Optimization of 2,4-D and Cytokinin Combination for The Growth of Vanda tricolor in Solid and Liquid Medium

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Abstract. Vanda tricolor orchid is an endemic plant. Yogyakarta is one of the distribution areas of the V. tricolor Lindl Suavis varieties in Indonesia, besides East Java, West Java, Bali and Sulawesi. The existence of the V. tricolor orchid on the slopes of Mount Merapi in Yogyakarta is threatened with extinction due to eruption and exploitation by humans. The existence of the diminishing V. tricolor orchid encourages efforts to conserve V. tricolor orchids into their natural habitat through conservation. One of the conservations carried out through in vitro propagation is somatic embryogenesis. The aim of this study is to determine the best combination of 2,4-D and cytokinin in solid and liquid medium for the growth of pro embryo V. tricolor. This research is a laboratory experiment using a single factor experimental method arranged in a completely randomized design. The treatments tested were a combination of 2,4-D (0, 2, 4 mg L\(^{-1}\)), cytokinin (Benzy1 Amino Purine and Thidiazuron, 0 and 0.5 mg L\(^{-1}\)) and the type of medium (solid and liquid). The basic medium used is New Dogashima Medium (NDM). Activated charcoal 0.2 g L\(^{-1}\) and Plant Preservative Mixture 0.5 ml L\(^{-1}\) were added to all treatments. The observed parameters included: Percentage of viable, contaminated and vitrified explants (%), number of leaves, number of pro embryos and number of roots. The results showed that treatment of liquid NDM without 2,4-D with the addition of 0.5 mg L\(^{-1}\)Thidiazuron was chosen as the best medium for the growth of V. tricolor shoots.

Keywords: Vanda tricolor, pro embryo, 2,4-D, cytokinin.

1. Introduction

Vanda tricolor Lind orchid plant. var. suavis is a superior horticultural commodity with a high enough selling value in the local market because of its various shapes and colors and its long-lasting flowers [1]. The existence of the V. tricolor orchid has been threatened with extinction since the eruption of Mount Merapi in 1994. The subsequent eruption of Merapi, namely in 2010, destroyed forests and V. tricolor orchids which scorched 80% of the original habitat of these orchids [1]. In addition, the exploitation of V. tricolor out of its natural habitat by the community for collection and selling it outside the region has also reduced the population of this orchid [2]. The diminishing presence of the V. tricolor orchid has encouraged efforts to protect the V. tricolor orchid in its natural habitat, especially on the slopes of Mount Merapi through conservation.

The Natural Resources Conservation Agency of Yogyakarta has made efforts to increase the population of the V. tricolor orchid by conducting a breeding business by forming 5 conservation farmer groups from 3 districts on the southern slopes of Mount Merapi. Inaccurate cultivation techniques have
resulted in slow growth and reproduction of *V. tricolor*. The conventional method of propagation by farmer groups has not been able to produce results by increasing the number of orchid populations. There were 80 orchids distributed to farmers, only 36 plants remained after 1 year [2]. Therefore, technological improvements need to be made to reproduce *V. tricolor* orchids.

One of the alternatives to conserve orchid diversity is propagation by in vitro culture. Rindang et al. [3] stated that propagation by in vitro culture is a method of propagation that is beneficial for rare plant species for conservation purposes. One of in vitro plant propagation techniques is somatic embryogenesis. Somatic embryogenesis is advantageous because the number of propagules produced is greater and obtained in a short time [4], especially for species that have high economic value [5] and the progenies are identical to the parent [6].

One of the factors that determine the success of somatic embryogenesis is auxin. Several studies stated that auxin stimulates the formation of embryogenic callus and somatic embryo structure so that it can stimulate explant growth [7, 8]. One of the plant growth regulators including auxin is 2,4-D (2,4-Dichlorophenoxyacetic acid). In addition, plant growth regulators that influence embryogenesis is cytokinin [7]. Thidiazuron (TDZ) and Benzyl Amino Purine (BAP) are cytokinin which are usually added to the medium with the addition of 2,4-D to induce embryo somatic in various plants.

Our previous studies found that BAP 0.5 mg L\(^{-1}\) on New Dogashima Medium (NDM) was the best cytokinin for multiplication of *V. tricolor* protocorm like bodies (PLB) indicated by the parameters the diameters of PLBs (1.83 mm) and the number of shoots (2.6 shoots). Meanwhile, Niknejad [10] reported that the use of PLB Phalaenopsis orchid explants in liquid NDM medium with the addition of 1 mg L\(^{-1}\) NAA added 0.1 mg L\(^{-1}\) TDZ resulted the highest percentage of callus growth (100%). This study was aimed to combine 2,4-D and cytokinin (BAP and TDZ) in solid and liquid medium to encourage the growth of *V. tricolor* shoots through embryogenesis.

### 2. Materials and Methods

This research is a laboratory experiment using a single factor experimental method arranged in a completely randomized design (CRD). The treatments tested were a combination of 2,4-D (0, 2, 4 mg L\(^{-1}\)), cytokinin (BAP and TDZ, 0 and 0.5 mg L\(^{-1}\)) and the type of medium (solid and liquid). The basic medium used is NDM. Activated charcoal 0.2 g L\(^{-1}\) and PPM (Plant Preservative Mixture) 0.5 ml L\(^{-1}\) were added to all treatments. Each treatment was repeated 9 times.

#### 2.1. Medium Preparation

The medium was made by mixing NDM, sucrose, vitamins, 2,4-D, BAP, TDZ, activated charcoal, Plant Preservative Medium according to the treatment. The pH of the medium was adjusted to pH, then the solution was added with Pytagel as a solidifier for solid and without the addition of Phytagel for liquid medium. The medium was sterilized in an autoclave with a temperature of 121°C and a pressure of 1 psi for 20 minutes. Furthermore, the medium is stored in the incubation room before use.

#### 2.2. Explant Preparation and Inoculation

The explants used in this study were sterile 1 year old shoots of *V. tricolor*. Planting was carried out by taking the shoots from the available seedling bottles, then, the explants were planted in a treatment medium using tweezers. Each bottle contains one explant. After planting, the bottles were covered with the aluminium foil, secured with a rubber band, coated with a wrap, then labelled the treatment and date of planting. Furthermore, the culture bottle containing the solid medium was placed on the incubation rack, while the culture bottle containing the liquid medium was placed on a shaker in the incubation room.

#### 2.3. Parameter Observed and Data Analysis

Observations were made from the beginning of planting until the 8th week after planting. The observed parameters included: Percentage of viable explants (%), percentage of contaminated explants (%),
percentage of vitrified explants (%), number of leaves, number of pro embryos and number of roots. The data were analysed using a 5% level of analysis of variance.

3. Result and Discussion

*V. tricolor* shoot explants showed a growth response after 1 week of planting. The explants continued to grow as shown by the emergence of shoots, callus, proembryo and leaf growth. The viable orchid explants are characterized by a light green color, not vitrified, free of contamination and browning.

### 3.1. Percentage of viable explants, percentage of contaminated explants, percentage of vitrified explants

The growth of explants was influenced by the culture medium and type of explants [11]. Percentage of viable, contaminated vitrified explants of *V. tricolor* shoots are presented in Table 1. Contamination of 11.11% only occurred in the treatment of liquid NDM medium supplemented with 4 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ TDZ, because the explants used were sterile *V. tricolor* shoots and planting was carried out in a sterile condition.

| Types of medium with 2,4-D and BAP/TDZ | Viable Explant (%) | Contaminated Explant (%) | Vitrified Explant (%) |
|--------------------------------------|---------------------|--------------------------|-----------------------|
| NDM Solid                            |                     |                          |                       |
| 0 mg L⁻¹ 2,4-D + 0 mg L⁻¹ BAP         | 55.56               | 0                        | 22.22                 |
| 0 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BAP       | 55.56               | 0                        | 22.22                 |
| 2 mg L⁻¹ 2,4-D + 0 mg L⁻¹ BAP         | 55.56               | 0                        | 22.22                 |
| 2 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BAP       | 100                 | 0                        | 0                     |
| 4 mg L⁻¹ 2,4-D + 0 mg L⁻¹ BAP         | 33.33               | 0                        | 11.11                 |
| 4 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BAP       | 33.33               | 0                        | 33.33                 |
| NDM Liquid                           | 100                 | 0                        | 0                     |
| 0 mg L⁻¹ 2,4-D + 0 mg L⁻¹ TDZ         | 88.89               | 0                        | 11.11                 |
| 2 mg L⁻¹ 2,4-D + 0 mg L⁻¹ TDZ         | 57.78               | 0                        | 42.22                 |
| 2 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ TDZ       | 44.44               | 0                        | 55.56                 |
| 4 mg L⁻¹ 2,4-D + 0 mg L⁻¹ TDZ         | 77.78               | 0                        | 22.22                 |
| 4 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ TDZ       | 22.22               | 11.11                    | 66.66                 |

Explants can be categorized as vitrified if the explants lost chlorophyll so that the leaves and stems color changed to white and transparent. Vitrification in this study was quite high, namely 0 - 66.66% (Table 1). The data in Table 1 shows that the vitrification of explants in liquid medium is higher than that of solid medium. This high rate of vitrification resulted in high explant mortality. Karyanti et al. [12] stated that the basic cause of vitrification lies in the water potential in the plant tissue, the solidifier concentration or the cytokinin used. In this study, there were two types of vitrification, namely direct and indirect vitrification. The explants undergo immediate vitrification where the green ones will turn transparent white. Direct vitrification is also due to the high-water content. Meanwhile, vitrification indirectly occurs in the previously browning explants.

In this study, indirect vitrification occurred 2-4 weeks after browning explants. The high phenolic content which causes browning in the explants decreasing the ability of the explants to grow. The water potential in the medium also causes vitrification which is indicated by the change of the explants color. Table 1 indicated that the addition of the same concentration of 2,4-D with 0.5 mg L⁻¹ TDZ resulted a
lower percentage of the explants vitrified. This is in accordance with the statement of Karyanti et al. [12] that the basic cause of vitrification is the water potential in the plant tissue. In this study, the medium used was liquid NDM. The water content in the liquid medium causes the water move into the explants with high intensity, resulting in a loss of the content of plant cells which results in the explants being unable to develop and die. The water potential in the medium and the weakening of the explant tissues caused a high intensity diffusion. The concentration of water on the outside of the cell which higher than the inside of the cell causes water to tend to move into the cell through the cell membrane at a very high intensity and result in loss of cell content in the explants. The weakening of the explant tissue caused by the water potential in the medium resulted in vitrification and resulted in the loss of chlorophyll and plant cell content in the explants so that the explants’ ability to absorb nutrients stopped which resulted in the explants being unable to grow and causing the explants to die. In addition, the use of 0.5 mg L\(^{-1}\) TDZ may be too excessive. This is in line with Fitriani [13] opinion that vitrification can be caused by high concentration of cytokinin.

### 3.2. Number of Leaves

The number of leaves plays an important role for the vegetative growth and the ability of plants to carry out photosynthesis processes and various other metabolisms. Leaf formation begins with the leaf primordia initiation followed by cell division, cell enlargement and differentiation [14]. Data on the increase in the number of leaves for each treatment is presented in Figure 1.

![Figure 1. Effect of medium, 2,4-D and cytokinin on the number of *V. tricolor* leaves at 8 weeks after planting](image)

**Note:**

- D1B1 : NDM Solid + 0 mg L\(^{-1}\) 2,4-D + 0 mg L\(^{-1}\) BAP
- D1B2 : NDM Solid + 0 mg L\(^{-1}\) 2,4-D + 0.5 mg L\(^{-1}\) BAP
- D2B1 : NDM Solid + 2 mg L\(^{-1}\) 2,4-D + 0 mg L\(^{-1}\) BAP
- D2B2 : NDM Solid + 2 mg L\(^{-1}\) 2,4-D + 0.5 mg L\(^{-1}\) BAP
- D3B1 : NDM Solid + 4 mg L\(^{-1}\) 2,4-D + 0 mg L\(^{-1}\) BAP
- D3B2 : NDM Solid + 4 mg L\(^{-1}\) 2,4-D + 0.5 mg L\(^{-1}\) BAP
- D1T1 : NDM Liquid + 0 mg L\(^{-1}\) 2,4-D + 0 mg L\(^{-1}\) TDZ
- D1T2 : NDM Liquid + 0 mg L\(^{-1}\) 2,4-D + 0.5 mg L\(^{-1}\) TDZ
- D2T1 : NDM Liquid + 2 mg L\(^{-1}\) 2,4-D + 0 mg L\(^{-1}\) TDZ
- D2T2 : NDM Liquid + 2 mg L\(^{-1}\) 2,4-D + 0.5 mg L\(^{-1}\) TDZ
- D3T1 : NDM Liquid + 4 mg L\(^{-1}\) 2,4-D + 0 mg L\(^{-1}\) TDZ
- D3T2 : NDM Liquid + 4 mg L\(^{-1}\) 2,4-D + 0.5 mg L\(^{-1}\) TDZ

According to Arteca [15], the presence of cytokinin influence the process of cell division and enlargement, which leads to the formation of organs such as leaves. The leaves formed are green, indicating that cytokinin can stimulate the development of chloroplasts, which affect the formation of chlorophyll.
The data in Figure 1 showed that all the liquid medium treatments resulted in a higher number of leaves than the solid medium. The number of leaves that appeared on the explants in each treatment combination varied. The varying number of leaves is due to the varied levels of endogenous hormones in each explant so that the response to the addition of growth regulators varies. According to Paramartha et al. [16], the use of high concentration of exogenous auxin or cytokinin can inhibit tissue growth due to its competition with endogenous auxins or cytokinin as a cell membrane signal receiver which influence the growth and development of cell.

The leaves also formed on the rooted explants. The roots arise in the medium supplemented with 2,4-D 2 mg L\(^{-1}\). Roots absorb nutrients in the culture medium which used for plant growth including leaf formation. Roots also can synthesize cytokinin so that the endogenous cytokinin content increases. Increased levels of endogenous cytokinin can increase the number of leaves formed. Wetherell [17] stated that the normal leaf formation and development depends on cytokinin which are normally synthesized in roots and transported to plant shoots. Salisbury and Ross [18] proved that cytokinin from roots were able to promote growth in leaves.

### 3.3. Number of Pro embryos

The number of pro embryos is very important, because the more pro embryos, the more potential shoots, root primordia and embryos are formed. The result of the analysis of the number of pro embryos is presented in Figure 2.

![Figure 2. Effect of medium, 2,4-D and cytokinin on the number of pro embryos V. tricolor at 8 weeks after planting.](image)

The data in Figure 2 showed that all liquid mediums produce a higher number of pro embryos than solid medium. The use of 0.5 TDZ influence the number of pro-embryos formed. According to Arteca [15], this is because TDZ as a cytokinin plays a role in the process of cell division and enlargement, which leads to organ formation. TDZ plays a very important role in stimulating morphogenesis in plant in vitro cultures, with several responses such as being able to induce callus formation from various explant sources, shortening the time for culture regeneration, and increasing the number of shoots per explant [19]. The more pro embryos that are formed, the higher the chances of shoot and root growing on the explants. In addition, NDM contains macro, micro, vitamin and amino acid nutrients which needed for explant growth. The age and size of the explants also influence explant growth. Chawla [20] stated that the explants from young and healthy tissues are more responsive and have highly cell regeneration.
3.4. Number of Roots
The number of roots indicates the ability of plant to absorb nutrients [21]. Plants with many roots will increase the absorption of nutrients and water which can support the growth of the plant as well. The results of the analysis of the increase in the number of roots are presented in Figure 3.

![Figure 3](image_url)

**Figure 3.** Effect of medium, 2,4-D and cytokinin on the number of *V. tricolor* roots at 8 weeks after planting.

Fig. 3 showed that all liquid medium treatments resulted in the increase in the number of roots more than the solid medium. The use of BAP in various concentrations was not able to produce many roots. It can be assumed that BAP application in culture medium is used more for shoot multiplication than for root formation. It was in line with Marlin [22] study on ginger explants where the addition of high concentration of BAP to the medium might inhibit the root formation. Low light intensity can stimulate endogenous growth regulators to work more actively in the process of root growth and development. According to Martin-Urdiroz et al. [23] bright conditions significantly affect the improvement of plantlet regeneration ability.

4. Conclusion
Treatment of New Dogashima Liquid medium without 2,4-D with the addition of 0.5 mg L$^{-1}$ Thidiazuron was chosen as the best medium for the growth of *V. tricolor* shoots.

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