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Characterization of pollen development and female reproductive structure of *Phalaenopsis amabilis* after pollination

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Abstract. *Udkhiawati I, Solicahatun, Pitoyo A. 2020. Characterization of pollen development and female reproductive structure of Phalaenopsis amabilis after pollination. Cell Biol Dev 4: 17-25.* Orchid is a superior commodity in ornamental plants. Enthusiasts of orchids cause the demand for varieties diversity. Orchids often get obstacles in pollination, so it is necessary to characterize the development of post-pollination pollen and female gametophyte structure. Characterization was conducted through in vitro and in vivo methods. This research aims to determine the development of pollen and female gametophyte structure of *Phalaenopsis amabilis* (L.) Blume after pollination and detect the potential of in vitro pollen germination. This research was explorative. It involves methods were in vitro pollen germination and preparation of anatomy of pollen and ovule of the 1st DAP (Day After Pollination), 2nd DAP, 3rd DAP, 4th DAP, 7th DAP, 20th DAP, 30th DAP, 50th DAP, and 60th DAP. The observations included in vitro culture of pollen cultured in Brewbaker and Kwack (mBK) media using a phase-contrast microscope and observation of anatomical development of post-pollination pollen tube and ovule using a fluorescence microscope. The observed data consisted of in vivo female gametophytes and pollen development in vitro and in vivo methods. The data were analyzed descriptively. From observation of post-pollination pollen, it was known that the pollen tube started to germinate on the 2nd -3rd DAP. At the 7th DAP, all pollens had germinated and formed the pollen tube, and then the pollen tubes continued to grow closer to the ovule. Primordial ovule began to form on the 7th DAP, then continued to develop a seed on 60th DAP, and then an embryo pouch formed. The in vitro pollen germination did not show any change in pollen after 30th DAC (Day After Cultivating).

Keywords: Characterization, in vitro culture, ovule, *Phalaenopsis amabilis*, pollen

INTRODUCTION

Orchids are a group of angiosperm plants, the leading commodity in ornamental plants. Data from the Central Statistics Agency in 2015 shows that orchid production in Indonesia has increased. In 2006, the production of orchids was 10,903,444 stalks/year, while in 2015, the production of orchids reached 21,514,789 stalks/year. The more orchid lovers in the ornamental plant industry market, the demand for novelty orchid varieties is increasing, so it is necessary to increase new superior varieties of orchids, one of which is by crossing. Crosses require parents with outstanding traits, so a combination of these traits will appear in the cross results. (Widyastoety et al., 2010).

Orchids can be crossed interspecies (between species in one genus), intraspecies (one species in one genus), or intergeneric (between genera) (Sarwono, 2002). Orchids have a vast potential to produce new varieties. However, crosses on orchids often encounter obstacles, namely pollination failure. Immature reproductive organs could cause pollination failure, failure of pollen germination on foreign stigmas (Marwoto et al., 2012), and cross-compatibility. According to (Widyastoety et al., 2010), pollen from small orchids will usually fail if pollinated on large orchids because the pollen tube cannot reach the embryo sac.

Orchids have a different reproductive system from other angiosperms. The structure of the ovule in orchids is not fully developed before pollination and even partially has not been formed. Pollination will trigger the formation and differentiation of ovules, so pollination is essential for regulating the ovule and embryo sac development. Due to the time difference between pollination and the maturity of the embryo sac, orchid fertilization takes place over a long period. In addition, the pollen and pollen tube must survive in the ovary for a long time. Therefore, post-pollination characterization is necessary to determine the development of post-pollination pollen and the role of pollen in the development of female gametophytes.

The characterization carried out included the time of the emergence of the pollen tube and the growth of the ovary and ovule. Characterization can be done in vitro and in vivo. Efforts to grow pollen tubes in vitro are expected to be a solution to overcome the crossbreeding problem due to the inability of the pollen tube to reach the embryo sac in the ovule. Fertilization through in vitro culture with pollen tube formation has also been reported in Brassica rapa (Lorbiecke, 2012). Moreover, this in vitro pollen tube culture was carried out by treating the stigma tissue extract on growth media. According to Chen and Fang (2016), stigma tissue extract is needed to initiate pollen germination in vitro. There are indications that the stigma network has molecules as signals for pollen tube germination.

Post-pollination characterization can also be carried out in vivo to determine the development of the pollen tube and

post-pollination ovule in vivo by making anatomical preparations for each stage of ovarian development. Post-pollination characterization is expected to provide additional information to overcome problems in generative propagation and quality improvement of orchids such as crosses. Research on plant reproduction, especially in orchids, has not been widely carried out, so there is a need for further research on the reproduction of angiosperms, especially in orchids.

The aims of this research were (i) to know pollen tube development and female gametophyte structure in *Phalaenopsis amabilis* (L.) Blume post-pollination, (ii) to provide a chemical signal that stimulates pollen tube growth, female gametophyte development, and to know pollen tube grows towards the post-pollination development and differentiation of ovule.

MATERIALS AND METHODS

Plant material

Phalaenopsis amabilis flower (Anggrek Bulan or moon orchid) was collected from an ornamental market in Surakarta, Central Java, Indonesia.

Procedure

Pollination of Phalaenopsis amabilis

To implant the pollinia, one must first open the anther cap on the flower with a sterile toothpick before inserting it into the stigma cavity.

Pollen collection

Pollinia were sterilized by immersion in 0.05% NaClO and then rinsed using distilled water. After sterilization, the pollinia were placed in sterile Petri dishes and then squashed to obtain pollen grains which would later be cultured as explants.

Extraction of stigma tissue of Phalaenopsis amabilis

Orchid stigma was cut using a scalpel and then sterilized by immersion in 70% ethanol for 20 seconds. The stigma pieces were immersed in 3 ml of distilled water and vacuumed to obtain the stigma tissue extract. The stigma tissue extract was mixed in the pollen germination medium.

In vitro culture of Phalaenopsis amabilis

The in vitro culture stage includes sterilization activities using an autoclave at a temperature of 121ºC and a steam pressure of 1.5 atm for 20 minutes, making Brewbaker and Kwack (mBK) media consisting of 10% sucrose, 100 mg/L H3BO3, 100 mg/ L CaCl2.2H2O, 100 mg/L MgSO4.7H2O, 100 mg/L KNO3, and 5 g agar. Sucrose was dissolved in 500 ml of distilled water and heated on a hot plate; after dissolving, it was added with 100 mg of H3BO3, 100 mg of CaCl2.2H2O, 100 mg of MgSO4.7H2O, 100 mg of KNO3, and the obtained stigma extract while stirring until homogeneous. After boiling, it was removed and cooled. A total of 500 ml of distilled water was heated, added 5 g of agar powder, and stirred until it boiled. After boiling, remove it and cool to a temperature of 60ºC. The cold media solution was added to the agar solution and stirred until homogeneous. The explants were planted in LAFC (Laminar Air Flow Cabinet).

Preparation of fresh preparations of Phalaenopsis amabilis

Post-pollinated orchid pollen on the stigma or ovary was cut into thin strips and stained with aniline blue. The fresh preparations were observed under a fluorescence microscope with a UV-1A filter.

Preparation of anatomical preparations of ovule development

Preparation of anatomical preparations for the development of orchid ovules using the paraffin method includes the stages of fixation, dehydration, clearing, infiltration, embedding, and staining.

Observation

In the in-vitro, culture observations were started at one DAC (Day After Cultivating) using a phase-contrast microscope. Observations were made periodically to see the direction of growth of the pollen tube. In vivo pollen observations were preceded by staining with aniline blue and then observed under a fluorescence microscope using a UV-1A filter. Observation of female gametophyte in vivo was carried out by making preserved preparations for the post-pollination stage of ovule development consisting of 1 DAP (Day After Pollination), 2 DAP, 3 DAP, 4 DAP, 7 DAP, 20 DAP, 30 DAP, 50 DAP, and 60 DAP using a fluorescence microscope using a UV-1A filter.

Data analysis

Data on pollen development, female gametophyte, and pollen tube growth are presented descriptively.

RESULTS AND DISCUSSION

Observation of morphological changes in the flower of *Phalaenopsis amabilis* Post-pollination

The results of observations on the success of pollination were indicated by changes in flower morphology after pollination. These changes include wilting of flower ornaments, changes in pigmentation, maturation of ovaries, differentiation of ovules, and development of female gametophytes.

Figure 1 shows the morphological changes of *P. amabilis* from 0 DAP (Days After Pollination) to 60 DAP. At 0 DAP, perianthium (flower decoration), the orchid looks fresh and clean white, consisting of 3 sepals and three petals where one petal is modified into a labellum structure. The gynostemium (organ structure consisting of androecium and gynoecium) is white, and a pollinium is covered by an anther cap located at the tip of the gynostemium. In gynostemium, there is a stigma hole as a way for pollinia to enter at the time of pollination. The ovary looks like a short segment directly related to the flower stalk, located at the end of the flower stalk below the gynostemium, and there are longitudinal lines.

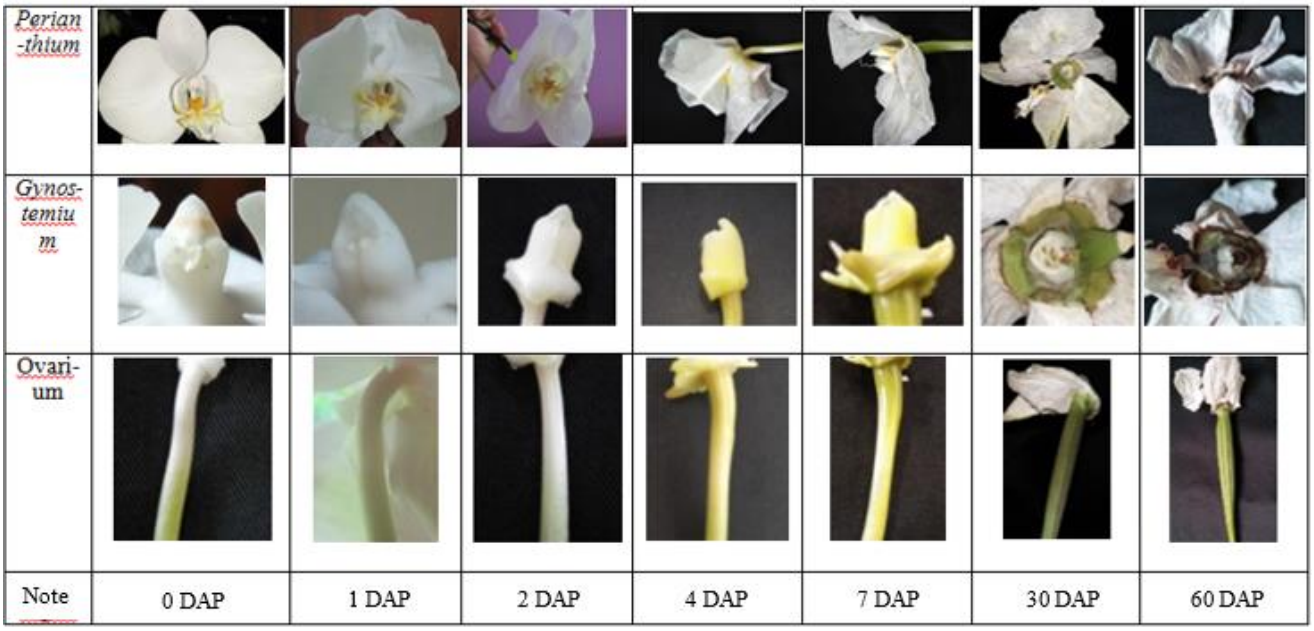

Figure 1. Morphological changes of *Phalaenopsis amabilis* post pollination; DAP (Days After Pollination)

At 1 DAP, the flower buds begin to wither; the stigma hole closes around the pollinia. The ovaries have not changed. At 2 DAP, the flower ornaments were wilting; the gynostemium started to swell. The swelling of the gynostemium is also thought to be due to cell division in gynostemium tissue. At this stage, there is stimulation of the development of the ovule, then the ovary enlarges and undergoes differentiation. At 4 DAP, the flower embellishments are wilting. Gynostemium continues to swell and begins to change color to yellow-green. The color change is thought to be due to the production of chlorophyll. The ovaries begin to swell and change color to yellow-green, as in gynostemium. Ovarian swelling is believed to be due to the accumulation of starch.

At 7 DAP, the withered flower buds became dry, the base of the corolla swelled, the gynostemium and ovary became swollen and enlarged due to the development of the female gametophyte structure. At 30 DAP, the flower and gynostemium adornment dried, and the ovary formed a fruit structure. At 60 DAP, the surface of the gynostemium was slightly blackened and dried; the fruit structure was growing. The results of post-pollination orchid flower morphology observations follow the explanation of O'neil et al. (1993), who stated that the success of pollination caused changes in orchid flowers. Curtis (1943) in Nadeau et al. (1993) explained that the gynostemium cells in orchids swell after pollination so that the gynostemium becomes prominent and the stigma hole closes around the pollinia. The wilting of floral ornaments are caused by the auxin-induced spike in ethylene from pollen during pollination (Hew and Young, 2004). According to Hsiang (1950), pollination causes swelling of gynostemium due to increased water absorption capacity, while most flower ornaments wither due to increased transpiration.

There were several morphological changes in post-pollination month orchid flowers in this observation. Changes in the morphology of orchids after pollination can be caused by several things, one of which is the interaction between hormones. Hew and Young (2004) stated that pollination causes the transfer of auxin from pollen to the stigma. As a result of this auxin transfer, growth hormone spreads into the stigma and induces ethylene production in the stigma tissue. Auxin induces the expression of genes encoding the enzymes ACC synthase and ACC oxidase. ACC synthase and ACC oxidase enzymes play a role in ethylene biosynthesis. The ethylene biosynthesis process begins with converting methionine to *S-adenosylmethionine* (SAM), then SAM is catalyzed by the ACC synthase enzyme to *1-aminocyclopropane-1-carboxylic acid* (ACC). The enzyme ACC oxidase catalyzes ACC to ethylene. Both enzymes are produced in the stigma, while in the flower jewelry, there is only ACC oxidase, so the ethylene synthesis in the flower jewelry depends on the transfer of ACC from the stigma. ACC stimulates ethylene production, causing wilting.

Growth of pollen tube flower of *Phalaenopsis amabilis* in vivo

Pollinia morphology of Phalaenopsis amabilis

Figure 2 is a morphological image of the *P. amabilis* pollinia observed under a fluorescence microscope at 100x magnification using a UV-1A filter.

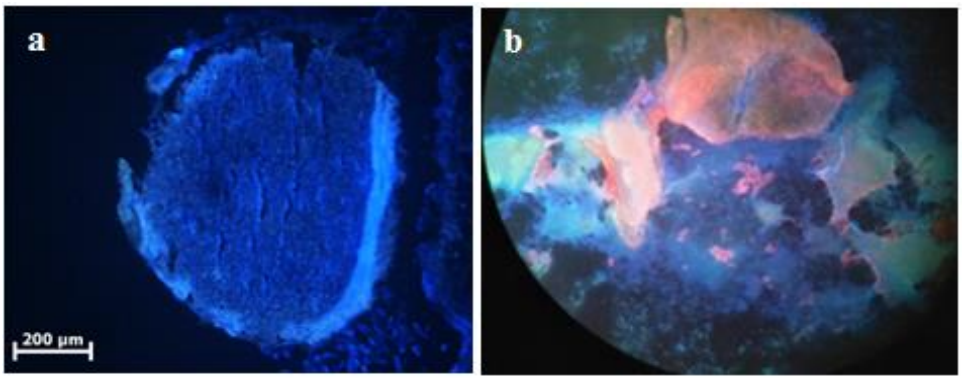

Figure 2. Morphology of *Phalaenopsis amabilis* pollinia. a. P.B. observations of *P. amabilis* pollinia on a fluorescence microscope using a UV-1A filter at 100x magnification; b. Observation of a fresh section of *P. amabilis* pollinia on a fluorescence microscope using a UV-1A filter at 100x magnification.

Figure 2. is a morphological image of the pollinia *P. amabilis*. Figure 2a is a cross-section of the *P. amabilis* pollinia observed under a fluorescence microscope with aniline blue staining. These observations indicate that the pollen of *P. amabilis* collected into a single structure called pollinia. In one pollinium, there are thousands of pollen. Figure 2b shows the color luminescence difference in *P. amabilis* pollinia after squash. *P. amabilis* pollinia preparations were viewed using a fluorescence microscope under a UV-1A filter. Pollen on the outer pollinia or surface glows with a red glow, while the pollen inside produces a blue glow. It happens because there is an exine on the surface of pollinia. In exine, there is elastoviscin which contains carotenoids. The exine will glow red when under a fluorescence microscope. The results of this observation indicate differences in the pollen constituents' walls on the pollinia's surface with the pollen inside the pollinia. In pollinia, an elastic material binds pollen together with pollinia called elastoviscin. Elastoviscin in the epidendrum is a lipid polymer. Elastoviscin in pollinia has the same role as pollenkitt in pollen. Paccini and Hesse (2005) explained that pollenkitt is an oily coat pollen layer. Pollenkitt's functions include keeping pollen from losing water, making it easier for pollen to spread, protecting it from U.V. filter radiation, protecting pollen from bacteria and fungi, and making it easier for pollen to reach the stigma.

Observation of Phalaenopsis amabilis

Figure 3 shows an image of the *P. amabilis* pollen grains observed under a fluorescence microscope at 100x magnification using three different filters. In Figure 3. it can be seen that the pollen grains that have been stained with aniline blue can be seen under a fluorescence microscope using a UV-1A filter. There is no visible presence of pollen grains in the emission of filters B-2A and G-2A. It happens because the excitation wavelength of the UV-1A filter is 360-370 nm, the B-2A filter is 450-490 nm, and the G-2A filter is 510-560 nm. In comparison, the maximum excitation wavelength can be absorbed by aniline blue is 370 nm. (Scordato and Schwartz, 2017). It also follows the statement of Vieira et al. (2015) that aniline blue has been used as a specific dye for Callose which can stain selectively and fluoresce under U.V. filters. A collection of substances called Callose is in the pollen wall and tube. Callose is one of the most dynamic components of the cell wall. Callose is synthesized and deposited on the outer surface of the plasma membrane by callose synthase present on the membrane. The function of Callose in the pollen wall is to control the balance of cell wall turgor pressure, resistance to pressure around the pollen tube wall, and water permeability (Parre and Geitmann, 2005). Kho and Baer (1968) explained that Callose is a polysaccharide in the form of β-1,3-glucans. Furthermore, Callose will brightly glow when stained with aniline blue and viewed under a U.V. filter (Jefferie and Belcher, 1974). Evans et al. (1984) stated that aniline blue could interact directly with the surface of β-1,3-glucans through the hydrophobic region.

Pollen tube growth

Figure 4 shows the growth of the *P. amabilis* pollen tube under a fluorescence microscope using a U.V. filter. Pollen tubes are formed after pollination as a sign of successful pollination. Pollination is the arrival of pollen on the stigma (Lorbiecke, 2012). No pollen tubes appeared at 1 DAP (Day After Pollination) observation; moreover, pollen is still in the form of granules attached to each other and gathered into one. At 2 DAP, the pollen grain size was more prominent, but the pollen tube was not visible. It indicates that hydration has occurred in the pollen. At 3 DAP, the pollen tube has started germinating, and even a pollen tube has begun to elongate. Following Bedinger's (1992) statement, the pollen tube begins to germinate through the pores on the exine surface, and then the pollen tube elongates rapidly. Therefore, pollen tube germination is suspected to begin at 2-3 DAP. According to Wihelmi and Preus (1997), after penetration of the pollen tube, it will begin to germinate and form a tube that grows between the stigma cells. Pollen tubes emerge from each pollen grain. At 4 DAP, the pollen tube progressively elongates within the stigma and begins to grow toward the ovary. At 7 DAP, all pollen tubes germinated and grew lengthwise into the ovary. In the observation results in Figure 4g, a collection of pollen tubes leads to the primordia of the ovule. At 20 DAP, several ovular primordia were formed in the ovary.

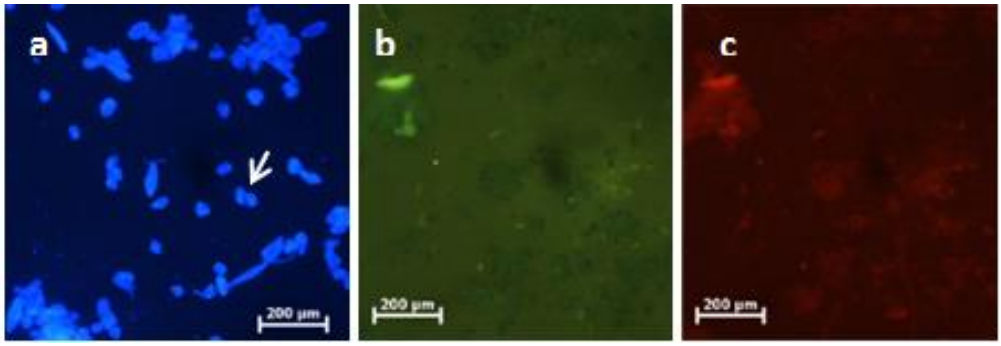

Figure 3. Observation of *Phalaenopsis amabilis* on a fluorescence microscope using different filters. Description: a. UV-1A filter; b. B-2A filters; c. G-2A filters. ; magnification 100x; arrows indicate pollen grains.

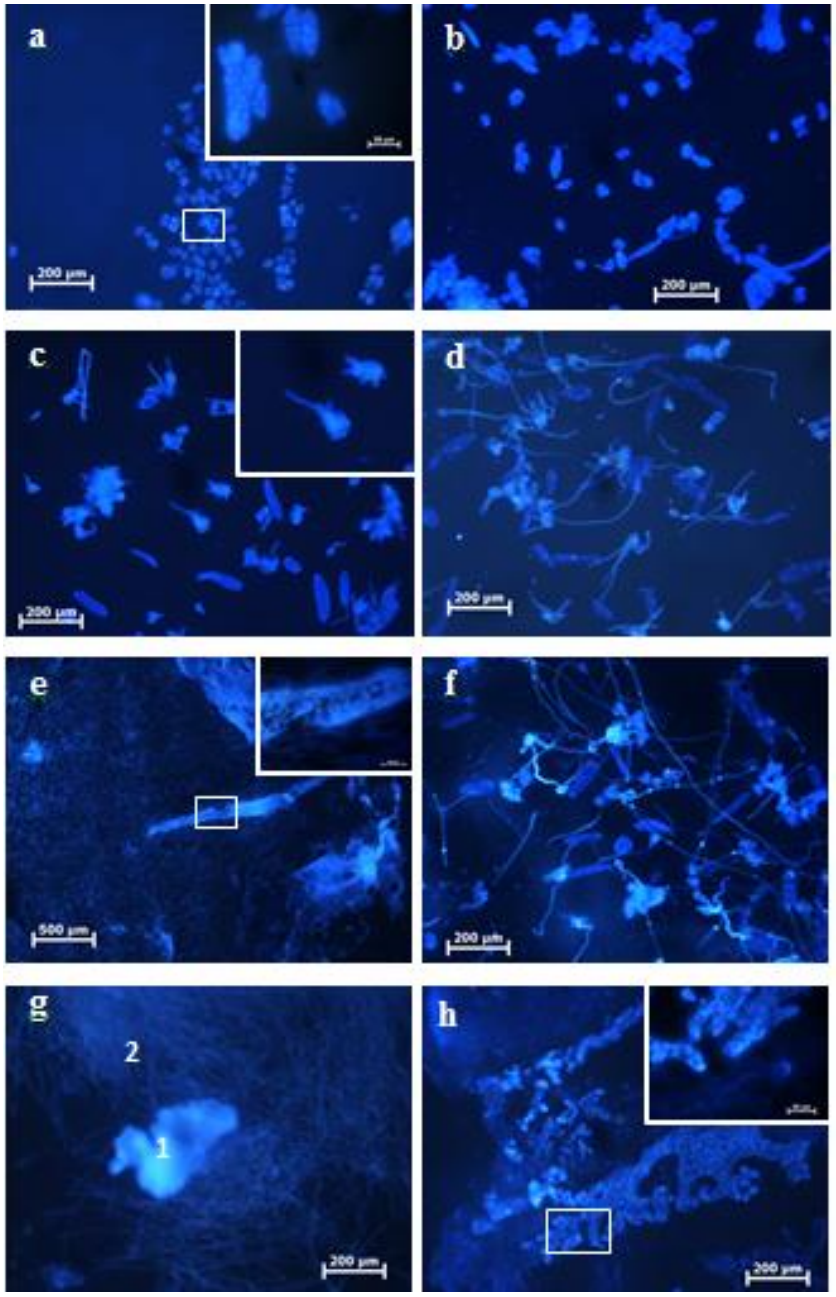

Figure 4. Observation of *Phalaenopsis amabilis* pollen tube growth using a fluorescence microscope at 100x magnification. Description: a. 1 DAP (Day After Pollination); b. 2 DAP; c. 3 DAP; d. 3 DAP; e. 4 DAP; f. 7 DAP; g. 7 DAP; h. 20 DAP; 1. Primordia ovule; 2. Pollen tube collection

After the interaction between pollen and stigma occurs, then pollen undergoes penetration. The pollen tube extends inside the stigma cell, whereas the pollen tube grows through the extracellular matrix in style. The extracellular matrix has hollow solid channels. It is supported by Wihelmi and Preus's (1997) statement that the matrix content includes lipids, proteins, carbohydrates, and small molecules. Presumably, these components provide signals to support the growth of pollen tubes. The pollen tube continues to grow in a nutrient-rich matrix where the female gamete secretes the nutrients. After the pollen tube leaves the style, it is suspected that it receives a signal from the ovule to arrive at the ovule. From this observation, it can be concluded that pollination is divided into several stages, namely (a) the arrival of pollen on the stigma, (b) penetration and germination of pollen, (c) growth of the pollen tube on the stigma and style (d) the pollen tube to the ovule and (d) the arrival of sperm, in the embryo sac.

The observations in this study showed the growth of pollen tubes after pollination. The growth of pollen tubes is thought to be stimulated by many factors. One of the factors that can stimulate the growth of pollen tubes is the molecules of the stigma. Wihelmi and Preus (1997) stated that stigma is also a factor that stimulates the growth of pollen tubes. The pollen tube germinates inside the stigma and grows towards the ovule via the style. During growth, the pollen tube receives several molecules from the female tissue (ovule). These molecules are ions, peptides, and glycoproteins. The movement of the pollen tube, along with style towards the ovule, is directed by a chemoattractant signal. Examples of signals that direct the pollen tube to the ovule has been found in several species, such as chemocyanin derived from *Lilium* and TTS protein derived from *Nicotiana tabaccum* (Higashiyama and Takeuchi 2015).

Ovule development of the Phalaenopsis amabilis *in vivo*

Figure 5 shows the development of the *P. amabilis* ovule post-pollination. Ovaries in orchids have not matured before pollination. The stages of ovule development in this study include (i) the development of the cell mass to form a primordial ovule, (ii) division of the placenta to form dichotomous branches, (iii) the formation of the integument, and (iv) the formation of the embryo sac. Figure 5a shows the mass cell development in the primordial ovule at 7 DAP. The primordial ovule is formed from the placenta, located in the innermost part of the ovary. The mass cell development forms a structure such as small protrusions grouped into a single unit. It is supported by the statement of Nadeau et al. (1996) that the first stage in the development of the orchid ovule is cell division in the cell wall area.

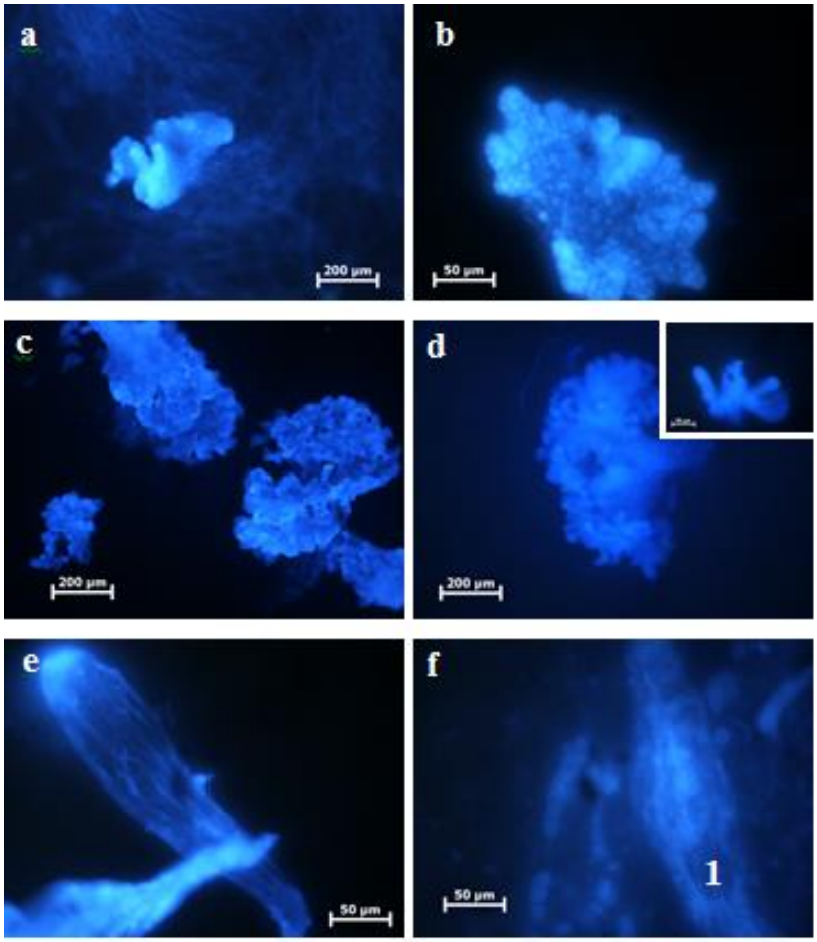

Figure 5. Observation of the growth of *Phalaenopsis amabilis* ovules using a fluorescence microscope. Description: a. 7 DAP (Days after Pollination) 100x magnification; b. 20 DAP 400x magnification; c. 30 DAP 100x magnification; d. 50 DAP 100x magnification; e. 60 DAP; f. 60 DAP 400x magnification; 1. Embryo sac

This area is elongated and forms dichotomous branches to produce thousands of ovular primordia. At this stage, the dermal and subdermal layers cells are densely packed with the cytoplasm. Figure 5b shows an ovule at the age of 20 DAP, and the appearance of the primordial ovule is growing into an elongated bulge-like structure. In Figure 5c, the ovule is 30 DAP, and it can be seen that the protrusion of the ovule is growing and looks lumpy and forms dichotomous branches. Duncan and Curtis (1942) stated that at 35 days after pollination, there was placental proliferation followed by elongation and swelling of the ovaries, supported by elongation of the pollen tube. The ovules differentiate between 30 and 40 days after pollination.

At 50 DAP, Figure 5d shows many ovule structures in the placenta in the form of dichotomous branches to form a finger-like structure. Nadeau et al. (1996) stated that the subdermal cells at the tip of the primordia enlarge to form archesporial cells. The deep integument begins to form a ring from periclinal cell division near the tip of the primordia. It is accompanied by asymmetric growth and cell division on one side of the primordia to form the anatrophic orientation of the ovule. The outer integument is formed after the integumentary and funiculus cells enlarge and turn into vacuolate. The archesporial cells enlarge to form megasporocytes directly. The meiotic division of the megasporocyte forms four cells, but the development of the megaspore crushes one cell near the chalaza. The vacuoles begin to unite in the surviving megaspores.

Further mitotic division occurs according to the development of the polygonum type, namely megaspore cells that divide three times in a row to produce eight nuclei. After the first division of the megagametophyte cell, the nuclei migrate to the ends of the coenocytic megagametophyte, where they divide twice to form three antipodes at the end of the chalaza, an egg cell, and two synergides at the end of the micropyle. The two remaining nuclei gather at the center of the cell to form the polar nucleus. After all mitotic divisions are complete, a cell wall forms between the nuclei at both ends of the megagametophyte but not around the polar nucleus. At 60 DAP, figure 5e shows the ovule has formed an integument, while Figure 5f shows a structure resembling an embryo sac. Niimoto and Sagawa (1962) stated that macrogametophyte maturation occurs at 60 DAP, and fertilization occurs at 65-70 DAP.

Morphological observation of pollen culture of the flower of *Phalaenopsis amabilis*

Figures 6A and 6B show observations of pollen tube cultures on two different media. Figure 6A is a pollen tube culture with Brewbaker and Kwack (mBK) media, while Figure 6B is a pollen tube culture with Brewbaker and Kwack (mBK) media and stigma extract. The observations for 30 days after planting concluded that pollen tube cultures performed on both media showed no pollen tubes growing. It is thought to be caused by several things, including the media and growing environment, that are not suitable for pollen germination and growth. Pacini and Hesse (2005) stated that pollen could only germinate if the media is mixed with stigma extract or lipids.

In this study, culture was carried out on two media, and one of the media was added with stigma extract, but there was no pollen tube growth in both media. This result is thought to have occurred because the stigmatic exudate mixed with the media did not meet the pollen needs to germinate. In Lorbiecke's research (2012), the pollen tube culture of Brassica rapa was successfully carried out using the semi-in Vivo method, where the pistil was cut and then cultured in pollen culture media after pollination that the need for stigma exudate was fulfilled.

The solid media use could also cause the failure of pollen tube growth in this study. Bar-Shalon and Mattson (1977) in Hodgkin (1987) stated that pollen from plants with a wet stigma type germinated more easily in liquid media. Pollen cultures in this study did not include ovules; furthermore, it is suspected that this also affects the germination of pollen tubes. Higashiyama and Takeuchi (2015) stated that several molecules from the female could stimulate pollen tube germination. These molecules could be ions, peptides, and glycoproteins. The nutrients contained in the media are also very influential in pollen tube growth.

In this study, the media used was Brewbaker and Kwack (mBK) media with a composition of 10% sucrose, 100 mg/L H_3BO_3, 100 mg/L $CaCl_2.2H_2O$, 100 mg/L $MgSO_4.7H_2O$, 100 mg/L KNO_3, 5 g instant agar, and distilled water. Johhri and Vasil (1961) stated that sucrose and boron influenced pollen tube germination. Sucrose plays a role in controlling the osmotic concentration in the media for pollen tube germination. In contrast, boron assists sugar absorption and metabolism, increasing oxygen absorption and being involved in synthesizing pectin to elongate pollen tube walls. It is suspected that the sucrose and boron in the media used did not meet the pollen needs to germinate. Several studies have used hormones, vitamins, carotenoids, antibiotics, and other organic salts in pollen tube cultures that play a role in pollen tube germination. This study did not use these components. Growing environmental factors are also very influential in pollen tube germination. The optimum temperature for pollen tube growth was 20º-30ºC, while the temperature used during the study was 18ºC; according to the temperature of the culture incubation room, it may be one of the causes of pollen failure to germinate and form a pollen tube.

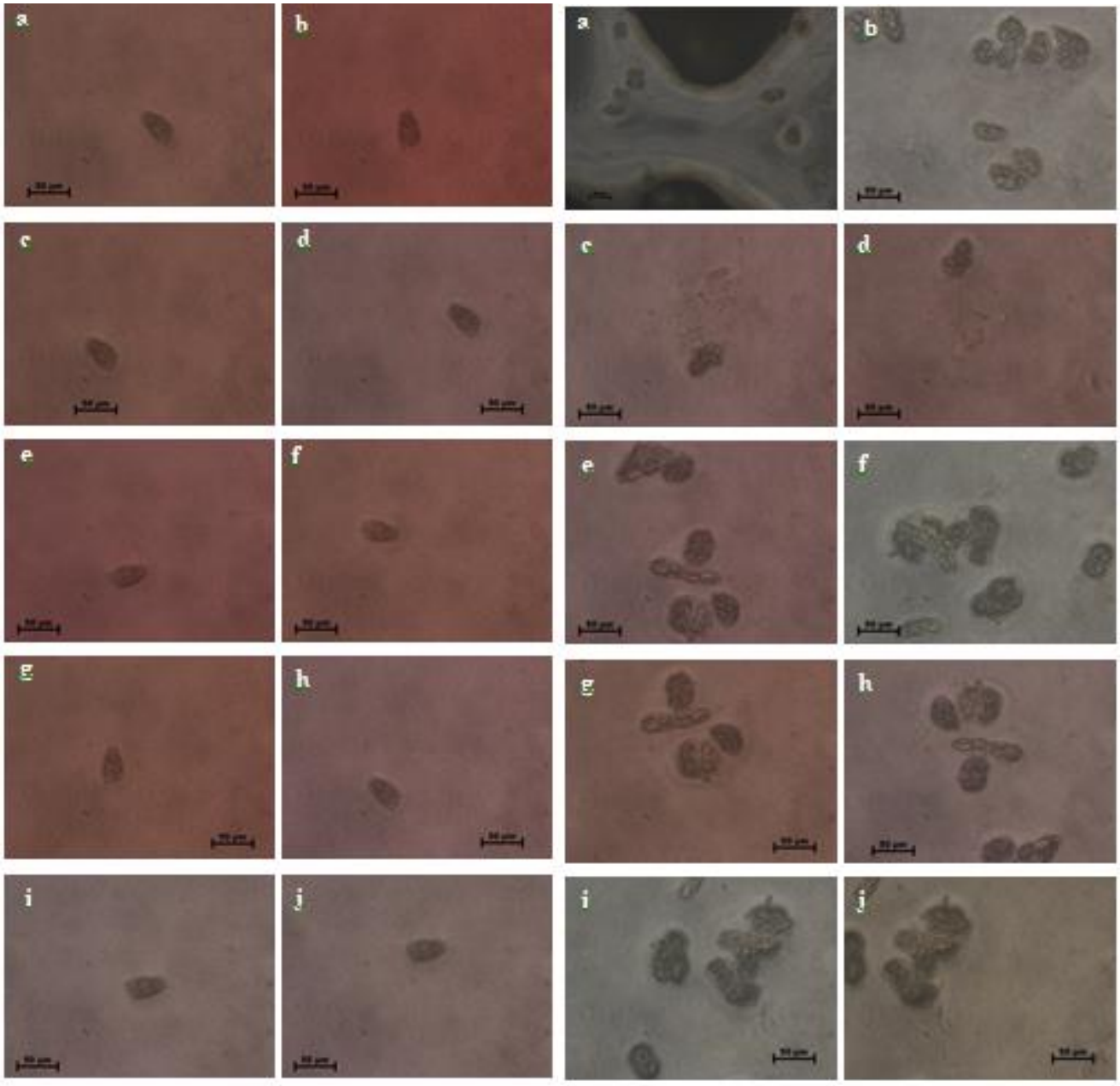

Figure 6. Observation of *Phalaenopsis amabilis* pollen culture on a phase-contrast microscope. **A**. On Brewbaker and Kwack Media (mBK). **B**. On Brewbaker and Kwack and stigma extracts. Description: a. 0 DAC (Day After Cultivating); b. 1 DAC; c. 2 DAC; d. 3 DAC; e. 4 DAC; f. 5 DAC; g. 6 DAC; h. 7 DAC; i. 20 DAC; j. 30 DAC; 400x magnification

In conclusion, pollen tube development and female gametophyte structure in *P. amabilis* post-pollination are interrelated. In addition to providing a chemical signal that stimulates pollen tube growth, female gametophyte development is also influenced by pollen tube growth. The pollen tube grows towards the post-pollination ovule while the ovule undergoes post-pollination development and differentiation. In vitro pollen tube culture has not been successful.

REFERENCES

Badan Pusat Statistik. 2015. Statistik Tanaman Hias Indonesia 2015. Badan Pusat Statistik : Jakarta. [Indonesian]

Bedinger P. 1992. The Remarkable Biology of Pollen. The Plant Cell 4: 879-887

Bercu R, Bavaru A, Broasca L. 2011. Anatomical aspects of *Phalaenopsis amabilis* (L.) Blume. Annals of RSCB 17 (2): 102-109.

Bhanwra RK, Vij SP, Chandel V, Ravikant, Dutt S. 2006. Development of Pollinium in Two Epidendroid Orchids. Current Science 90 (10) : 1384-1388.

Catalogueoflife.org. 2010. Catalogue of Life : 2010 Annual Chhecklist. http://www.catalogueoflife.org. Diakses 22 Desember 2016.

Chaturvedi M, Chaturvedi AK. 2017. Concept of Plant Tissue Culture inAncient Science W.S.R Vriksha- Ayurveda. Imperial Journal of Interdiscilinary Research 3 (1) : 1495-1498.

Chen, Jhun-Chen, Fang S. 2016. The Long Pollen Tube Journey and In Vitro Germination of Phalaenopsis Orchids. Plant Reprod. 29 (1-2): 179-188. DOI: 10.1007/s00497-016-0280-z

Chen JT, Chang WC. 2000. Efficient Plant Regeneration Through Somatic Embryogenesis From Callus Cultures of Oncidium (Orchidaceae). Plant Science 160 (1) : 87-93. DOI: 10.1016/s0168-9452(00)00367-8

Dagla HR, Nair S, Vyas DK, Upendra JM. 2014. Climate Change and Plant abiotic Stress Tolerance,First Edition. Wiley-VCH Verlag GmbH & Co. KgaA.

Darmono, Widiastoety D. 2003. Agar Anggrek Rajin Berbunga. Panebar Swadaya : Bogor. [Indonesian]

Duncan RE, Curtis JT. 1942. Intermittent Growth of Fruits of Phalaenopsis. A Correlation of The Growth Phases of an Orchid Fruit With Internal Development. Bulletin of The Torrey Club. 69: 75-166.

Ederer T. 2017. How can I Get Some Seed?. http://www.orchideenvermehrung.at. 18 November 2017.

Evans NA, Hoyne PA, Stone BA. 1984. Characteristics and Specificity of the Interaction of a Fluorochrome from Aniline Blue (Siroflour) withh Polysaccharides. Elsevier, Amsterdam. 4 : 215-230.

George EF. 2008 . Plant Propagation by Tissue Culture 3rd Edition. Springer.

Gunawan LW. 1988. Teknik Kultur Jaringan Tanaman. Pusat Antar Universitas (PAU) Bioteknologi Institut Pertanian Bogor, Bogor, Indonesia. [Indonesian]

Hew CS, Yong JWH. 2004. The Physiology Of Tropical Orchids In Relation to The Industry Second Edition. World Scientific Publishing : Singapore.

Higashiyama T, Takeuchi H. 2015. The Mechanism and Key Molecules Involved in Pollen Tube Guidance. The Annual review of Plant Biology. 66 (17) : 1-21

Hodgkin T. 1987. A Prosedure Suitable for In Vitro Pollen Selection in *Brassica Oleracea*. Euphytica. 36: 153-159

Hong-QI Z, Croes AF. 1982. A New Medium For Pollen Germination In Vitro. Acta Bot Neerl. 31: 113-119.

Hsiang T.T. 1950. Physiological and Biochemical Changes Accompanying Polination in Orchid Flowers. American Society of Plant Biologist

Iswanto, Hadi. 2010. Petunjuk Praktis Merawat Anggrek. AgroMedia Pustaka : Jakarta Selatan. [Indonesian]

Jefferies, C.J. and A.R. Belcher. 1974. A Fluorescent Brightener Used for Pollen Tube Identification in vivo. The Williams and Wilkins Co. 49 (4) : 199-202

Johnson SD, Edwards TJ. 2000. The Structure and Function of Orchid Pollinaria. Plant Syst Evol 222: 243-269.

Johri BM, Vasil IK. 1961. Phyisiology Of Pollen. Botanical Review 27 (3) : 325-381

Kauth PJ, Johnson TR, Stewart SL, Kane ME. 2008. A Classroom Exercise in Hand Pollination and in Vitro Asymbiotic Orchid Seed Germination. Plant Cell, Tissue and Organ Culture 93 (2): 223-30. DOI: 10.1007/s11240-008-9365-1

Kho YO, Baer J. 1968. Observing Pollen Tubes by Means of Fluorescence. Euphytica 17: 298-302. DOI: 10.1007/BF00021224

Kwack BH, Kim I. 1967. Effects of Calcium Ion and The Protective Action on Survival and Growth Inhibition of Pollen. Physiol Plant. 20: 73-82.

Lorbiecke, Rene. 2012. Plant Reproduction & the Pollen Tube Journey-How the Females Lure the Males. Am Biol Teach. 74 (8) : 575-580.

Marwoto D, Badriah DS, Dewanti M, Sanjaya L. 2012. Persilangan Interspesifik dan Intergenerik Anggrek Phalaenopsis Untuk Menghasilkan Hibrid Tipe Baru. Balai Penelitian Tanaman Hias. 2012: 101-116

McCormick S, Yang H. 2005. Is There More Than One Way to Attract a Pollen Tube?. Elsevier, Amsterdam 20 (20) : 1-3.

Nadeau JA, Xhang XS, Nair H, O'Neill SD. 1993. Temporal and Spatial Reghulation of 1-Aminocyclopropane-1-Carboxylate Oxidase in the Pollination-Induced Senescence of Orchid Flowers. Plant Physiol 103: 31-39. DOI: 10.1104/pp.103.1.31

Nadeau JA, Zhang XS, Li J, O'Neill SD. 1996. Ovule Develpment : Identification of Stage-Specific and Tissue-Spesific cDNAs. The Plant Cell 8 : 213-239. DOI: 10.1105/tpc.8.2.213

Niimoto DH, Sagawa Y. 1962. Ovule Development in Phalaenopsis. Caryologia 15 (1) : 89-97. DOI: 10.1080/00087114.1962.10796051

O'Neill SD, Nadeau JA, Zhang XS, Bul AQ, Halevy AH. 1993. Interorgan Regulation of Ethylenen Biosynthetic Genes by Polination. The Plant Cell. 5 : 419-432 DOI: 10.1105/tpc.5.4.419

Pacini E, Hesse M. 2005. Pollenkitt-its composition, forms and functions. Elsevier, Amsterdam 5: 399-415. DOI: 10.1016/j.flora.2005.02.006

Parre E, A. Geitmann. 2005. More Than a Leak Sealant. The Mechanical Properties of Cllose in Pollen Tubes. Plant Physiology. 37: 274-286.

Rakosy D, Speckmaier M, Weber A, Huber W, Weissenhofer A. (eds). 2013. Orchids: Botanical Jewels of the Golfo Dulce Region, Costa Rica. Verein zur Förderung der Tropenstation La Gamba : Vienna, Austria.

Roberts DL, Dixon K. 2008. Orchids. Curr Biol 18 (8) : 325-329.

Rodriguez-Enriquez MJ, Mehdi S, Dicinson HG, Grant-Downtown RT. 2012. A Novel Method for Efficient In Vitro Germination and Tube Growth of *Arabidopsis thaliana* Pollen. New Phytologist. 197 : 668-679. DOI: 10.1111/nph.12037

Roxana D, Maria B. 2013. Cross Pollination on Randomly Chosen Commercial Varieties of Phalaenopsis. J Hortic Sci Biotechnol 17(4) : 28-31.

Rukmana H.R. 2008. Budidaya Anggrek Bulan. Kanisius, Yogyakarta. [Indonesian]

Rupawan, I Made, Basri Z, Bustami M. 2014. Pertumbuhan Anggrek Vanda (*Vanda* sp.) pada Berbagai Komposisi Media Secara *In Vitro*. e-J Agrotekbis. 2 (5): 488-494. [Indonesian]

Russle SD. 2017. Reproduction and Biodiversity. Encyclopedia of Apllied Plant Science. 2: 262-268.

Sarwono B. 2002. Mengenal dan Membuat Anggrek Hibrida. Agromedia Pustaka : Depok, Indonesia. [Indonesian]

Scordato A, Schwartz S. 2017. Ultraviolet Excitation Filter Sets. https://www.microscopyu.com. 18 November 2017.

Sukla MR, Singh AS, Piunno K, Saxena PK, Jones AMP. 2017. Application of 3D Printing to Prototype and Develop Novel Plant Tissue Culture System. Plant Methods. 13 (6) : 1-10. DOI: 10.1186/s13007-017-0156-8.

ThePlantList. 2017. The Plant List A Working List of All Plant species. http//www.plantlist.org. Diakses pada 02 Februari 2017. [Indonesian]

Tuhuteru S, Hehanussa MI, Raharji SHT. 2012. Pertumbuhahhn dan Perkembangan Anggrek Dendrobium anosmum Pada Media Kultur *In Vitro* Dengan Beberapa Konsentrasi Air Kelapa. Agrologia. 1 (1) : 1-12. [Indonesian]

Vieira LJ, Santana JRF, Alves AAC, Ledoand CAS, FVD. Souza. 2015. Use of Aniline Blue Stain to Observing Pollen Tubes Development in Different Manihhot Mill. Spesies. Afr J Agric Res 10 (15) : 1805-1809. DOI: 10.5897/AJAR2014.8976

Widiastoety, Dyah, Solvia N, Soedarjo M. 2010. Potensi Anggrek Dendrobium Dalam meningkatkanVariasi dan Kualitas Anggrek Bunga Potong. Jurnal Litbang Pertanian 29 (3) : 101-106. [Indonesian]

Wihelmi LK, Preuss D. 1997. Pollen Tube Guidance in Flowering Plants. Plant Physiol. 113 : 307-312.

Wimmer R. 2017. Symmetry. https://www.medicinalplantsarchive.us. 05 November 2017.

Yam TW, Arditti J. 2009. History of Orchid Propagation : A Mirror of The History of Biotechnology. Plant Biotechnol 3: 1-56. DOI: 10.1007/s11816-008-0066-3

Yu H, Goh CJ. 2001. Molecular Genetics of Reproductive Bioloogy in Orchids. Scientific Correspondence. 127 : 1390-1393. DOI: 10.1104/pp.010676

Zenkteler M. 1980. *Intraovarian and In Vitro Pollination*. Academic Press. Institute of Biology, Adam Mickiewicz University : Poland

Zhang XS, O'Neill SD. 1993. Ovary and Gametophyte Development are Coordinately Regulated by Auxin and Ethylene Following Pollination. The Plant Cel 5: 403-418. DOI: 10.1105/tpc.5.4.403.