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Enhancing Plantlet Growth of *Vanda floresensis* Motes through Acclimatization Chamber, Chitosan Spraying, and Mixed Medium

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Abstract. The conservation of *Vanda floresensis* Motes has been conducted using *in vitro* techniques and has produced a large number of plantlets. These plantlets require acclimatization, but the optimal method has not yet been found. The aims of the research were to develop an acclimatization protocol by verifying the effectiveness of an acclimatization chamber (AC), chitosan spraying, and mixed medium composition on plantlet growth. The research was conducted in a screen house of Universitas Negeri Semarang, using a factorial randomized block design with three factors. The AC factor consisted of two levels: inside and outside the AC. The chitosan concentration factor consisted of five levels, namely 0.0, 1.5, 3.0, 4.5, and 6.0 ppm. The composition of the mixed medium consisted of three levels. The observed variables included the increase in number, length, and width of the leaf, total chlorophyll content, and wilted leaf. Data were analyzed by ANOVA and Duncan test. It can be concluded that the use of AC, chitosan spraying, and mixed medium composition was effective in increasing leaf growth and total chlorophyll content and reducing wilted leaf. Spraying chitosan of 1.5 - 3.0 ppm with the use of AC and spraying chitosan of 1.5 ppm with a mixed medium of brick pieces, coconut fibre, and tree bark chips with a ratio of 1:2:1 resulted in the highest leaf growth and total chlorophyll content, and the lowest wilted leaf. The results of this study can be used as an acclimatization protocol for orchid propagation for conservation purposes.

Key words: acclimatization chamber; chitosan; leaf growth; mixed medium; *Vanda floresensis*

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INTRODUCTION

Vanda floresensis Motes is an epiphytic natural species orchid and grows primarily in the wet tropical biome of Flores Island, East Nusa Tenggara, Indonesia. The orchid was first published in Nat. Gen. Vanda: 159 (2021). It has beautiful petals and a fragrant aroma. As an orchid species with a limited population, *V. floresensis* needs to be conserved. Efficient conservation can be achieved through *in vitro* propagation. This technique has been carried out and produced a large number of plantlets that need to be acclimatized before being planted in the natural environment. An optimal acclimatization protocol for *V. floresensis* has not yet been established. Consequently, seedlings are in short supply, and conservation activities have not been carried out as expected.

Acclimatization is a key factor that determines the success of *in vitro* plant propagation. Acclimatization is a step of providing transitional conditions between the *in vitro* room and the natural environment to prevent plantlets from stress due to drastic environmental changes (Grzelak et al., 2024). A common problem faced during acclimatization is that plantlets wilt easily. Our observation showed that the wilted plantlet occurs especially due to the sharp difference in air humidity between the *in vitro* room (80-95%) and the natural environment (45-65%).

The wilted plantlets can be prevented by regulating the humidity in the acclimatization room from dropping drastically compared to the culture room. Current acclimatization protocols

regulate the humidity by spraying water or covering the plantlets with plastic bags (Bani et al., 2022; Mullin et al., 2022), but this technique is less efficient. An acclimatization chamber (AC) is more efficient in controlling humidity. The AC, which has been developed, has drawbacks, including poor drainage techniques, impractical use, and a lack of humidity monitoring (Mohammed et al., 2023). Therefore, the AC needs to be improved.

The wilted plantlets can also be prevented by spraying chitosan solution on the leaves. Chitosan has been proven as an anti-transpiration agent and affects the levels of abscisic acid, which regulates the closure of stomata (Cheba, 2020; Reshad et al., 2021). It also increases photosynthesis, consequently improving plant growth and yield (Walled Fouad, 2023). Spraying of 0.5~1.0% chitosan effectively increased the leaf area and plant height of *Pinellia ternate* (Chen et al., 2023).

Another common problem during acclimatization is plantlet slow growth, which is believed because to a single medium material. Each medium material has a specific role (Nasution et al., 2020). Organic materials are able to retain water and maintain good aeration, while inorganic materials have a good mechanical support for plantlet establishment. The advantages of each component can be combined through a mixed medium(Irsyadi, 2021; Zanello et al., 2022).

The improvement of plantlet growth in this research will be assessed by leaf growth, as leaf plays a key role in supporting plantlet growth. The improvement will be enhanced through three factors: the use of AC, chitosan spraying on the leaves, and the use of a mixture of organic and inorganic materials in the medium. Therefore, the aims of the research were to analyze the effectiveness of AC and determine the optimal concentration of chitosan sprayed on the leaf and medium composition to improve the leaf growth of *V. floresensis*.

METHODS

The research materials were *V. floresensis* plantlets of 6-7 cm in height, with ≥2 leaves and ≥2 roots (Figure 1A). The AC was constructed from a plastic box consisting of a plate, a perforated tray, and a transparent lid. Four vents were installed on both sides of the lid, equipped with sliding windows (Figure 1B), allowing for easy, gradual opening. Consequently, air humidity decreases gradually. In addition, a thermo-hygrometer was installed on the AC roof (Figure 1B) to monitor temperature and humidity. The AC was used to place the acclimatization pots.

Research Design

The research design used was a factorial randomized block design with three factors: AC, chitosan concentration, and mixed medium composition. The AC factor consisted of two levels: inside the AC (A1, Figure 2A) and outside the AC (A2, Figure 2B). The chitosan concentration factor consists of five levels, namely 0.0 ppm (C1), 1.5 ppm (C2), 3.0 ppm (C3), 4.5 ppm (C4), and 6.0 ppm (C5). The medium composition consists of three levels, namely M1, M2, and M3. In the three medium compositions studied, the inorganic component was identical, namely crushed bricks. The variation among the three medium compositions was the type and proportion of organic materials. Specifically, M1 contained coconut fibres and dry moss but no bark fragments, M2 contained coconut fibres and bark fragments, and M3 contained dry moss and bark fragments (Table 1). Each combination of treatment levels was replicated four times. The research unit consisted of a single pot planted with a single plantlet (Figure 2C/D/E).

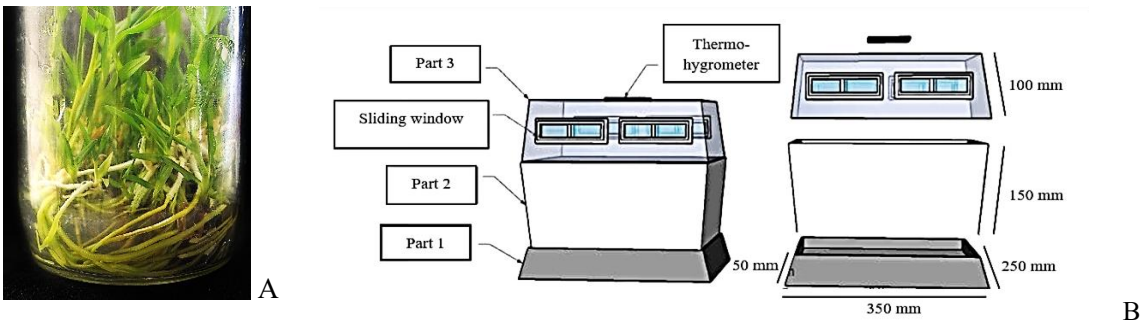

Figure 1. The plantlet and AC. A. Plantlets in the culture bottle. B. The AC parts and size

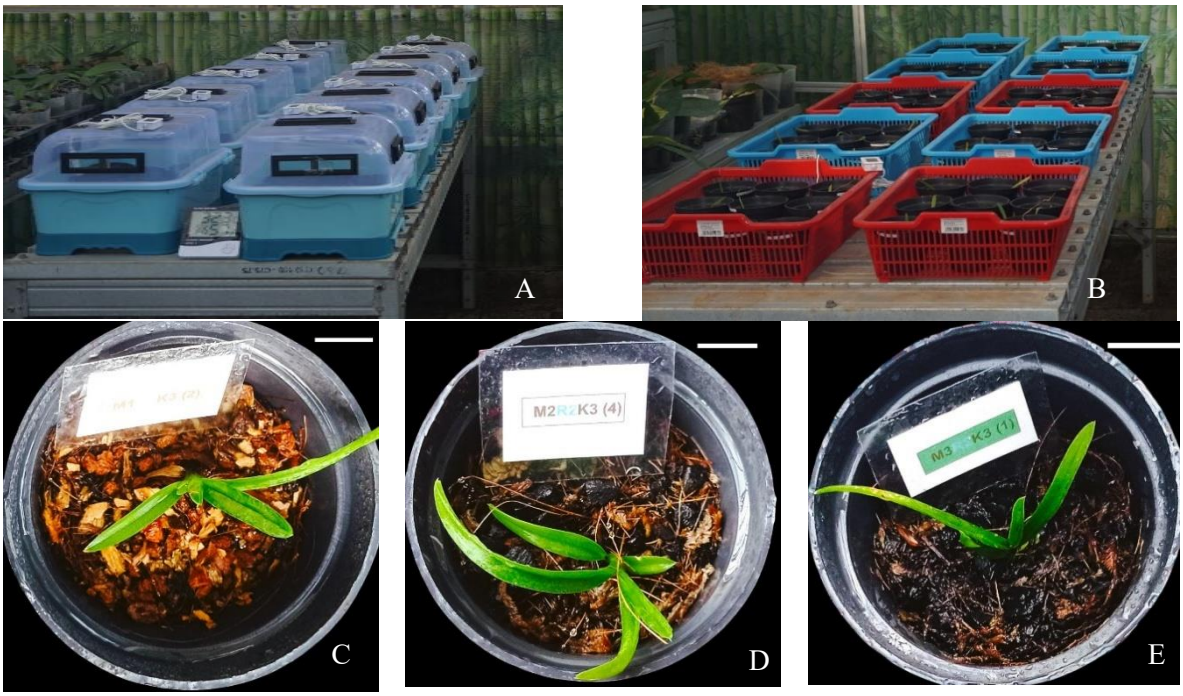

Figure 2. Placement of pots and planting plantlets in various medium compositions. A. Pots placed inside the AC, B. Pots placed in baskets outside the AC. C. Medium M1, D. Medium M2, E. Medium M3.

Table 1. Comparison of the medium composition

Medium composition	Various materials and ratios				Water content at field capacity (%)
	Crushed bricks	Coconut fibber	Dry moss	Tree bark fragments	
M1	1	2	1	0	46.20
M2	1	2	0	1	70.37
M3	1	0	1	2	65.77

Medium Preparation

Each medium material was soaked in clean water for two days, then rinsed and chopped into pieces approximately 0.5 cm in size. The mixture of medium materials was placed into 10 cm diameter plastic pots until three-quarters full. The pots were watered with distilled water until they reached field capacity (FC). The FC was obtained by pouring water evenly until water came out of the holes in the pot bottom, and waiting until no more water came out of the holes. The water content at FC of each medium composition was measured by the formula:

$$\text{FC} = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}} \times 100\ \%$$

The pots were placed in an AC on shelves according to the experimental design in a screen house with 60% lighting. Chitosan was sprayed five times per plant every 2 days at the appropriate concentration for each treatment level. During acclimatization, the plantlets are watered every 3 days with Aquadest in the field capacity or approximately 10 ml (Prayoga et al., 2024)

The observed variables included the increases in leaf number, length, and width at week 12. The number of fully opened leaves was counted. The leaf length is the distance between the base and tip of the largest leaf, while the leaf width is the distance from one edge to the other at the widest point of the largest leaf. In addition, a score or level of wilted leaf was calculated visually (Table 2). Each individual plantlet was assigned a wilted leaf score, and then the average was calculated for each treatment combination level.

Table 2. Description of the visual characteristic and wilting stage used in the visual assessments of wilted leaf

Score	Wilting stage	Visual characteristics
1	Normal (not wilted)	No signs of wilting or drought stress
2	Slightly wilted	Slight leaf angle changes but no folding, rolling, or changes in leaf surface structure
3	Wilted	Strong leaf angle change or protrusion of veins on the leaf surface, but no cell death
4	Severely wilted	Very strong change of leaf angle or protrusion of veins on the leaf surface with the beginning of necrosis
5	Nearly dead	Most leaves are necrotic, some young leaves are still green near the midrib, and leaf angles are mostly near 0°
6	Dead	All above-ground parts are dead, no resprouting after rewatering at the end of the experiment.

*Engelbrech et al. (2007)

In addition, total chlorophyll content was also measured. Chlorophyll content was calculated at 12 weeks after planting using a UV-VIS spectrophotometer (Hach DR-5000) at wavelengths of 649 nm and 665 nm. The spectrophotometer absorbance value was then calculated using the formula Ritchie (2008) as follows:

$$\text{Total chlorophyll } (\mu g/ml) = 20.2 \times (OD649) + 6.1 \times (OD665)$$

OD649 : spectrophotometer absorption value at 649 nm wavelength

OD665 : spectrophotometer absorption value at 665 nm wavelength

Data analysis

Statistical analyses were performed using SPSS version 25.0. Data normality and homogeneity were assessed with the Kolmogorov-Smirnov and Levene's test, respectively. The main and interaction effects of AC, chitosan concentration, and mixed medium composition were evaluated through a Three-Way analysis of Variance (ANOVA). Significant effects were further examined using Duncan's Multiple Range Test (DMRT) to compare treatment means.

RESULTS AND DISCUSSION

The data on the increase of leaf number, leaf length, and leaf width; total chlorophyll content, and score of wilted leaves were normally distributed and homogeneous. Based on the ANOVA result, it can be generally said that the AC, chitosan concentration, and medium composition separately had significant effects on the data measured. The interaction between the AC and chitosan concentration and between the medium composition and chitosan concentration also had significant effects on all parameters measured, except the wilted leaf score. Conversely, the interaction between the AC and medium composition and the interaction of the three factors did not have significant effects (Table 3).

Table 3. The significance value (sig) of the ANOVA results for the influence of the AC, chitosan concentration, media composition, and their interaction on the increase of leaf growth, chlorophyll content, and leaf wilting score

Factor	Sig of an increase in leaf growth			Sig of chlorophyll content (µg/ml)	Sig of leaves wilting score
	Leaf number	Leaf length (mm)	Leaf width (mm)		
Acclimatization chamber (A)	0.02	0.02	0.01	0.00	0.00
Chitosan concentration (C)	0.03	0.01	0.00	0.03	0.00
Media composition (M)	0.23	0.01	0.01	0.03	0.04
AxC	0.02	0.03	0.04	0.04	0.10
CxM	0.02	0.05	0.03	0.03	0.09
AxM	0.21	0.13	0.24	0.19	0.14
AxCxM	0.15	0.21	0.22	0.27	0.08

* Sig ≤ 0.01 : very significant effect; 0.01 < sig ≤ 0.05 : significant effect ; sig > 0.05 : not significant effect

Table 4. The optimal AC on the increase of leaf growth, chlorophyll content, and wilted leaf score

AC	Increase in leaf growth			Chlorophyll content (µg/ml)	Wilted leaf score
	Leaf number	Leaf length (mm)	Leaf width (mm)		
A1 (inside)	2.29 [a]	5.1 [a]	2.10 [a]	3.54 [a]	1.00 [a]
A2 (outside)	2.17 [b]	3.7 [b]	0.97 [b]	1.92 [b]	2.25 [b]

*) Numbers in one column followed by a different letter mean significantly different based on the 5% DMRT results.

The Effect of AC on Growth and Wilted Leaf

Plantlets grown inside the AC had a higher increase in leaf number (2.29) than those grown outside the AC (2.17) (Table 4). The increase in leaf number is determined by the amount of leaf primordia formed in the first stage of leaf development. The leaf primordium develops from the shoot apical meristem (SAM), which contains different functional regions, including a central zone (CZ) that generates pluripotent cells and a peripheral zone (PZ) from which lateral organs are formed. Leaf primordium is formed from periclinal cell divisions in the three outermost cell layers of the SAM, namely L1, L2, and L3, followed by cell elongation, resulting in an increase in cell size (Lv et al., 2023). The formation of a primordium will be followed by the next primordium, and as a result, increasing the number of leaves.

Plantlets grown inside the AC had a higher increase in leaf length (5.1 mm) than those grown outside AC (3.7 mm). Plantlets grown inside AC also had a higher increase in leaf width (2.10 mm) than those grown outside AC (0.97 mm) (Table 4). The increase in leaf length and width occurs in young leaves that have completely formed from the leaf primordium. The increase in leaf length occurs due to periclinal cell division, while the increase in width occurs due to anticlinal cell division. The cell division, followed by cell expansion, will result in the bigger cells and finally the leaves getting longer or wider (Jathar et al., 2022). Therefore, it can be stated that the increase in the leaf number, length, and width involves both cell division and expansion.

Cell division requires the activity of cytokinin; proteins, nucleic acids, and other organic components of cells; and enzymes that play a role in the formation of organic substances (Huang et al., 2023). Besides, cell elongation requires the auxin hormone to activate the cellulase enzyme to break down cellulose. In turn, this mechanism causes the cell walls to be more flexible, and water is absorbed into the cell, which increases cell turgor pressure (Wu et al., 2021; Xiong et al., 2021). Therefore, it can be stated that cell division and elongation require the activity of hormones and enzymes, the addition of water, and the accumulation of organic matter.

The activity of hormones and enzymes is affected by some environmental factors, such as temperature (Kabir & Ju, 2023). Compared to the culture room, the temperature inside the AC increased by 6.9°C, while the temperature outside the AC increased by 9.2°C (Table 4). This lower temperature inside the AC prevented temperature shock to the hormone and enzyme, and consequently maintained optimal performance and activity. This matter enabled optimal cellular metabolism, resulting in higher leaf growth inside the AC compared to the outside.

Another environmental factor that affects cell metabolism and water availability is humidity. Compared to the culture room, the humidity inside the AC decreased by 8.6%, while outside the AC decreased higher as much as 32.3% (Table 4). The air humidity affects the rate of transpiration (Salman et al., 2023). A relatively low decrease in air humidity will maintain optimal transpiration, and, respectively, influence the optimal cellular water availability, biochemical reactions, and cellular metabolism within the plantlet. According to (Chia & Lim, 2022), plant growth improves with increasing humidity, as higher humidity conditions help to keep the stomata open to maintain the photosynthesis process and minimize the evaporation process of the plant.

Table 5. Differences of temperature, humidity, and light intensity between the inside culture room (ICR) and inside AC (IAC) and outside AC (OAC)

Environmental factors	ICR	IAC	OAC	Difference between ICR and IAC	Difference between ICR and OAC
Temperature (°C)	23.0	29.9	32.2	6.9	9.2
Humidity (%)	95.6	87.0	63.3	8.6	32.3
Light intensity (lux)	1578	2160	2348	582	770

The total leaf chlorophyll levels of plantlet grown inside the AC (3.54 µg/ml) significantly higher than outside the AC (1.92µg/ml) (Table 4). This may be due to light intensity difference between inside and outside the AC (Table 5). The light intensity influences chloroplast development. In leaf development, chloroplasts are formed from proplastid. Light is strictly required for chloroplast formation and directly regulates the expression of chloroplast-related genes. Light also modulates the levels of several hormones that control chloroplast development, particularly during early stages of plant development (Cackett et al., 2022). The increase of light intensity inside the AC (582 lux) was lower than that outside the AC, as much as 760 lux (Table 5). A relatively small increase in light intensity still ensures normal chloroplast development; conversely, a sharp increase will have a negative impact. This affected chlorophyll formation, resulting in significantly higher total chlorophyll levels inside the AC than outside.

Plantlets grown inside the AC had a lower wilted leaf score than those grown outside the AC (Table 4). Leaf wilting occurs when a plant loses more water than it can absorb, causing its cells to lose turgor pressure and become flaccid. When turgor pressure decreases, cells lose their rigidity, causing leaves to droop or curl (Rascio et al., 2023). One of the main factors influencing cell turgor pressure includes high temperatures, which cause cell water to evaporate, and turgor pressure decreases. This study revealed that the temperature inside the AC was lower than the outside the AC, resulting in fewer wilted leaf. The lower wilted leaf was associated with the increase in leaf number and size, as well as higher total chlorophyll content in the AC.

The Effect of Chitosan Concentration on Growth and Wilted Leaf

Chitosan concentration affected leaf growth and wilted (Table 3)**.** The increase of leaf growth, total chlorophyll content, and leaf wilting at one concentration is not always the same as at another concentration (Table 6). Chitosan sprayed onto the leaf surface will form a semipermeable film or thin layer and act as an anti-transpiration agent. The chitosan also increases the production of abscisic acid, which reduces transpiration rate (Reshad et al., 2021). In addition, the chitosan is able to diffuse into mesophyll cells, the nitrogen structure undergoes metabolism to produce various amino acids that can be used for the formation of phytohormones, proteins, pectin, and others. As a result, chitosan can increase the growth of plantlets (Bani et al., 2022). As a biostimulant, chitosan's influence on nutritional efficiency (Bhupenchandra et al., 2020), induces the activities of genes responsible for various events in plant life processes, such as photosynthesis, plant defense system, hormone metabolism, and alteration of protein metabolism, resulting in increased storage protein content (Shahrajabian et al., 2021).

The chitosan also increases endogenous cytokinin and auxin levels. In the early stages of leaf development, cytokinin and auxin enhance the growth of SAM, which provides stem cells for the formation of leaf primordia (Mawale & Giridhar, 2024). The cytokinin increases nucleic acid endoreduplication, shortens the cell replication cycle, promotes cell proliferation, which increases the number of leaf cells in a short time (Wu et al., 2021). On the other hand, the auxin plays a very important role in cell wall flexibility, increasing turgor pressure, inducing cell elongation in meristematic tissue, and cell differentiation, which is useful for leaf growth (Xiong et al., 2021).

Table 6. The optimal chitosan concentration for the increase of leaf growth, chlorophyll content, and wilted leaf score

Chitosan concentration (ppm)	Increase in leaf growth			Chlorophyll content (µg/ml)	Wilted leaf score
	Leaf number	Leaf length (mm)	Leaf width (mm)		
C1 (0.0)	2.21 [b]	2.69[c]	1.43 [b]	2.15 [b]	1.50 [b]
C2 (1.5)	2.32 [a]	5.70[a]	1.58 [a]	3.61 [a]	1.12 [a]
C3 (3.0)	2.21 [b]	4.78[b]	1.64 [a]	3.01 [a]	1.62 [b]
C4 (4.5)	2.20 [b]	4.50[b]	1.39 [b]	2.54 [b]	1.62 [b]
C5 (6.0)	2.22 [b]	3.89[b]	1.40 [b]	2.37 [b]	2.12 [c]

*) Numbers in one column followed by a different letter mean significantly different based on the 5% DMRT results.

Table 7. The optimal medium composition for the increase of leaf growth, chlorophyll content, and wilted leaf score

Medium composition	Increase in leaf growth			Chlorophyll content (µg/ml)	Wilted leaf score
	Leaf number	Leaf length (mm)	Leaf width (mm)		
M1	2.19 [a]	3.99 [c]	1.50 [c]	2.85 [b]	1.42 [b]
M2	2.24 [a]	5.66 [a]	1.95 [a]	3.14 [a]	1.17 [a]
M3	2.25 [a]	4.83 [b]	1.73 [b]	2.99 [b]	1.29 [ab]

*) Numbers in one column followed by a different letter mean significantly different based on the 5% DMRT results.

The increase in leaf growth and total chlorophyll content in chitosan treatments at certain concentrations tended to be the highest compared to other concentrations. The highest increase in leaf number, length, and width was at 1.5 ppm chitosan concentration; besides, the highest total chlorophyll content was 1.5 ppm and 3.00 ppm. The lowest leaf wilting was observed at 1.5 ppm chitosan (Table 6). This result is in accordance with the statement that the effect of chitosan is determined by its concentration; when it is too low will cause a suboptimal condition, while when it is too high will cause osmosis, or the withdrawal of water from leaf cells (Salman et al., 2023). Therefore, an optimal concentration is needed for normal metabolism and growth.

The results indicated that at the chitosan concentrations of 1.5 and 3 ppm, the layer formed by chitosan spraying can prevent transpiration but did not cause osmosis, thus maintaining relatively fresh leaves. Fresh leaves have adequate water content and allow the metabolism of chlorophyll formation. In the next process, high chlorophyll level optimizes photosynthesis and growth.

The results of this study are in line with previous studies. Rahman et al., (2018) conclude that foliar application of chitosan to strawberries improved plant growth and fruit yield. A significant increase in levels of anthocyanins, carotenoids, flavonoids, and phenolic compounds was observed when plants were sprayed with a chitosan solution compared to the untreated control. Another study reported that the biomass and number of flowers of tomato plants were improved by foliar spraying with chitosan (El Amerany et al., 2020).

The Effect of Medium Composition on Growth and Wilted Leaf

Medium composition had significant effects on the leaf growth, total chlorophyll content, and wilted leaf score. In general, the M2 medium results in the highest leaf growth and total chlorophyll levels than to M1 and M3; and the lowest wilted leaf score (Table 7). The M2 medium consisted of crushed bricks that have a good role as mechanical support for plantlet growth, coconut fibers that have a good role in water retention and aeration, and two parts of bark fragments, more than M3 (one part) and M1 (without this component) (Table 1). Tree bark fragments provided identical conditions to the natural habitat of the epiphytic orchid *V. floresensis*, suggesting the plantlets would be more adaptable during acclimatization.

Moreover, the M2 medium has the highest water content at field capacity (70.37%) compared to the others (Table 1). Field capacity (FC) refers to the amount of water held in the soil after excess water has drained away. It represents the upper limit of water storage, while the lower limit is called the permanent wilting point (PWP). The total amount of water available for plant uptake is the plant available water (PAW), which is the difference between FC and PWP and is often expressed as a percent by volume (volume of water/volume of soil) (Giap & Ahmad, 2022). The FC significantly impacts plant growth. At FC, water is readily available for plants to absorb, promoting healthy growth and development. When soil is at or near FC, water is readily accessible to plant roots, facilitating nutrient uptake and overall plant health (Gavrilescu, 2021).

These findings confirmed previous results reported by Febriyani et al. (2019) that wood shavings were the best medium for pencil orchid acclimatization. The addition of wood and bark chips to soil substrate as an upper layer improved the subsequent growth and development of *Dendrobium nobile* (Mirani et al., 2019).

The Effect of the Interaction of AC and Chitosan Concentration on Plantlet Leaf Growth

The interaction between AC and chitosan concentration significantly affected plantlet growth and total chlorophyll content. This result indicated that the effect of the AC used was determined by the concentration of chitosan

sprayed onto the leaves, and vice versa. The use of AC aims to reduce the temperature and humidity difference between the culture room and natural conditions, thereby reducing transpiration. Meanwhile, spraying chitosan on the leaf surface also aims to reduce transpiration. Consequently, the effect of the two factors interacted.

In general, the A1C2 interaction resulted in significantly higher leaf growth compared to the other interactions, especially in leaf number, length, and width (Table 7). This result was supported by the highest total chlorophyll content. Similar results were achieved by the A1C3 interaction. Plantlets also look fresher and greener (Figure 4A, 4B). This means that plantlet growth under AC and sprayed with 1.5 or 3.0 ppm chitosan resulted in the highest total chlorophyll content, resulting in higher leaf growth compared to the other interactions.

The Effect of Interaction of Medium Composition and Chitosan Concentration on Plantlet Leaf Growth

A significant interaction between medium composition and chitosan concentration was observed for plantlet growth and total chlorophyll content (Table 3). This finding suggests that the impact of medium composition was modulated by the concentration of foliar chitosan spraying and vice versa. Variations in medium composition alter water-holding capacity at field capacity (FC), thereby affecting plantlet water uptake and subsequent water availability within the leaf cells. Chitosan application reduces transpiration and contributes to maintaining leaf water status. Consequently, medium composition and chitosan concentration act synergistically to influence plantlet growth and chlorophyll accumulation.

Table 7. The optimal interaction between AC and chitosan concentration on the increase of leaf growth and total chlorophyll content

Interaction of AC and chitosan	Increase in leaf growth			Chlorophyll content (µg/ml)
	Leaf number	Leaf length (mm)	Leaf width (mm)	
A1C1	2.22 ^{b}	2.69 ^{c}	2.14 ^{a}	2.35 ^{b}
A1C2	2.41 ^{a}	7.20 ^{a}	2.06 ^{a}	4.17 ^{a}
A1C3	2.30 ^{ab}	5.28 ^{b}	2.39 ^{a}	3.81 ^{a}
A1C4	2.25 ^{b}	5.05 ^{b}	1.94 ^{ab}	3.73 ^{a}
A1C5	2.29 ^{ab}	4.21 ^{b}	1.93 ^{ab}	3.64 ^{a}
A2C1	2.20 ^{b}	2.69 ^{c}	1.12 ^{b}	1.96 ^{bc}
A2C2	2.23 ^{b}	4.20 ^{b}	1.10 ^{b}	3.05 ^{ab}
A2C3	2.13 ^{c}	4.28 ^{b}	0.90 ^{b}	2.21 ^{bc}
A2C4	2.14 ^{c}	3.95^{b}	0.85 ^{b}	1.36 ^{c}
A2C5	2.14 ^{c}	3.57 ^{bc}	0.87 ^{b}	1.11 ^{c}

Numbers followed by the same letter mean not significantly different based on the DMRT 0.05

Table 8. The optimal interaction of medium composition and chitosan concentration for the increase in leaf growth and chlorophyll content

Interaction of medium composition and chitosan concentration	Increase in leaf growth			Chlorophyll content (µg/ml)
	Leaf number	Leaf length (mm)	Leaf width (mm)	
M1C1	2.20 ^{c}	2.25 ^{d}	0.97 ^{c}	1.98 ^{c}
M1C2	2.22 ^{c}	5.52 ^{b}	1.78 ^{b}	3.87 ^{a}
M1C3	2.15 ^{c}	4.50 ^{b}	1.89 ^{ab}	2.81 ^{b}
M1C4	2.17 ^{c}	3.43 ^{c}	1.62 ^{bc}	3.03 ^{ab}
M1C5	2.11 ^{cd}	4.27^{bc}	1.26 ^{c}	2.56 ^{bc}
M2C1	2.25 ^{b}	4.35 ^{b}	2.54 ^{a}	3.07 ^{ab}
M2C2	2.48 ^{a}	7.11 ^{a}	2.00 ^{a}	4.03 ^{a}
M2C3	2.30 ^{b}	6.89 ^{a}	1.74 ^{b}	2.89 ^{b}
M2C4	2.35 ^{b}	5.88 ^{ab}	1.44 ^{c}	3.28 ^{a}
M2C5	2.30 ^{b}	4.08 ^{c}	2.02 ^{a}	2.41 ^{bc}
M3C1	2.18 ^{c}	1.47 ^{e}	1.38 ^{c}	1.40 ^{c}
M3C2	2.26 ^{bc}	4.47 ^{bc}	0.96 ^{c}	2.93 ^{b}
M3C3	2.18 ^{c}	2.95 ^{d}	1.29 ^{c}	3.33 ^{a}
M3C4	2.08 ^{d}	4.19 ^{c}	1.11 ^{c}	1.31 ^{c}
M3C5	2.25 ^{bc}	3.12 ^{d}	0.92 ^{c}	2.14 ^{c}

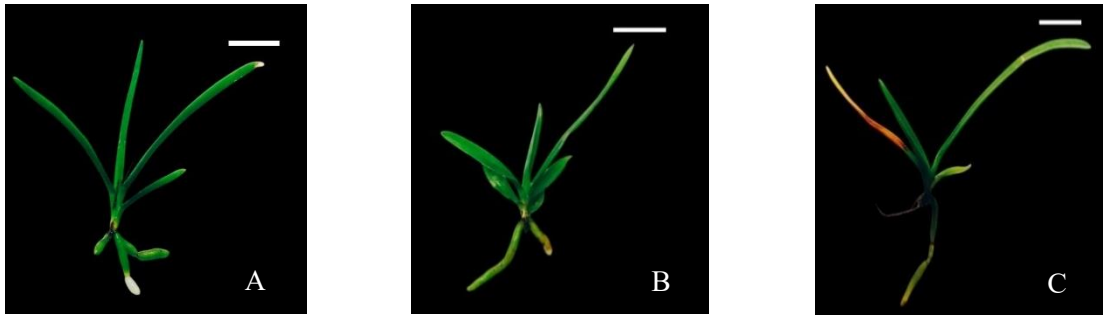

Figure 4. The performance of plantlets grown in the various interactions of AC, chitosan concentrations, and medium composition. A. Inside the AC, chitosan concentration of 1.5 ppm, and M2 medium. B. Inside the AC, chitosan concentration of 3.0 ppm, and M1 medium. C. Outside AC, chitosan concentration of 4.5 ppm, and M3 medium. Scale bar: 10 mm

In general, it can be stated that the M2C2 interaction resulted in significantly higher leaf growth compared to other interactions (Table 9). This result is supported by the total chlorophyll content, which is also the highest. This result showed that the growth of plantlets in the M2 medium and sprayed with 1.5 ppm chitosan resulted in the highest total chlorophyll content and leaf growth. Plantlets grown outside of the AC and sprayed with chitosan at more than 3 ppm showed slightly yellowing and even browning of the leaves (Figure 4C).

The study found that AC, chitosan, and mixed medium, either individually or in combination, can support plantlet growth during acclimatization. This research is novel compared to other studies because it combines all three factors, whereas other studies have only examined one. These findings can be developed into an acclimatization protocol for a rare orchid.

The novelty of this research is that it combines three factors at once in the orchid acclimatization technique, namely AC usage, foliar chitosan spraying, and mixed media. The previous research only examined one or two factors. The research's benefits are to enrich the acclimatization protocol for wild orchid species, which is crucial for *V. floresensis* conservation, and contribute to supporting the achievement of the 15th Sustainable Development Goals (SDGs), namely life on land (terrestrial ecosystems).

CONCLUSION

The AC, foliar chitosan spraying, and mixed medium significantly influenced leaf growth and total chlorophyll content, wilted leaf score of *V. floresensis* plantlets. The leaf growth and total chlorophyll content significantly increase when grown in an AC, sprayed with 1.5 ppm and 3.0 ppm chitosan, and planted on a mixed medium of brick pieces, coconut fibres, and tree bark chips in a 1:2:1 ratio. Practically, the best acclimatization protocol of *V. floresensis* plantlets was implemented in an acclimatization chamber, planted on the mixed medium consisting of brick pieces, coconut fibres, and tree bark chips in a 1:2:1 ratio, and foliar spray with 1.5 ppm chitosan. This study focused on morphological growth parameters. Further research is needed to assess biochemical and physiological changes, such as photosynthetic efficiency, antioxidant activity, and secondary metabolite production, to better understand the mechanisms underlying chitosan's effects.

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AUTHOR CONTRIBUTION STATEMENT

All authors contributed significantly to the work reported in this manuscript. ESR conceived and designed the study, NAH conducted the data analysis, and YUA supported in the literature review. DM and NDM were responsible for data collection and assisted data visualization. FP provided substantial feedback during the manuscript drafting process. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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