

STERILIZATION AND INDUCTION CALLUS OF KLUWIH TEAK (*Tectona grandis* f. *abludens*) USING IN VITRO CULTURE

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Received: 27 June 2025, Revised: 5 July 2025, Accepted: 20 March 2026

STERILIZATION AND INDUCTION CALLUS OF KLUWIH TEAK (*Tectona grandis* f. *abludens*) USING IN VITRO CULTURE. *Tectona grandis* f. *abludens* or kluwih teak is a variety of teak endemic to Java, especially found in Desa Selang Gunung Kidul and Desa Loh Putih, Kecamatan Dlingo Bantul -Yogyakarta, where currently only six trees are found. Due to very low population size of this teak variety, efforts to conserve them are indispensable. The action conservation was achieved through *in vitro* propagation methods (embryogenesis). However, the main problems with *in vitro* techniques are known especially with explant sterilization and callus induction. This study aimed to analyze the response of mercury chloride (HgCl₂) immersion time to explant sterilization and analyze the response of a combination of 2,4-D Plant Growth Regulators (PGR) and kinetin to embryogenic callus formation. The data analysis method used a Completely Randomised Design (CRD) for explants sterilization consisting of 300 mg/L HgCl₂ treatment in 0; 15; 20; 25 and 30 minutes and Complete Randomised Factorial Design (CFRD) consisting of 2,4-D: 0; 1.5; 3 mg/L and kinetin: 0; 0.5; 1; 1.5 mg/L for callus induction. The results of the explant sterilization study showed that immersion treatment of 300 mg /L HgCl₂ for 15 minutes resulted in the highest sterile explants of 90% at 15 day after planting (DAP). The results of callus induction research showed that 0 ppm 2,4-D treatment combined with 1.5 mg/L kinetin resulted in the highest callus percentage of 73.33% and the highest callus formation score was 1.53 at 12 WAP (week after planting).

Keywords: *Tectona grandis* f. *abludens*, kluwih teak. *in vitro*, sterilization, embryogenic callus induction

STERILISASI DAN INDUKSI KALUS JATI KLUWIH (*Tectona grandis* f. *abludens*) PADA KULTUR IN VITRO. *Tectona grandis* f. *abludens* atau jati kluwih merupakan forma jati yang unik serta endemik Jawa khususnya di Gunung Kidul dan Bantul Daerah Istimewa Yogyakarta yang hanya ditemukan enam individu pohon. Kendala utama regenerasi alami forma tersebut diduga karena memiliki hambatan fisik dan fisiologis. Aksi konservasi nyata agar forma tersebut tidak punah dapat dilakukan melalui pendekatan propagasi *in vitro* (embriogenesis) yang diharapkan menjadi salah satu solusi. Permasalahan utama dalam riset propagasi *in vitro* adalah sterilisasi eksplan dan induksi kalus. Riset ini bertujuan menganalisis respon waktu perendaman merkuri klorida (HgCl₂) terhadap sterilisasi eksplan dan menganalisis respon kombinasi Zat Pengatur Tumbuh (ZPT) 2,4-D dan kinetin terhadap pembentukan kalus embriogenik. Metode analisis data yang digunakan adalah Rancangan Acak Lengkap (RAL) untuk sterilisasi eksplan yang terdiri atas perlakuan HgCl₂ 300 mg/L dalam waktu 0; 15; 20; 25 dan 30 menit serta Rancangan Acak Lengkap Faktorial (RALF) yang terdiri atas 2,4-D: 0; 1,5; 3 mg/L dan kinetin: 0; 0,5; 1; 1,5 mg/L untuk induksi kalus. Hasil penelitian sterilisasi eksplan menunjukkan perlakuan perendaman 300 mg/l HgCl₂ selama 15 menit menghasilkan eksplan steril tertinggi 90% pada 15 hari setelah tanam (HST). Hasil penelitian induksi kalus menunjukkan perlakuan 0 ppm 2,4-D yang dikombinasikan 1,5 ppm kinetin menghasilkan persentase kalus tertinggi 73,33% dan skor perkembangan kalus tertinggi sebesar 1,53 pada 12 MST (Minggu Setelah Tanam).

Kata kunci: *Tectona grandis* f. *abludens*, jati kluwih, *in vitro*, sterilisasi, induksi kalus embriogenik

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I. INTRODUCTION

Teak (*Tectona* sp.) is a commercially important wood species widely employed in the transportation (Tripathi et al., 2016), building (Herzog et al., 2012), and furniture and craft sectors (Bastian & Rahadiyanti, 2023). In 2019, the teak wood sector in Indonesia had substantial growth of 8.35%, with an export value of US\$1.95 billion (Gunadi, 2021). Teak species are indigenous to Southeast and South Asia, particularly Java, with a limited distribution outside Java. Fauzi et al. (2021) recognized four species of teak globally: *Tectona grandis*, *T. hamiltoniana*, *T. philippinensis*, and *T. grandis* f. *abludens* (teak kluwih), the last being native to Indonesia. Fauzi et al. (2021) states that kluwih teak is indigenous to Gunung Kidul and Bantul (Yogyakarta). *Tectona grandis* f. *abludens*, locally known as "jati kluwih," is found primarily in the Dlingo, Bantul, and Selang areas of Gunungkidul, Yogyakarta. Jati kluwih can reach a height of 10–20 m, with a canopy that tends to branch heavily and has a rounded umbrella shape with wavy leaves resembling those of kluwih (*Artocarpus incise*) or breadfruit (*Artocarpus altilis*). Jati kluwih was first discovered and identified by Santi and Rujiman as one of four types of teak that differ from ordinary teak (*T. grandis*). These differences can be seen in leaf shape, tree cross-section, bark shape, petiole length, leaf edge shape, ovary size, and epidermis shape. The results of surveys and observations conducted by the author in 2020–2022 in Selang and Dlingo Villages, Yogyakarta, found only six individuals in community yards/gardens.

Insufficient understanding of kluwih teak's ecology, phenology, reproduction, and culture hinders its advancement and utilization. Particularly concerning reproduction, kluwih teak is believed to have challenges in seed and embryo production. Ex situ conservation and artificial propagation are essential strategies for sustaining its existence. Using in vitro methods to grow plants from callus (somatic embryogenesis) is a good way to quickly produce a lot of seedlings (Thorpe, 2007).

The primary challenge in *in vitro* propagation is the generation of sterile explants from mature trees utilized as explant sources. Research by Fauzan (2017) found that soaking *T. grandis* side shoot cultures in 300 mg/l HgCl₂ (mercury chloride) for 3 minutes was the best way to sterilize them, resulting in an 85% success rate in keeping them free from contamination. Furthermore, the efficacy of embryogenic callus induction is significantly affected by the combination of growth regulators, necessitating the optimization of the planting media formulation. Previous studies on somatic embryogenesis in annual woody plants were done by Supriyati et al. (2016), Faizy et al. (2019), Tripathi et al. (2021), Bisht et al. (2024), Liu et al. (2024), and the *Tectona* genus (*T. grandis*) by Arsyad et al. (2020) using auxin (2,4-D) and cytokinin (BAP, Kinetin) formed the basic groundwork for this research on growing kluwih teak. The in vitro method of embryogenesis commences with the callus phase. Callus comprises a conglomeration of undifferentiated cells (Hartmann et al., 2014). Auxin and cytokinin, as growth regulators, are employed to generate embryogenic callus. Auxin helps with callus growth, making chlorophyll, forming roots and shoots, and creating embryos, while cytokinin encourages cell division, the formation of organs, and the growth of shoots.

Research and development of the kluwih teak (*T. grandis* f. *abludens*) is currently still in its early stages, particularly in terms of in vitro propagation through tissue culture. Although numerous studies have been conducted on its close relative, *Tectona grandis*, specific research on the kluwih teak has never been reported, either on physiological mechanisms or the effects of plant growth regulators (PGRs) on cell and tissue development. The current research approach, somatic embryogenesis, is considered a promising strategy, as it is expected to produce large numbers of plants while maintaining their genetic purity. In addition, several studies on the controlled use of mercuric chloride as a sterilant have demonstrated its effectiveness and

safety for use. This study aimed to (i) analyze the effect of HgCl₂ immersion duration on the sterilization of teak kluwih explants and (ii) examine the interaction of growth regulators, namely auxin (2,4-D) and cytokinin (kinetin), on the induction of *T. grandis* f. *abludens* callus.

II. MATERIALS AND METHODS

A. Material

Young stem explants were obtained from duplicate mother plants constructed from grafting and planted in the greenhouse of the BRIN Chemistry and Bioprocess Laboratory, BJ. Habibie Science and Technology Area, Serpong, Tangerang Selatan in July 2022 to produce many adventitious shoots. The *T. grandis* f. *abludens* scion was obtained from the Manggala Wanabakti collection of the Ministry of Environment and Forestry, Jakarta, planted in 1981, located at latitude and longitude $-6^{\circ}12'25.56''$ S, $106^{\circ}47'58.56''$ E ($\pm -6.2071, 106.7996$), elevation $\pm 16-17$ m asl, with an average annual rainfall of 2100–2150 mm per year.

Creating duplicate mother plants began with scion preparation by pruning plagiotropic branches with pruning shears and allowing them to grow for two months. The scion is then grafted onto a three-month-old teak rootstock and grown in a plastic incubator within a greenhouse at a temperature of 27–43°C, with humidity exceeding 70%, and a light intensity of 2000 – 6000 lux. The stem sections from the new shoots that grew after one month were then used as explants for sterilization in the laboratory.

B. Experiment 1: Sterilization

The sterilization experiment is categorized into two steps: outside of the LAF (Laminar Air Flow) and inside the LAF. The outside of the LAF is prepared by excising the explant hairs stem using a scalpel. The explants were subsequently sectioned, cleansed with detergent, and thoroughly rinsed. Sterilization within the LAF starts with washing the explants three times with sterile water. The explants were

subsequently immersed in HgCl₂ (MERCK) at a concentration of 300 mg/L for a specified duration 0, 15, 20, 25, 30 minutes. The explants were subsequently cleaned with sterile water three times, submerged in a 3% clorox solution for five minutes, and then rinsed again with sterile water three times. The explants were subsequently immersed in a 3% clorox solution for 2 minutes, washed three times with sterile water, and then dried using sterile tissue paper. The initiation and planting of sterilized explants were conducted on Murashige and Skoog media (MS0).

C. Experiment 2: Embryogenic callus induction

The explants utilized were sterile specimens from the explant sterilization experiment, aged three weeks post-initiation. The explants were subsequently subcultured in the designated callus induction medium. The planting was done in a clean airflow cabinet, and then the subcultures were kept in a controlled environment at 25°C, with 76% humidity, and in the dark.

Designing experiments of sterilization used a Completely Random Experimental Design (CRD) to test how long to soak the explants in HgCl₂, with times of 0, 15, 20, 25, and 30 minutes. Each treatment was conducted 27 to 30 times, contingent upon the availability of explants. Each replicate was grown in an individual test tube utilizing MS0 medium. Designing experiments Induction of embryogenic callus was a Completely Factorial Randomized Experimental Design (CFRD). Factor 1 is the concentration of auxin (2,4-D), including three levels: 0, 1.5, and 3 ppm. Factor 2 is the amount of cytokinin (kinetin) used, which has four levels: 0, 0.5, 1, and 1.5 ppm. This creates 12 different treatment combinations, and each combination is tested three times, with each test involving 5 experimental units.

D. Data Collection

d. 1 Exp. 1: Sterilization of explants. Observations were conducted every three days during a period of fifteen days. The variables

monitored included the quantity of explants infected with fungus, bacteria, instances of browning, and the number of sterile explants.

d.2 Exp. 2: Induction of embryogenic callus. We conducted observations weekly for a duration of 12 weeks. The recorded parameters included the quantity of explants survive, number of callus, callus development, color, and a cellular examination of the callus. At the end of the examination, we conducted observations using a VHX 970 F digital microscope with a magnification range of 100-200X.

E. Analysis

Observation data were analyzed using Microsoft Excel to ascertain the proportion of explants contaminated with fungus, contaminated with bacteria, exhibiting browning, remaining sterile, as well as the percentages of viable and callus explants. The parameter of each treatment was analyzed using the analysis of variance (ANOVA) test using SPSS 27, followed by the Duncan Multiple Range Test (DMRT) at $\alpha=0.01$. The examination of callus development characteristics employed the Kruskal-Wallis test, followed by the Dunn post hoc test (Bonferroni-Dunn test) at $\alpha=0.01$. Data analysis was accomplished using the following formulas (Fauzan et al., 2017).

1. Explants sterilization

The percentage of fungal contamination (%) =

$$\frac{\text{the number of contaminated fungi}}{\text{The number of planted}} \times 100\% \dots\dots\dots(1)$$

The percentage of bacterial contamination (%) =

$$\frac{\text{The number of bacteria contaminated}}{\text{The number of planted}} \times 100\% \dots\dots\dots(2)$$

The percentage of browning (%) =

$$\frac{\text{The number of browning}}{\text{The number of planted}} \times 100\% \dots\dots\dots(3)$$

The percentage of steril (%) =

$$\frac{\text{The number of browning}}{\text{The number of planted}} \times 100\% \dots\dots\dots(4)$$

2. Induction of embryogenic calli

The percentage of survive (%) =

$$\frac{\text{The number of survive}}{\text{The number of planted}} \times 100\% \dots\dots\dots(5)$$

The percentage of callus (%) =

$$\frac{\text{The number of callus}}{\text{The number of planted}} \times 100\% \dots\dots\dots(6)$$

3. Callus development

The assessment of callus development (callus structure) is conducted using scoring, as adapted from Karomah (1998):

- 0 The callus has not formed or the explant is dead
- 1 Callus structure like sponge/cotton
- 2 A compact callus structure is formed
- 3 A compact nodular callus structure is formed
- 4 A freeable callus structure is formed (easily separable and grainy like sand)
- 5 A nodular, freeable callus structure is formed

III. RESULTS AND DISCUSSION

A. Sterilization of Explant

The ANOVA test (Table 1) indicated that none of the treatments significantly affected the percentage of explants infected with fungus or exhibiting browning. All treatments showed a minimal incidence of fungal contamination. Explants under the H0 T0 treatment exhibited fungal presence at 3 days after planting (DAP), whereas the H300 T15 treatment had fungal indications after 6 DAP. Initial symptoms included the appearance of white hyphae (fine threads) on the explants.

Likewise, the browning remains minimal across all treatments. The H0 T0 and H300 T15 treatments exhibited a browning percentage of 3.33%, the H300 T20 treatment displayed a browning percentage of 14.07%, the H300 T25 treatment recorded a browning percentage of 3.70%, and the H300 T30 treatment showed a browning percentage of 0.00%.

Explants exhibiting browning (Figure 1C) are distinguished by the emergence of a brown color on the explants, which subsequently causes the medium to likewise turn brown over time. Prolonged sterilization with HgCl_2 will adversely impact the explants due to cellular damage (Farooq et al., 2001)

Table 1 indicates that all the treatments exerted a highly significant influence on both the parameter of explants infected with bacteria and the percentage of sterile. Bacterial contamination is characterized by the presence of white or yellow mucus (Figure 1 B). The prolonged soaking period in HgCl_2 resulted in a larger percentage of bacterially infected explants and a reduced number of sterile explants.

The DMRT findings in Table 2 for the H300 T15 treatment indicated optimal outcomes, with bacterial explants at 3.33% and sterile explants at 90.00%. The treatment exhibited no significant difference compared to the control without HgCl_2 , although it was considerably different from the treatments H300 T20, H300 T25, and H300 T30. The H300 T15 treatment

achieved the highest sterilization rate and the lowest contamination rate. This result indicates that immersion in HgCl_2 for 15 minutes decreases the bacterial percentage and enhances the sterile percentage relative to the absence of HgCl_2 immersion. In contrast to the treatment of H300 T20, H300 T25, and H300 T30, there is a propensity for prolonged soaking to elevate the proportion of contaminated bacteria. The soaking of explants facilitates the ingress of pollutants into them. During soaking, explants may sustain injuries from cutting, which can serve as entry points for pollutants.

Some study uses HgCl_2 as a sterilant agent because it is associated with several mechanisms in killing bacteria and fungi. According to dos Santos (2018), Hg^{2+} ions can disrupt glutamate transport, resulting in excessive production of ROS (O^{2-} and H_2O^{2-}). Heavy metals such as Hg^{2+} ions can inhibit DNA polymerase and RNA polymerase enzymes, resulting in disruption of DNA replication and gene transcription (Ren et al., 2024). Due to the effects mentioned above, HgCl_2 can be used as a surface sterilization

Table 1. Summarizes the findings of the ANOVA from the sterilization experiment

Parameters	HgCl_2
Percentage of explants contaminated with fungi	0.2500 ^{ns}
Percentage of explants contaminated with bacteria	0.0001 ^{**}
Percentage of explants browning	0.0800 ^{ns}
Percentage of sterile explants	0.0001 ^{**}

Note: ns = not significant, * = significant, ** = very significant

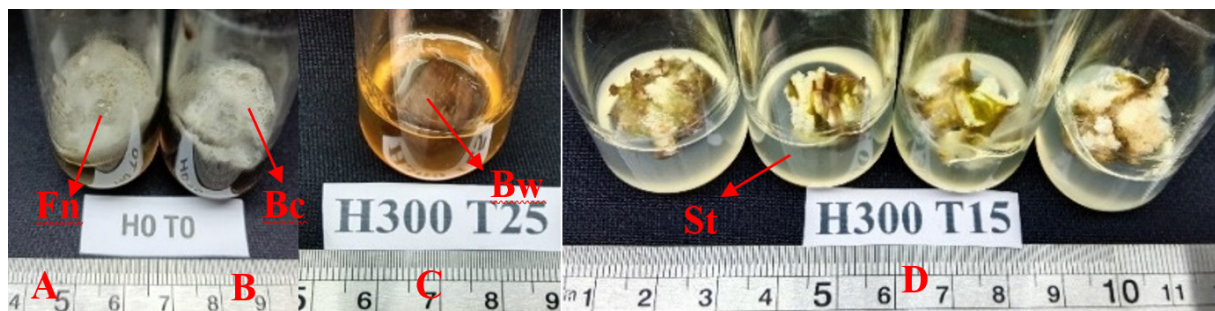


Figure 1. Fungal (A), bacterial (B), browning (C), and sterile (D) explants. Notes: Fn: fungal, Bc: bacterial, Bw: browning, St: sterile

Table 2. DMRT results of the percentage of bacterial contaminated explants and sterile explants

HgCl ₂ immersion time (minutes)	Bacterial contamination (%)	Fungus Contamination (%)	Browning (%)	Sterile (%)
0 minutes (H0 T0)	16.67 ^b	3.57 ^a	4.28 ^a	73.33 ^a
15 minutes (H300 T15)	3.33 ^b	6.07 ^a	2.50 ^a	90.00 ^a
20 minutes (H300 T20)	85.93 ^a	0.00 ^a	14.28 ^a	0.00 ^c
25 minutes (H300 T25)	77.78 ^a	0.00 ^a	3.57 ^a	18.52 ^{bc}
30 minutes (H300 T30)	65.00 ^a	0.00 ^a	0.00 ^a	35.00 ^b

Notes: Numbers in the same column, followed by the same letter, indicate that they are not significantly different at the 1% test level (DMRT).

agent for plant explants. In addition, HgCl₂ can also cause explant death, so controlling the dose and optimal exposure time is important.

Both internal and environmental variables influence the success rate of sterilization. The internal factors that influence sterilization include the type of plant, the part of the plant used, and the size of the explant. A significant of explants infected with bacteria is believed to result from the presence of fine stem hairs (trichomes) on the explant source. Explant components with trichomes, when utilized as a source of explants, may serve as a habitat for bacteria. Consequently, trichomes on the explants must be excised and sterilized (Yuliani et al., 2018). Furthermore, trichomes serve as a physical barrier, impeding the penetration of sterilant into the explant tissue.

The explant portion also influences the success rate of sterilization. Hardarani dan Nisa (2022) assert that node explants have a higher level of contamination compared to leaf explants. The results of a study by Webster et al. (2003) also stated that using leaf explants resulted in lower levels of fungal and bacterial contamination compared to shoot explants. Furthermore, the findings of Hand et al. (2016) also showed that the position of nodes on the explant significantly influenced the occurrence of bacterial and fungal contamination and the ability to grow. In node explants, the sterilant solution fails to penetrate the folds of the book,

allowing pollutants in these areas to persist. Therefore, initial treatment is necessary before sterilization in the laboratory. Nugraheni et al. (2019) used the fungicide Pyraclostrobin 50 g.kg⁻¹ + Metiram 550 g.kg⁻¹ by spraying it on the explants every week for three months, which was proven to be effective in increasing the success of sterilization of sengon shoots. Conversely, in leaf explants, there exists optimal surface contact between the sterilant and the explant. The size of the explant also influences the outcomes achieved. Explants that are excessively tiny lead to the rapid demise of meristem tissue, while those that are overly large possess an increased surface area that may harbor more contaminants.

Another important factor is that there was no preparation before sterilizing the explants, such as growing *T. grandis* f. *abludens* in a greenhouse and continuously using bactericides and fungicides on it. The sterilization process does not involve any previous treatment. This is in accordance with the statement of Yu and Reed (1995) and Hand (2016) that using plants grown in greenhouses aims to reduce contaminants. The degree of worker precision correlates with the efficacy of the sterilization procedures conducted. Employees with elevated precision will enhance the efficacy of sterilization. Additional affecting elements include the utilization of instruments and a sterile atmosphere (Onwubiko et al., 2013).

B. Callus Induction

1. Percentage of survive

The ANOVA findings for the percentage of living cells and callus formation during the callus induction of *T. grandis* f. *abludens* at week twelve are displayed in Table 3. The treatment of D0K0 and D0K1 exhibits a percentage of life that is not substantially different from D0K1.5; however, the treatment of D0K0.5 demonstrates a considerable difference when compared to the other three treatments. The survival rate in the D0K0.5 treatment was 8.33%. This is attributable to bacterial contamination. Bacterial contamination in

the D0K0.5 treatment was identified two weeks post-planting, and by four weeks, the majority of the explants perished. The bacterial contaminants in the D0K0.5 The treatment are presumed to be internal pollutants. Internal pollutants manifest during a timeframe of a few days to one month post-planting. Internal pollutants cannot be eliminated with surface sterilization, as this contamination resides within the plant tissue (Lestari, 2011). The signs of internal contamination resulting from the D0K0.5 treatment included the emergence of yellowish-white mucus exudates on the explant pieces, leading to chemosis as seen in Figure 2.

Table 3. Recapitulation of ANOVA of callus induction of *T. grandis* f. *abludens*

Dependent of Variable: Percentage of survive					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	64475.000 ^a	12	5372,917	38,493	0,000 ^{**}
Auksin (2,4-D)	34234,722	2	17117,361	122,632	0,000 ^{**}
Sitokinin (Kinetin)	4374,306	3	1458,102	10,446	0,000 ^{**}
Auksin * Sitokinin (2,4-D + Kinetin)	8748,611	6	1458,102	10,446	0,000 ^{**}
Error	3350,000	24	139,583		
Total	67825,000	36			
a. R Squared = .951 (Adjusted R Squared = .926)					
Dependent Variable: Percentage of callus					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	19675.000 ^a	12	1639,583	7,949	0,000 ^{**}
Auksin (2,4-D)	6612,500	2	3306,250	16,030	0,000 ^{**}
Sitokinin (Kinetin)	3252,083	3	1084,028	5,256	0,006 ^{ns}
Auksin * Sitokinin (2,4-D + Kinetin)	6504,167	6	1084,028	5,256	0,001 ^{**}
Error	4950,000	24	206,250		
Total	24625,000	36			
a. R Squared = .799 (Adjusted R Squared = .698)					

Notes: ^{ns} = not significant, * = significant, ** = very significant

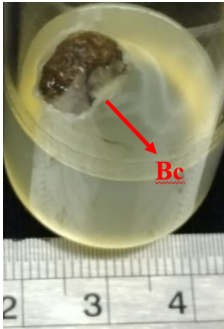


Figure 2. D0K0.5 treatment affected by bacteria at 2 weeks after planting (WAP). Bc : Bacterial

Table 4. DMRT outcomes regarding the impact of the combined treatment of 2,4-D and kinetin on the percentage of survive of *T. grandis* f. *abludens*

Tretaments	2,4-D (ppm)	Kinetin (ppm)	Survive (%) (Sterile)
D0K0	0	0	86.67 a
D0K0.5	0	0.5	8.33 b
D0K1	0	1	86.67 a
D0K1.5	0	1.5	80.00 a
D1.5K0	1.5	0	0.00 b
D1.5K0.5	1.5	0.5	0.00 b
D1.5K1	1.5	1	0.00 b
D1.5K1.5	1.5	1.5	0.00 b
D3K0	3	0	0.00 b
D3K0.5	3	0.5	0.00 b
D3K1	3	1	0.00 b
D3K1.5	3	1.5	0.00 b

Notes: Numbers in the same column, followed by the same letter, indicate that they are not significantly different at the 1% DMRT level

The DMRT in Table 4 shows that using 2,4-D at 0 ppm with kinetin at 0-1.5 ppm significantly increases the number of healthy explants compared to other treatments. The incorporation of 2,4-D concentrations ranging from 1.5 to 3 ppm, in conjunction with kinetin at 0 to 1.5 ppm, appears to diminish the proportion of viable explants. The balance of auxin and cytokinin concentrations greatly influences the ability of explants to maintain and sustain life and grow well. These two hormones regulate metabolism, cell division, and the response to oxidative stress. Auxin plays a crucial role

in maintaining tissue viability by regulating the polar flow of hormones and enhancing the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) (Montalvo et al., 2022).. Furthermore, optimal auxin-cytokinin concentrations are also known to modulate epigenetic pathways and ROS metabolism, thereby protecting cells under active metabolic conditions without inducing oxidative stress, and thus contributing to the maintenance of explant vitality in vitro (Zhang et al., 2025).

A combination of 2,4-D at 0 ppm with kinetin between 0 and 1.5 ppm results in a high survival rate, likely because the natural auxin in the explant tissue is strong enough, and adding kinetin helps cells divide. According to the research conducted by Illahi et al. (2022), the incorporation of 2,4-D at a concentration of 6 mg/l results in a 12% increase in the browning of explants, whereas a concentration of 4 mg/l leads to a 4% increase in browning in callus from *Diospyros discolor* leaves. Akkenapally (2024) also found that in *Madhuca longifolia* var. *latifolia* (Roxb.) plants, using more than 2 mg/L of 2,4-D led to less callus growth. The elevated concentration of 2,4-D in the medium induces toxicity in the explants, resulting in mortality.

The treatment of D1.5K0; D1.5K0.5; D1.5K1; D3K0; D3K0.5; and D3K1.5 is believed to cause mortality, as indicated by explants that fail to grow and develop and display browning, due to excessive application of the ZPT 2,4-D. Giving 2,4-D in high amounts creates harmful phenolic chemicals that can reduce the health of explants or cause browning, which might result in their death (Fitriani et al., 2019). Potent exogenous auxin 2,4-D poses a significant risk due to its capacity to trigger the production of ethylene gas (Waryastuti et al., 2017). Liu et al. (2024) found that the browning of callus from the woody plant *Juglans mandshurica* from China may be linked to the interaction between phenylalanine ammonia-lyase (PAL), polyphenols, and polyphenol oxidase (PPO). In this process, PAL helps produce polyphenols, which PPO then oxidizes to create brown quinones, instead of just increasing the number of polyphenols. In this process, PAL helps create polyphenols, which are then changed by PPO into brown quinones, instead of just increasing the number of polyphenols.

2. Percentage of Callus

The DMRT in Table 5 shows that using 2,4-D at 0 ppm with kinetin at 0.5 - 1.5 ppm increases the amount of callus explants. The best callus percentage happens with 2,4-D at 0 ppm and kinetin at 1.5 ppm, reaching 73.33%.

The findings of Arsyad et al. (2022) on *Tectona grandis*, which utilized 2 ppm thidiazuron to achieve 100% callus formation with a favorable structure, stand in contrast. Adding 2,4-D in amounts from 1.5 to 3 ppm along with kinetin at 0.5 to 1.5 ppm seems to reduce the number of survival explants. (Kim et al., 2025) found that using more than 3 mg/L of 2,4-D can reduce the success of callus production in coffee plants by up to 28.57%, while the best results come from using 0.5 mg/L of 2,4-D combined with 1 mg/L of kinetin, which gives a success rate of 86.84%. The best callus percentage happens when 2,4-D is at 0 ppm and kinetin is at 1.5 ppm, resulting in 73.33%. Adding 2,4-D in amounts between 1.5 to 3 ppm, along with kinetin at 0.5 to 1.5 ppm, seems to reduce the number of healthy explants. Explants of *T. grandis* f. *abludens* showed a reaction two weeks after being planted, which was marked by swelling around the edges of the damaged explants (Figure 3). This happens because the growth of the cells is influenced by hormones from within the plant and by kinetin added to the growth medium. Endogenous and exogenous auxin influence cell wall plasticity, leading to cell expansion and elongation. Auxin works by turning on specific proteins in the plant's outer layer, which help move H⁺ ions into the cell wall, causing the cells to grow and stretch (Taiz and Zeiger 2012). H⁺ ions trigger specific enzymes, which disrupt certain hydrogen bonds within the cellulose chain that forms the cell wall. As a result, water permeates the plant's cells via osmosis, leading to their elongation.

The D0K0 treatment (control) shows an average callus percentage of 0.00%, probably because there aren't enough natural hormones in the explant needed to encourage callus growth. Adding exogenous kinetin to the media can help activate the natural hormones in the cells, which encourages the growth of the explant tissue. The D0K0.5, D0K1, and D0K1.5 treatments can produce callus in kluwih teak without needing 2,4-D. The callus growth in these treatments has enough auxin for callus formation and only needs kinetin to

Table 5. Results of DMRT on the effect of the combination treatment of 2,4-D and kinetin on the percentage of callus in *T. grandis* f. *abludens*

Treatments	2,4-D (ppm)	Kinetin (ppm)	Callus (%)
D0K0	0	0	0.00 b
D0K0.5	0	0.5	8.33 b
D0K1	0	1	33.33 b
D0K1.5	0	1.5	73.33 a
D1.5K0	1.5	0	0.00 b
D1.5K0.5	15	0.5	0.00 b
D1.5K1	1.5	1	0.00 b
D1.5K1.5	1.5	1.5	0.00 b
D3K0	3	0	0.00 b
D3K0.5	3	0.5	0.00 b
D3K1	3	1	0.00 b
D3K1.5	3	1.5	0.00 b

Notes: Numbers in the same column, followed by the same letter, indicate that they are not significantly different at the 1% DMRT level

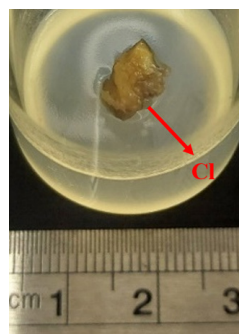


Figure 3. The first signs of the callus appearing. Cl : Callus

develop. The D0K1.5 treatment exhibited a substantial difference from all other treatments, achieving the greatest callus percentage of 73.33% on day 12 post-subculture. The notable rise in the proportion of explants producing callus in the D0K1.5 treatment is attributed to the compatibility of endogenous hormone concentrations with exogenous kinetin in promoting cell division in the leaf tissue of kluwih teak plants.

The aforementioned is distinct from the treatment. D1.5K0; D1.5K0.5; D1.5K1; D1.5K1.5; D3K0; D3K0.5; D3K1; D3K1.5. The inclusion of 2,4-D often does not induce explant callus development (0.00%) but results in the demise of all explants, evidenced by a color shift from green to brown. The brown

color is believed to result from an excessive concentration of 2,4-D. This aligns with the findings of (Wijaya et al., 2024), which indicate that providing 2,4-D at excessive concentrations suppresses callus development and induces toxicity in the explants, thereby diminishing their capacity to generate callus.

Physiologically, callus formation in plant explants is the result of the interaction between auxin and cytokinin in cells that regulate dedifferentiation and cell reprogramming so that they can live (totipotent). Auxin plays a role in inducing cell division through the activation of the AUX/IAA-ARF signal transduction pathway, which triggers the expression of division-related genes such as CYCD3 and WOX11, thereby restoring the

parenchymal cells to a young state (Montalvo et al., 2022). Cytokinin functions to regulate cell differentiation and morphogenesis by activating the CRE1/AHK–ARR-B type pathway, which increases the synthesis of nuclear division regulatory proteins and prevents the formation of lignin in the tissue (Liu et al., 2025). When the ratio of auxin and cytokinin is in a balanced state, a hormonal signal crosstalk occurs that stabilizes the expression of the LBD16 and BBM genes, thereby accelerating dedifferentiation and the formation of embryonic callus that is competent for organogenesis (Andrade et al., 2025)). Current research indicates that to maintain the stable pluripotent status of callus, a combination of auxin and cytokinin is administered to modulate ROS metabolism and epigenetic pathways, such as DNA methylation in regenerative gene promoters (Zhang et al., 2025). Therefore, it can be concluded that auxin and cytokinin, working together and synergistically, determine the direction of callus formation and play a crucial role in preparing cells for subsequent regeneration.

3. Callus development

The observed callus's development encompasses its structure, coloration, and cellular characteristics. The Kruskal-Wallis analysis (Table 6) indicates that all treatments exert a highly significant influence on the parameters of callus formation. The asymptotic significance value of 0.005, which is below 0.01, indicates this.

The results from the Dunn post hoc test (Table 7) show that treatment D0K1.5 is significantly different from treatment D0K0 (Control) and from treatments with 2,4-D at concentrations between 1.5 ppm and 3 ppm along with kinetin at 0 to 1.5 ppm. However, treatment D0K1.5 does not show a significant difference when compared to treatments D0K0.5 and D0K1. Still, treatment D0K1.5 has a better average callus score than treatments D0K0.5 and D0K1. However, treatment D0K1.5 does not demonstrate a significant difference when compared to treatments D0K0.5 and D0K1. Nonetheless, treatment D0K1.5 has a superior average callus score relative to treatments D0K0.5 and D0K1.

Table 6. Results of the Kruskal-Wallis test on the development of callus *T. grandis* f. *abludens*

Statistical test			
Kruskal-Wallis H			26.494
df			11
Asymp.Sig			0.005
Monte Carlo Sig	Sig		0.001 ^c
	99% Confidence Interval		
	Lower Bound		0.000
	Upper Bound		0.002

Table 6. RResults of Dunn's test on the development of calus *T. grandis* f. *abludens*

No.	Treatment	P-value	Result
1	D0K0-D1.5K0 (2,4-D 0 Kinetin 0 – 2,4-D 1.5 Kinetin 0)	1.00	Not significant
2	D0K0-D1.5K0.5 (2,4-D 0 Kinetin 0 – 2,4-D 1.5 Kinetin 0.5)	1.00	Not significant
3	D0K0-D1.5K1 (2,4-D 0 Kinetin 0 – 2,4-D 1.5 Kinetin 1)	1.00	Not significant
4	D0K0-D1.5K1.5 (2,4-D 0 Kinetin 0 – 2,4-D 1.5 Kinetin 1.5)	1.00	Not significant
5	D0K0-D3K0 (2,4-D 0 Kinetin 0 – 2,4-D 3 Kinetin 0)	1.00	Not significant
6	D0K0-D3K0.5 (2,4-D 0 Kinetin 0 – 2,4-D 3 Kinetin 0.5)	1.00	Not significant

No.	Treatment	P-value	Result
7	D0K0-D3K1 (2,4-D 0 Kinetin 0 – 2,4-D 3 Kinetin 1)	1.00	Not significant
8	D0K0-D3K1.5 (2,4-D 0 Kinetin 0 – 2,4-D 3 Kinetin 1.5)	1.00	Not significant
9	D0K0-D0K0.5 (2,4-D 0 Kinetin 0 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
10	D0K0-D0K1 (2,4-D 0 Kinetin 0 – 2,4-D 1 Kinetin 1)	0.037	Not significant
11	D0K0-D0K1.5 (2,4-D 0 Kinetin 0 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
12	D3K0.5-D3K1 (2,4-D 3 Kinetin 0.5 – 2,4-D 3 Kinetin 1)	1.000	Not significant
13	D3K0.5-D3K1.5 (2,4-D 3 Kinetin 0.5 – 2,4-D 3 Kinetin 1.5)	1.000	Not significant
14	D1.5K0-D0K0.5 (2,4-D 1.5 Kinetin 0 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
15	D1.5K0.5-D0K0.5 (2,4-D 1.5 Kinetin 0.5 – 2,4-D 0 Kinetin 0)	0.325	Not significant
16	D1.5K1-D0K0.5 (2,4-D 1.5 Kinetin 1 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
17	D1.5K1.5-D0K0.5 (2,4-D 1.5 Kinetin 1.5 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
18	D3K0-D0K0.5 (2,4-D 3 Kinetin 0 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
19	D3K0.5-D0K0.5 (2,4-D 3 Kinetin 0.5 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
20	D3K1-D0K0.5 (2,4-D 3 Kinetin 1 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
21	D3K1.5-D0K0.5 (2,4-D 3 Kinetin 1.5 – 2,4-D 0 Kinetin 0.5)	0.325	Not significant
22	D3K1-D3K1.5 (2,4-D 3 Kinetin 1 – 2,4-D 3 Kinetin 1.5)	1.000	Not significant
23	D1.5K0-D0K1 (2,4-D 1.5 Kinetin 0 – 2,4-D 0 Kinetin 1)	0.037	Not significant
24	D1.5K0.5-D0K1 (2,4-D 1.5 Kinetin 0.5 – 2,4-D 0 Kinetin 1)	0.037	Not significant
25	D1.5K1-D0K1 (2,4-D 1.5 Kinetin 0.5 – 2,4-D 0 Kinetin 1)	0.037	Not significant
26	D1.5K1.5-D0K1 (2,4-D 1.5 Kinetin 1.5 – 2,4-D 0 Kinetin 1)	0.037	Not significant
27	D3K0-D0K1 (2,4-D 3 Kinetin 0 – 2,4-D 0 Kinetin 1)	0.037	Not significant
28	D3K0.5-D0K1 (2,4-D 3 Kinetin 0.5 – 2,4-D 0 Kinetin 1)	0.037	Not significant
29	D3K1-D0K1 (2,4-D 3 Kinetin 1 – 2,4-D 0 Kinetin 1)	0.037	Not significant
30	D3K1.5-D0K1 (2,4-D 3 Kinetin 1.5 – 2,4-D 0 Kinetin 1)	0.037	Not significant
31	D1.5K0-D0K1.5 (2,4-D 1.5 Kinetin 0 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
32	D1.5K0.5-D0K1.5 (2,4-D 1.5 Kinetin 0.5 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
33	D1.5K1-D0K1.5 (2,4-D 1.5 Kinetin 1 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
34	D1.5K1.5-D0K1.5 (2,4-D 1.5 Kinetin 1.5 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
35	D3K0-D0K1.5 (2,4-D 3 Kinetin 0 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
36	D3K0.5-D0K1.5 (2,4-D 3 Kinetin 0.5 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
37	D3K1-D0K1.5 (2,4-D 3 Kinetin 1 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
38	D3K1.5-D0K1.5 (2,4-D 3 Kinetin 1.5 – 2,4-D 0 Kinetin 1.5)	0.001	Significant
39	D1.5K0-D1.5K0.5 (2,4-D 1.5 Kinetin 0 – 2,4-D 1.5 Kinetin 0.5)	1.00	Not significant
40	D1.5K0-D1.5K1 (2,4-D 1.5 Kinetin 0 – 2,4-D 1.5 Kinetin 1)	1.00	Not significant
41	D1.5K0-D1.5K1.5 (2,4-D 1.5 Kinetin 0 – 2,4-D 1.5 Kinetin 1.5)	1.00	Not significant
42	D1.5K0-D3K0 (2,4-D 1.5 Kinetin 0 – 2,4-D 3 Kinetin 0)	1.00	Not significant
43	D1.5K0-D3K0.5 (2,4-D 1.5 Kinetin 0 – 2,4-D 3 Kinetin 0.5)	1.00	Not significant
44	D1.5K0-D3K1 (2,4-D 1.5 Kinetin 0 – 2,4-D 3 Kinetin 1)	1.00	Not significant

No.	Treatment	P-value	Result
59	D1.5K1.5-D3K1 (2,4-D 1.5 Kinetin 1.5 – 2,4-D 3 Kinetin 1)	1.00	Not significant
60	D1.5K1.5-D3K1.5 (2,4-D 1.5 Kinetin 1.5 – 2,4-D 3 Kinetin 1.5)	1.00	Not significant
61	D3K0-D3K0.5 (2,4-D 3 Kinetin 0 – 2,4-D 3 Kinetin 0.5)	1.00	Not significant
62	D3K0-D3K1 (2,4-D 3 Kinetin 0 – 2,4-D 3 Kinetin 1)	1.00	Not significant
63	D3K0-D3K1.5 (2,4-D 3 Kinetin 0 – 2,4-D 3 Kinetin 1.5)	1.00	Not significant
64	D0K0.5-D0K1 (2,4-D 0 Kinetin 0.5 – 2,4-D 0 Kinetin 1)	0.269	Not significant
65	D0K0.5-D0K1.5 (2,4-D 0 Kinetin 0.5 – 2,4-D 0 Kinetin 1.5)	0.017	Not significant
66	D0K1-D0K1.5 (2,4-D 0 Kinetin 1 – 2,4-D 0 Kinetin 1.5)	0.199	Not significant

Table 8. Average callus developmen

Treatments 2,4-D (ppm)	Kinetin (ppm)				Average
	K0 = 0	K0.5 = 0.5	K1 = 1	K1.5 = 1.5	
D0 = 0	0.00	0.17	0.67	1.53	0.59
D1.5 = 1.5	0.00	0.00	0.00	0.00	0.00
D3 = 3	0.00	0.00	0.00	0.00	0.00
Average	0.00	0.06	0.23	0.51	

The mean values presented in Table 8 reflect the progression of callus (callus quality), demonstrating a range of values from 0 to 1, indicating that the callus development is predominantly necrotic and occurs in the sponge or cork phase. In the D0K0 treatment, callus development receives an average score of 0, indicating the absence of callus formation. In the D0K0 treatment, the majority of explants exhibited no growth or development, resulting in a standstill. This may result from the extremely low levels of endogenous hormones in the D0K0 treatment, which impede the growth activity necessary for callus formation.

In treatment D0K0.5, the callus formation has an average score of 0.17. The average, which is near zero, results from the significant mortality of explants in treatment (D0K0.5) caused by bacterial contamination. The bacterial contamination in treatment D0K0.5 is believed to have originated from the explant tissue itself. Treatment D0K1 exhibits callus formation with an average score of 0.67. The diminished

average in treatment D0K1 is attributable to the insufficient application of kinetin, a plant growth regulator, leading to inadequate callus formation.

In treatment D0K1.5, the callus development exhibits an average score of 1.53, with the callus morphology illustrated in Figure 4B. The callus seen in Figure 4B has a dense structure. Compact callus is characterized by the presence of nodular protrusions and cannot be separated into single cells because the small-sized cells are very close together and have a dense and rigid texture (Julianti et al., 2021). This type of callus is a type 2 primary callus consisting mainly of elongated parenchymal cells (101-165 µm), with large intercellular spaces, developing vacuoles, and cell walls about 1.9 mm thick between two adjacent cells and showing intense and irregular growth (Bartos et al., 2018). These callus characteristics are an early indicator of the potential for further growth in somatic embryogenesis. Callus texture can vary from compact to crumbly, depending on the type of

plant, the composition of the nutrient media, growth regulators, and the environmental conditions of the culture (Pierik, 1997). Crumbly callus is usually more widely used for tissue propagation (Sitorus et al., 2011). The majority of the callus in treatment D0K1.5 exhibits a compact structure (score 2) at 12 WAP; however, one explant displays a nodular compact callus morphology (Figure 5) at the same time point, potentially influenced by various factors, including the genotype of the explant and its individual responsiveness. Furthermore, Isah (2016) stated that embryogenic tissue induction depends on many factors, including the selection of appropriate explants (juvenile or mature), culture media, added plant growth regulators, other growth substances, and the physical environment.

The color of the callus serves as an indication of the cellular status, reflecting whether the cells are actively dividing or have

ceased proliferation (Afiah et al., 2022). The D0K0.5 treatment yielded a whitish-green callus. The presence of green callus signifies ongoing cellular division, suggesting that if it progresses to the regeneration stage, it can advance to further phases.

The D0K1 treatment, the majority of the calli exhibited a brown coloration at 12 WAP. The brown color was attributed to the media's stress, which suggested that the calli would have a deeper hue compared to fresh calli. The D0K1.5 treatment, the majority of the calli exhibited a yellow color at 12 WAP. The yellow hue signifies that the calli possess proficient division capability. Yellow calli indicate that there are flavonoid pigments in the cells of the callus (Qahtan et al., 2022). A little portion of the calli in the D0K1.5 treatment exhibited a white coloration. White calli signify that the embryonic tissue is devoid of chloroplasts, yet possesses a substantial starch content.

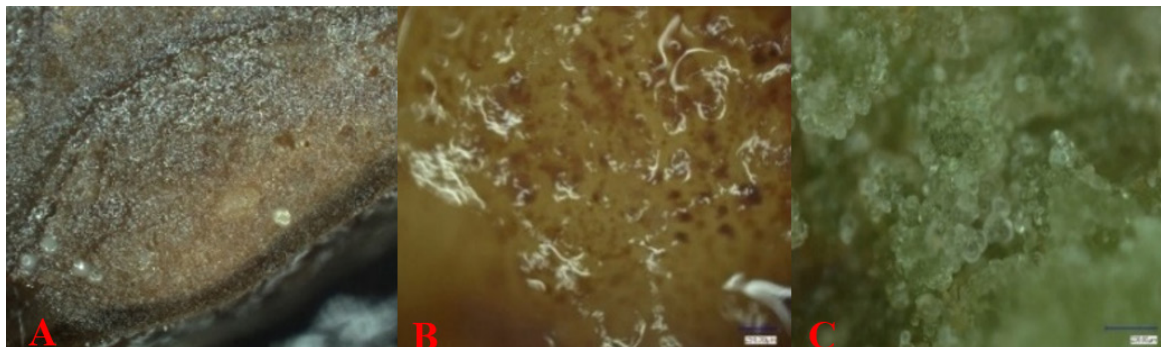


Figure 4. Callus shapes under the microscope; Score 0 (A), Score 2 (B), Score 3 (C)

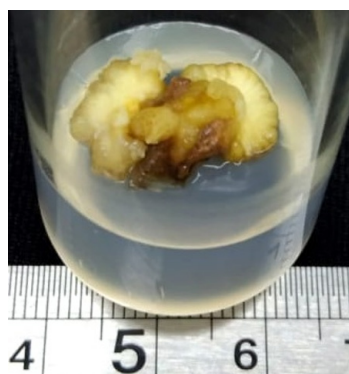


Figure 5. Score 3 (Compact nodular callus)

Stasolla et al. (2002) said that in some conifers, embryogenic tissue is made up of a group of small, clear cells full of cytoplasm that have underdeveloped filamentous embryos with well-formed suspensors and distinct embryos, while non-embryogenic tissue has brown or green parenchymal cells that lack any special structure. The earlier findings indicate that the callus is mainly in the growth stage, but a small amount of round callus with the ability to develop into embryos was also observed. Subsequent callus formation remains under ongoing observation. Further work on technological factors, including subculture in new mediums and the optimization of growth regulator use, is necessary.

Callus form substantially influenced somatic embryo development and callus proliferation, according to (Chang et al., 2025). A detailed study on the species *Quercus alina* showed that the combination of callus shape, genetic type, and growth medium together explained 60.9% of the differences observed. Specifically, the shape of the callus was the biggest factor, explaining 50.2% ($p < 0.001$) of the differences, indicating it had the strongest effect on how fast the callus grew. The genetic type also played a significant role, explaining 10.1% ($p < 0.001$), suggesting that differences in genetics within the species can greatly affect the growth process. Specifically, callus morphology was the primary component, accounting for 50.2% ($p < 0.001$) of the variation, hence demonstrating the most significant influence on the proliferation rate. Genotype had a significant effect on the differences observed, making up 10.1% ($p < 0.001$), which indicates that the genetic differences within the species can greatly affect how well it grows. In contrast to the other two variables, the impact of medium formulation was minimal, accounting for -0.1% ($p = 0.395$) of the variation (Chang et al., 2025).

IV. CONCLUSION

The H300 T15 treatment (300 ppm HgCl_2 for 15 minutes) is the best method for sterilizing young kluwih teak stems, producing

the highest number of sterile explants at 90% by 15 days after planting. The optimal concentration for callus induction was achieved with a combination of 0 ppm, 2,4-D, and 1.5 ppm, resulting in the greatest callus percentage of 73.33% and a callus development score of 1.53. Utilizing HgCl_2 at a concentration of 300 ppm for a duration of 15 minutes is preferable for the sterilization of immature kluwih teak stems. Preliminary treatment is required prior to the sterilization of explants, including their placement in a greenhouse and the application of bactericides and fungicides. Optimization of 2,4-D at a concentration of <1.5 ppm and kinetin > 1.5 ppm is essential for the induction of jati kluwih callus. More research is needed on the fungi and bacteria that affect kluwih teak to improve how well sterilizing materials work. The results of this research represent an initial step that has the potential to significantly contribute to future conservation and vegetative propagation strategies for kluwih teak. Scientifically, this technology helps develop efficient in vitro culture methods and is a basis for studies on the physiology and molecular basis for somatic embryo formation. These findings are also helpful for plant breeding programs using biotechnological approaches, such as genetic transformation or somaclonal selection, to produce superior genotypes. They can be implemented in mass seed propagation, thus supporting efforts to develop national genetic resources.

ACKNOWLEDGEMENT

We would like to thank the RIIM (Riset Inovasi Indonesia Maju) Batch VI LPDP program SK. Deputi Bidang Fasilitas Riset dan Inovasi No. 40/II.7/HK/2024 and BRIN (Badan Riset dan Inovasi Nasional) that has supported all of this research.

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