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Response of *Pinus merkusii* Jung. & Devr. Megagametophyte Explants Cultured on DCR Medium with Different 2,4-D and Kinetin Combination

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ABSTRACT

Pinus merkusii Jung. & Devr. is a multipurpose tree species that is widely cultivated for timber, resin production, and land conservation. Nearly all parts of the tree are utilized, making it highly valuable. However, the high demand for its products is not met by its low natural regenerative capacity, which poses challenges for sustainable management. To address this, somatic embryogenesis, an in vitro propagation technique, has been explored as an alternative method for multiplying conifer species like *Pinus merkusii*. This study aims to assess the in vitro response of megagametophyte explants cultured on DCR medium with various combinations of 2,4-D and kinetin. The concentrations of 2,4-D tested were 0 µM, 7 µM, 9 µM, and 11 µM, while kinetin was tested at 0 µM, 2 µM, 3 µM, and 4 µM. The explants used were megagametophytes from female strobili measuring 5-7 cm, containing young zygotic embryos in the preembryo stage. After two months of cultivation in the dark, the explants exhibited responses such as enlargement, callus formation, and germination. The highest response rates for explant enlargement, callus formation, and germination were observed in the combinations of DK00 and DK04 (33.33%), DK70 and DK114 (33.33%), and DK72 and DK114 (44.44%). No somatic embryo formation was observed, possibly due to the age of the explants.

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1. INTRODUCTION

Pinus merkusii Jung. & Devr. is a pine species commonly found in Indonesia and is the only one that grows naturally in the country. This species was first discovered in the Sipirok region, South Tapanuli, North Sumatra, by a German botanist, Dr. F. R. Junghuhn, in 1841 (Harahap, 2006). *Pinus merkusii* is considered a multipurpose tree species that continues to be developed and its cultivation expanded for timber production, resin extraction, and land conservation (Siregar, 2005). The increasing use of *Pinus merkusii* has led to a growing demand for its yearly products. However, the high demand for its products is not matched by low regenerative capacity, which is due to the long life cycle of *Pinus merkusii*, approximately 20-50 years (Hidayat and Hansen, 2001).

Tissue culture, as the application of micropropagation, is considered one of the solutions to address challenges in the cultivation of *Pinus merkusii*. In vitro propagation methods can produce clonal seedlings in large quantities and in a relatively short time (Riyadi, 2017). One of the most promising in vitro techniques, both economically and in terms of the number of propagules produced, is somatic embryogenesis (Gupta, 1988).

Somatic embryogenesis is the process by which somatic embryos are formed, which are embryos derived not from a zygote, but from somatic cells (Gunawan, 1992). This process can occur directly, forming proembryos or embryo-like structures from explant tissue, known as direct embryogenesis, or it can involve the formation of a callus first, known as indirect embryogenesis (Suryowinoto, 1990). Plant regeneration via somatic embryogenesis can be divided into four stages: induction of somatic embryos from primary explants, proliferation of embryogenic cultures, maturation of somatic embryos, and plant regeneration from somatic embryos (von Arnold et al., 1996).

A study on the induction of somatic embryos in Pinus merkusii using Douglas Cotyledon Reserve (DCR) medium with combinations of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) by Nurdini in 2005 showed very low results, with an induction rate of only 3.67%. Rahmadani in 2007 was able to improve this result in her study, achieving the highest somatic embryo induction percentage of 20%. However, the somatic embryo induction rate for Pinus merkusii remains low. The success of somatic embryogenesis is influenced by several factors, one of which is the type of Plant Growth Regulators (PGRs) that used in tissue culture media (Purnamaningsih, 2002).

The PGRs that commonly used in plant propagation through somatic embryogenesis are auxins and cytokinins (Utami et al., 2007). Combination of auxin dan cytokinine that most frequently used to induce somatic embryos are 2,4-D (2,4-Dichlorophenoxyacetic acid) and Benzyl Amino Purine (BAP) (Purnamaningsih, 2002). Another cytokinin commonly used in somatic embryogenesis is 6-furfuryl amino purine (kinetin). The addition of kinetin to the culture medium has been shown to increase callus proliferation and regeneration (Wan and Liang, 1988). Thus, the use of 2,4-D and kinetin in the culture medium is believed to promote somatic embryogenesis due to the synergistic effects of these two growth regulators. Kinetin has also been used as a PGR combination with 2,4-D in initiation and proliferation media for *Pinus halepensis* (Montalban et al. 2013).

Somatic embryogenesis in pine is a continuation of natural cleavage polyembryony (Becwar and Pullman, 1995), which involves the formation of more than one embryo, eventually developing into Embryo Suspensor Masses (ESM). The formation of ESM is achieved by culturing young megagametophytes containing zygotic embryos in the proembryo stage on induction media. Culturing megagametophytes on induction medium for ESM formation produces various responses, as found by Rahmadani (2007) and Dinar (2007).

Based on that background, this study was conducted to analyze the response of *Pinus merkusii* megagametophytes cultured on DCR medium with different combinations of 2,4-D and kinetin.

2. METHODS

The research was conducted using a descriptive research design. The study was carried out from December 2017 to July 2018 at two research locations. The sample collection was conducted in the Pondok Hijau area, North Bandung. The planting and observation of explants were carried out in the Tissue Culture Laboratory of the Botanical Garden, FPMIPA, Universitas Pendidikan Indonesia. The explants used were megagametophytes from female strobili measuring 5-7 cm with a shiny green color. The medium used was DCR medium (Gupta and Durzan, 1985) with the addition of growth regulators 2,4-D (7, 9, 11 µM) and Kinetin (2, 3, 4 µM).

The research involved sterilizing tools and materials, preparing DCR medium and explants, and planting the sterilized explants. Data on explant responses, including enlargement, callus formation, germination, and somatic embryo development, were collected weekly for eight weeks and analyzed descriptively.

3. RESULTS AND DISCUSSION

The results of explant cultivation on DCR medium with the addition of 2,4-D and kinetin for 8 weeks showed responses in the form of explant tissue enlargement, callus formation, and germination. In this study, no response for somatic embryo formation was observed as we can see in **Figure 1**.

Table 1. Response of megagametophytes cultured on DCR medium with the addition of 2,4-D and Kinetin.

ZPT Combination	Responses (%)			
	Tissue Enlargement	Callus formation	Germination	Somatic Embryo
DK00	33,33	0	33,33	0
DK02	22,22	0	11,11	0
DK03	11,11	0	11,11	0
DK04	33,33	11,11	44,44	0
DK70	0	33,33	33,33	0
DK72	22,22	22,22	44,44	0
DK73	0	22,22	33,33	0
DK74	11,11	0	22,22	0
DK90	0	11,11	33,33	0
DK92	22,22	11,11	44,44	0
DK93	0	22,22	22,22	0
DK94	22,22	0	22,22	0
DK110	11,11	0	0	0
DK112	11,11	22,22	33,33	0
DK113	22,22	22,22	11,11	0
DK114	0	33,33	22,22	0
Average	**13,89**	**13,19**	**26,39**	**0**

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3.1 Explant Enlargement Reponse

Based on **Tabel 1**, we can see that the explant enlargement responses, in this case, it is marked by the enlargement of the megagametophyte (**Figure 1**). This megagametophyte enlargement appeared in almost all medium combinations as a result of the interaction between the explants and the added combinations of 2,4-D and kinetin growth regulator concentrations in the medium. The highest percentage was obtained from megagametophytes cultured on media with the combinations of DK00 (0 µM 2,4-D and 0 µM kinetin) and DK04 (0 µM 2,4-D and 4 µM kinetin), which was 33.33%. This indicates that these two medium combinations were the most effective in initiating explant enlargement. In the medium combinations DK70, DK73, DK90, DK93, and DK114, no explant enlargement response was observed. This may be related to the low levels of endogenous auxin and cytokinin in the explants, suggesting that higher exogenous auxin and cytokinin concentrations are required in the culture medium to initiate enlargement. Explant enlargement is associated with the presence of PGRs in the culture medium. Auxins (2,4-D) can trigger cell elongation and enlargement, while cytokinins (kinetin) can stimulate cell division. The elongation and division of cells lead to an increase in both the size and number of cells in the megagametophyte explant. We can see in **Figure 1.**

Figure 1. Enlargement of megagametophyte explants after 7 weeks

Auxins, in this case, 2,4-D, are known to play a role in cell development. At certain concentrations, auxins can increase osmotic pressure, enhance cell permeability to water, reduce pressure on the cell wall, stimulate protein synthesis, and increase cell wall plasticity and expansion (Abidin, 1982; Gunawan, 1992). The effect of auxins on tissue growth, as stated by Gunawan (1987), involves inducing the secretion of H^+ ions out of the cell through the cell wall. The acidification of the cell wall leads to the uptake of K^+, reducing the water potential inside the cell, causing water to enter the cell and resulting in cell enlargement. This cell enlargement contributes to the overall growth of the tissue.

The presence of cytokinins in the medium also plays a role in tissue enlargement. Cytokinins, in this case kinetin, are a crucial group of plant growth regulators involved in cell division and morphogenesis (Gunawan, 1987). Tissue enlargement may also occur due to an increase in the number of cells within the explant tissue. This is highly likely because cytokinins act as a specific trigger for cell division (Devlin and Witham, 1983). A study conducted by Sukamto et al. (2018) showed that the application of plant growth regulators, auxins and cytokinins, in treatment P1 (BAP 1.5 ppm + NAA 0.05 ppm) was the treatment that induced the most swelling response in the young leaf explants of rubber plants.

3.2 Callus Formation Response

The response in the form of callus formation with the highest percentage (33.33%) was obtained from megagametophytes cultured on media with the combinations of DK70 (7 µM 2,4-D and 0 µM kinetin) and DK114 (11 µM 2,4-D and 4 µM kinetin). The addition of exogenous 2,4-D and kinetin resulted in an auxin to cytokinin ratio within the tissue that was in the range capable of inducing callus formation, which is an equilibrated ratio of auxins and cytokinins. According to Yusnita (2003), an equilibrated ratio of cytokinin and auxin can promote callus formation. Rahayu and Anggarwulan (2003) added that the addition of 2,4-D in the medium stimulates cell division and enlargement in *Acalypha indica L*. leaf explants, thereby promoting callus formation and growth.

In vivo, callus generally forms at wound sites caused by microbial infections, such as *Agrobacterium tumefaciens*, insect bites, and nematode attacks. Callus can also form as a response to stress (Gunawan, 1988). In vitro, callus formation due to stress can be induced by deliberately wounding the explants. Hendaryono and Wijayani (1994) stated that this occurs because some cells on the wounded surface of the explants undergo proliferation.

The callus formed from megagametophytes cultured on media with the addition of 2,4-D and kinetin exhibited two different colours. The callus formed in the DK70 and DK73 combinations was white, while the callus from megagametophytes cultured in DK93 medium was yellowish (**Figure 2**). The white and yellowish colours of the callus are suspected to be closely related to the storage of megagametophyte explants in dark conditions, preventing the induction of chloroplast formation. According to Salajova et al. (1998), explants cultivated in darkness will not form leaf pigments (chlorophyll).

Figure 2. Callus formation response after 6 weeks of cultivation with DK70 combination (a) (2.68x); DK73 combination (b) (2.07x); DK93 combination (c) (2.75x)

The colour of the callus indicates the presence of chlorophyll in the tissue; the greener the callus, the higher its chlorophyll content. Light or white colours may indicate that the callus is still in good condition (Fatmawati, 2008). White callus is embryogenic tissue that has not yet developed chloroplasts but contains a high level of starch grains (Ariati, 2012). Leupin (2000) added that white callus contains plastids with starch grains that gradually develop into distinct membrane systems, eventually forming chlorophyll grains when exposed to light, turning the callus green. According to Kresnawati (2006), the colour differences in callus indicate the stage of its development, whether the cells in the callus are still actively dividing or have died. The varying colours of the callus are due to light pigmentation and the origin of the explant.

During its development, the callus, which remains on the culture medium for an extended period, will undergo physiological degradation or a decline in the physiological state

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of the plant due to a deficiency of nutrients or growth hormones. This is commonly indicated by a color change from bright to brown or often called as Browning Process (**Figure 3**).

a **b**

Figure 3. Color changes in callus: Callus at the initial planting (a) (1.87x); callus after 6 weeks of cultivation (b) (1.48x)

The colour change of the callus to brown not only indicates the synthesis of phenolic compounds but is also caused by the increasing age of the cells or tissues of the callus. High concentrations of 2,4-D and the absence of cytokinin in the medium can trigger cell senescence, which can inhibit the growth process of the callus. This is caused by cytokinins which play a role in slowing down the cells senescence process by inhibiting the breakdown of chlorophyll and protein grains within the cells (Wattimena, 1991). The browning event occurs due to the metabolism of toxic phenolic compounds, which are often triggered by the sterilization process of the explant, inhibiting growth or even causing tissue death (Yusnita, 2004).

3.3 Response to Seedling Formation

Based on the research results in Table 1, all medium combinations showed a response in seedling formation (**Figure 4**), with the highest percentage (44.44%) obtained from megagametophyte planted in combinations of DK04, DK72, and DK92 media. Naturally, seedling formation does not require the addition of growth regulators. This can be proven by the formation of seedlings on a medium without the addition of growth regulators, with a percentage reaching 33.33% (**Table 1**). The availability of water in the culture medium is a factor that can trigger seedling formation. Water acts as a carrier of oxygen and a source of energy needed for germination.

Kusfebriani et al. (2010) state that germination is the process of growth and development of the embryo and the early stage of development of a plant, especially seed plants. During germination, the embryo inside the seed, initially in a dormant state, undergoes several physiological changes that cause the embryo to develop into a young plant. This young plant is known as a seedling. The result of germination is the emergence of a small plant from the seed. Ardian (2008) mentioned several factors affecting seed germination, including internal factors (seed maturity, embryo imperfection, seed coat permeability to water and oxygen and external factors (temperature, water, oxygen, and light). Germination cannot occur if the seed does not absorb water from the environment. Harjadi (2002) stated that

water is an essential requirement for germination. The amount of water required varies depending on the species. A study by Kosmiatin et al. (2016) showed that agarwood seeds (*Aquilaria malaccensis* Lank) germinated better on simple media, such as MS medium without the addition of vitamins, growth regulators, or other compounds like PVP.

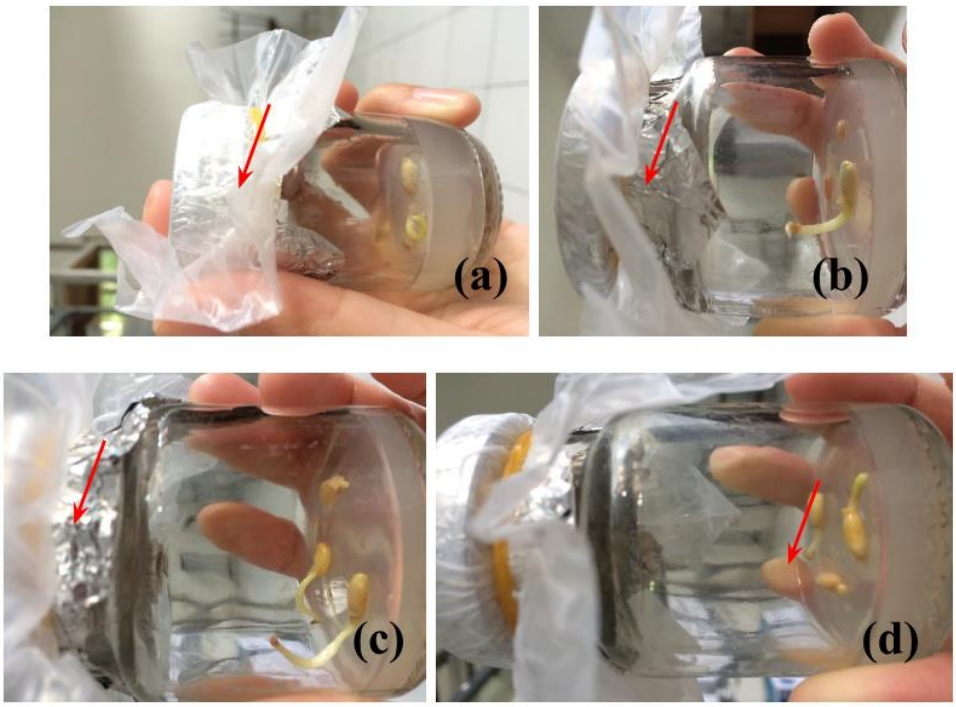

Figure 4. Germination response after 2 weeks of cultivation: DK00 combination (a) (1.34x); DK04 combination (b) (1.41x); DK70 combination (c) (1.29x); DK73 combination (d) (1.73x)

The response in seedling formation in this study should not have occurred if the seeds used were immature. This is because, in immature seeds, the embryo is still in the proembryo phase. In this phase, the cotyledon primordia, as well as the epicotyl and hypocotyl, have not yet formed. The formation of seedlings is suspected to be due to seeds that have entered the late globular phase or pre-cotyledon stage. In this phase, the cells inside the seed have undergone differentiation, and their future developmental fate has been determined. Seeds in this phase already have cotyledon primordia (Vasishta, 1983).

3.4 Response to Somatic Embryo Formation

Based on the research, no somatic embryo formation response was observed in any of the megagametophyte explants cultured on any of the combinations of growth regulators used (**Table 1**). This is different from the results of the pre-study, where somatic embryo formation was observed in *Pinus merkusii* megagametophyte explants cultured on DCR medium with a combination of 9 µM 2,4-D and 3 µM BAP after three weeks of cultivation (**Figure 5**). Megagametophyte explants induced to form somatic embryos did not subsequently develop or proliferate. The somatic embryos eventually turned brown and died in the following week. This relates to the development period of the megagametophyte explants in the culture medium, which, over time, will experience physiological degradation or a decline in the physiological state of the plant due to a lack of nutrients or growth hormones. This is typically marked by a color change from bright to brown.

The absence of somatic embryos with the characteristic of a thin thread-like protrusion, which serves as the suspensor of the megagametophyte micropyle, as reported

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by Lelu et al. (1999), is an issue that needs further investigation to determine the cause. The appearance of the response in the form of seedling formation in megagametophyte explants in this study indicates that the explants were not suitable for somatic embryo induction. This is because the megagametophytes used contained embryos that had already entered the late globular phase or pre-cotyledon stage, while it is known that the most suitable zygotic embryos for induction into somatic embryos are those in the proembryo stage.

Figure 5. Induced megagametophyte explants in March, cultured on DCR medium with a growth regulator combination of 9 µM 2,4-D and 3 µM BAP (1.47x).

Based on this research, explant selection is a critical factor determining the success of somatic embryo induction in *Pinus merkusii*. As noted by Nurani (2004), Umar (2005), and Nurdian (2004), strobili that are 5–7 cm in size with light brown seed coats do not guarantee that the embryo inside is in the proembryo phase. Within a single strobilus, seed age can vary due to differences in pollination time. Variations in pollination time could also be influenced by the differing maturation periods of the megagametophytes. The most accurate determination of the embryo phase is made using the color characteristics of the megagametophyte. The megagametophyte color should be a milky white that is not too dense or too clear (**Figure 6**), as somatic embryogenesis in the pre-study conducted between December 2017 and April 2018 occurred in megagametophytes with these characteristics.

The emergence of a response in the form of seedling formation is not the only factor indicating that the explants used were not in a developmental stage suitable for somatic embryo induction. The lack of induced megagametophyte explants may also be related to the pollination season of *Pinus merkusii*. Based on the pollination calendar developed from intensive monitoring of pollen and spore concentrations of anemophilous plants in Lennoxville, Sherbrooke, throughout the years 2006, 2007, and 2008, particularly during the pollination season (spring and summer), Levac (2001) reported that Pinus typically undergoes pollination from mid-May to mid-June. In Indonesia, the pollination season for Pinus merkusii occurs between March and June (Hidayat and Hansen, 2001). By understanding the pollination season, it is possible to estimate the best time for collecting megagametophyte explants containing zygotic embryos in the proembryo stage.

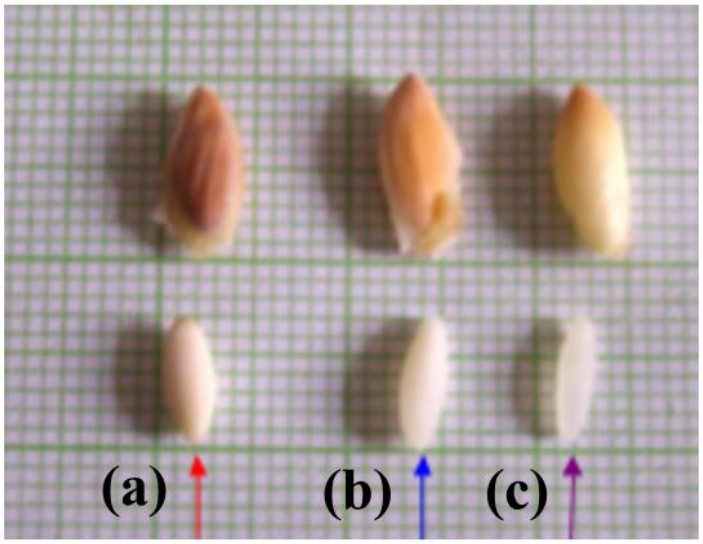

Figure 6. Colour of megagametophytes that may serve as a reference for determining the developmental stage of the zygotic embryo in *Pinus merkusii*: mature megagametophyte (a); megagametophyte containing proembryo phase embryo (b); overly young megagametophyte (c) (Rahmadani, 2007).

According to Becwar and Pullman (1995), somatic embryogenesis in Pinus in vitro is a continuation of cleavage polyembryony in vivo, suggesting that a significant amount of time is required after the pollination season (March–June in Indonesia) to obtain megagametophyte explants containing zygotic embryos in the proembryo development stage. In the second year, the ovum reaches the fertilization stage and forms a zygote that develops toward the third year. The zygote then undergoes division and forms polyembryonic cleavage by mid or late in the year, leading to the formation of mature embryos. This could explain why all samples collected in May and June were already in the stage of embryo maturation or were continuing cleavage after fertilization, resulting in megagametophyte explants forming callus and seedlings. The pollination season and regeneration patterns are also supported by Rahmadani (2007), who suggested that strobilus collection should be done by the end of June.

4. CONCLUSION

Based on the research results, it can be concluded that the response obtained from planting megagametophyte explants on Douglas Cotyledon Reverse (DCR) medium with a combination of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-furfuryl amino purine (kinetin) was the enlargement of explants, callus formation, and seedling formation. The highest percentage of response in explant enlargement (33.33%) was obtained from the megagametophyte planted on the medium combinations DK00 and DK04. The highest percentage of callus formation response (33.33%) was obtained on the combinations of DK70 and DK114. The highest percentage of seedling formation response (44.44%) was obtained from the combinations of DK70, DK72, and DK114. No response in the form of somatic embryo formation was found in any of the growth regulator (ZPT) combinations used. This is suspected to be related to the age of the megagametophyte, which had already passed the proembryo phase, that was used as the explant.

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6. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. Authors confirmed that the paper was free of plagiarism.

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