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In Vitro Callus Induction from Leaf Explants of *Vanda* sp Stimulated by 2,4-D

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Abstract

The addition of growth regulator is one of the critical success factors in in vitro cultures. 2,4-D as a plant regulator in media can stimulate the cell division, enlargement of the explants and promotes the formation and growth of callus. The purpose of this study was to determine the time of callus formation and to determine the best concentration of 2.4-D in inducing the growth of callus from leaf explants of *Vanda* sp. This research was conducted by experiment with completely randomized design, which consists of six levels of treatment concentration of 2,4-D i.e. 0 ppm; 1 ppm; 1.5 ppm; 2 ppm; 2.5 ppm; and 3 ppm. The parameters observed were the percentage of callus formation and the form of callus from *Vanda* sp leaf explants. The results were statistically analyzed by using MINITAB program version 17. Analysis of variance (ANOVA) was performed and the difference between means score/value was separated by F test at $p < 0.05$. The results showed that 2,4-D treatment give significant effect ($F_{5,12} = 3{,}20$; $p = 0{,}046 < 0{,}05$) on the callus growth time and its percentage. Application of 2 ppm 2.4-D was the best concentration for accelerating the callus growth time (14.3 days after planting) and increasing the percentage of callus formation (83.3%). Most of callus type were proliferative callus (36.11%) and senescence callus (11.11%). The results of this research are very important to grow the callus from Vanda leaves orchid explant because it is very diffucult to grow.

How to Cite

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INTRODUCTION

Vanda as one of orchid species is cultivated because it has beautiful flowers and valuable, so the seedlings need to be produced in large quantities. According to Dohling *et al.* (2012), the multiplication of an orchid can be done in various ways, conventional and non conventional (*in vitro* culture). In conventional method, the multiplication of orchid can be done by using vegetative and generative organ. Vegetative propagation is commonly used by cutting of shoot. Generative propagation by using seed has low success rate, because the orchid seed have no endosperm (Jainol & Gansau, 2017). Conventional method through seed propagation is sometimes problematic as seed germination in natural environment is a slow process and it requires mycorrhizal or fungal contribution (Parthibhan *et al.,* 2015). Moreover, any disturbance in the habitat or physical environment during the germination will destroy the whole population (Martin & Madassery, 2006). Naturally, germination of orchid seed must be in symbiosis with mycorrhiza (Nasiruddin *et al.,* 2003), so to solve this problem, it can be done by using *in vitro* culture propagation (Hasanah *et al.,* 2014).

In vitro culture has proven as a better method for plant propagation that gives a great hope in plant cultivation especially orchid because this method can be used for orchid propagation in large quantities in a relative short of time (Naing *et al.,* 2011). The succeed of *in vitro* culture depends on many factors, such as the explants selection, medium type, growth regulator, and nutrient availability. Explant is a part of plant (can be cell, tissue, or organ) as a starting material grown in *in vitro* culture medium (Aktar *et al.,* 2008). *In vitro* propagation of orchids by shoot tip culture is a well-developed method. However, by using shoot tip as an explant means that the most significant part that is actively growing has to be sacrificed. Therefore, a technique of reproduction using other insignificant parts such as leaves of the plant would be beneficial (Gow *et al.,* 2009).

In *In vitro* culture, especially callus culture, part of the explants which is used for explants should has capability to divide *i.e.* shoot, leaves, buds, stem short and tip of root. Those materials can increase the success of *in vitro* culture because it has a high regeneration (Romeida & Ganefianti, 2016). The application of growth regulator is very important for the success of *in vitro* culture. Application of 2,4-D in medium will stimulate the cell division and enlarge the explants that stimulate the formation and growth of callus. 2,4-D is a strong auxin that is often used alone for callus induction in various plant tissues. Auxin has been characterized as a plant morphogen due to its ability to control leaf initiation in the meristem, stimulate the root formation, guide the tropic responses and organize the tissue patterns within the developing embryo. Indolacetic acid (IAA) is the most common naturally occurring auxin. Synthetic auxins include compounds such as naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Auxins are primarily synthesized in the shoot apex and move in a polar, basipetal mode through the stem, and acropetally in the root with a transition to basipital flow from the root tip which often described as a fountain-like flow (Novak *et al.,* 2014).

Successful cultures of plantlet from leaf culture of many orchid species has been reported. The species among them are *Aerides* (Devi *et al.,* 2013), *Aranda* (Gantait *et al.,* 2012), *Phalaenopsis* (Mayer *et al.,* 2010), *Vanda* and *Dendrobium* (Tee *et al.,* 2010) (Hasanah, 2014), and *Oncidium* (Mayer *et al.,* 2010). In leaf cultures, for example in *Renanthera* (Wu *et al.,* 2012) and *Vanilla* (Parthibhan *et al.,* 2015), wound surface of leaf explants had been reported to produce callus. Wu *et al.* (2012) reported that 99.67 % of plantlet formation was obtained from callus originated from leaf segments of *Renanthera* orchid. Protocorm-like bodies (PLBs) generally have a high potency to proliferate rapidly and regenerate into a whole plantlet (Nasiruddin *et al.,* 2003). However, there are lack information of 2,4 D application on callus induction of *Vanda* sp. Recent studies of callus induction has been done by Romeida and Ganefianti (2016) in *Concidium*, Nasiruddin *et al.* (2003) in *Dendrobium tribolium* and in *Spathoglottis plicata* (Novak *et al.,* 2014).

Based on the description above, we would like to find out the issues of how long does it take for callus to create formation from the leaf explants of *Vanda* sp, and how many 2,4-D concentration for callus formation in leaf explants of *Vanda* sp. The purpose of this research was to find out the duration of callus formation from leaf explants of *Vanda* sp by 2,4-D application and to know the best concentration for callus induction of leaf explants *Vanda* sp. The result of this research is very important because the information of plant regulator to stimulate the growth of *Vanda* sp callus is still limited (Tee *et al.,* 2010).

METHODS

Experimental

This research was conducted in Labo-

ratory of Plant Physiology, Faculty of Biology, Jenderal Soedirman University Purwokerto from February to June 2016. The method used was an experimental method by using Completely Randomized Design (CRD). The treatment included six levels of 2,4-D concentration,consisted of 0 ppm (D0); 1 ppm (D1); 1.5 ppm (D2); 2 ppm (D3); 2.5 ppm (D4) and 3 ppm (D5). Each treatment level was repeated 3 (three) times, so there were 18 experimental units. The medium used was a formulating media from Vacin and Went.

Procedure

The leaf explants for culture was taken from orchid culture collection in the Laboratory. *Vanda* sp plantlet was removed from the culture bottle then cut 1 mm^2 in size. The plantlet was then planted in the treatment medium and placed in a dark room. The dark condition was obtained by placing the culture petri dish in the incubator. Temperature in the incubator was 24-27°C with the humidity of 45%. Planting technique was done by using petri dish containing 20 ml of VW media which was then incubated. Five explants were planted in each petri dish. Variables measured were the time of callus formation (days), and the number of callus formed.

Data Analysis

The data analyzed were the time of callus formation (days), and the percentage of callus formed. The growth time of callus was determined when it appears on the growing explants of unorganized cells. The percentage of callus formed was obtained by the following formula:

$$\text{Percentage of callus} = \frac{\text{number of eksplan that callus formated}}{\text{number of all explant}}$$

The results were statistically analyzed by using MINITAB program version 17. The analysis of variance (ANOVA) was performed by F test at $p < 0.05$, then the mean values were separated by Duncan's Multiple Range Test (DMRT) at level of significance (α) = 0.05

RESULTS AND DISCUSSION

In the present experiment, 100% of the explant successfully produced callus within 14 – 20 days after the planting period. The shortest time of callus formation was at the treatment of 2 ppm 2,4-D (14,3 ± 1,5) and the longest time was at a control group (20 ± 2,0). This may due to the 2,4-D application in media which is able to improve auxin endogen concentration needed by explants to form callus. An appropriate amount of auxin inside can stimulate the formation of callus tissue were face the injury (Rahayu & Anggarwulan, 2003). The application of various concentration of 2,4-D in leaf explants of *Vanda* sp give different response in days after planting callus formation than 2,4-D application was able shortime in callus formation compared to medium without 2,4-D (control) (Figure 1).

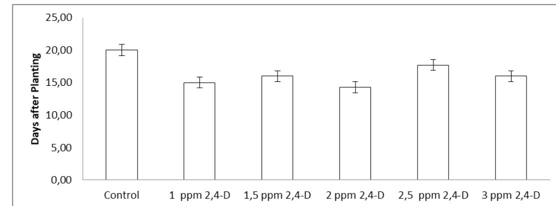

Figure 1. Callus formation in the application of 2,4-D on *Vanda* sp leaf explant.

The beginning of callus formation in *Vanda* sp slice explants is characterized by the appearance of swelling in the area around the incision leaves, then the callus cover the wound area in 15-22 days after planting (Figure 2). That swelling explants cells caused an elongation due to the application of 2,4-D,, even though not all media give the same effect.

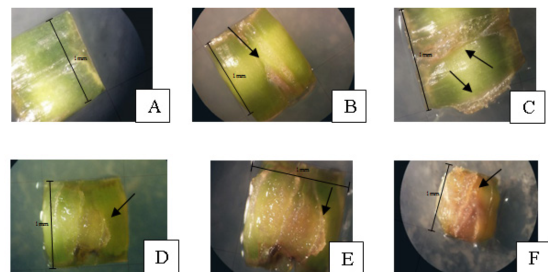

Figure 2. Time of callus formation (A). 0 day after planting, (B) 13 days after planting, (C) 15 days after planting, (D) 17 days after planting, (E) 19 days after planting, and (F) 22 days after planting (Photos : Amalia, 2016).

The data shows that the highest percentage of callus formation is found in the treatment with 2 ppm of 2,4-D. The application of 2,4-D gives significant effect in percentage callus formation. 2,4-D significantly increase the percentage of callus formation compared to the other treatments (Table 1).

Table 1. The effect of 2,4-D in different concentration on the percentage of callus formed

Treatment	Replication			Means
	1	2	3	
Control	75	75	25	58.3 [b]
2,4-D 1 ppm	50	25	25	33.3 [bc]
2,4-D 1.5 ppm	50	50	25	41.7 [bc]
2,4-D 2 ppm	100	75	75	83.3 [a]
2,4-D 2.5 ppm	25	25	25	25.0 [c]
2,4-D 3 ppm	50	50	25	41.7 bc

Note : The mean value ± SD followed by different letters in the same column indicate significant difference according to Duncan's Multiple Range Test (DMRT) at a significance level α = 0.05.

The application of 2 ppm 2,4-D gives the callus formation in 14,3 days after planting (Figure 1). This is due to combination of endogenous concentration of auxin and 2,4-D application can stimulate callus formation. The same result was also reported by Saputra (2012) that application of 2 mg/L 2,4-D is the best concentration for initiate somatic embryo in *Phalaenopsis* sp at three weeks after the subculture. Rianawati *et al.* (2009) reported that 0.5 mg/L 2,4-D is an optimum concentration in *Phalaenopsis* sp characterized by the emerging of swelling. The same result also reported by Sugiyarto and Kuswandi (2014) that 2,4-D is effective for callus swelling in binahong (*Anredera cordifolia*) leaf. In this study, 2,4-D is effective for callus induction, although sometimes cytokinin is also required for callus proliferation. This is in line with the statement of Rianawati *et al.* (2009) and Gill *et al.* (2004), that swelling explants caused an elongation of cells. The similar result was also reported by Rahayu and Anggarwulan (2003) that 2,4-D application culture medium will stimulate all division and enlargement of explants, so that it can stimulate the formation and growth of callus.

The application of 2,4-D gives a higher percentage in callus formation compared with medium without 2,4-D (control). Figure 3 showed that 2,4-D may increase the percentage of callus formation that indicate that the treatment of 2 ppm 2,4-D give a significant different comparde to the other treatment. The application of 2 ppm 2,4-D were able to increase the percentage of callus formation from *Vanda* sp leaf explants, with the average percentage of 83,3%. The higher concentration of 2,4-D will inhibit callus formation. The same result also reported by Romeida and Ganefianti (2016) that application of 1 mg 2,4-D give highly percentage of callus formation of Pencil Orchid (*Papilionanthe hookeriana* Rchb.f.).

The cell enlargement increasing the cell wall plasticity and the formation of cellulase enzyme that can dissolve cell wall cellulose, it causes oxygen, water and mineral passing by for growth and enlargement of cell. Auxin enhance the concentration of intracellular fluid by promoting the accumulation of material structure and chemical component in cytoplasm. Callus is an unorganized cell mass from cell that divides continually. In *in vitro* culture, callus can be formed at the incision site because the same cell on surface of incision will undergo the proliferation process. There are some type of callus : embryonic callus, proliferative callus, and senescence callus. Based on 5 weeks of observation, media with different combination of 2,4-D produce different type of callus. Mean percentage of explants formed callus was 47.22%. They are consisting of 36.11% proliferative type of callus and 11.11% senescence type of callus, without embryonic callus (Figure 3). This result is because 2,4-D strongly stimulate the cell division but inhibit the organogenesis. No cytokinin added is also one of the reasons of the absence of embryonic callus in callus formation. According to Ariati (2012), 2,4-D is effective for stimulating cell proliferation, inhibit the organogenesis and maintaining the growth of callus.

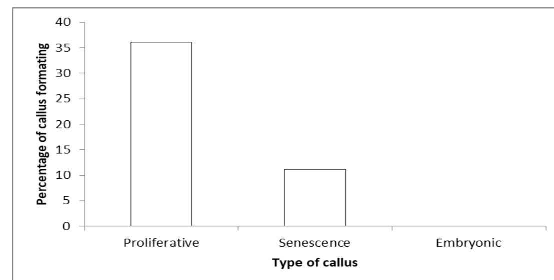

Figure 3. Effect of 2,4-D in type of callus formed

The characteristic of proliferative callus is yellowish-white, clear and watery. Morphology callus is brownish. According to Nisa (2005), proliferative callus has an ability to grow, but it cannot develop through the organogenesis or embryogenesis. Senescence callus have a slowly growth, with the characteristic of no chlorophyll, beige or brown in color. Some senescence callus start from proliferative callus due to the long time incubation without a subculture. That statement is supported by Saputra (2012) that the appearance of proliferative and senescence callus were long time in culture incubation, nutrient in medium become decrease, anoxia due to the increasing of CO_2, limited O_2 and accumulation of ethylene in the medium as well.

Jitsopakul *et al.* (2013) reported that the color of callus indicates the presence of chlorophyll. The green callus indicates more chlorophyll content. White and clear callus indicates that callus is still in a quite good condition. Raghavan (1986) and Parthibhan *et al.* (2015) explained that the good quality callus is the one with the green color. The green color of callus is due to the effect of cytokine in chlorophyll formation.

The result of this study is important as it gives an information about the use of 2,4-D to accelerate and increase the percentage of callus formation on *Vanda* sp leaf explant. This information can be used to help the propagation of *Vanda* sp in more effective way.

CONCLUSION

Based on the study result, it can be concluded that 2,4-D 2 ppm treatment is the most effective to increase the speed of callus formation as well as increase the percentage of callus formed in *Vanda* sp leaf explants. The average callus formation time was 14.3 days after planting. The best concentration for callus formation in *Vanda* sp of leaf explants was 2,4-D 2 ppm treatment. Number of callus formed is 83.3%.

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