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Effects of mannitol-induced drought stress on the growth of *Phalaenopsis amabilis* (L.) blume plantlets under in vitro conditions

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Abstract

Phalaenopsis amabilis (L.) Blume is an ornamental orchid with high aesthetic and economic value and is recognized as the national flower of Indonesia. However, its growth and physiological performance are highly susceptible to drought stress. This study aimed to evaluate the effects of mannitol-induced drought stress on the growth of *P. amabilis* plantlets under in vitro conditions and to determine the highest mannitol concentration that could be tolerated. Mannitol was applied as an osmotic agent at five concentrations (0, 50, 100, 150, and 200 mM) in Vacin and Went (VW) medium using a completely randomized design with five replications. The evaluated parameters included plantlet survival percentage, morphological characteristics, chlorophyll content, and stomatal density. Data were analyzed using analysis of variance (ANOVA) at the 5% significance level, followed by *Tukey's* test when significant differences were detected. The results showed that all treatments maintained a 100% plantlet survival rate throughout the four-week culture period. Mannitol application had no significant effect on morphological characteristics or chlorophyll content. In contrast, stomatal density was significantly reduced with increasing mannitol concentrations. Plantlets cultured on medium containing 50 mM mannitol exhibited the greatest tolerance and maintained normal growth, whereas concentrations of 150–200 mM induced more pronounced drought stress responses. These findings indicate that 50 mM mannitol is the optimum concentration for simulating mild drought stress while maintaining the growth of *P. amabilis* plantlets under in vitro conditions.

Keywords: *Phalaenopsis amabilis*; Drought Stress; In Vitro Culture; Mannitol; Osmotic Stress.

1. Introduction

The *Phalaenopsis amabilis* (L.) Blume orchid is a group of ornamental plants that has extraordinary appeal due to the beauty of its shape, color, and variety of flowers. Taxonomically, orchids belong to the Orchidaceae family, one of the largest families in the world of flowering plants. Indonesia as a tropical country with a high level of biodiversity is home to an abundant wealth of orchid species [1]. Ecologically, natural orchids have an important role in maintaining the balance of tropical rainforest ecosystems, especially because of their diverse life patterns, ranging from epiphytes to lithophytes. [2].

Drought is a condition where there is a lack of water in the soil, which can ultimately result in plant failure [3]. In vitro culture methods are currently widely used in orchid propagation to increase plant propagation capacity. In vitro-cultivated orchids are always susceptible to environmental conditions, requiring adaptation or adaptation to the climate. Mannitol is used as a solution to maintain cell stability and regulate water potential in plant media. Mannitol is a sugar alcohol that controls the osmotic potential of the growing medium and stimulates drought stress conditions [4].

Conducted a study on the effect of osmotic stress induced by mannitol on the physiological and biochemical responses of tea plants (*Camellia sinensis* L.) under in vitro culture conditions. In this study, mannitol was used as a water potential

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reducing compound to create drought conditions so that the plantlets experience osmotic stress. Various mannitol concentrations of 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 300 mM were applied to observe the tolerance limits of plant tissues to stress that gradually increased. The results obtained showed that increasing mannitol concentrations were associated with significant changes in several physiological parameters, such as decreased chlorophyll content, changes in antioxidant enzyme activity, and decreased tissue growth. Mannitol is effectively used as a stress-inducing agent in the study of plant resistance to water shortage conditions. Plant adaptation mechanisms emerge when under prolonged osmotic stress, especially in plant tissue culture systems [5,6].

This study was conducted to determine how variations in mannitol concentration affect the growth of moon orchid plantlets. Observations were made on parameters such as the percentage of surviving plantlets, morphology (number of leaves, root length, stem height), stomatal content, and chlorophyll content, which can indicate the level of plantlet adaptation to stress conditions. This study aimed to determine the mannitol concentration that can still be tolerated by plantlets and the level of stress that causes growth inhibition.

2. Material and methods

2.1. Equipment and Materials

The tools used in this study include culture bottles, Laminar Air Flow Cabinet (LAF), autoclave, tweezers, analytical balance, measuring cylinder, measuring flask, beaker glass, volumetric pipette and sterile pipette, petri dish, cover glass, UV spectrophotometer, spatula, stirring rod, micropipette, test tube rack, syringe, funnel, mortar and pestle, scalpel, scissors, weighing paper, label paper, clear tape, object glass, microscope, aluminum foil, bunsen, magnetic stirrer, Optilab camera, and documentation tool (cell phone camera).

The materials used in this study included *Phalaenopsis amabilis* (L.) Blume orchid plantlets, ready-to-use Vacin and Went (VW) media for 500 mL of media, sucrose, agar, 70% alcohol, sterile distilled water, 80% acetone, 1 N KOH solution, 1 N HCl solution, mannitol ($C_6H_{14}O_6$), Whatman No. 1 paper, litmus paper, transparent nail polish, sodium hypochlorite, rubber bands, plastic wrap, sterile tissue .

2.2. Experimental Design

This study used a completely randomized design (CRD) with five levels of mannitol concentration: 0 mM, 50 mM, 100 mM, 150 mM, and 200 mM. Each treatment was replicated five times. Observed parameters included the number of viable plantlets, chlorophyll content, morphology such as roots, stems, and leaves, and stomatal density.

2.3. Procedure

2.3.1. Sterilization of Equipment and Work Area

All glass and metal instruments were washed with detergent, dried, then wrapped and sterilized in an autoclave at 121°C for 15–20 minutes. Tweezers and scalpels were sterilized by immersing them in 70% alcohol and heating them in a Bunsen burner. The work area was sterilized with a UV lamp in Laminar Air Flow (LAF) for approximately 30 minutes, then the table was cleaned using 70% alcohol with sterile tissue.

The work area was sterilized inside a laminar airflow (LAF) cabinet. The ultraviolet (UV) lamp was switched on for 30 min prior to use and then turned off before the blower and fluorescent light were activated. The working surface of the

LAF cabinet was sprayed with 70% ethanol and wiped with sterile tissue paper to ensure aseptic conditions before inoculation.

2.3.2. Preparation of Mannitol Stock Solution

A mass of 18.22 g of mannitol was weighed using an analytical balance to prepare the stock solution. The mannitol was then dissolved in approximately 70 mL of sterile distilled water and homogenized until completely dissolved. After dissolving, sterile distilled water was added to the solution until a final volume of exactly 100 mL was reached. The mannitol solution was then stored in a sterile bottle and labeled with the concentration and date of preparation.

2.3.3. Preparation of Growing Media

The basic Vacin and Went (VW) medium was prepared with 1.63 g of VW medium in distilled water, then added 15 g/L of sucrose and 2 g of activated charcoal. The pH of the medium was adjusted to 5.6-5.8 using HCl or KOH. After that, 7 g

of agar was added and the medium was heated until homogeneous. The medium was then divided into five mannitol concentration treatments (0 mM, 50 mM, 100 mM, 150 mM, 200 mM), each amounting to 200 ml, then sterilized using an autoclave at a temperature of 121°C and a pressure of 1 atm for 15 minutes [7-8].

2.3.4. Preparation for Planting Plantlets

Moon orchid plantlets were aseptically sterilized using 0.5% sodium hypochlorite for three minutes, then rinsed with sterile distilled water three times. Plantlets were planted on VW media with different mannitol concentrations, one plantlet per culture bottle. The cultures were stored on a culture rack with 16 hours of light and 8 hours of darkness [9].

2.4. Observations Parameters

Observations were made on four-week-old moon orchid plantlets after planting. Growth observations were conducted to determine the plantlets' response to mannitol-induced drought stress, with parameters such as the percentage of surviving plantlets, chlorophyll content, and morphological characteristics such as roots, stems, and leaves, and stomatal density.

2.4.1. Plantlet Survival and Visual Appearance

Observations on the survival percentage of moon orchid plantlets were carried out every week until they reached week 4. The level of plantlet viability was calculated based on the percentage of plantlets that were able to survive in each plantlet.

$$\%\ Live\ planlets = \frac{\text{Number of living plantlets}}{\text{Total number of planlets}} \times 100\ \%$$

2.4.2. Morfologis (roots, stems, and leaves)

Morphological observations were conducted to assess the vegetative growth response of plantlets to various concentrations of mannitol administered as drought stress treatments. Parameters observed included roots, stems, and leaves as the main indicators of growth.

2.4.3. Stomatal Density

Stomata preparations on moon orchid leaves were made using the replica method. The leaves were washed thoroughly and dried using tissue. Clear nail polish was applied to the underside, middle, and tip of the leaves, then air-dried. Clear tape was attached to the nail polish-treated parts of the leaves, the tape was slowly removed, and the specimens were placed on a glass slide for observation using a B-510-2F optical microscope at 100x magnification. The stomatal density value was calculated using the formula*:*

$$KS = \frac{\text{Number of Stomata}}{\text{Wide field of view } (\text{mm}^2)}$$

2.4.4. Chlorophyll Content

Chlorophyll content analysis was carried out using the extraction method using 96% ethanol. 0.1 g of leaf samples were extracted, then the absorbance was measured using a UV spectrophotometer at wavelengths of 648 mm and 664 mm.

2.5. Data Analysis

Data obtained from the growth of moon orchid plantlets consisted of quantitative and qualitative data. Qualitative data were presented descriptively and comparatively and supplemented with documentation in the form of photos of plantlet morphology, such as root, stem, and leaf length, leaf color changes related to chlorophyll content, and photos of stomatal preparations from microscopic observations. Meanwhile, quantitative data were analyzed using analysis of variance (ANOVA) at a 5% significance level. If the analysis results showed significant differences, further *Tukey's* test was performed at a 5% confidence level to determine the average difference between treatments.

3. Results

3.1. Percentage of Live Plantlets and Plantlet Visualization

Observations on the number of live orchid plantlets were conducted from the first week to the fourth week of planting. Observations on orchid plantlets grown using mannitol on VW medium were conducted at concentrations of 0 mM (control), 50 mM, 100 mM, 150 mM, and 200 mM. The percentage of live plantlets and visualization of orchid plantlets are presented in **tables 1** and **table 2**.

Table 1 Percentage of the Number of Living Moon Orchid Plantlets

Mannitol Concentration (mM)	**Percentage of Number of Plantlets per Week (%)**			
	I	II	III	IV
0	100	100	100	100
50	100	100	100	100
100	100	100	100	100
150	100	100	100	100
200	100	100	100	100

Based on the results in **Table 1**, it is known that with concentrations of 0 mM (control), 50 mM, 100 mM, 150 mM, 200 mM, the survival rate of the planlets was 100% until the 4th week.

Table 2 Visualization of Moon Orchid Plantlets with Various Concentrations

Mannitol Concentration (mM)	**Visualization of the Number of Plantlets per Week (%)**							
	I		II		III		IV	
	H	HK	H	HK	H	HK	H	HK
0	100	0	100	0	100	0	100	0
50	100	0	100	0	100	0	50	50
100	100	0	100	0	50	50	30	70
150	100	0	30	70	20	80	20	80
200	20	80	20	80	10	90	10	90

Description: H: Green; HK: Yellowish Green

Based on **Table 2**. visualization of plantlets tends to change along with increasing mannitol concentration, plantlets at 0 mM (control), and 50 mM are still green (H) until week 3, but show yellowish green in week 4. However, at a concentration of 100 mM, the color change from green (H) to yellowish green began to appear in the third week. Meanwhile, the 150 mM treatment showed a change to yellowish greenish yellow (HK) since the second week. At a concentration of 200 mM, the plantlets experienced a decrease from leaf green (H) to yellowish green (HK) and in the first week to the fourth week the decline in visualization quality became increasingly apparent.

Plantlets that were able to survive showed better visual conditions, while plantlets that experienced stress showed visual changes in color and tissue condition as presented in **Figure 1**.

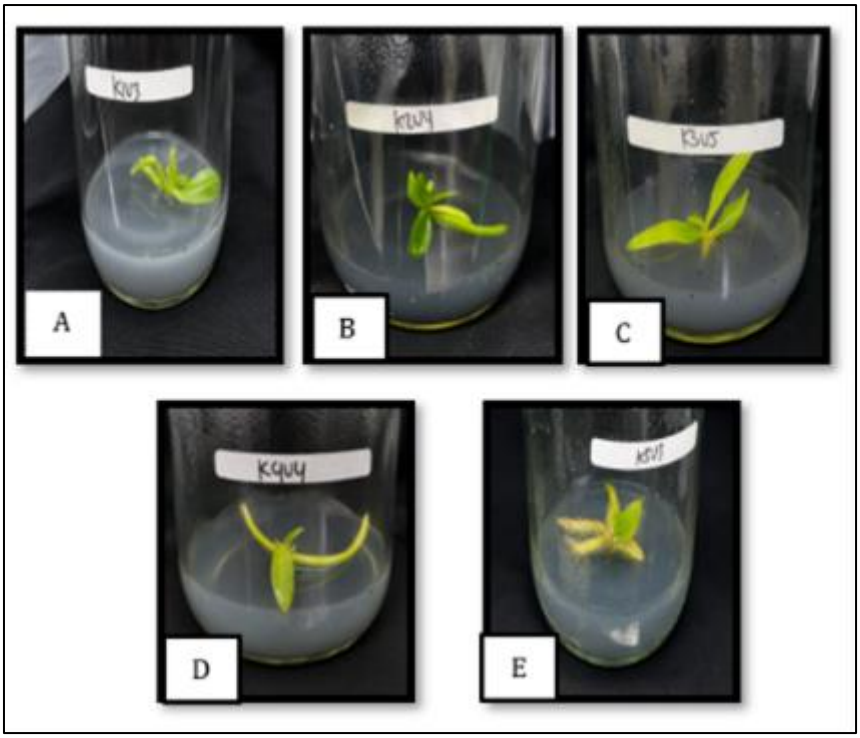

Figure 1 Moon orchid plantlets *Phalaenopsis Amabilis* (L.) Blume grown on VW media with manito concentration in the 4th week. A. Concentration 0 mM, B. Concentration 50 mM, C. Concentration 100 mM, D. Concentration 150 mM, E. Concentration 200 mM.

3.2. Morfologis (roots, stems, and leaves)

Measurements of root length, stem height, and leaf blades indicate that the application of mannitol at various concentrations had no significant effect. Based on the results of observations that have been made, the effect of mannitol concentration on the morphology of moon orchid plants is presented in **Table 3.**

Tabel 3 The Effect of Mannitol Concentration on The Morphology of Moon Orchid

Mannitol Concentration (mM)	**Morphology of the Moon Orchid**		
	Root Length (cm)	Stem Height (cm)	Number of Leaves (strands)
0	2.34	0.5	2
50	2.22	0.44	3
100	2.5	0.5	2
150	2.62	0.26	2.4
200	2.18	0.28	1.8

Based on **Table 3**, the administration of mannitol at various concentrations showed differences in plant growth responses, as seen in root length, stem height, and leaf number. However, the ANOVA test results showed that these differences were not significant. Each mannitol concentration was considered not to have a significant effect on the treatment.

3.3. Stomata Density

Based on the results of the analysis of variance (ANOVA), it was seen that variations in mannitol concentration did indeed affect changes in stomatal density. After treatment with various concentrations, including the average values and the results of the Tukey follow-up test at the 5% level, are presented in **Table 4.**

Table 4 Stomata Density of Moon Orchid Plantlets After Administration of Mannitol at Various Concentrations

Mannitol Concentration (mM)	Stomata Density (mm^2) μ = (Ȳ ± SE)
0	34.36 ± 2.58 [a]
50	28.44 ± 0.53 [ab]
100	27.04 ± 0.82 [b]
150	18.02 ± 1.13 [c]
200	16.05 ± 1.06 [c]

Description: μ = Ȳ ± SE; Ȳ = Average Stomatal Content; SE = Standard Error; Numbers followed by different letters indicate significant differences between treatments.

Based on **Table 4**. the results of the analysis of mannitol administration on moon orchid plantlets show that mannitol administration on moon orchid plantlets has a significant effect on stomatal density. The highest stomatal density value was obtained in the 0 mM treatment (control) which was 34.36 ± 2.58, while the lowest value was obtained in the 200 mM treatment which was 16.05 ± 1.06. In general, it can be seen that the higher the concentration of mannitol given, the stomatal density tends to decrease. Observations on the underside of moon orchid leaves with various concentrations of mannitol are presented in **Figure 2.**

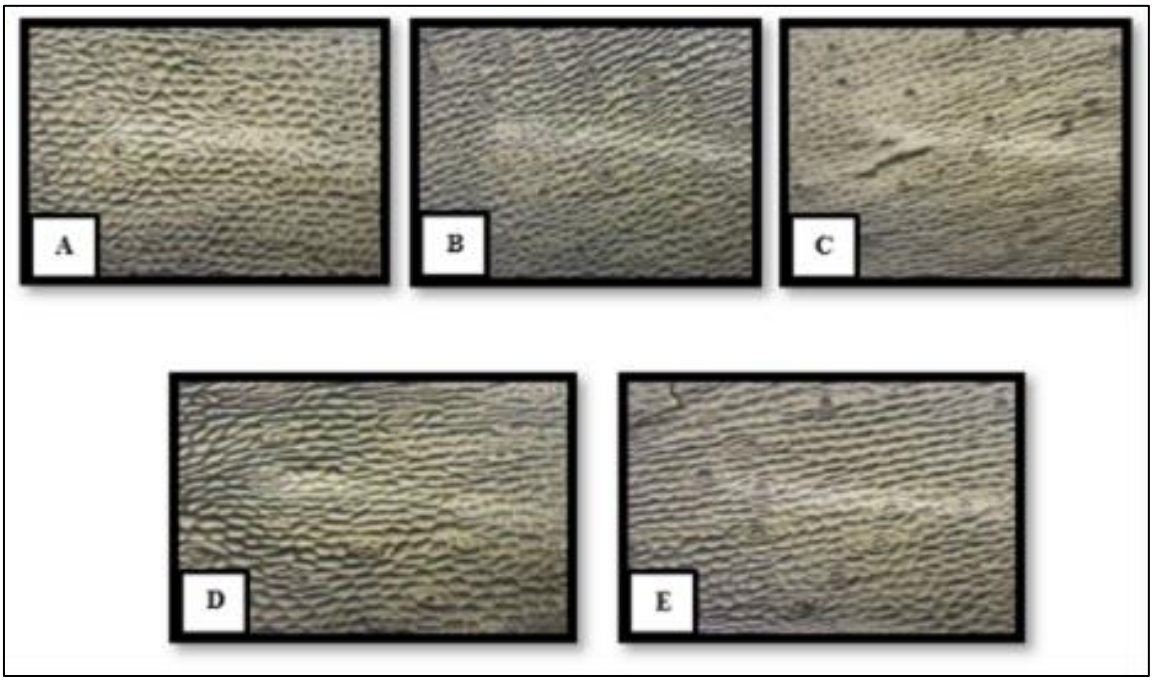

Figure 2 Stomata density of moon orchid leaves with 100x magnification, A. Concentration 0mM, B. Concentration 50 mM, C. Concentration 100 mM, D. Concentration 150 mM, E. Concentration 200 mM.

3.4. Chlorophyll Content

Chlorophyll content analysis was conducted to determine the effect of mannitol treatment on the photosynthetic capacity of moon orchid plantlets, which is related to their growth. Plantlets were grown on VW medium, then treated with various concentrations of mannitol to simulate drought stress in vitro. The chlorophyll content analyzed included chlorophyll a, chlorophyll b, and total chlorophyll. The test was carried out through pigment extraction using organic solvents, then the absorbance value was measured with a spectrophotometer.

The results of chlorophyll content measurements of chlorophyll a, chlorophyll b, and total chlorophyll showed that the treatment of mannitol administration at various concentrations had no significant effect. These results indicate that the differences between treatments were not statistically significant, as presented in **Table 5.**

Table 5 Average Chlorophyll Content of Moon Orchids in Week 4 after Mannitol Concentration Treatment

Mannitol Concentration (mM)	**Chlorophyll Content** (mg/L)		
	Chl a	Chl b	Chl Total
0	4.641	3.904	3.824
50	4.734	3.725	3.639
100	3.635	2.993	2.938
150	4.851	3.922	3.237
200	4.345	3.592	3.488

Based on **Table 5** The data of chlorophyll a, chlorophyll b, and total chlorophyll showed variations in values at each mannitol concentration. However, the results of the analysis of variance (ANOVA) showed that the administration of mannitol did not have a significant effect on the three parameters so that the differences that occurred were not significant. The 150 mM treatment produced the highest chlorophyll a and chlorophyll b values, respectively, at 4.851 and 3.922, while the 100 mM treatment produced the lowest values, namely 3.635 for chlorophyll a and 2.993 for chlorophyll b. The highest total chlorophyll was found in the 0 mM treatment at 3.824, while the lowest value was found in the 100 mM treatment at 2.938. These results indicate that mannitol affects the content of photosynthetic pigments in moon orchid plantlets. The decrease in values in the 100 mM treatment indicates that osmotic stress can disrupt the formation or stability of photosynthetic pigments.

4. Discussion

Observations on the number of surviving moon orchid plantlets were conducted from the first week to the fourth week of planting. Observations on moon orchid plantlets grown using mannitol in VW medium were conducted at concentrations of 0 mM (control), 50 mM, 100 mM, 150 mM, and 200 mM. It was found that with concentrations of 0 mM (control), 50 mM, 100 mM, 150 mM, and 200 mM, the survival rate of the plantlets was 100% until the fourth week.

Green to yellowish-green plantlets indicate good physiological condition and fresh tissue, and are therefore categorized as live plantlets. Brown plantlets indicate browning or tissue damage, and are therefore categorized as dead plantlets [10].

The visual appearance of plantlets tended to change with increasing mannitol concentration. Plantlets at 0 mM (control) and 50 mM remained green (H) until week 3, but showed a yellowish-green color in week 4. However, at a concentration of 100 mM, the color change from green (H) to yellowish-green began to appear in week 3. Meanwhile, the 150 mM treatment showed a change to yellowish-green (HK) from week 2. At a concentration of 200 mM, the plantlets experienced a decline from green to yellowish-green (HK), and from week 1 to week 4, the visual quality decline became increasingly apparent.

The visual appearance of orchid plantlets is influenced by the plant's ability to adapt to the growing environment. These symptoms not only reduce the plant's visual quality but also potentially reduce its long-term survival. Increasing mannitol concentration increases the osmotic pressure of the medium, limiting water availability to plant tissues and triggering physiological stress [11]. Visualization of Dendrobium sp. orchid plantlets. shows the morphological response to drought stress due to the administration of PEG 6000. Plantlets that were able to survive showed better visual conditions, while plantlets that experienced drought stress [12].

The root length of the moon orchid does not always follow the same pattern as the stem length. In this study, root length showed considerable variation, ranging from 2.18 cm to 2.62 cm. This variation is thought to be a form of plantlet adaptation to limited water availability. These results differ from studies that reported that PEG administration at a concentration of 15% increased black orchid root count, from 1.94 roots in the control treatment to 2.38 roots in the 15% PEG treatment [13].

Variations in the stem length of moon orchid plantlets showed a response to the administration of mannitol at various concentrations. Increasing the concentration of mannitol reduced the length of the plantlets' stems from 0.44 cm in the 50 mM treatment to 0.28 cm in the 200 mM treatment. This change occurred due to increased osmotic pressure of the

media which disrupted the water balance in plant tissues. The results of this study are in line with who reported that the administration of PEG reduced the increase in the height of black orchid plants from 0.42 cm in the control treatment to 0.11 cm in 5% PEG and 0.07 cm in 10% PEG, while 15% and 20% PEG did not result in an increase in plant height [14].

The number of leaves of moon orchid plantlets showed variations in each mannitol treatment. The 50 mM mannitol treatment produced the highest number of leaves, namely 3, while the 200 mM mannitol treatment produced the lowest number of leaves, namely 1.8. Increasing the concentration of mannitol inhibits leaf formation by increasing osmotic stress which limits water availability for plants. The results of this study are in line with. who reported that the 5% PEG treatment produced the highest number of leaves in black orchids, namely 6.44. Conversely, increasing the concentration of PEG to 25% reduced leaf growth due to inhibited water absorption by plants.

Mannitol administration to moon orchid plantlets significantly affected stomatal density. The highest stomatal density value was obtained in the 0 mM (control) treatment, at 34.36 ± 2.58, while the lowest value was obtained in the 200 mM treatment, at 16.05 ± 1.06. In general, it appears that the higher the mannitol concentration, the lower the stomatal density.

The 0 mM (control) treatment was significantly different from the 100 mM, 150 mM, and 200 mM treatments, but not significantly different from the 50 mM treatment. The 50 mM treatment was not significantly different from either the 0 mM or 100 mM treatments, but was significantly different from the 150 mM and 200 mM treatments. Meanwhile, the 100 mM treatment was not significantly different from the 50 mM treatment, but was significantly different from the 150 mM and 200 mM treatments. The 150 mM and 200 mM treatments showed no significant differences in response. Based on the stomatal density values, the 50 mM concentration is still tolerable for moon orchid plantlets [15].

In *Phalaenopsis amabilis*, PEG 6000 treatment increased stomatal density as the concentration increased from 0% to 40%, from 185 ± 74.1 to 580 ± 48.0. These results differ from this study, which showed a decrease in stomatal density due to the administration of mannitol. This difference in response is thought to occur due to the different types of osmotic agents used, namely mannitol and PEG 6000. Both osmotic agents affect the physiological conditions of plants through different mechanisms. Nevertheless, the results of both studies indicate that osmotic stress affects stomatal density as a response to plant adaptation to water limitations [16].

Chlorophyll a, chlorophyll b, and total chlorophyll data showed variations in values at each mannitol concentration. However, analysis of variance (ANOVA) results indicated that mannitol administration had no significant effect on these three parameters, resulting in insignificant differences. The 150 mM treatment produced the highest chlorophyll a and chlorophyll b values, at 4.851 and 3.922, respectively, while the 100 mM treatment produced the lowest values, at 3.635 for chlorophyll a and 2.993 for chlorophyll b. The highest total chlorophyll value was found in the 0 mM treatment at 3.824, while the lowest value was found in the 100 mM treatment at 2.938. These results indicate that mannitol affects photosynthetic pigment content in moon orchid plantlets. The decrease in values in the 100 mM treatment indicates that osmotic stress may disrupt the formation or stability of photosynthetic pigments

The moon orchid (*Phalaenopsis amabilis*) with PEG 6000 treatment. The study showed that increasing the concentration [2] of PEG 6000 from 0% to 40% reduced the content of chlorophyll a, chlorophyll b, and total chlorophyll. In this study, 100 mM mannitol treatment also produced the lowest content of chlorophyll a, chlorophyll b, and total chlorophyll. However, 150 mM mannitol treatment increased the content of chlorophyll a and chlorophyll b again. This pattern indicates that the response of the moon orchid to mannitol is not linear. This difference in response is thought to occur due to the type of osmotic agent, concentration level, and different plant adaptation capabilities [17].

5. Conclusion

Based on the results of the study on the effect of mannitol application as an inducer of drought stress on the growth of moon orchid *Phalaenopsis amabilis* (L.) Blume plantlets, it can be concluded that different concentrations of mannitol significantly affected the stomatal density of the plantlets. Based on the stomatal density parameter, a mannitol concentration of 50 mM was the highest concentration that could still be tolerated by moon orchid plantlets under in vitro conditions.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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