectrophotometer at wavelengths (λ) 646 nm and 663 nm for disease resistance selection, with repetition of each sample 3 times. Chlorophyll content was calculated using the following formula.:

Total chlorophyll = 17,3 λ_{646} + 7,18 λ_{663} mg/l
Chlorophyll a = 12,21 λ_{663} – 2,81 λ_{646} mg/l
Chlorophyll b = 20,13 λ_{646} – 5,03 λ_{663} mg/l

Note:
A646 = absorbance at a wavelength of 646 nm
A663 = absorbance at a wavelength of 663 nm

For selection of drought stress resistance, absorption readings with UV spectrophotometer at wavelengths (λ) 649 nm and 665 nm, with 3 repetitions per sample. Chlorophyll content was calculated using the following formula:

Chlorophyll a = 13.36 A665 – 5.19 A649$\left(\frac{v}{w\ x\ 1000}\right)$
Chlorophyll b = 27.43 A649 – 8.12 A665$\left(\frac{v}{w\ x\ 1000}\right)$
Total chlorophyll = 22.24 A649 – 5.24 A665$\left(\frac{v}{w\ x\ 1000}\right)$

Note:
A665 = absorbance at a wavelength of 665 nm
A649 = absorbance at a wavelength of 649 nm
V = volume of ethanol
W = leaf weight

III. Results and Discussion

The effect of giving FA as an inducer on *P. amabilis* plantlet can be known through the chlorophyll content of the plantlet. The chlorophyll content of *P. amabilis* plantlet was observed by comparing the plantlet without FA and the plantlet which was induced using FA with concentrations of 10 ppm, 20 ppm, 30 ppm, and 40 ppm. Analysis of chlorophyll content in this study using the Harbourne method (1987).

The addition of FA to VW medium with various concentrations significantly affected the content of chlorophyll A, chlorophyll B, and total chlorophyll of *P. amabilis* plantlet. The results of the analysis of *P. amabilis* plantlet chlorophyll content known to be an increase in the content of chlorophyll A, chlorophyll B, and total chlorophyll. The increased chlorophyll content of *P. amabilis* plantlet occurs along with the increased concentration of FA given. The results of the analysis showed that the mean comparison of total chlorophyll A, chlorophyll B, and total chlorophyll content between controls with the four concentrations of FA was significantly different. The results showed that the highest chlorophyll A, chlorophyll B, and total chlorophyll content of *P.amabilis* plantlet were at the concentration of FA at 40 ppm. The results of the analysis of the content of chlorophyll A, chlorophyll B, and total chlorophyll *P. amabilis* plantlet by planting in Vacin & Went (VW) medium added with FA with various concentrations are presented in Table 1.

Table 1The chlorophyll content of *P.amabilis* plantlet results of induced with fusaric acid

Fusaric Acid Concentration(ppm)	Chlorophyll A content (mg/g tissue)	Chlorophyll B content (mg/g tissue)	Chlorophyll total content (mg/g tissue)
0 (control)	0,053±,003180[b]	0,067±,002728[b]	0,119±,006028[b]
10	0,262±,018889 [a]	0,122±,018448[a]	0,397±,025621[a]
20	0,244±,026577 [a]	0,129±,005044[a]	0,404±,028416[a]
30	0,293±,016737 [a]	0,137±,004177[a]	0,429±,020851[a]
40	0,324±,015875 [a]	0,164±,004177[a]	0,488±,021835[a]

Note: Numbers followed by the same letter, not significantly different at 95% confidence level

Chlorophyll is a very important part in a plant. Chlorophyll plays a role in the process of photosynthesis, with the main function of utilizing solar energy, and processing it into carbohydrates. In theory, healthy plants will continue to produce chlorophyll as plants age, but due to several factors the presence of chlorophyll will decrease. When all environmental factors are in the right conditions, the presence of chlorophyll will be very high in a plant. When the presence of chlorophyll in a plant is low, it can be explained that the presence of pathogens or plant-disturbing organisms that interfere with plant physiology. Increase or decrease in the value of chlorophyll content can indicate the level of resistance of a variety of downy mildew in

maize (Agustamia *et al*., 2016). The results of chlorophyll content analysis in this study are in line with research conducted by Andari and Nurcahyani (2018) and Isharnani, *et al.* (2015) showed an increase in the content of chlorophyll A, chlorophyll B, and total chlorophyll *Spathoglottis plicata* plantlet with increasing concentration of FA.

Chlorophyll A, chlorophyll B, and total chlorophyll levels in atonic and PEG treatments with various concentrations obtained the lowest results in the combination of atonic treatments 0 mL/L and PEG 6000 with a concentration of 10%, this shows that plants are able to defend themselves under stress conditions drought. The chlorophyll content in the leaves will affect photosynthesis. Little chlorophyll levels certainly will not make the maximum photosynthesis reaction. Lack of water will cause absorption of nutrients is inhibited, so that affects the availability of elements N and Mg which play an important role in the synthesis of chlorophyll (Syafi, 2008). The results of this study are in line with studies of Ai (2011) and Banyo *et al*. (2013) showed that the chlorophyll content decreased under water deficit conditions in ginger and rice plants. Previous studies have shown that water deficit induced by PEG 8000 with potential water media (WP) -0.25 and -0.5 MPa reduces the total chlorophyll and chlorophyll A content in local rice in North Sulawesi (Nio *et al*., 2019). The results of the analysis of chlorophyll A, chlorophyll B and total chlorophyll in atonic and PEG treatments with various concentrations are presented in Tables 2, 3, and 4.

Table2. Chlorophyll A content of *P. amabilis* plantlet in a combination of atonic and PEG 6000

PEG (%)	Atonic (mL/L) (v/v)		
	0	**2**	**3**
0	0,060 ±0,00890[a]	0,031±0,00503[a]	0,040 ±0,00219[ab]
5	0,038 ±0,00229[ab]	0,050 ±0,00313[ab]	0,043 ±0,00794[ab]
10	0,027 ±0,00093[b]	0,049 ±0,00263[ab]	0,049 ±0,00980[ab]

The highest chlorophyll A content in *P. amabilis* plantlet is found in the combination of atonic treatment 0 mL/L and PEG 6000 concentration of 0%, while the smallest chlorophyll A content in the combination of atonic treatment is 0 mL/L and PEG 6000 with a concentration of 10%.

Table3. Chlorophyll B content of *P. amabilis* plantlet in a combination of atonic and PEG 6000

PEG (%)	Atonic (mL/L) (v/v)		
	0	**2**	**3**
0	0,030±0,00439[a]	0,020±0,00083[a]	0,022±0,00103[a]
5	0,021±0,00232[a]	0,024±0,00152[a]	0,028±0,00185[a]
10	0,020±0,00080[a]	0,025±0,00071[a]	0,025±0,00315[a]

The highest chlorophyll B content in *P. amabilis* plantlet is found in the combination of atonic treatment of 0 mL/L and PEG 6000 concentration of 0%, while the smallest chlorophyll B content in the combination of atonic treatment is 0 mL/L and PEG 6000 with concentration of 10%.

Table4. Chlorophyll Total content of *P. amabilis* plantlet in a combination of atonic and PEG 6000

PEG (%)	Atonic (mL/L)		
	0	**2**	**3**
0	0,035±0,00251[a]	0,028±0,00080[b]	0,027±0,00110[b]
5	0,025±0,00091[bc]	0,022±0,00393[cd]	0,021±0,00252[cd]
10	0,019±0,00089[d]	0,019±0,00215[d]	0,027±0,00368[b]

The highest total chlorophyll content in *P. amabilis* plantlet is found in the combination of atonic treatment 0 mL/L and PEG 6000 concentration of 0%, while the smallest chlorophyll b content in the combination of atonic treatment is 0 mL / L and PEG 6000 with a concentration of 10%.

Chlorophyll is the main component of chloroplasts for photosynthesis, and the relative chlorophyll content has a positive relationship with the rate of photosynthesis. In general, the higher the chlorophyll content, the higher the rate of photosynthesis (Anjum *et al*., 2011). Water deficit will affect changes in metabolic function, especially reducing the synthesis of chlorophyll pigments (Jaleel *et al*., 2009). Decrease in leaf chlorophyll concentration is one of the physiological responses of plants to the lack of water that causes inhibition of chlorophyll formation, decreased rubisco enzyme, and inhibition of nutrients, especially nitrogen and magnesium which play an important role in chlorophyll synthesis (Ai and Banyo, 2011).

Conclusion

Based on the results of the study, scaling resistance by using fusaric acid of 10, 20, 30 and 40 ppm can increase the content of chlorophyll a, b, and total *P. amabilis* plantlets, with the highest yield at 40 ppm fusaric acid

concentration. Then for the selection of drought stress resistance, the lowest chlorophyll a, chlorophyll b and total chlorophyll content in the combination of atonic treatment is 0 mL/L and PEG 6000 with a concentration of 10%.

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