Benzyl amino purine enhances multiplication of *Vanda tricolor* protocorm like bodies

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Abstract. *Vanda tricolor* orchids are endemic to the slopes of Mount Merapi, Yogyakarta Province, Indonesia. The exploitation of *Vanda tricolor* out of its natural habitat by the community for collection or selling it outside the area has reduced the orchid population. Therefore, efforts should be carried out to improve technology to reproduce and regenerate *Vanda tricolor* orchids. The objective of this study was to determine the best type and concentration of cytokinin for the multiplication of *Vanda tricolor* Protocorm Like Bodies (PLBs). The experiment was arranged in a completely randomized design with single factor consisting of 7 treatments namely medium without cytokinin, BAP 0.5 mgL\(^{-1}\), BAP 1 mgL\(^{-1}\), TDZ 0.5 mgL\(^{-1}\), TDZ 1 mgL\(^{-1}\), Kinetin 0.5 mgL\(^{-1}\) and Kinetin 1 mgL\(^{-1}\). Parameters observed in this study were percentage of viable and abnormal explant, PLBs diameter, growth time of shoots and roots, number of explant sprouting and shoots. The data were analyzed by using The Analysis of Variance and further tested using Duncan Multiple Range Test (DMRT) at \(\alpha\) 0.05. The results showed that BAP 0.5 mg / L was the best cytokinin for multiplication of *Vanda tricolor* PLBs as shown by the parameters the diameters of PLBs (1.83 mm) and the number of shoots (2.6 shoots).

1. Introduction

*Vanda tricolor* orchids are endemic to the slopes of Mount Merapi, Yogyakarta Province, Indonesia. White-flowered orchids with patches of reddish-purple spots live epiphytically and are often found sticking to tree trunks in the forests of Mount Merapi. However, bursts of hot clouds, forest fires on the mountain slopes and eruptions in 2006 have destroyed 80% of the habitat and threatened the existence of these orchids. In addition, the exploitation of *Vanda tricolor* out of its natural habitat by the community for collection or selling it outside the area has reduced the orchid population [1]. Gardiner [2] stated that the main restriction to get *Vanda tricolor* for research sample due to the lack of populations in the wild available for sampling.

*Vanda tricolor* conservation efforts have been carried out by the Natural Resources Coordinating Board of Yogyakarta Province by giving these orchid plants to farmer groups around the Merapi Volcano area. However, maintenance and conventional propagation methods carried out by farmer groups have not been able to increase the number of orchid populations, even on the contrary the percentage of plant death is still quite high. Only 45 percent of orchid plants were left after 1 year of conservation [1]. Merapi eruption in 2010 further reduced the *Vanda tricolor* orchid population in the Mount Merapi region. Therefore, efforts should be carried out to improve technology to reproduce and
regenerate *Vanda tricolor* orchids. One of alternative to regenerate *Vanda tricolor* is using multiplication technique through in vitro cultures.

In vitro or plant tissue culture is a technique of isolating parts of a plant, growing it in artificial media that contains complete nutrients in a sterile environment so that parts of the plant grow into perfect plants [3, 4]. Multiplication is done by multiplying the number of shoots from various explants such as shoots, leaves and protocorm like bodies (PLB). Growth and multiplication of shoots can be obtained from the medium given cytokinin such as BAP, Thidiazuron, Kinetin and 2-iP. Cytokinins can encourage multiplication of buds by stimulating the formation of enzymes needed in mitosis. Tokuhara and Mii [5] have produced more than 10,000 Phalaeonopsis and Doritaenopsis orchid PLB for 1 year by culturing shoot cuttings explants on New Dogashima Medium (NDM) medium containing 1 mgL\(^{-1}\) BAP and 0.1 mgL\(^{-1}\) NAA. NDM medium contains several vitamins and organic ingredients that encourage the formation of PLB in orchid explants.

This study aims to determine the best type and concentration of cytokinin to multiply *Vanda tricolor* orchid buds from PLBs explants. The plantlet production technique is expected to be an alternative to provide better, more and faster planting material for the conservation or return of *Vanda tricolor* orchids to their natural habitat.

2. Materials and Methods
The study was conducted using a single factor experiment with seven treatments which arranged in a completely randomized design (CRD). The treatments were the types and concentration of cytokinin (medium without cytokinin, Benzyl Amino Purine-BAP 0.5 mgL\(^{-1}\), BAP 1 mgL\(^{-1}\), Thidiazuron-TDZ 0.5 mgL\(^{-1}\), TDZ 1 mgL\(^{-1}\), Kinetin 0.5 mgL\(^{-1}\) and Kinetin 1 mgL\(^{-1}\)). Each treatment was replicated ten times. Each treatment medium was supplemented with Naphtalene Acetic Acid (NAA) 0.5 mgL\(^{-1}\), activated charcoal 0.2 gL\(^{-1}\) and Plant Preservative Mixture (PPM) 0.5 mlL\(^{-1}\). The explants used were 2-months-old PLB, derived from *Vanda tricolor* orchid seeds were previously germinated in culture medium. The basic medium used is New Dogashima Medium (NDM).

2.1. Medium Preparation
The medium was prepared by supplementing NDM powder 1.96 gL\(^{-1}\), sucrose 30 gL\(^{-1}\), PPM 0.5 mlL\(^{-1}\), activated charcoal 0.2 gL\(^{-1}\) into beaker glass and cytokinin according to treatment, namely BAP 0.5 mgL\(^{-1}\), BAP, BAP 1 mgL\(^{-1}\), Thidiazuron-TDZ 0.5 mgL\(^{-1}\), TDZ 1 mgL\(^{-1}\), Kinetin 0.5 mgL\(^{-1}\) and Kinetin 1 mgL\(^{-1}\). Furthermore, the medium was autoclaved at 121°C and pressure of 1 psi. TDZ was added to the treatment medium after the medium was autoclaved. The addition of TDZ into the treatment medium was carried out using millipore in a laminar air flow cabinet.

2.2. Explant Preparation and Inoculation
The explants used were 2-months-old *Vanda tricolor* PLBs. Only one PLB explant was inoculated in each bottle. Incubation was carried out in a culture room with a temperature of 20-28°C. Parameters observed in this study were a percentage of viable, browning and contamination explant, the diameter of PLB, the emergence of shoots, the percentage of explant sprouting, the number of buds, the emergence of roots. The data were analyzed by using The Analysis of Variance at \(\alpha=5\%\) and further tested using Duncan Multiple Range Test (DMRT) at \(\alpha=5\%\).

3. Results and Discussion

3.1. The Percentage of Viable, Browning and Contaminated Explant
The growth of explant is influenced by the percentage of viable, browning and contaminated explant. The higher the percentage of viable and the lower the percentage of browning and contaminated explants can increase the success of multiplication of *Vanda tricolor* buds from PLBs. The results showed that the percentage of viable explants was 100%. The high percentage of viable explants because the explants used were PLBs derived from sterile cultures, so the possibility of contamination was low.
In addition, the use of PPM in the medium can prevent the growth of bacteria and fungi. This is because PPM is one of biocide materials in liquid culture which belongs to the isothiazolone group which can inhibit microbes and fungi [6]. The high percentage of viable explant was also caused by the medium used containing nutrients and complete organic compounds, so that it can encourage explants to survive. The percentage of explants browning was 0%, because explants did not release phenol compounds, so oxidation did not occur. This was in line with Rineksane and Sukarjan [7] who stated that the growth of explant can be inhibited by phenol compounds released by explants because they react with oxygen which results in browning on the surface of explants. The low percentage of explants browning on the explant was due to explant responses on the compounds or growth regulating substances in the medium that encourage the cell division.

The success of Vanda tricolor PLBs culture without browning was also due to the addition of activated charcoal to each treatment medium. Activated charcoal can reduce browning medium due to high heating after sterilization [8]. Activated charcoal can also absorb phenol compounds released by the explant [8]. In addition, the use of young explants such as PLB causes the low browning of explants, as stated by Rineksane et al. [9] that young explants release very few phenolic compounds.

### 3.2. The Diameter of Protocorm Like Bodies (PLBs)

The enlarged PLB diameter can be caused by the increasing number of cells or enlarged cell size due to the content of growth regulators as cytokinins in the medium. The results of the analysis of PLB diameter are presented in Figure 1.

**Figure 1.** Effect of types and concentration of cytokinin on PLB *Vanda tricolor* diameter at eight weeks. Bars followed by the same letter showed no significant difference based on the DMRT test at α level of 5%. Ten replicates for each treatment were used in this histogram.

Based on the analysis of variance there were significant differences between BAP 0.5 mgL$^{-1}$ treatment with all treatments given as presented in Figure 1. This is presumably because BAP is a cytokinin which active in the development and division of cells and work optimally in low concentration. The best of PLB diameter (1.83 mm) was obtained in BAP 0.5 mgL$^{-1}$ treatment. Low concentration of BAP has a very large role in cell division and optimum work, so that it can accelerate cell division quickly in treated explants. Increasing the diameter of PLB is also due to cytokinin interactions for plant growth as stated by Gunawan [10], that the addition of exogenous hormones will affect the amount and activity of endogenous hormones to encourage growth and development of explants. Gill *et al.* (2004) in Fibrianty [11] stated that swelling of explants in plants gives an indication of cell elongation or
enlargement caused by cytokinins. Increasing the diameter of *Vanda tricolor* PLB is also due to PLB absorbing water and nutrients from the medium. This is consistent with the research of Rineksane and Sukarjan [7] that swelling in explants is caused by imbibition that shows explants absorb water and nutrients.

### 3.3. The Growth Time of Shoots

The growth time of shoots is one indicator of the growth of explants in response to the treatment given. The sooner the shoots are formed, the more nutrients will be absorbed by the explants so that they will accelerate the formation of new individuals. Based on the results of the analysis of variance, it was found that there was no significant difference among the treatment on the growth time of shoots of *Vanda tricolor*. This means that all explants produce shoots in a period of 1.7-2.8 weeks. The data of the growth time of shoots are presented in Figure 2.

![Figure 2](image.png)

**Figure 2.** Effect of types and concentration of cytokinin on the growth time of shoots of *Vanda tricolor* at eight week. Ten replicates for each treatment were used in this histogram.

The data in Figure 2 shows that treatment without cytokinins tends to produce shoot which relatively faster (1.7 weeks) compared to other treatments (1.9 - 2.8 weeks). This is because PLB explants have shoots primordia, those shoots primordia appear as a response to the absorption of explants on nutrients in the NDM medium. On the other hand, the increasing of cytokinins results in slower growth time of shoots, because cytokinins which absorbed by explants are used for cell division, which then multiplies shoots, as stated by Putri [12] that the main activity of cytokinins is to promote the cell division.

Among the cytokinins used, BAP 0.5 mgL⁻¹ tends to produce shoots that are relatively fast compared to other treatments. This is because BAP is an active and stable cytokinin. In addition, the explant response to cytokinins in the medium tends to be slow, as stated by Rineksane and Sukajan [7], that the factor causes no development on the explant is the response of *Vanda tricolor* plant which is very slow, so there is no significant development.

### 3.4. The Percentage of Explants Sprouting

The percentage of explants sprouting shows the ability of shoot explants to form new shoots in response to cytokinins added to the treatment medium. The data showed that all treatments had high percentage of sprouting explants which tended to be 90-100%. This is presumably due to the presence of cytokinins such as BAP, Thidiazuron and Kinetin which are active in the process of cell division and stimulate shoot growth. This is in accordance with the opinion of Wareing and Philips [13], that cytokinins stimulate plant cell division. In addition, nutrients in the medium encourage the activity of substances
exchange between one cell or overall in the plant's tissues and causing the plant cells to divide to form shoots.

The high percentage of sprouting explants is also due to the absence of contamination and browning in PLB, so PLB absorbs nutrients from the medium very well. Sabar [14] stated, that shoot formation normally occurs in explants that are free from contamination and browning, normally developing shoots have a higher chlorophyll content caused by perfect absorption of nutrients. PLB is a young tissue which still actively dividing to encourage the growth and development of shoots. In addition, NDM contains a lot of organic elements, so that explants able to absorb the nutrient in the medium for growth.

3.5. The Number of Shoots

New shoots are formed through the process of cell division as an explant reaction to cytokinins absorbed from the medium. The dividing cells subsequently undergo differentiation to form new shoots. The effect of the type and concentration of cytokinins on the number of *Vanda tricolor* shoots are presented in Figure 3.

![Figure 3](image_url)

**Figure 3.** Effect of types and concentration of cytokinin on the number of shoots of *Vanda tricolor* at eight weeks. Ten replicates for each treatment were used in this histogram.

The results of analysis of variance showed no significant difference between treatments for the number of *Vanda tricolor* shoots (Figure 3). This is due to the fact that PLB explants have shoots primordia, so that the availability of nutrients in the medium absorbed by plants can encourage the growth of shoots primordia. However, the addition of BAP 0.5 mgL\(^{-1}\) into the medium tends to produce the highest number of shoots (2.6 shoots) compared to other treatments. Increasing of BAP concentration of 1 mgL\(^{-1}\) causes a decrease in the number of shoots produced. Likewise in the treatment with the addition of Kinetin. This means that the use of higher BAP and Kinetin concentrations inhibits the addition of shoots to explants. Conversely, increasing of the amount of TDZ added to the medium, causes increasing in the number of shoots formed. Aini et al. [15] which stated that increasing the number of chromosomes in the cell nucleus prolongs the interphase phase which results in slow cell division processes that inhibit shoot growth.

Suryowinoto [16] stated that BAP is a group of cytokinins that play a role in encouraging cell division and inducing shoot formation. BAP is the most effective group of cytokinins for shoot formation. Those results are supported by the study of Latip et al. [17] using BAP (0.5 - 3.5 mgL\(^{-1}\)) added in the NDM medium to multiply the *Phalaenopsis gigantia* orchid protocorm. BAP is a group of stable cytokinins
to induce the formation of adventitious shoots on many plants [17]. Figure 4 shows the addition of BAP 0.5 mgL$^{-1}$ resulting in a large number of shoots compared to other treatments.

| Treatment     | 2-month-old | 9-month-old | Treatment     | 2-month-old | 9-month-old |
|---------------|-------------|-------------|---------------|-------------|-------------|
| BAP 0.5 mgL$^{-1}$ | ![Image](image1) | ![Image](image2) | BAP 1 mgL$^{-1}$ | ![Image](image3) | ![Image](image4) |
| TDZ 0.5 mgL$^{-1}$ | ![Image](image5) | ![Image](image6) | TDZ 1 mgL$^{-1}$ | ![Image](image7) | ![Image](image8) |
| Kinetin 0.5 mgL$^{-1}$ | ![Image](image9) | ![Image](image10) | Kinetin 1 mgL$^{-1}$ | ![Image](image11) | ![Image](image12) |
| Without Cytokinin | ![Image](image13) | ![Image](image14) | | ![Image](image15) | ![Image](image16) |

**Figure 4.** Effect of types and concentration of cytokinin on the number of shoot of *Vanda tricolor* at two and nine months.

### 3.6. The growth time of roots

The growth time of roots showed the rapidity of explants to grow roots. The effect of the type and concentration of cytokinins on the growth time of *Vanda tricolor* roots are presented in Figure 5.

The results of analysis of variance showed that there were significant differences between treatments for the growth time of roots (Figure 5). The fastest growth time of roots was shown by the BAP treatment of 1 mgL$^{-1}$ (0.2 weeks) and was not significantly different from the Kinetin treatment of 0.5 mgL$^{-1}$ (0.4 weeks). On the other hand, BAP 0.5 mgL$^{-1}$ showed the longest time of root growth. This is because the BAP 0.5 mgL$^{-1}$ treatment has the most shoots, so the growth leads to shoot growth compared to root growth. The roots are formed if there is a balance between cytokinins and auxins. PLB explants that are used already have endogenous cytokinins and auxins that can encourage root formation. However, the addition of exogenous cytokinins encourages PLB to form more shoots than it forms roots. Lisnandar *et al.* [18] stated that explants can produce their own cytokinins, in addition it is thought to have the ability to produce auxin, but not as much as cytokinin production.

Figure 5 showed that BAP treatment of 1 mgL$^{-1}$ resulted the fastest growth time of roots, namely 0.20 weeks. This is presumably because explants prioritize root appearance in BAP treatment of 1 mgL$^{-1}$, roots that appear will absorb the nutrient in the medium and the addition of exogenous cytokinins will interact with endogenous auxins in explants. This proves that in vitro plant growth is controlled by balance and interaction between growth regulating substances both contained in the explants themselves (endogenous) and those absorbed from the medium (exogenous).
The treatment of BAP 0.5 mgL\(^{-1}\) tends to be slow to the growth time of roots namely 2.00 weeks. This is presumably because BAP is an active synthetic cytokinin which functions more to encourage shoot formation [4], so that there is an antagonistic interaction between auxin and cytokinin which will inhibit root growth [19]. This can also be caused by the time needed for the root formation process is longer. In accordance with Nazi [20], the time needed for root formation is longer than the time needed for leaf formation.

4. Conclusion
Cytokinin BAP 0.5 mgL\(^{-1}\) enhances the multiplication of Vanda tricolor orchids as shown by the parameters the diameters of PLBs (1.83 mm) and the number of shoots (2.6 shoots)

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