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Optimization of Plant Growth Regulators for Tobacco Anther Culture Regeneration

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Abstract. Tobacco is one of the most important plantation crops and is used as raw material by the cigarette industry. Improvements in the quantity and quality of tobacco crops are needed, one of which can be achieved by using superior hybrid varieties. However, the production of hybrid varieties requires pure-line plants, which are generally produced through conventional methods that take approximately 7–8 generations. Anther culture has emerged as an alternative method to accelerate the production of pure-line plants within 1–2 generations by using haploid plants. Although several studies have investigated the effects of auxins and cytokinins on anther culture in Solanaceae species, the optimal combination of 2,4-D and Kinetin for tobacco (Nicotiana tabacum L.) anther culture regeneration remains unclear. Therefore, this study aimed to optimize the combination of these plant growth regulators to improve regeneration efficiency in tobacco anther culture. The success of anther culture can be enhanced by using Plant Growth Regulators (PGRs) such as 2,4-D and Kinetin. This study aimed to determine the optimal concentration of 2,4-D and Kinetin for regenerating tobacco anther culture explants. The experiment was designed using a completely randomised design (CRD) with two factors: 2,4-D (0, 0,1, 1, and 2 mg/L) and Kinetin (0, 0,1, 0,2, and 0,4 mg/L). The interaction between 2,4-D and Kinetin significantly affected swelling initiation, callus initiation, and callus formation ($P < 0.05$). The D1K3 treatment (0.1 mg L^{-1} 2,4-D + 0.4 mg L^{-1} Kinetin) produced the fastest swelling initiation (3.67 DAI) and callus initiation (28.33 DAI), whereas D3K3 produced the highest callus formation (73.33%). Kinetin at 0.4 mg L^{-1} significantly accelerated shoot (98 DAI) and root initiation (105 DAI). These findings demonstrate that optimizing the balance between auxin and cytokinin is essential for improving tobacco anther culture regeneration and provide useful information for doubled haploid production. However, a single application of Kinetin produced optimal effects on explant regeneration, resulting in the emergence of shoots and roots.

Keywords: callus induction; explant regeneration; haploid production; in vitro culture; plant growth regulators

1. Introduction

The tobacco plant (Nicotiana tabacum L.) is an annual crop in the Solanaceae family that is commonly used as a raw material in the cigarette manufacturing industry, with its leaves having high economic value (Azhari et al., 2024). The potential of tobacco plants makes this commodity a contributor to Indonesia's economic growth, as evidenced by the high value of tobacco exports. According to the Directorate General of Plantations (2023), the export value of tobacco plants in 2020 reached US$ 196.01, increasing to US$ 213.41 in 2021 and to US$ 266.026 in 2022. An increase in the quantity and quality of tobacco plants is needed; one way is to use hybrid tobacco varieties with superior properties. Hybrid plants typically originate from the first generation (F1) resulting from the crossbreeding of purebred parents that are homozygous and possess superior characteristics compared to their parents (Armandoni, Akhmad et al., 2022). However, hybrid plants are generally obtained using conventional methods, namely 7-8 generations of self-crossing to produce pure lines, which takes a long time (Iqbal & Möllers, 2019). This indicates that conventional breeding methods tend to be less effective at producing purebred plants quickly, so if the formation process takes a

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long time, it will be comparable to acquiring hybrid varieties, which also take longer. Therefore, a method is needed to shorten the cycle time, one of which is anther culture using haploid plants. According to Ningsih et al. (2016), haploid plants from anther culture can shorten the time required to obtain pure lines to one or two generations.

Anther culture is a tissue culture technique that involves culturing flower anthers containing young pollen (Dewi & Purwoko, 2016). Anther culture can also be referred to as a haploid culture because it can produce large numbers of haploid (n) in vitro plants from field plants with a diploid (2n) chromosome number and heterozygous genotypes, thereby accelerating the acquisition of pure lines. According to Lu et al. (2020), anther culture is one of the most effective methods for haploid plant production because it enables the rapid production of pure homozygous plants. One of the keys to successful anther culture is the use of plant growth regulators (PGRs). Among Indonesian tobacco cultivars, Deli Sumatra is an important cigar tobacco variety with high economic value. It is widely used as breeding material for its desirable agronomic and leaf-quality characteristics. However, its response to anther culture remains highly genotype-dependent, and regeneration efficiency is still relatively low. Therefore, optimization of culture conditions, particularly the concentration and balance of plant growth regulators, is required to improve regeneration efficiency in Deli Sumatra tobacco. Therefore, selecting stage-2 Deli Sumatra tobacco anthers in the present study was intended to maximize the regenerative competence of microspores before the application of different plant growth regulator combinations (Walbot & Egger, 2016; Nyirahabimana et al., 2025). Plant growth regulators (PGRs) are compounds that play an important role in promoting, directing, stimulating, or inhibiting the growth and development of plant cells (Khozin & Mona, 2025). A supporting factor for the high success rate of anther culture is the type and concentration of PGRs, including both auxins and cytokinins, such as 2,4-Dichlorophenoxyacetic acid (2,4-D) and Furfurylamino Purine (Kinetin). The PGs 2,4-D and Kinetin certainly have different roles: 2,4-D belongs to the auxin group and stimulates callus and root growth. In contrast, Kinetin belongs to the cytokinin group and stimulates shoot growth and cell division (Winarto, 2011).

Auxin (2,4-D) and cytokinin (Kinetin) regulate different developmental pathways during in vitro morphogenesis. 2,4-D primarily promotes dedifferentiation and callus induction, whereas Kinetin stimulates cell division and shoot organogenesis. Their interaction determines the developmental fate of cultured explants; therefore, optimizing their concentration ratio is essential for successful regeneration (Phillips & Garda, 2019; Karami et al., 2023). It is also supported that 2,4-D has more stable properties because it is not easily damaged by light or heat (Chainda, 2023). Meanwhile, Kinetin stimulates cell division during regeneration, including when buds or leaves form on an explant such as an anther. Therefore, among the cytokinin group, one PGR that can support pollen growth and development in plant anther culture is furfuryl amino purine (Kinetin) (Olszewska et al., 2014). Thus, both can also work together when combined or interacted at the right concentration. According to Hidayati & Subroto (2018), the combination of auxin and cytokinin PGs can interact positively at appropriate concentrations, working together to stimulate the formation, growth, and regeneration of explants.

This means that the application's concentration needs to be considered to achieve optimal growth and development, including during the regeneration process. The interaction between PGs and explants, particularly at different auxin and cytokinin concentrations, will lead to different directions of explant development (Mastuti et al., 2017). Explant regeneration through anther culture has been widely used across

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several Solanaceae families. Research by Aboshama & Atwa (2019) on in vitro potato anther culture treated with 2,4-D and BA PGs found that a single treatment of 2,4-D at 2 mg/L in Chu's (N6) medium produced the highest percentage of callus induction, namely 56%, compared to other treatments. Additionally, in the study by Matsubara et al. (1992) on eggplant anther culture, it was found that the pollen capable of producing the most callus was found in the interaction treatment between 2,4-D (0.1 mg.L^{-1}) and Kinetin (0.1 mg.L^{-1}) treatment, with 58 out of 162 pollen grains, representing a percentage of 35.8%. Although several studies have evaluated the effects of auxins and cytokinins on anther culture in Solanaceae species, most investigations have focused on potato, eggplant, and pepper. At the same time, information on optimizing 2,4-D and Kinetin concentrations specifically for Deli Sumatra tobacco remains very limited. Furthermore, previous studies have primarily focused on callus induction without comprehensively evaluating subsequent regeneration responses, including shoot and root formation. Recent studies demonstrated that optimizing the in vitro culture environment through basal medium modification and silver nanoparticle supplementation significantly improved tobacco anther regeneration efficiency (Khozin, Ahnaf et al., 2025; Khozin et al., 2026). However, these studies did not specifically optimize the interaction between 2,4-D and Kinetin. Therefore, this study addresses this research gap by systematically evaluating combinations of 2,4-D and Kinetin to identify the optimal concentrations for complete tobacco anther regeneration, thereby providing an improved regeneration protocol that supports doubled haploid production in tobacco breeding. Therefore, this study aims to determine the effect of different concentrations of 2,4-D and Kinetin on explant regeneration in tobacco anther culture.

2. Materials and Methods

2.1 Description Research Location, Materials, and Tools

The study was conducted at the Ecophysiology and Tissue Culture Laboratory, University of Jember, Indonesia, from September to December 2024. The explants used were anthers from Deli Sumatra tobacco plants in stage 2 of development with a corolla length of 9–12 mm, as shown in Figure 1.

Figure 1. Tobacco plant flowers and anthers in growth stage 2

The anther explants were sterilized before planting. The sterilization method involved soaking the anther explants in 70% alcohol for 30 seconds, then in 1% chlorine for 15 minutes, followed by three rinses with distilled water for 2 minutes each. Other materials included Chu's culture medium (N6), 30 g/L sugar, 8 g/L agar, 1 mL/L vitamins, and ZPT. The tools used included tweezers, scalpels, culture bottles, autoclaves, laminar air flow (LAF), petri dishes, etc.

2.2 Treatments

The anthers used were at stage 2 of development in the Deli Sumatra variety, with a flower corolla length of 9-12 mm. This study used a completely randomized design (CRD) with treatments at different concentrations of 2,4-D and Kinetin, each with 4 levels, repeated 3 times. The 2,4-D concentration treatments were D0 (0 mg/L), D1 (0.1 mg/L), D2 (1 mg/L), and D3 (2 mg/L), while the Kinetin concentration treatments were K0 (0 mg/L), K1 (0.1 mg/L), K2 (0.2 mg/L), and K3 (0.4 mg/L). Each

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experimental unit consisted of one culture bottle containing five anthers as explants. Therefore, a total of 240 anthers were used in this study (16 treatment combinations × 3 replications × 5 anthers).

2.3 Data Collection

In this study, the research parameters included swelling, callus formation, sprouting, and rooting, which were measured from the beginning of planting until their appearance, in days after inoculation (D.A.I.). The percentage of swelling and callus formation was calculated at the end of the observation period. The calculations are as follows: 1) Initiation Time of *Swelling* and Callus Explants (Days After Inoculation/D.A.I). The parameters of explant *swelling* and callus formation were observed visually, and the results were recorded when the explants first showed a response. Explants that experienced *swelling* were characterized by increased size and appeared swollen/wrinkled. Meanwhile, callus-forming explants appeared to have irregular cells. 2) Percentage of Swelling/Callus Explants (%). This parameter measures the number of explants that exhibit swelling or calcification. The results are recorded by treatment and calculated at the end of the observation period. The calculation is performed using Equation 1.

$$\text{Percentage of swelling/callus explants} = \frac{\text{Number of anthers swelling/callus}}{\text{Number of anthers planted}} X\ 100\% \quad \ldots\ldots (1)$$

The culture bottles were randomly assigned to treatment combinations according to a Completely Randomized Design (CRD). To minimize positional effects during incubation, the culture bottles were randomly arranged on the culture racks and their positions were rotated weekly throughout the culture period. All inoculation procedures were performed aseptically inside a laminar airflow cabinet using sterilized instruments. Culture media and glassware were sterilized by autoclaving at 121°C and 15 psi for 20 min before use. To minimize contamination, all explants were subjected to the sterilization procedure described above, and contaminated cultures were immediately discarded and excluded from further observation. Environmental conditions, including temperature and photoperiod, were maintained uniformly for all treatment combinations throughout the experiment. After inoculation, cultures were incubated at 25 ± 2°C under a 16 h light/8 h dark photoperiod with a light intensity of approximately 40–50 μmol m^{-2} s^{-1}.

All media preparation, explant inoculation, and subculture procedures were carried out under aseptic conditions inside a laminar airflow cabinet. Sterile instruments were flame-sterilized with 70% ethanol before each manipulation, and all culture media were autoclaved at 121°C for 15 min before use to minimize microbial contamination. To minimize experimental bias, all observations were performed using identical evaluation criteria throughout the experiment. Culture bottles were coded before observation, and all treatments were maintained under identical incubation conditions, including temperature, photoperiod, and light intensity.

In addition, descriptive qualitative parameters, including the color and texture of the callus, are visually analyzed using Munsell Color for Plant Tissues as a comparative indicator of callus color across treatments. The callus color parameter is observed daily using a stereo microscope. Meanwhile, callus texture is assessed using indicators such as crumbly, compact, and intermediate. Callus texture is observed visually starting 7 days after the explant begins to develop callus. The results are recorded for further descriptive processing. Another qualitative parameter is the analysis

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of anthers using a Scanning Electron Microscope (SEM) to assess their morphology. For SEM observation, freshly collected anthers were fixed in 2.5% glutaraldehyde for 24 h at 4°C, rinsed three times with 0.1 M phosphate buffer (pH 7.2), and dehydrated through a graded ethanol series (30, 50, 70, 80, 90, 95, and 100%). The samples were then air-dried, mounted on aluminum stubs using carbon adhesive tape, sputter-coated with gold, and observed using a Scanning Electron Microscope at magnifications of 35×, 300×, and 500×.

For scanning electron microscopy (SEM) analysis, representative anther samples were collected from each treatment at the callus induction stage. The samples were immediately fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C, followed by three rinses with phosphate buffer (10 min each). Samples were then post-fixed in 1% osmium tetroxide for 2 h and dehydrated through a graded ethanol series (30, 50, 70, 80, 90, 95, and 100%), with each step lasting 15 min. The dehydrated samples were dried, mounted on aluminum stubs using carbon adhesive tape, sputter-coated with gold, and observed using a Scanning Electron Microscope at an accelerating voltage of 10–15 kV. Micrographs were obtained to examine surface morphology and structural changes of the cultured anthers.

2.4 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) to evaluate the effects of 2,4-D, Kinetin, and their interaction. Before ANOVA, the data were examined to ensure that the assumptions of normality and homogeneity of variance were reasonably satisfied. When significant differences were detected ($P < 0.05$), treatment means were compared using Duncan's Multiple Range Test (DMRT) at the 95% confidence level. Qualitative observations, including callus morphology and SEM images, were analyzed descriptively.

3. Results and Discussion

3.1 The Effect of 2,4-D and Kinetin on Initiation Time of Swelling, Callus, Shoot, and Root Explants

Based on Figure 2, the interaction of 2,4-D and Kinetin concentrations affected the initiation time of swelling, as shown in Figure 3A. The average initiation times for swelling at each treatment concentration of 2,4-D and Kinetin differ. The best result was observed in the treatment interaction between the concentrations of 2,4-D (D1) and Kinetin (K3), which showed the shortest average swelling initiation time of 3,67 Days After Inoculation (D.A.I). This result was likely due to the synergistic effect of both PGRs, which accelerated the initiation which accelerated the initiation of the swelling response, as shown in Figure 2A. When auxin and cytokinin are applied at balanced or appropriate concentrations, they promote the differentiation process of explant cells, not only for swelling initiation but also for the rapid emergence of callus.

The rapid swelling response observed in the D1K3 treatment may indicate that an appropriate balance between auxin (2,4-D) and cytokinin (Kinetin) is associated with the initiation of cellular reprogramming during the early stage of androgenesis. However, this mechanism was not directly investigated in the present study. Previous studies have suggested that auxin promotes cell dedifferentiation through auxin-responsive signaling pathways, whereas cytokinin enhances mitotic activity and cell cycle progression (Phillips & Garda, 2019). These mechanisms were not directly examined in the present study. The coordinated action of these two plant growth regulators accelerates cell enlargement and the transition of microspore-derived cells toward callus initiation. Similar responses have been reported in tobacco and other Solanaceae species, where balanced auxin–cytokinin ratios enhanced morphogenic competence (Phillips & Garda, 2019; George et al., 2008).

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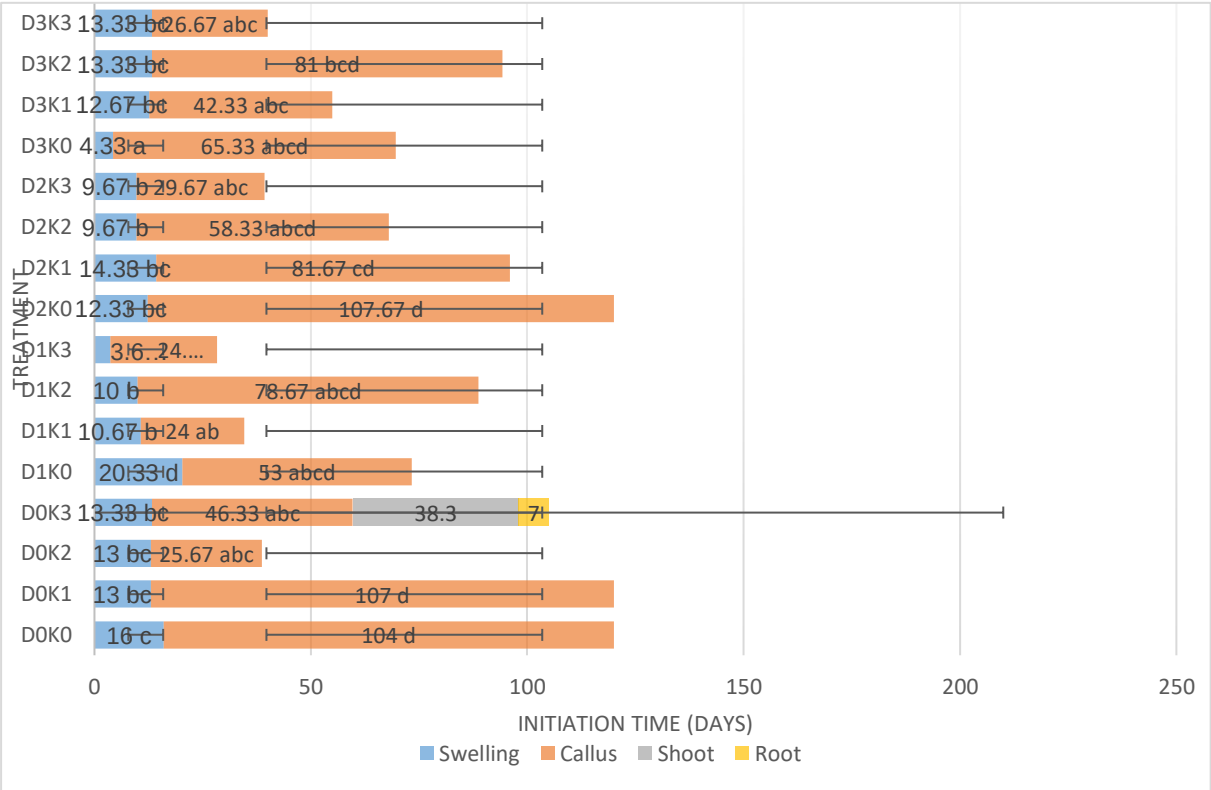

Figure 2. Mean initiation time (days after inoculation, DAI) of swelling, callus, shoot, and root formation in tobacco anther explants cultured under different combinations of 2,4-D and Kinetin. Values are presented as mean ± SD (n = 3 biological replicates). Different lowercase letters indicate significant differences among treatments according to Duncan's Multiple Range Test (DMRT) at $P < 0.05$.

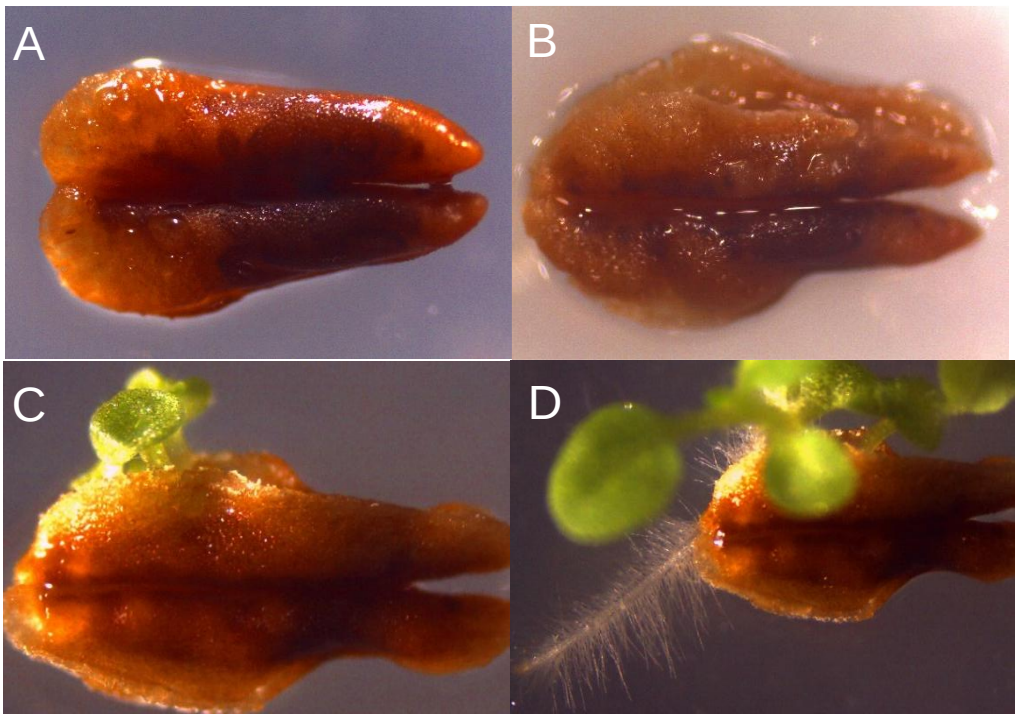

Figure 3. A. Visual of swelling initiation, B. Visual of callus initiation, C. Visual of shoot initiation, and D. Visual of root initiation

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The interaction between auxin and cytokinins at an appropriate concentration further stimulated and accelerated callus formation, including the early response of swelling initiation (Mastuti et al., 2017). On the other hand, the interaction between 2,4-D and Kinetin did not significantly affect the rapid emergence of shoots and roots, as shown in Figure 2. Additionally, the treatment using only 2,4-D (D3K0) also produced a response that was not significantly different from the D1K3 treatment, with an average swelling initiation time of 4,33 Days After Inoculation (D.A.I). The role of 2,4-D helped accelerate the swelling response by strongly stimulating growth and dedifferentiation of explant cells (Ai & Hanny, 2021). However, the effectiveness of 2,4-D in accelerating swelling initiation depended on its concentration; if applied at too low a concentration, it could delay the differentiation process, as in treatment D1K0, which had an average swelling initiation time of 20,33 D.A.I. A low concentration of PGRs could result in cells and tissues failing to differentiate optimally, leading to a delayed response in explant growth and development (George et al., 1996).

Callus formation was the subsequent stage after the swelling response of the explants. According to Duri et al. (2024), callus was defined as a mass of cells that had not yet undergone complete differentiation, characterized by an irregular (amorphous) structure and actively dividing cells. Similar to swelling, the acceleration of callus initiation could be influenced by the application of PGRs, particularly when auxin and cytokinin were applied in an interactive or balanced manner, as shown in Figure 3B. This condition enhanced callus formation, as seen in the D1K3 treatment, which had the fastest average callus initiation time of 28,33 D.A.I. The balance between auxin and cytokinin, when applied at appropriate concentrations, could affect the rate of callus explant emergence and influence explant morphogenesis or growth (Mastuti et al., 2017). These findings are consistent with those reported by Khozin et al. (2026), who demonstrated that optimizing the in vitro culture environment substantially increased callus induction and shoot regeneration in tobacco anther culture. Their study indicated that regeneration efficiency depends on interactions between the culture medium and growth-promoting compounds, underscoring the importance of optimizing plant growth regulators in the present study. A possible explanation is the synergistic interaction between auxin and cytokinin during the callus proliferation phase, as previously reported by Phillips & Garda (2019). These two hormones worked synergistically when applied under balanced concentrations.

Callus initiation is generally considered to represent the transition from cellular dedifferentiation to active proliferation. During this stage, previous studies have suggested that 2,4-D may maintain cells in a highly mitotic state by stimulating the activity of auxin-responsive transcription factors. In contrast, Kinetin promotes cytokinesis and the establishment of new meristematic tissues. Consequently, the balanced interaction between auxin and cytokinin may contribute to faster callus initiation and improved regenerative competence, as suggested by previous studies on developing calluses. (Phillips & Garda, 2019). However, contrasting results have been reported in previous studies. Matsubara et al. (1992) observed that the optimum combination for eggplant anther culture was 0.1 mg L^{-1} 2,4-D combined with 0.1 mg L^{-1} Kinetin, whereas AboShama & Atwa (2019) reported that potato anther culture achieved the highest callus induction using 2 mg L^{-1} 2,4-D without cytokinin supplementation. These differences indicate that the optimal balance between auxin and cytokinin is highly dependent on plant genotype, explant developmental stage, basal medium composition, and in vitro culture conditions. In the present study, Deli Sumatra tobacco responded most favorably to the combination of 0.1 mg L^{-1} 2,4-D and 0.4 mg L^{-1} Kinetin,

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suggesting that this cultivar requires a relatively higher cytokinin concentration to achieve efficient regeneration. This observation is consistent with the recent review by Nyirahabimana et al. (2025), which emphasized that androgenetic responses vary considerably among species and cultivars because regeneration efficiency is influenced by genotype, developmental stage of microspores, culture medium composition, and combinations of plant growth regulators. Meanwhile, in treatments with Kinetin without 2,4-D, such as D0K1 or D0K3, the average initiation time for callus formation was relatively long. This occurred because Kinetin alone was less effective at accelerating callus induction. When Kinetin was applied without the addition or interaction of auxin-type PGRs, it generally resulted in less optimal or slower callus formation (Sholehah et al., 2024).

In contrast to the results of swelling and callus initiation, the interaction between 2,4-D and Kinetin did not significantly affect the acceleration of shoot and root emergence. However, the application of Kinetin alone showed a positive effect, accelerating the initiation of shoots and roots, as shown in Figure 2. This was observed in the K3 treatment, which had an average shoot initiation time of 98 D.A.I was visually represented in (Figure 3C). The present results suggest that Kinetin was more effective in promoting shoot and root initiation under the experimental conditions used in this study. According to Sarita et al. (2022), cytokinin groups such as Kinetin functioned as regulators that enhanced cell division and elongation, especially during the formation of new organs (morphogenesis). Furthermore, the K3 treatment used a relatively high concentration of Kinetin, which remained within the optimal range for tobacco anther explants, thereby accelerating organogenesis. Increasing the concentration of Kinetin could promote shoot growth and, if the concentration remains within the optimal threshold, stimulate faster emergence of lateral shoots (Munthe et al., 2022). Basically, kinetin played a major role in shoot initiation but was less effective at promoting root formation. However, as shown in Figure 3D, the D0K3 treatment still initiated root formation, with an average emergence time of 105 D.A.I. A possible explanation is that the applied Kinetin interacted with endogenous hormonal pools already present in the anther tissues, thereby promoting organogenesis. Although endogenous hormone concentrations were not quantified in the present study, previous reports have demonstrated that the balance between endogenous auxin and exogenous cytokinin plays a decisive role in shoot and root differentiation during in vitro regeneration (Reddy, 2023; George et al., 1996). Therefore, this interpretation should be considered in the context of previous physiological studies rather than in light of direct evidence from the present experiment.

According to Reddy (2023), the balance of interactions between active growth substances (including endogenous hormones) and PGRs could influence the formation of new organs (organogenesis) in plants. Therefore, one possible explanation is that the D0K3 treatment may have provided a favorable balance between exogenous Kinetin and endogenous hormones. This balance allowed both hormones to work synergistically to promote cell activity. The application of PGRs interacted positively with endogenous hormones when both were present at optimal concentrations (Andany & Ratnasari, 2023). If the concentrations of endogenous hormones and ZPT are appropriate, they could interact positively and significantly to stimulate growth and development processes such as root formation. The interaction between applied PGRs and the endogenous hormones of explants could determine both the direction and the speed of growth and development, including during the regeneration process (Yusron, 2020).

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3.2 The Effect of 2,4-D and Kinetin on Percentage of Swelling and Callus Explants

The percentages of swelling and callus formation showed the influence of PGR application, both when applied individually and in combination, resulting in different responses. The effect of PGRs on the swelling response percentage was illustrated in the graph presented (Figure 4).

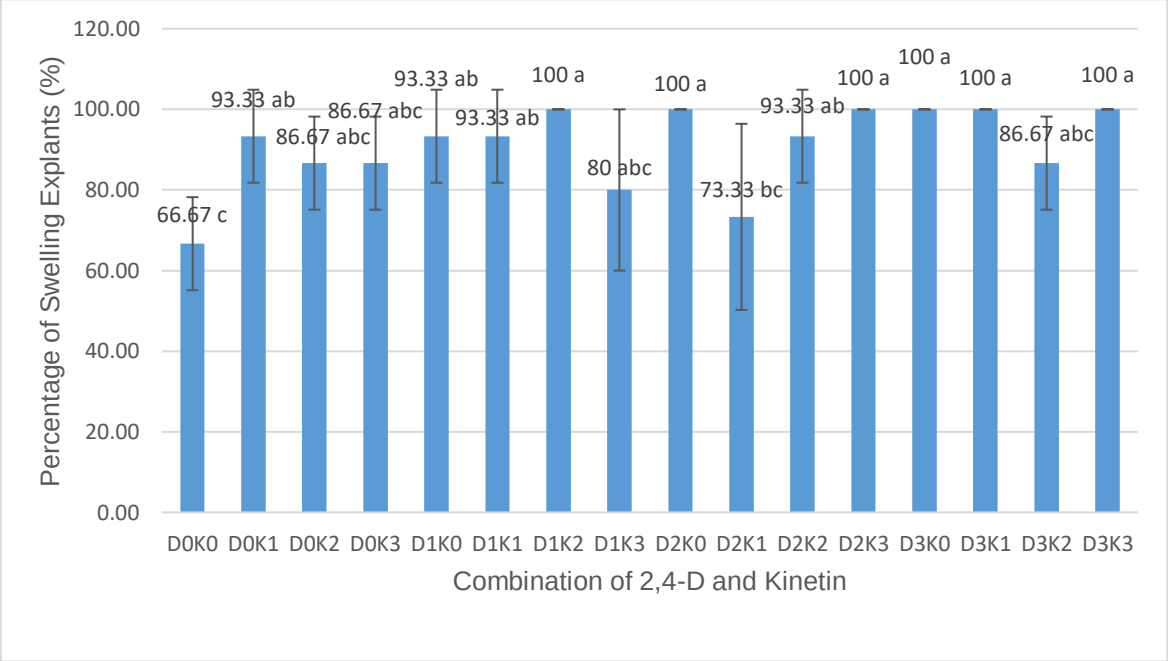

Figure 4. Percentage of swelling explants in each treatment

The treatment with the highest average swelling percentage was (D1K2), which reached 100%. This was also supported by the optimal results observed with each 2,4-D concentration increase. This indicated that 2,4-D played a significant role and strongly promoted cell enlargement in the explant. Therefore, its application, either alone or in combination with cytokinins, optimized the frequency of swelling responses. According to Henry & de Buyser (1990), 2,4-D was classified as an auxin hormone that was more efficient than NAA and IAA in producing high-quality embryos in anther culture, although it had lower regenerative potential. The application of 2,4-D at an appropriate or high concentration primarily induced cell swelling responses, but was not optimal for further regeneration processes. Therefore, interaction with the cytokinin Kinetin was required to optimize the role of 2,4-D in promoting both cell division and enlargement, as observed in the (D1K2) treatment.

The interaction between 2,4-D and Kinetin at appropriate concentrations further enhanced the frequency of swelling and encouraged faster callus proliferation. The interaction between auxin and cytokinin has been shown to stimulate cellular activity and increase callus formation, including the frequency of swelling responses, particularly under balanced concentration conditions (Nur Afiyah et al., 2022). These results suggest that the interaction between 2,4-D and Kinetin at optimal concentrations produced a synergistic effect on cell proliferation, including an increased frequency of swelling responses in explants. Auxin played a role in cell enlargement, while cytokinin stimulated regeneration through cell division. The interaction

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between the two can increase cells' ability to mount a swelling response more quickly and form more callus (Anggraeni et al., 2022). Meanwhile, the D0K0 treatment had the lowest average swelling percentage among all treatments, at 66,67%. However, the D0K0 treatment resulted in the lowest percentage among the treatments. This is because the growth and development of explants relied solely on endogenous hormones and nutrients from the culture medium, which were generally insufficient to increase cellular activity and to elicit optimal cell enlargement responses (Lubis & Harahap, 2024).

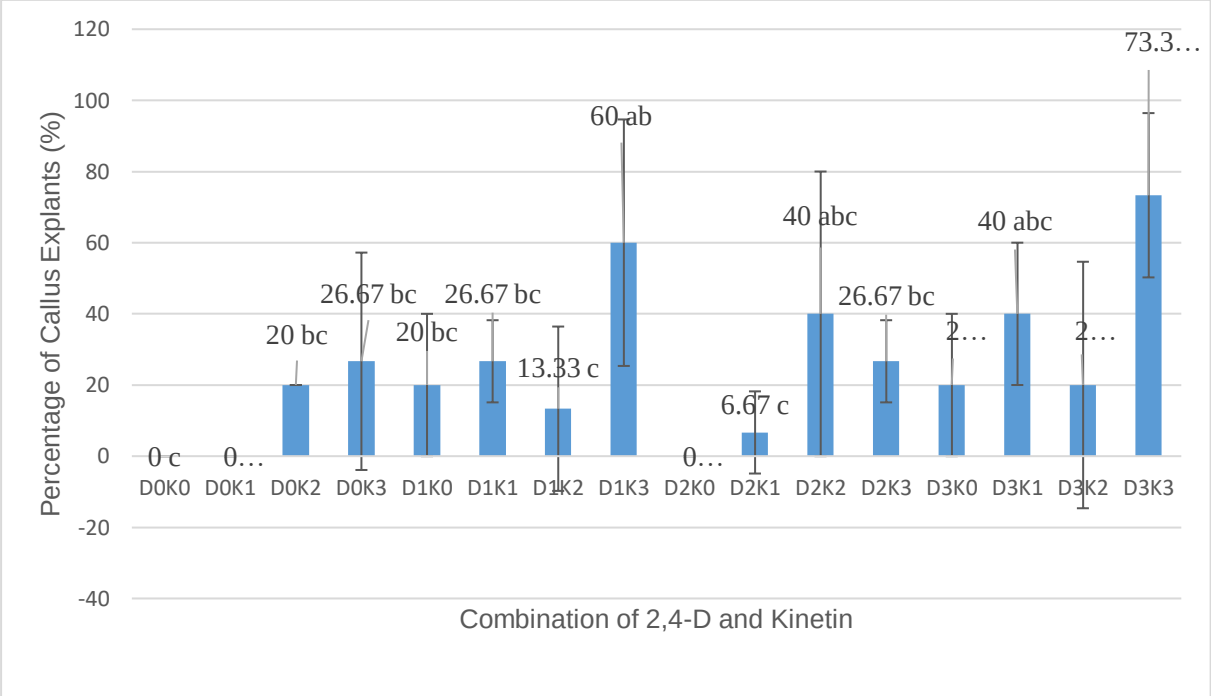

Figure 5. Percentage of callus explants in each treatment

As can be seen from Figure 5 above, the treatment with the highest percentage of callus formation was (D3K3) at 73,33%, which was significantly different than (D2K1) at 6,67% and (D1K2) at 13,33%. Similarly, the interaction between 2,4-D and Kinetin increased the average callus percentage. The treatment with the highest average callus percentage was D3K3, at 73,33%. This result may be attributed to 2,4-D's ability to promote cell differentiation. The activity of 2,4-D was considered potent due to its ability to activate auxin-responsive genes, which contributed to stable cell division (Karami et al., 2023). However, the single application of 2,4-D was not optimal in producing regenerative effects on explants. It thus required the addition of cytokinin substances such as Kinetin to complement and synergize its action, particularly in enhancing callus formation.

Kinetin functioned more as a stimulant to support auxin-induced cell division and callus regeneration (Tarigan et al., 2023). Additionally, the results of research by Mastuti et al. (2017), who reported that the interaction of 2,4-D and Kinetin produced more callus than other treatments, indicate a positive role for the two PGRs in increasing cell proliferation, particularly in callus formation. Although the present study successfully identified an effective combination of 2,4-D and Kinetin for tobacco anther regeneration, several limitations warrant consideration. The experiment was conducted with a single tobacco cultivar and focused primarily on morphological responses, without molecular confirmation of

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doubled-haploid formation. Therefore, additional physiological and molecular investigations are required to verify the genetic stability and regeneration mechanisms underlying the observed responses.

3.3 The Effect of 2,4-D and Kinetin on Callus Colour and Texture

The callus colour parameter was visually analysed and matched with the colour in the Munsell Colour Chart for Plant Tissue book. The colour and texture variables of the callus were used to assess cellular activity during cell division, thereby enabling visual evaluation of the extent of callus growth (Putri et al., 2024). The difference in callus colour indicates the transformation occurring during callus development, suggesting that applying different hormone concentrations could also affect this process. According to Prashariska et al. (2021), different callus colours can indicate varying levels of growth and development. Additionally, callus texture also serves the same purpose as colour and is categorised into three indicators: friable, compact, and intermediate. Table 1 shows the results of the callus colour and texture analysis for each treatment.

Table 1. Callus colour and texture in each treatment of 2,4-D and kinetin concentration

Auxin/Cytokinin (mg/L)		Callus Colour	Callus Texture
2,4-D	Kinetin		
0	0	Yellow-Red	+
0	0,1	Yellow-Red	+
0	0,2	Yellow-Red	+
0	0,4	Yellow-Red	+
0,1	0	Yellow-Red	+
0,1	0,1	Yellow-Red	+
0,1	0,2	Yellow-Red	+
0,1	0,4	Yellow-Red	++
1	0	Yellow-Red	+
1	0,1	Yellow-Red	+
1	0,2	Yellow-Red	+
1	0,4	Yellow-Red	+
2	0	Yellow-Red	+
2	0,1	Yellow	+++
2	0,2	Yellow-Red	+
2	0,4	Yellow-Red	+++

Note: Callus Texture of Compact (+), Friable (++), Intermediate (+++)

Table 1 shows that the best treatment in this experiment, based on the callus colour variable, was the interaction of 2,4-D and Kinetin at the (D3K1) concentration. The D3K1 treatment produced the best-coloured callus, characterized by a bright yellowish-green colour. Nevertheless, it can be concluded that the callus had potential to be embryogenic, with optimal growth and developmental capacity influenced by the addition of Plant Growth Regulators (PGRs). Embryogenic callus is generally characterized by bright colours, such as white, green, or yellowish tones, which indicate high starch content and actively dividing cells, thereby facilitating optimal differentiation (Wulandari, 2022). Embryogenic callus exhibited high metabolic

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activity, particularly in terms of cell division, and had not yet experienced cellular senescence. The metabolic activity of embryogenic callus, especially about carbohydrates, was significantly higher than that of non-embryogenic callus, providing the energy necessary for further cell division (Caeiro et al., 2023). This makes the regeneration potential of embryogenic callus quite optimal compared with that of non-embryogenic callus. Conversely, most of the treatments produced callus with a yellow-red hue and slightly darker tones, which likely indicated the presence of phenolic compounds resulting from prolonged exposure to 2,4-D in the medium or from a high-concentration application. Brownish callus indicates oxidation of phenolic compounds, which was associated with cell senescence, leading to impaired cell division and growth stagnation. This occurred because browning of the callus in the presence of phenolic compounds can hinder the explant's nutrient absorption, thereby inhibiting callus growth and development (Khozin & Mona, 2025).

The application of Plant Growth Regulators (PGRs) also affected callus texture, resulting in friable, compact, or intermediate forms. The D1K3 treatment produced a friable texture in the callus, which could be attributed to PGRs, particularly auxins. A friable texture was induced by auxins such as NAA or 2,4-D, which increased the plasticity of the cell wall, allowing water to be absorbed more easily (Millenia et al., 2022). Friable-textured callus was considered for callus propagation because its cells can be easily separated into single cells. Friable-textured callus was easy to separate due to large intercellular spaces and the absence of lignification in the cell walls, thereby facilitating dissociation into single cells (Khozin et al., 2026). In contrast, compact-textured callus, which appeared across several treatments, was more difficult to separate. This was due to lignification, a process in which lignin compounds were synthesised and deposited into the callus cell wall, causing the callus to accumulate and harden (Teresia et al., 2024). Meanwhile, intermediate-textured callus, such as that observed in the D3K1 and D3K3 treatments, was also considered to have embryogenic potential, although they were not as friable as typical friable callus. According to Fahira et al. (2023), callus with intermediate to friable texture could exhibit embryogenic potential without the characteristic brown colour.

3.4 Scanning Electron Microscope (SEM) Analysis of Tobacco Anther

The SEM observations presented in Figure 6 were obtained from anthers prepared according to the protocol described in the Materials and Methods section. Scanning Electron Microscope (SEM) analysis generally aims to observe the surface structure of a sample. Each species has distinct visual characteristics and surface-structure morphology (Figure 6).

SEM can obtain high-resolution images by firing electrons at the sample surface. The SEM observations presented in Figure 6 were obtained from samples prepared according to the protocol described in the Materials and Methods section. This enables SEM testing to determine the surface structure in detail, starting from the epidermis and other components. As shown in Figure 6A, the visual condition of the tobacco anther was used as a sample for SEM analysis. Figures 6B, 6C, and 6D show the complete results of the SEM analysis, displaying the surface structure of the tobacco plant anther.

Based on the SEM results (Figure 6b) at 35x magnification, the anther as a whole is shown, with two lobes, each containing two pollen grains (microsporangia). In addition, several structural layers of the anther were visible, including the epidermis, septum (the dividing line between the lobes), released or broken pollen grains, and the cuticle. Generally, the anther structure consisted of multiple layers, including the epidermis, an endothecium, a middle layer, a tapetum, and vascular layers, each with a distinct role (Wilson et al., 2011). The outer layer has a

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tight, smooth epidermis. So it can be said that the anther has not yet experienced a significant or optimal developmental response. In line with Falcão et al. (2016), the anther epidermis of Solanum (Solanaceae) plants is generally thin, smooth, and composed of small, closely spaced cells. However, at 300x magnification, as shown in (Figure 6c), the surface texture of the tobacco anther exhibited a wavy structure, which may be associated with the developmental phase preceding pollen release. Dehiscence is the process by which pollen grains are released, involving a change in the anther's surface structure. Therefore, the surface texture morphology changes during this phase (Wilson et al., 2011).

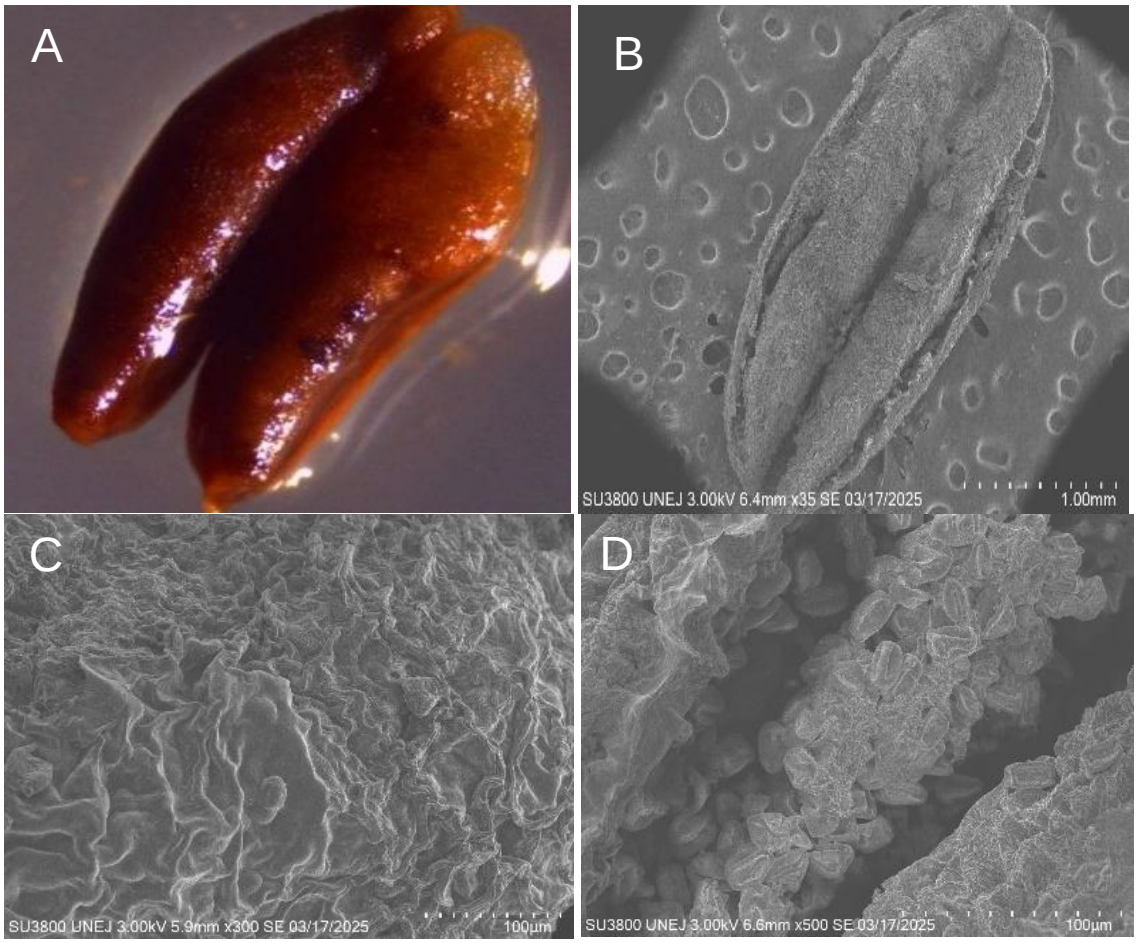

Figure 6. A. Visual condition of the anther before SEM analysis, B. Tobacco anther with two lobes at 35x magnification, C. Anther surface at 300x magnification, D. Surface pattern structure of the pollen wall (exine) at 500x magnification.

The dehiscence process could be influenced by Plant Growth Regulators (PGRs), particularly 2,4-D, which promotes the release of pollen or microspores from the anther wall. The auxin. Previous studies have reported that 2,4-D may enhance cellular activity involved in pollen release (Karami et al., 2023). Previous studies have proposed that cell wall-modifying enzymes, including cellulase and pectinase, contribute to anther wall loosening during dehiscence. However, enzymatic activity was not examined in the present study; therefore, this explanation should be regarded as a literature-based interpretation rather than direct experimental evidence (Bonner, Lynda J.; Dickinson, 1990). The surface morphology observed at higher magnification may reflect developmental changes associated with anther maturation. Previous studies have

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suggested that anther dehiscence is regulated by hormonal balance and cell wall-modifying enzymes; however, these processes were not directly evaluated in the present study (Szarejko, 2003; Wilson et al., 2011).

According to previous studies, dehydration of the anther wall is associated with structural changes during pollen release. Because physiological measurements were not performed in the present study, this mechanism is discussed only as background information from the literature (Bonner & Dickinson, 1990; Wilson et al., 2011). This determines the anther opening area and shrinks the epidermal layer, enabling pollen to be released easily and grow (Bonner, Lynda J.; Dickinson, 1990). Furthermore, Figure 6d showed an anther at 500x magnification, with an anther on one side that likely displayed the structural pattern of the tobacco pollen wall, such as elongated pores or particles. In general, the pollen walls (exine) of dicotyledonous plants, such as tobacco, are patterned with elongated lines, while those of monocotyledonous plants are reticulate (Zhang et al., 2011). The findings of the present study provide a basis for integrating optimized combinations of plant growth regulators with previously developed regeneration systems based on basal medium optimization and silver nanoparticle supplementation (Khozin et al., 2025; Khozin et al., 2026). Collectively, these studies indicate that successful tobacco anther regeneration depends on the combined optimization of culture medium composition, growth-promoting additives, and the balance of plant growth regulators. Such an integrated approach has considerable potential to improve regeneration efficiency and support the development of a more robust doubled-haploid production platform for tobacco breeding. Overall, the SEM observations in the present study provide morphological evidence of the structural characteristics of tobacco anthers. However, the physiological and molecular mechanisms underlying these structures were beyond the scope of this study and require further investigation.

4. Limitations and Future Directions

This study has several limitations that should be considered when interpreting the results. First, the experiment was conducted with a relatively small number of anther explants per treatment, which may have increased variability in regeneration responses. Increasing the number of explants in future studies would improve the statistical robustness and reliability of the results.

Second, this study evaluated only one tobacco cultivar (Deli Sumatra). Since androgenic response and in vitro regeneration capacity are highly genotype-dependent, the optimum concentrations of 2,4-D and Kinetin identified in this study may not be directly applicable to other tobacco cultivars. Therefore, future studies should validate the proposed regeneration protocol using diverse tobacco genotypes to determine its broader applicability.

Third, this study focused primarily on morphological responses, including swelling, callus formation, shoot emergence, and root formation, without molecular confirmation of the regenerated plantlets. Consequently, the ploidy status and genetic stability of the regenerated plants were not verified. Future studies should incorporate molecular and cytological approaches, such as flow cytometry, chromosome counting, molecular marker analysis (SSR or SNP), gene expression analysis, and endogenous hormone profiling, to confirm doubled-haploid formation and better understand the mechanisms regulating tobacco anther regeneration.

Furthermore, only the interaction between 2,4-D and Kinetin was evaluated in this study. Future research should investigate additional combinations of auxins and cytokinins, including NAA, IAA, IBA, BA, TDZ, and zeatin, as well as their interaction with different basal media, carbon sources, and growth-promoting compounds such as silver nanoparticles or polyamines, to further improve regeneration efficiency and reproducibility.

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Finally, the regeneration protocol developed in this study was evaluated only under controlled laboratory conditions. Before practical application, the protocol should be validated across multiple breeding populations and under commercial tobacco breeding programs to assess its scalability, reproducibility, genetic stability, and effectiveness for routine doubled haploid production.

5. Conclusion

The present study demonstrated that the interaction between 0.1 mg/L 2,4-D and 0.4 mg/L Kinetin (D1K3) produced the most favorable regeneration response among the treatments evaluated, as indicated by faster swelling initiation, earlier callus formation, and improved regeneration of tobacco anther explants. In addition, the single application of 0.4 mg/L Kinetin was associated with earlier shoot and root emergence, suggesting that Kinetin may contribute to the organogenesis phase of tobacco anther regeneration under the conditions evaluated in this study. These findings provide an optimized combination of plant growth regulators for tobacco anther culture in the Deli Sumatra cultivar and offer a useful basis for improving regeneration protocols. Future studies involving molecular validation, additional combinations of plant growth regulators, and evaluation across different tobacco genotypes and breeding conditions are required before broader application. Overall, this study provides a practical foundation for optimizing tobacco anther regeneration through plant growth regulator manipulation and represents an important step toward the development of efficient doubled haploid production systems for tobacco breeding.

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Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this manuscript, the author(s) used ChatGPT as an AI-assisted tool to support language editing, paraphrasing, and improving the clarity of the manuscript. After using this tool, the author(s) carefully reviewed, revised, and validated all content and took full responsibility for the accuracy and integrity of the final manuscript.

Authorship Contribution Statement

Mohammad Nur Khozin: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization; Imam Aditya Agung Mulyono: Methodology, Investigation, Data curation, Writing – review & editing; Didik Pudji Restanto: Supervision, Validation, Formal analysis, Writing – review & editing; Aran Patarapuwadol: Methodology, Validation, Formal analysis, Writing – review & editing; Iryono: Resources, Investigation, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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