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Effect of Benzyl Amino Purine (BAP) on the Leaf Growth of *Vanda limbata* Blume Orchid *In Vivo*

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Abstract

Vanda limbata is a natural orchid with slow growth and is often taken directly from the forest in large quantities, threatening its sustainability. Ex-situ preservation efforts are needed for this orchid, one of which is through cultivation with the addition of Benzyl Amino Purin (BAP), known to increase shoot growth. This research aims to analyze the effect of BAP and determine its optimal concentration on the growth of *V. limbata* orchids. This research used a single-factor completely randomized design in BAP concentrations of 0, 25 and 50 mg l^{-1} with 5 replications. The method used was spraying BAP solution once a week on all parts of adult *V. limbata* orchids aged ±2 years. The research was conducted for 3 months in the experimental garden with parameters observed in the form of the number of new leaves, elongation of old leaves, the length of new leaves, increase in the width of old leaves, the width of new leaves, stomatal density and the number of roots. The results showed that the most effective BAP concentration was 50 mg l^{-1} for the number of new leaves which increased by 200%, the length of old leaves which increased by 168.66% and the length of new leaves which increased by 800%, but resulted in less stomatal density than the control. BAP can increase the growth of *V. limbata* orchid leaves. This research can be a recommendation for ex-situ conservation efforts that have the potential to be developed as a means of orchid cultivation.

Keywords: cytokinin; orchid preservation; plant growth regulator

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INTRODUCTION

Orchids are ornamental plants that are favored by many people because of the beauty of their flowers (Kasutjianingati and Firgiyanto, 2018), one of the genus is *Vanda*. There are about 60 species of the genus *Vanda* distributed throughout Indonesia. Almost half of the 60 species are native to Java, including *Vanda limbata*. *V. limbata* has a monopodial growth pattern with long and thick roots. The orchid has red-dominated flowers with yellow spots and dark red sepals (Dwiyanto et al., 2017). The beauty of the flowers makes *V. limbata* much sought after by orchid enthusiasts, even though the cultivation of this orchid species is still very limited, so generally the types of *Vanda* sold in the market are taken directly from the forest in large quantities. The limited cultivation of *V. limbata* is caused by its very long juvenile period or slow growth. Setiaji et al. (2021) stated that *Vanda* orchid cultivation takes more than 3 years from juvenile to flowering. If this condition continues, it is feared that it will have an impact on the lack of orchid populations in nature. Therefore, modification of *V. limbata* cultivation is needed to improve its growth and conservation. One method that is known to accelerate plant growth is the application of plant growth regulators (PGR).

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PGR are chemical substances that affect the growth and differentiation of plant cells, tissues and organs (Hira and Bijaya, 2019). One method that can be done to increase shoot growth is the addition of cytokinins. Márquez-López et al. (2019) and Shimotohno et al. (2021) stated that cytokinins generally play a role in 2 stages of the cell cycle, it is increasing the transition from the G1 phase to S and the G2 transition to the M phase so that the formation of shoots into new leaves occurs faster. One type of cytokinin that is often used is Benzyl Amino Purin (BAP). Farber et al. (2016) added that BAP can affect the transpiration of tomato plants by regulating stomatal density and leaf size due to increased cell division followed by cell differentiation into stomata. The advantages of BAP are that it has more stable activity (Khadr et al., 2020), as well as is resistant to oxidation and cheaper than other cytokinins (Bryksová et al., 2020). In addition, in sustainable agriculture, it is necessary to use environmentally friendly materials, hence the choice of BAP-type cytokinins because their content is similar to the natural plant hormones that are friendly to the environment. According to Campolongo et al. (2020), BAP is considered a compound that is very similar to endogenous cytokinins because it presents a very low risk to the environment, as it is non-toxic to mammalian and arthropod species. Based on this, BAP is suitable for supporting sustainable agriculture.

The use of PGRs *in vivo* by Nambiar et al. (2012) showed that spraying BAP 200 mg l^{-1} caused the highest number of new leaves and induced flowering in *Dendrobium* Angel White orchids. Burhan (2017) reported that spraying BAP 100 and 200 mg l^{-1} produced the largest pseudo-stem diameter, more new leaves and induced flowering in hybrid *Dendrobium* orchids. Lee et al. (2021) showed that spraying BAP 100 mg l^{-1} on *Phalaenopsis* orchids increased leaf width, stem diameter, accelerated flowering and emergence of lateral bud growth. The three studies used high concentrations of BAP on various types of orchids. This is different from the statement of Albrecht and Argueso (2016) that BAP in low concentrations can already increase the shoot growth of a plant. The statement was proven by Putri et al. (2017) that spraying 50 mg l^{-1} BAP was able to increase plant height, number of leaves and leaf area of *Adenium arabicum* compared to the control treatment. Rahmah et al. (2023) also proved that spraying 50 mg l^{-1} BAP was able to increase plant height and number of branches of edamame (*Glycine max* L. Merr) compared to the control treatment. Spraying BAP at low concentrations has not been widely done on adult orchids, especially on *V. limbata*. Therefore, in this research, BAP spraying at a lower concentration was carried out to increase leaf growth of *V. limbata* to support its conservation.

MATERIALS AND METHOD

Place and time of research

The research was conducted at the Tembalang experimental garden and the Biological Laboratory of Plant Structure and Function, Department of Biology, Universitas Diponegoro, from October 2022 to March 2023.

Research procedure

Preparation of *V. limbata* orchids aged ±2 years obtained from a seller in the Trenggalek area, East Java. Preparation of BAP stock solution with a concentration of 1000 mg l^{-1} was made by dissolving 0.5 g BAP with a few drops of 10N HCl, then added with 200 ml aquadest. Then, the BAP solution was made in accordance with the treatment level every week when spraying. The treatment was given by spraying using a hand sprayer on all parts of the *V. limbata* orchid plant until wet (±20 ml each). BAP spraying was done once a week for 3 months in the morning. The care was carried out by watering every day in the morning with well water (±750 ml for all treatments) and giving vitamin B1 at intervals of two weeks for all treatments. Vitamin B1 used was 1.5 ml diluted in 750 ml of well water. Vitamin B1 in this study only served as a starter for orchid growth, considering that these orchids were taken directly from the forest so they needed to adjust to the new environment. In accordance with Fitzpatrick and Chapman (2020), vitamin B1 appear to have vital roles in plant health and metabolism, both through a role as an essential enzymatic coenzyme and as molecules for plant stress resistance.

Research observation

Observations were made at the beginning before treatment, then once every two weeks for 3 months. The parameters observed in this research were the number of new leaves, elongation of old leaves, length of new leaves, increase in width of old leaves, width of new leaves, number of roots and stomatal density. Stomatal density observation begins with the making of semi-permanent preparations. For making preparations, the leaf surface was cut longitudinally as thin as possible, fixed in alcohol, stained with safranin, washed with aquadest, placed on a glass object and dripped with glycerin, and then covered with a glass cover. The preparations were then observed on an Olympus CX23 microscope using an Optilab Viewer 2.2 camera at 10x magnification and a field of view of 1.03 mm^2. The calculation of stomatal density used Equation 1, based on Nurunisa et al. (2018).

$$\text{Stomatal density (stomata mm}^{-2}\text{)} = \frac{\text{Number of stomata}}{\text{Field of view (mm}^2\text{)}} \quad (1)$$

Experimental design and data analysis

The research was conducted experimentally using a completely randomized design (CRD) with a single factor of BAP concentration consisting of 3 treatment levels, 0 (control), 25 and 50 mg l^{-1}. Each treatment level was given 5 replicates. The data obtained were analyzed using analysis of variance (ANOVA). If there was a significant effect on each treatment, then further test analysis was carried out with Duncan's multiple range test (DMRT) at the 5% level.

RESULTS AND DISCUSSION

Number of new leaves

Based on data analysis, BAP has a significant effect and there is a real difference between treatments. The observation results showed that the treatment of 50 mg l^{-1} contributed to the highest average number of new leaves (2 leaves), followed by the treatment of 25 mg l^{-1} with the average of 1.33 leaves. Meanwhile, the control treatment did not produce any new leaves (Figure 1). These results showed that spraying BAP solution was able to increase the number of leaves up to 133% at 25 mg l^{-1} and 200% at 50 mg l^{-1} compared to the control. In the control treatment, no new leaves appeared at all (Figure 1 and 2), presumably due to the endogenous cytokinin content insufficient for the formation of new leaves. Therefore, when BAP was added as an exogenous cytokinin in the 25 and 50 mg l^{-1} treatments, it was able to increase the growth of new leaves. This happens because of the work of BAP, which can accelerate cell division in leaf primordia, so that leaf buds grow faster. This is in accordance with the statement of Wybouw and De Rybel (2019) that cytokinin hormones have an important role in cell division through the process of protein synthesis. The growing cells then develop into the constituent tissues of the shoots.

The action of cytokinin in protein synthesis is in the translational process by increasing the performance of mRNA in translating codons into amino acids that make up proteins in ribosomes (Karunadasa et al., 2020). One of the proteins produced is the phosphatase enzyme, which plays a role in the cell cycle. Furthermore, cytokinin plays a role in 2 stages of the cell cycle, in the G1 to S and G2 to M phases. In the G1 to S phase, cytokinin helps cyclin to activate cyclin-dependent kinase (CDKs) so that cell phosphorylation occurs and the cell cycle continues. Whereas in the G2 to M phase, 2 phosphates in CDKs make them inactive, so to reactivate CDKs, the phosphatase enzyme produced from protein synthesis plays a role in removing these inactive phosphates and the cell cycle can continue to the M phase (Márquez-López et al., 2019; Shimotohno et al., 2021). Based on this mechanism, cytokinins can increase shoot growth by accelerating cell division.

Based on Figure 1, BAP 50 mg l^{-1} is the most effective concentration to increase the number of leaves of *V. limbata*. These results prove the statement of Albrecht and Argueso (2016) that BAP in low concentrations can already increase shoot growth. In this research, the concentration of 50 mg l^{-1} is said to be low because there are similar studies that use BAP at high concentrations. Nambiar et al. (2012) sprayed 150 mg l^{-1} BAP on adult *Dendrobium* Angel White orchid leaves *in vivo* increasing the number of leaves of 40.9%. A similar response was observed by Burhan (2017) on adult hybrid *Dendrobium* orchid leaves *in vivo* which had the

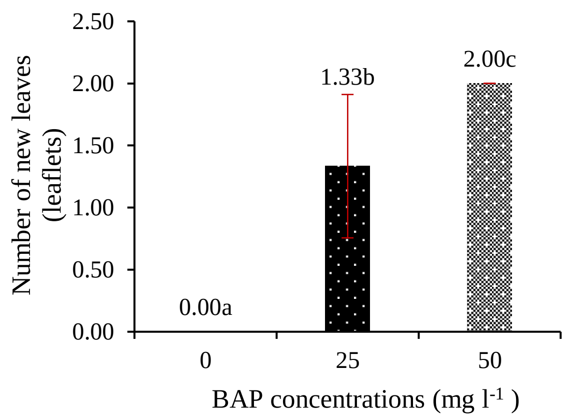


Figure 1. Number of new leaves of *V. limbata* after the spraying of BAP at the concentrations of 0, 25 and 50 mg l^{-1} for 3 months

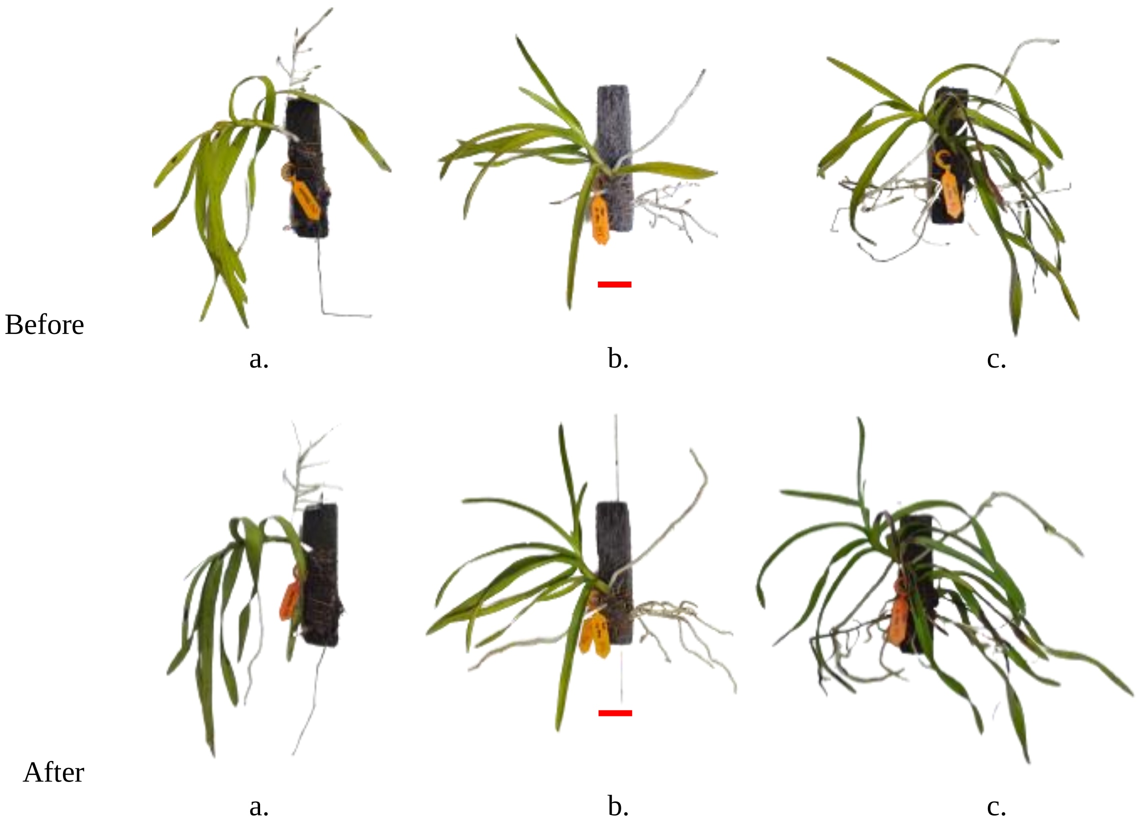


Figure 2. Comparison of leaf number of *V. limbata* before and after the spraying of BAP at the concentrations of 0 (a), 25 (b) and 50 (c) mg l^{-1} for 3 months (Red bar: 5 cm)

highest number of new leaves in the 200 mg l^{-1} BAP treatment.

Length and width of leaves

In the observation of leaf length, the parameters observed were the elongation of old leaves and the length of new leaves. Besides affecting the length of new leaves, BAP spraying also affects the elongation of old leaves. Based on the results of data analysis, spraying BAP on the parameter of elongation of old leaves showed a significant effect and significantly different results between treatments (Figure 3). The results disclosed that the elongation of the longest leaves was highest in the 50 mg l^{-1} treatment, which reached 12.7 cm, while the control treatment was only able to increase the length of the leaves by 1.93 cm. The 50 mg l^{-1} treatment was the most effective in this parameter, increasing the length of old leaves by 168.66% compared to the control treatment, which only increased by 29.38%. In the new leaf length parameter, the results of data analysis also showed significant effects and significant differences between treatments (Figure 4). The observation results indicated the longest new leaf length in the 50 mg l^{-1} treatment, which reached 8 cm, while in the control treatment, no new leaves appeared so there was no increase in the length of new leaves. The 50 mg l^{-1} treatment was also the most effective in this parameter, increasing the length of new leaves by 800%, longer than the control treatment, which had no increase in length at all (0%).

Based on the observations, BAP spraying affected the growth of leaf length in *V. limbata* orchids, both on old and new leaves. In this case, a similar mechanism occurred in new leaf initiation, in which BAP increased cell division. Cytokinin may also boost endogenous auxin in leaf cell elongation. Based on this, spraying BAP causes the leaf elongation process to be faster by accelerating cell division at the base of the leaf and endogenous auxin, which plays a role in cell elongation in the middle of the leaf. This is following the statement of Larekeng et al. (2020) that BAP positively regulates cell division in growing leaves. Wu et al. (2021) also added that leaf development in monocots is spatially regulated, in which cell division occurs mainly at the base of the leaf, cell expansion in the middle of the leaf, and cell maturation at the tip of the leaf. Kurepa et al. (2019) also reinforce that cytokinin and auxin hormones work synergistically, with cytokinin increasing cell division through mitosis and auxin increasing

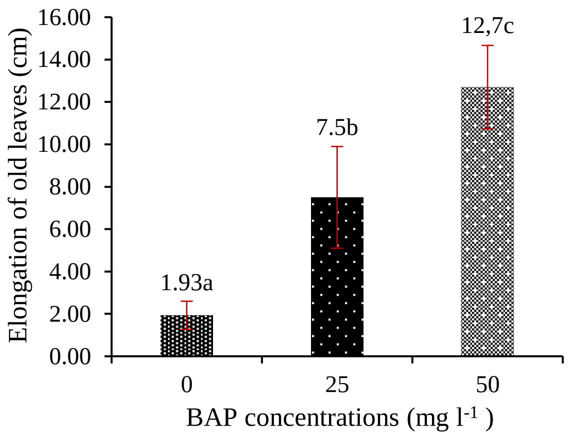


Figure 3. Elongation of old leaves of *V. limbata* after the spraying of BAP at 0, 25 and 50 mg l^{-1} for 3 months

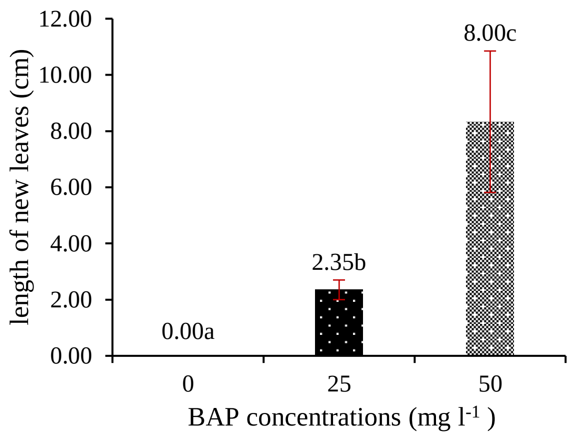


Figure 4. Length of new leaves of *V. limbata* after spraying BAP 0, 25 and 50 mg l^{-1} for 3 months

cell elongation through the process of cell osmosis.

In the parameter of elongation of old leaves and new leaf length, spraying BAP 50 mg l^{-1} was the most effective treatment, but not optimal for *V. limbata* orchids. This is supported by similar studies revealing that BAP also has an effect at higher concentrations. The results of research by Lee et al. (2021) showed that applying BAP 100 mg l^{-1} to adult *Phalaenopsis* orchids *in vivo* resulted in an increase in leaf length with an average of 20.4 cm.

BAP application can significantly affect the length of the leaves, but does not significantly affect the width of the leaves (Figure 5). The results of data analysis showed there were not significantly differences on old leaves for all treatments (Figure 6a), while the new leaves showed a significant difference between the control treatment and the treatment of 25 and 50 mg l^{-1} (Figure 6b). The significant differences in new leaves occurred because no new leaves appeared in the control treatment. Meanwhile, the treatments of 25 and 50 mg l^{-1} showed no significant difference. In this case, it means that the *V. limbata* orchid has reached the maximum growth of its leaf width. This finding is supported by Gerry et al. (2020) that in general, the width of *Vanda* orchid leaves is no more than 3 cm, while the length can continue to grow up to 45 cm. These results indicate that the morphological characteristics of plants also affect the growth response of plants when given exogenous hormones. For example, the research of Lee et al. (2021) disclosed that the application of 100 mg l^{-1} BAP to adult *Phalaenopsis* orchids *in vivo* produced the widest leaves, reaching 8.3 cm. The difference in results is due to the morphological characteristics of *Phalaenopsis* leaves, which can develop wider than the leaves of *V. limbata*.

Stomatal density

The results of data analysis disclosed that the application of various concentrations of BAP affected the stomatal density (Figure 7). The average stomatal density after the spraying of BAP at the concentrations of 0, 25 and 50 mg l^{-1} was 44.98, 40.45 and 34.95 stomata mm^{-2}, respectively. The stomatal density of *V. limbata* in all treatments is considered low. This is in line with the report of Susilowati et al. (2022) that stomata are categorized into 3 based on their density, which are low density (< 300 mm^{-2}), medium density (300 to 500 mm^{-2}) and high density (> 500 mm^{-2}). Low stomatal density

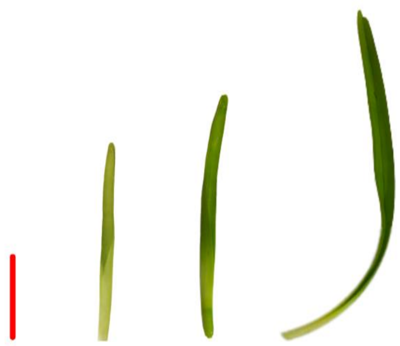


Figure 5. Comparison of *V. limbata* old leaf morphology after the spraying of BAP at various concentrations, Control (a), 25 (b) and 50 (c) mg l^{-1} for 3 months (Red bar: 3 cm)

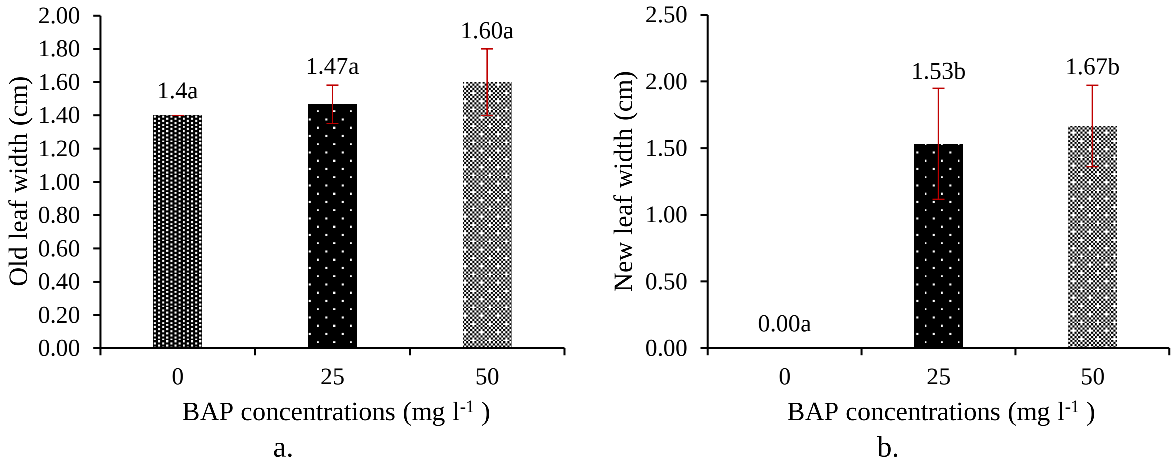


Figure 6. Width of old (a) and new (b) leaves of *V. limbata* after the spraying of 0, 25 and 50 mg l^{-1} BAP for 3 months

indicates that the orchid is in a good environmental condition. In this research, all treatments obtained homogeneous light intensity because they were placed in the same shaded location and sufficient water availability. According to Driesen et al. (2020), stomatal density is generally influenced by environmental factors, such as light intensity, temperature, humidity and CO_2 concentration in the air. It is also supported by the statement of Caine et al. (2019) that the low density of stomata indicates plants in environmental conditions with sufficient water availability. This is in line with the report of Dunn et al. (2019) that leaves with lower stomatal density have the advantage of reduced water loss in terms of loss of assimilation rate in cereal plants.

The 50 mg l^{-1} BAP treatment resulted in lower stomatal density on *V. limbata* (Figure 7 and 8). This result indicates that when cell division and elongation occur in growing leaves, they are not followed by cell differentiation into stomata. Stomatal cell differentiation followed by an increase in stomatal density is more influenced by environmental factors, so that BAP spraying has not been able to affect the increase in stomatal density. This is supported by the statement of Driesen et al. (2020) that stomatal density is influenced by environmental conditions, such as light intensity, temperature and humidity. In addition, it is suspected that the endogenous cytokinin concentration is enough for stomatal development so that when BAP is sprayed, it causes excess cytokinin concentration in orchids and inhibits stomatal development. This is supported by Maulia et al. (2021) that exogenous BAP concentrations were too high actually inhibit plant growth.

Stomatal cell differentiation that did not occur in this research was not only influenced by environmental factors, but also by genetic factors and adaptations of the orchid. *V. limbata* is a type of orchid that does not require a lot of evaporation to maintain its moisture so that there is no increase in stomatal density when the environmental factors are supportive. Suradinata et al. (2017) stated that the increase in the number of stomata is a form of plant adaptation to the environment due to the need for greater evaporation. This designates that leaf expansion is not necessarily followed by an increase in stomatal density.

Number of roots

The results showed that BAP spraying did not affect the number of roots in all treatments (Table 1). This demonstrates that BAP has a greater effect on the growth of leaf buds than the number

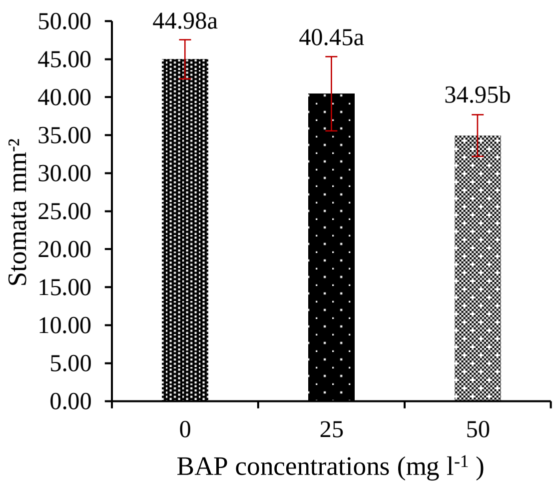
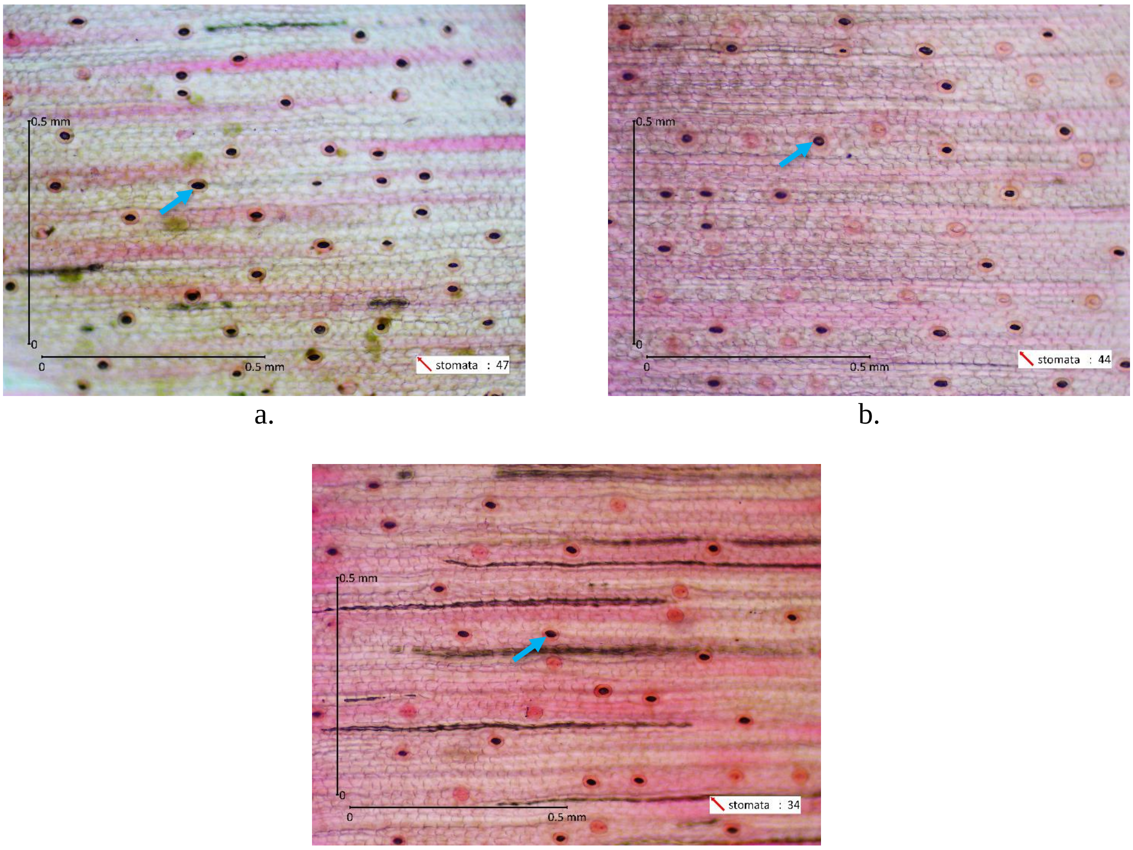


Figure 7. Stomatal density of *V. limbata* after the spraying of BAP at the concentrations of 0, 25 and 50 mg l^{-1} for 3 months



c.

Figure 8. Comparison of stomatal density of *V. limbata* after the spraying of BAP at various concentrations and at 10x magnification (a. Control, b. 25, c. 50 mg l^{-1}) for 3 months (Blue arrows: stomata)

of roots. BAP is a class of cytokinins that can only increase cell division in the shoots, while increased root growth is a class of auxins. Cytokinin in the roots only synthesizes cytokinin; then cytokinin will be transported to the nodes for branching and leaves. This is supported by Campolongo et al. (2020) that cytokinins are mainly synthesized in the roots, then move through the stem xylem to the shoot apical meristem and increase cell division to form new shoots. The results of this research are similar to the results of the study by Hadiati (2011) on the immersion of BAP concentrations of 200 to 600 mg l^{-1} in pineapple (*Ananas comosus* L.) stem cuttings, in which the immersion of BAP did not have a significant effect on the number of pineapple roots at 17 weeks of age.

Table 1. Number of roots of *V. limbata* before and after the spraying of BAP for 3 months

	BAP concentrations (mg l^{-1})		
	0	25	50
Before treatment	2	3	5
After treatment	2	3	5

Root and shoot growth have a close relationship. This is because the synthesis of cytokinin and auxin hormones occurs in the roots. According to Matteo et al. (2015), the cytokinin signaling pathway has the main control in the roots, as well as auxin, so that root and shoot growth are closely coordinated. Kurepa et al. (2019) and Kurepa and Smalle (2022) explain that the ratio of auxin and cytokinin greatly affects the growth of roots and shoots. If auxin is higher than cytokinin, there will be a difference that leads to root growth. In contrast, cytokinin that is higher than auxin will encourage shoot formation. The results of the research have proven that spraying BAP causes the cytokinin contents to be higher than the endogenous auxin of *V. limbata* orchid, leading to no increase in the number of roots. If the auxin to cytokinin ratio is higher, root growth will increase.

CONCLUSIONS

BAP can increase *V. limbata* leaf growth in terms of the number of new leaves and leaf length, but contribute to lower stomatal density than the control. The most effective concentration of BAP on the growth of *V. limbata* orchid is 50 mg l^{-1}. These results are expected to provide scientific information on the use of exogenous PGR, particularly BAP, on the growth of *V. limbata*. This is also a recommendation for ex-situ conservation efforts that have the potential to be developed as a means of orchid cultivation. Future research is recommended to use a combination of PGRs to support root growth and flower induction, not only leaf growth, to achieve both conservation and cultivation of this ornamental orchid in a sustainable agricultural method.

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