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The effect of auxin and auxin inhibitor application on induction and proliferation of protocorms in immature fruit *Phalaenopsis amabilis* in vitro culture

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Abstract. *Cahyaningsih AP, Pitoyo A, Solichatun. 2019. The effect of auxin and auxin inhibitor application on induction and proliferation of protocorms in immature fruit* Phalaenopsis amabilis in vitro *culture. Cell Biol Dev 3: 49-55.* Auxin is a hormone that plays an important role in the ovulum development of moth orchid *Phalaenopsis amabilis* after pollination. Using immature fruit 60 days after pollination as explants are presumed to cause cessation of endogenous auxin distribution due to early fruit harvesting. The research was aimed to investigate the effect of exogenous auxin addition on induction and proliferation protocorm in moth orchid immature fruit explant through in vitro culture. This study used NAA (α-Naphthaleneacetic acid) as auxin in MS medium with 4 treatments: (i) control, (ii) NAA 1 ppm, (iii) NAA 5 ppm, and (iv) TIBA (2,3,5- triiodobenzoic acid) 5 ppm. Observed data include explants' color change, development, anatomy after planting, and protocorm development. Morphological and anatomical data were presented descriptively, and protocorm measurement data were analyzed using One Way ANOVA and followed by a DMRT test of 5% level. The results showed that the culture of immature fruit *P. amabilis* 60 DAP with the addition of NAA 1 and 5 ppm in immature fruit culture induced protocorm. Still, the protocorm was not able to develop further. Without adding exogenous hormone-induced protocorm formation, immature fruit culture developed some protocorm to form leaf primordia. Next, adding TIBA as auxin transport inhibitor, 5 ppm induced protocorm and developed all protocorms to form leaf primordia.

Keywords: Auxin, immature fruit culture, *Phalaenopsis amabilis*, protocorm

Abbreviations: DAP: Days After Planting; NAA: α-Naphthaleneacetic acid; TIBA: 2,3,5- triiodobenzoic acid; FAA: Formalin-Aceto-Alcohol

INTRODUCTION

Phalaenopsis amabilis (L.) Blume or *moon orchid* (Indon.: *anggrek bulan*) is one of the flowers that are popular among lovers of ornamental plants and are still primadonna, and the economic value is quite high. Consumer interest in moon orchids also leads to high production to meet market demand. However, the high interest in the moon orchid does not seem to balance its productivity. That is possible because of obstacles to the propagation of orchids. The unique structure in orchid flowers makes it difficult to pollinate; naturally, seeds produced from pollination are seeds that do not have an endosperm, so germination must be assisted by mycorrhizal (Bazand et al. 2014). Therefore, various problems encourage the research development to optimize orchid cultivation methods.

The plant tissue culture technique is an alternative method of propagation that can overcome the constraints of orchid propagation with a high rate of propagation success (Balilashaki et al., 2014). Propagation in orchids is not only demanded in terms of quantity but also terms of quality. Vegetative propagation carried out has not been able to be used to get orchid plants with superior variations. In-vitro generative propagation can be used to solve the limitations of vegetative propagation.

In vitro generative propagation is one solution to get a superior orchid plant and varies in large quantities. The propagation starts from pollination to ripening of the resulting fruit and seeds and then grows in vitro. However, this generative propagation also has limitations where getting mature seeds takes a long time, at least 90 days after pollination (Yusnita and Handayani 2011). Only explants of moon orchids with a minimum age of 90 days after pollination can grow, and at that age, protocorm is produced in in-vitro culture (Mweetwa et al. 2008).

On the other hand, some research is on auxin's role in the orchid development process; moreover, endogenous auxin plays an important role in the development of orchids, from pollination to ovule development (Novak et al. 2014). The presence of auxin that accumulates after pollination causes continued development in the ovaries and ovules in orchid fruits. The auxin accumulation will encourage the ovaries and ovules to develop properly, so the fertilization process will also succeed in forming embryos.

Based on Novak et al. (2014) research, the failure to use orchid fruit explants under the age of 90 days after pollination other than because it does not have embryos can also be due to the dynamics of the auxin. Fruits harvested

early cause the distribution of auxin for the development of the ovaries and ovules to stop. Low levels of auxin in the immature orchid fruit explants can cause the failure of ovule development. Next, the failure of ovule development in in-vitro cultures causes the explants to be unable to grow.

The use of immature fruit explants as culture explants can provide many benefits. Immature fruits under 90 days after pollination used as explants can shorten the harvest time. In addition, if used as culture explants, immature fruits undergoing ovule development can be induced to produce haploid plants when fertilization has not occurred. Another advantage is that it is possible to produce uniform diploid plants that are not the result of the fusion of gametes. Based on the advantages of using immature orchid fruits, it is necessary to optimize their development to be used as in vitro culture explants.

Harvesting immature fruit 60 days after pollination causes the distribution of endogenous auxin to stop. It must be overcome because auxin plays an essential role in the development of the ovule. In this study, exogenous auxin was given to meet the needs of stopped endogenous auxin. This study aims to determine the effect of the exogenous application hormone auxin on tissue culture media on the induction and proliferation of protocorm in in-vitro cultures of immature moon orchid fruits.

MATERIALS AND METHODS

Plant material

The material needed is the moon orchid plant (*Phalaenopsis amabilis*) that has flowered. The moon orchid plant used comes from PT. Eka Karya Graha Flora and obtained at orchid nursery, Surakarta.

Procedure

This study used a Complete Randomized Design with variations in NAA concentration as a treatment on 1/2 MS medium. Immature orchid fruits used as explants were fruits 60 days after pollination. The treatment consisted of 4 levels (control, 1ppm NAA, 5 ppm NAA, 5ppm TIBA) with 5 replications. Next, the determination of auxin concentration was based on the results of Gurel and Gurel's research (1998); Kalimuthu et al. (2006); Ori et al. (2014); and Parmar and Pant (2015), and modifications were made through preliminary tests.

Pollination and orchid fruit collection

Orchid pollen is taken with a toothpick along with anther. The anther is removed, and pollen attached to the toothpick is inserted into the stigma cavity (pistil) (Parnata 2005). Orchid fruits aged 60 days after pollination were collected by cutting fruit stalks using sterile scalpels.

In vitro culture

Tools sterilization

Glass and dissection utensils were wrapped in straw paper, and culture bottles were covered with aluminum foil and sterilized by wet heating using an autoclave at 121ºC, a pressure of 1 atm for 1.5 hours.

In vitro *culture media*

Media without hormone treatment was made using an instant 1/2 MS (Murashige-Skoog) media in which 1 liter of media was made by dissolving 2,215 grams of MS, 30 grams of sucrose, and 8 grams in 1 liter of aquades. The media solution was also adjusted at a pH of 5.8. If the pH is less than 5.8 1N NaOH was added, and if it is more than 5.8 1N HCl was added. Media with hormone treatment were added with NAA and TIBA solution according to the concentration used. The media were then heated to boiling and poured into a culture bottle, covered with aluminum foil, then sterilized using an autoclave at 121º C, a pressure of 1 atm for 15 minutes.

Sterilization of materials

Orchid fruits used as explants were washed with running water for 5 minutes. The orchid fruit was then soaked in sterile material for 5 minutes of each ingredient while stirring. Sequentially, the orchid fruits were soaked in sterile materials containing soapy water, 5% Clorox, and 10% povidone-iodine. Each immersion in sterile material, orchid fruits were rinsed with sterile aquades. The material was sterilized in LAFC by soaking 70% alcohol for 5 minutes and rinsed with sterile aquades 3 times.

Explants planting

Explant planting was carried out inside the LAFC (Laminar Air Flow Cabinet), which had previously been sterilized with UV and LAFC spraying with 70% alcohol. Orchid fruits as explants were cut using scalpels and tweezers with a transverse position along the ± 0.5 cm - 1 cm. The pieces of explants were planted in culture bottles containing treatment media and then covered with aluminum foil and plastic wrap.

Explants maintenance

Explants planted in culture bottles were stored in the incubation room and placed on a culture rack. The culture rack was equipped with fluorescent lamps; the lamp was placed at a distance of 40-50 cm from the culture bottle with an intensity of 1,000-4,000 lux. The temperature in the culture incubation room was maintained in the range of 16-20ºC using the air conditioner.

Explants anatomy preparations

The explants were cut 1 cm long, and then fixation was done with an FAA solution consisting of formalin, glacial acetic acid, and 70% alcohol with a ratio of 5:5:90 for 24 hours. First, the explant pieces were washed with graded alcohol (70%, 80%, 90%, 95%, and absolute alcohol) and vacuum suctioned the air bubbles for ten minutes at each washing stage. Next, the preparations were dehydrated with a mixture of alcohol/xylol at a ratio of 3:1, 1:1, and 1:3, followed by absolute xylol twice with each stage for 10 minutes and followed by xylol/paraffin (1:9) for 24 hours. The preparations were then infiltrated with pure paraffin for 24 hours and then made blocks and attached o the holder. The preparation material in the block was then cut

with a sliding microtome; the resulting tape was attached to the glass of the object. Finally, preparations were observed using a fluorescence microscope.

Data observation

Observation of morphological data in the form of color changes of the explants was carried out every week for 21 days after planting. The color changes of the explants in browning or vitreous were observed through a stereomicroscope. The explants' development in protocorm formation was observed 42 days after planting. The protocorm morphology formed in each treatment was observed through a stereomicroscope. The protocorms diameter in each treatment was measured using the ImageJ application. The observed data were in the form of explants' anatomy before and after planting. Anatomical data before culture was obtained from the explants of the immature fruit *P. amabilis* aged 60 days after pollination, and anatomical data after culture was obtained from explants planted at 7 and 21 days after planting. The Protocorms that had formed 100 days after planting from each treatment were observed in their development and morphology.

Data analysis

The morphology of the explants, the development of the explants, the anatomy of the explants before and after planting, and the development of the protocorms were presented descriptively. The description is done by describing and comparing the results obtained between treatments. The results of protocorm measurements through the ImageJ application were quantitatively analyzed using One Way ANOVA, and further tests were conducted with a DMRT level of 5%.

RESULTS AND DISCUSSION

Changes in the color of *P. amabilis* immature fruit culture explants for 21 days after planting

Changes in the color of the explants were observed to determine the response of immature fruit culture growth to the administration of growing regulatory substances and their effects on the results of the development of subsequent ovule cultures. Figure 1 shows the changes in the color of the explants during the 21 days of planting. Changes in the color that occurs in the explants were indicated by the presence of browning and vitreous (transparent). Browning was found in the control treatment, adding 1 and 5 ppm of NAA. The explants with the addition of 5 ppm TIBA underwent vitreous. Browning that occurred in explants could be caused by cutting immature fruit explants. Such cuts resulted in the release of phenolic compounds as signaling molecules and induced the activity of the polyphenol oxidase (PPO) enzyme (Boeckx et al., 2015). PPO enzymes can be induced by activating pro-enzymes already present in previous explants (Haruta et al. 2001). The activity of these enzymes produced a brownish color that indicated the accumulation of phenolic compounds. The accumulated and oxidized phenolic components cause browning (Guardo et al., 2012).

Browning increased during the 21 days of planting on control treatment and NAA application. The cultured explants underwent gradual browning and showed increasingly obvious browning. It indicates prolonged PPO activity has an increasingly strong browning effect (Chuanjun et al., 2015). Browning on NAA treatment looked slightly stronger compared to control. Gunes (2000) reveals a link between the auxin and the activity of the peroxidase enzyme that produces phenolic compounds. The peroxidase enzyme can catalyze the oxidation activity of the IAA. The presence of endogenous auxin in the form of IAA that naturally exists in the ovule (Novak et al. 2014) is thought to affect the occurrence of browning. The addition of exogenous auxin causes the auxin accumulated in the explants to get higher and increases the activity of the enzyme peroxidase. Increased activity of peroxidase enzymes allowed the formation of high phenolic compounds and led to stronger browning.

The color change that occurred in applying 5 ppm TIBA (Figure 1.D) was in the form of vitreous, the color of the explants that became transparent. The color changes of the explants became transparent 21 days after planting, accompanied by the appearance of protocorms. Vitreous or hyperhydration in tissue cultures is caused by high air humidity, reduced transpiration, and excessive water uptake in tissues (Sowa 2012). Vitrification of explants can also occur due to disturbed polyamine metabolism and trigger lignin biosynthesis failure (Piqueres et al. 2002). Explants that undergo vitrification in in-vitro culture due to anti-auxin TIBA treatment also occurred in the study by Detrez et al. (1988). Research by Detrez et al. (1988) reports on research on petiole culture (leaf stalk) *Beta vulgaris* on IM culture media (containing 1 ppm TIBA), resulting in a high frequency of bud formation with explants that undergo vitrification. However, that study did not explain the mechanism of TIBA in inducing vitrification in explants.

Development of ovules from immature fruit cultures of *P. amabilis* at 42 days after planting

The development of ovules from immature fruit cultures of *P. amabilis* orchids can be seen from the formation of a globular structure called a protocorm in each treatment given. This observation was conducted to determine the effect of treatment on the protocorm that was successfully induced and proliferated. Figure 2 shows that the ovules in all treatments developed to form a protocorm. However, there were several variations in the protocorm produced in each explant. These variations include formation time, color, and size of the protocorm.

The time of the emergence of the protocorm varied in each treatment; at 5 ppm, TIBA treatment, Explants experienced the fastest protocorm formation 21 days after planting (Figure 1). Explants on control and application of 1 and 5 ppm NAA simultaneously formed a protocorm 42 days after planting. The color of the protocorm was also different in each treatment. At 42 days after planting (Figure 2), it could be seen that the 5 ppm TIBA treatment

had a light yellowish green protocorm with a fine hair structure on its surface. Protocorm in the control treatment and administration of 1 and 5 ppm NAA had a white color with a slight brownish color due to browning.

Size became a significant difference in the protocorms resulting from each treatment (Figure 3). The protocorm of the 5 ppm TIBA treatment had the widest circular area and had a significant difference compared to other treatments. Although the protocorm in the control treatment and 5 ppm NAA had the same size, the protocorm in the 1 ppm NAA treatment had the smallest size. There was no significant difference in the size of the protocorm produced in the control treatment and the administration of NAA. Table 1 shows that although the protocorm in the 1 ppm NAA treatment had the smallest protocorm size, the results did not significantly differ between the control treatment and 5 ppm NAA.

Table 1. The average protocorm size produced in each treatment

Treatments	42 DAP	
	Average protocrome diameter ± SD	Average protocrome area circumference ± SD
Kontrol	0.26[a] ± 0.03	0.05[a] ± 0.01
NAA 1 ppm	0.24[a] ± 0.06	0.04[a] ± 0.03
NAA 5 ppm	0.27[a] ± 0.05	0.05[a] ± 0.02
TIBA 5 ppm	0.32[b] ± 0.13	0.23[b] ± 0.09

Note: Numbers followed by the same letter do not show a significant difference according to the results of the DMRT follow-up test at the 5% level in the same column. SD: standard deviation

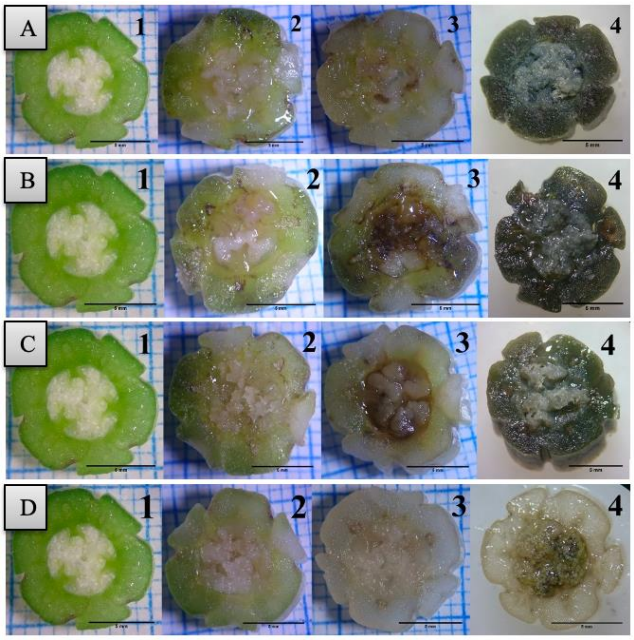

Figure 1. Changes in the color of the immature fruit culture of *P. amabilis* on various growth regulators treatments. A. control; B. 1 ppm NAA treatment; C. 5 ppm NAA treatment; D. 5 ppm TIBA treatment; 1. culture at 0 DAP (Days After Planting); 2. culture at 7 DAP; 3. culture at 14 DAP; 4. culture at 21 DAP

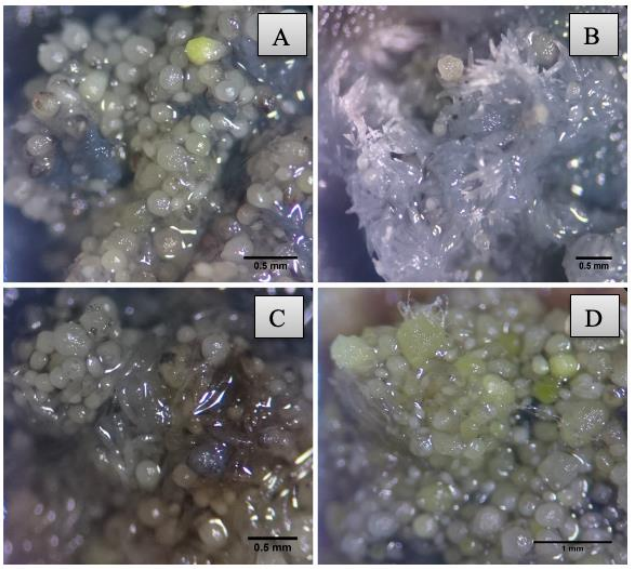

Figure 2. Development of ovules from immature fruit cultures of *P. amabilis* to form a protocrome at 42 DAP by observing through a 40X magnification stereo microscope. A.protocrome on control; B.protocrome at 1 ppm NAA treatment; C. protocrome at 5 ppm NAA treatment; D. protocrome at 5 ppm TIBA treatment

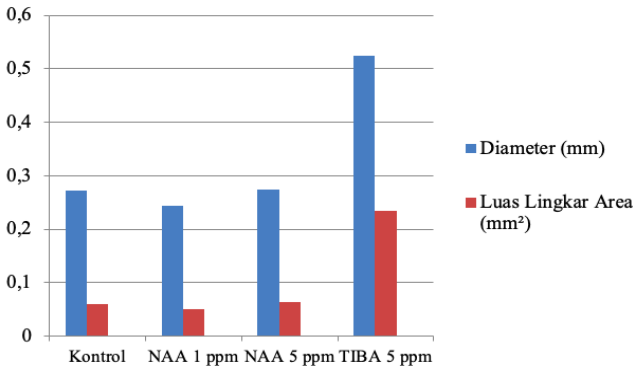

Figure 3. The average diameter and circumference of the protocorm area for each treatment

In the control treatment and the administration of 1 and 5 ppm NAA, each was able to form a protocorm simultaneously, the same size and brownish-white color. It indicates that the explants were able to produce protocorm under browning conditions. Furthermore, this is similar to the research results by Yamazaki and Miyosi (2006) regarding the germination of immature *Cephalanthera falcata* orchid seeds. Research by Yamazaki and Miyosi (2006) reports a higher frequency of germination in 60-80 days after pollination cultures experienced browning than in 90-120 days after pollination seed cultures that were not browning or slightly browning on culture media. Research on cultures that experienced browning was similar to that of Mii (1976), where the growth of explants was in line with the occurrence of browning in tobacco pollen cultures. Yamazaki and Miyosi (2006) suggest the need for further

experiments to evaluate whether browning in culture has a stimulatory effect on orchid germination.

The TIBA 5 ppm treatment in this study showed the ability of explants to produce protocorm with a faster time, yellowish-green color, and larger size than other treatments. However, this shows that the application of TIBA, which functions as an anti-auxin in the media, produces a protocorm with the best results from all treatments. The administration of TIBA did not inhibit the protocorm's formation but promoted the protocorm's induction and proliferation. It is similar to Chen and Chang's research (2004) regarding the positive response of TIBA to the in vitro culture of *Oncidium* orchid leaves. Chen and Chang (2004) report that were applying 0.5 µM TIBA on Oncidium leaf culture produced the highest average number of embryos.

Explants that produced a positive response to in vitro culture due to TIBA treatment also occurred in several studies. Tetu et al. (1990) report that was giving TIBA with a concentration of 0.2 µM increased shoot proliferation in the meristematic and cotyledon areas of *Pisum sativum* zygotic embryo culture. Sreenath et al. (1995) report that 1 and 5 ppm TIBA administration encouraged the fastest callus proliferation in coffee integumentary cultures. That is possible because the antagonist effect of TIBA can determine the balance of auxin and/or cytokinin for the growth of explants. The study also indicates that cells produce sufficient amounts of endogenous growth hormone without the exogenous addition of auxins or cytokinins.

This study showed that the administration of TIBA positively responded to the immature fruit culture of *P. amabilis*, which is possible that the application of TIBA is able to overcome the excessive level of auxin due to the production of endogenous auxin from explants in high quantities. TIBA is a specific IAA transport inhibitor (Sreenath et al. 1995). TIBA can interfere with IAA activity by competing for the same binding site as IAA, moving along the same path as IAA's polar transport, and having auxin competitor properties (Katekar and Geissler 1980). In addition, TIBA acts as an inhibitor of the carrier structure during auxin efflux inhibitor (Michniewicz et al. 2007). It is also possible that TIBA, an auxin competitor and auxin efflux inhibitor, can balance the need for auxin and/or other hormones in the cells. Hence, it provides a positive response to in vitro cultured explants. Further research is needed to determine how the TIBA mechanism can provide a positive response to tissue culture research.

P. amabilis ovule anatomy at 21 days after planting

Figure 4 shows the cross-sectional structure of the globular structure that was successfully induced and proliferated in each treatment. Anatomical data revealed that at 7 days after planting, the seed structure could develop into a globular structure at 21 days after planting for all treatments. The development of the seed structure to form a globular structure is characterized by the presence of a swollen embryo that is oval.

Observation results of the anatomy of *P. amabilis* ovules after 21 days of culture showed that the globular structure was successfully formed from the ovule structure 60 days after pollination was grown in vitro. The globular structure is characterized by the embryo's development in the integumentary structure. The development of the globular structure was similar to the structure of the seeds 99 days after pollination in *P. aphrodite* species. Jean et al. (2011) reported that the structure of *P. aphrodite* seeds aged 99 days after pollination showed the presence of seeds protected by seed coats with developing embryos.

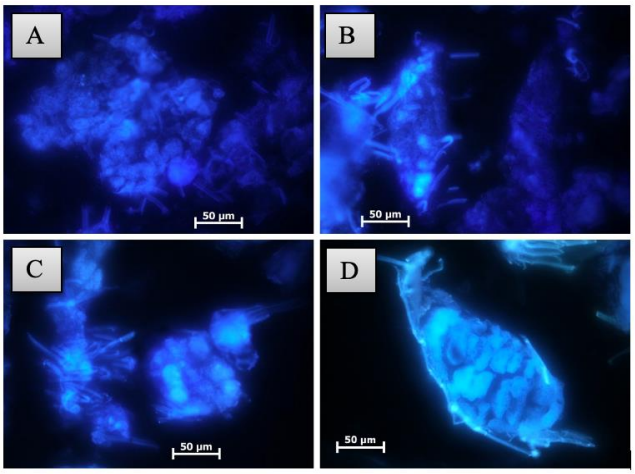

Figure 4. Cross-section of the globular structure of *P. amabilis* at 21 DAP by fluorescence microscopy. A. control treatment protocorm; B. protocorm of 1 ppm NAA treatment; C. protocorm of 5 ppm NAA treatment; D. protocorm of TIBA 5 ppm

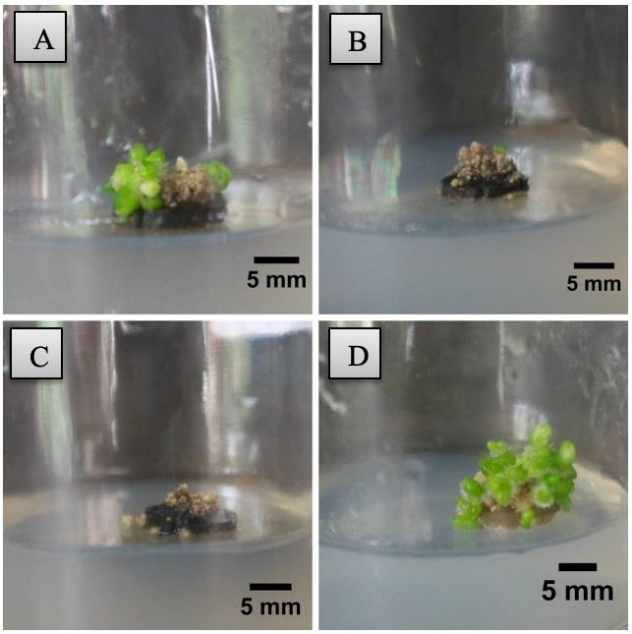

Figure 5. Protocorm development of *P. amabilis* immature fruit culture at 100 DAP. A. protocorm in the control treatment; B. protocorm of 1 ppm NAA treatment; C. protocorm of 5 ppm NAA treatment; D. protocorm of 5 ppm TIBA treatment

Development of protocorm *P. amabilis* at 100 days after planting

The protocorm that was induced and proliferated due to the development of the *P. amabilis* ovule was observed to continue its development. Furthermore, it was done to determine the effect of each given media treatment on the development of the protocorm. This observation was also carried out to determine the protocorm regeneration potential of *P. amabilis* immature fruit culture in in-vitro.

The protocol for the treatment with NAA (Figures 5.B and C) showed different results from the control treatment and the application of 5 ppm TIBA. The protocorm in the NAA treatment did not experience further development, and the protocorm formation stage seemed to have stopped. In the 5 ppm of NAA treatment, the growth of the explants showed a decline, and the protocorm produced was also less than in the 1 ppm NAA treatment. The protocorm in this treatment stopped at the formation of the protocorm, which was round and brownish white.

The treatment of giving NAA concentrations of 1 and 5 ppm in this study was considered toxic to the protocorm. The concentration of exogenous auxin and the level of endogenous auxin that is too high may cause failed embryogenesis (Sreenath et al. 1995). In addition, the phenolic compounds produced during culture can inhibit further protocorm development. Furthermore, this is similar to the research of Chen et al. (2015), in which the seeds of *Paphiopedilum spicerianum* grown on RE media (Robert Ernst medium) produced a protocorm that remained white, did not develop chlorophyll further and turned brown, and died. Moreover, ER media presence, which is toxic to some embryonic cells, is thought to affect the formation of chlorophyll.

Failure of protocorms development in NAA treatment may also be possible due to inappropriate exogenous auxin concentrations. Exogenous administration of auxin has varying results for each species, and explants are used where concentration is of utmost importance (Pradan et al., 2014). Therefore, the combination of concentration and ratio between auxin and cytokinin is very important. Some researchers found that low auxin concentrations can increase germination, and high auxin concentrations can inhibit orchid germination (Novak et al., 2014). The study showed that inappropriate hormone concentrations could inhibit the development and germination of orchid seeds.

The protocorm in the control treatment (Figure 5.A) showed a development in which some of the protocorms produced changed color from white to yellowish-green. In addition, some control treatment protocols could develop chlorophyll further and produce leaf primordia. The formation of meristems in orchid protocorms also requires an auxin organizing center that can regulate shoot development through polar auxin transport (Novak et al., 2014). Therefore, it can allow for the success of the control treatment protocorms, which can develop apical meristems due to sufficient endogenous auxin for protocorms development.

The 5 ppm TIBA treatment (Figure 5.D) showed that all the protocorms produced could change color from white to yellowish-green by showing the development of chlorophyll accompanied by the appearance of leaf primordia in the apical part. With the role of auxin efflux inhibitor from the addition of TIBA, the regulation of endogenous auxin transport in shoot development may become more balanced so that the protocorm can develop better.

This study found that the administration of 1 and 5 ppm NAA in the immature fruit culture of *P. amabilis* could only induce the formation of protocorms. Still, the protocorms were not able to develop further. The application of 5 ppm TIBA as an auxin transport inhibitor induced the formation of protocorms and developed all protocorms to form primordia leaves. Ovule culture, without the addition of hormones, induced the formation of protocorms and developed some of the protocorms to form primordia leaf. Further research is needed to obtain information on the ploidy of orchid plants produced from the culture of the immature fruit of *P. amabilis*, aged 60 days after pollination.

In conclusion, the immature fruit culture of the 60 days after pollination of *Phalaenopsis amabilis* orchid with the addition of exogenous auxin in the form of NAA with a concentration of 1 and 5 ppm was able to induce protocorms. Still, the protocorms were unable to develop further. Immature fruit cultures without the addition of hormones were able to induce protocorm and developed some protocorms to form primordial leaves. Finally, applying an auxin transport inhibitor in the form of TIBA with a concentration of 5 ppm induced the protocorms and developed all the protocorms to form primordial leaves.

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