Callus Induction of Javanese Endemic *Tectona grandis* f. *abludens*

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Abstract: Callus induction in kluwih teak wood represents a crucial stage of indirect plant propagation through somatic embryogenesis. This study aims to examine the effect of a combination of 2,4-D and kinetin on callus induction from three propagule types: leaf, petiole, and stem derived from in vitro culture. A completely randomized factorial design was applied with two factors: propagule type and medium formulation. The medium consisted of 2,4-D and kinetin combinations at concentrations of 0.00, 0.50, 1.00, 1.50, and 2.00 mg/L. Variables observed included propagules survival, percentage of callus formation, initiation time, proportion of propagules forming callus, texture, color, and fresh weight of callus. Significant interactions between explant type and medium formulation were observed for the percentage of callus formation, initiation time, and callus color. In contrast, the survival rate, proportion of callus-forming explants, and callus fresh weight were influenced by single factors. Leaf explants cultured in a medium containing 0.5 mg/L 2,4-D plus 0.5 mg/L kinetin achieved the highest callus formation rate and the fastest initiation, producing friable callus with a yellowish-white appearance, so that the combination was identified as the most effective treatment for in vitro callus induction of kluwih teak wood.

Keywords: Callus color; Callus texture; Initiation time; The percentage of callus formation

Introduction

Tectona grandis f. *abludens* (locally known as "jati kluwih" or "kluwih teak") is a Javanese endemic germplasm with a very limited presence. It is endemic to Java, primarily found in the Special Region of Yogyakarta in Jati Mulyo Village (Bantul) and Selang Village (Gunungkidul), and is not found in natural forests (Fauzi et al., 2021). Generative propagation of teak kluwih is hampered by suspected physical and physiological barriers that cause problems with flowering and fruiting of the mother plant. This puts the species at high risk of extinction, necessitating ex-situ conservation efforts, one of which is through tissue culture, which can provide a sustainable source of planting material. One potentially effective approach is the application of tissue culture techniques. This technique is carried out aseptically in a closed, transparent container so that plant parts can reproduce and regenerate into complete plants (Sakinah et al., 2024). The callus induction stage is a crucial initial step, determining the success of subsequent regeneration stages.

Callus induction is the process of forming meristematic tissue that can further develop through organogenesis or somatic embryogenesis. Callus formation is influenced by various factors, particularly the type of propagule and the composition of plant growth regulators (PGRs) in the culture medium (Iriani et al., 2025; Setiawati et al., 2019). Combinations of auxin and cytokinin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, are known to be effective in stimulating callus formation (Khoiriyah et al., 2023). For example, research by Afifah (2024) reported that a concentration of 0.50 mg/L of 2,4-D and 0.50 mg/L of

kinetin was the optimal combination for callus induction in parica plants. However, the response to this PGR combination can vary between plants, necessitating specific testing in teak kluwih.

Research on callus induction in kluwih teak is still very limited, particularly in evaluating the response of various propagule types—leaves, petioles, and stems—to the combination of 2,4-D and kinetin. No studies have specifically compared the performance of these three propagules in initiating and forming callus in this species. This information gap hampers the development of tissue culture protocols necessary to support the conservation and propagation of teak kluwih. Therefore, this study aimed to generate baseline data on the initial morphological response of kluwih teak to plant growth regulator (PGR) treatments as a first step in formulating a tissue culture protocol.

This study specifically focused on the callus induction stage without microscopic embryogenic characterization, so the results obtained provide an initial overview of the callus formation capacity of various propagules. Therefore, this study plays a crucial role in providing a scientific basis for ex situ conservation efforts and the development of kluwih teak propagation methods through tissue culture approaches. This study aimed to analyze the effect of a combination 2,4-D and kinetin on callus formation in stem, petiole, and leaf cultures of kluwih teak.

Method

Location and Time

This research was conducted from February 2025 to June 2025. The laboratories used were the Biotechnology Laboratory, Applied Botany Research Center, BRIN, BJ Habibie Research Institute, Serpong, South Tangerang.

Experimental Design

A completely randomized factorial design (CRDF) was used with two factors. The first factor was the type of propagules, consisting of three levels: stem, petiole, and leaf. The second factor was the combination of 2,4-D and kinetin, consisting of five levels: no PGR (control) 2,4-D 0.50 mg/L + kinetin 0.50 mg/L; 2,4-D 1.00 mg/L + kinetin 1.00 mg/L; 2,4-D 1.50 mg/L + kinetin 1.50 mg/L; and 2,4-D 2.00 mg/L + kinetin 2.00 mg/L, resulting in 15 treatment combinations. Each treatment combination was replicated three times. Each replicate consisted of three culture bottles, and each bottle contained three propagules. In this experiment, the culture bottle served as the experimental unit, because it was the smallest unit that independently received treatment. The propagules inside each bottle were treated as observation units (subsamples) rather than independent experimental units. In total, the experiment consisted of 135 experimental units (135 bottles) and 405 observation units (405 propagules). All statistical analyses were conducted using the bottle as the experimental unit to avoid pseudoreplication.

Work Procedures Callus Induction

The propagules (stems, petioles, and leaves) were obtained from a 14-month-old in vitro culture previously maintained under sterile conditions, thus no additional surface sterilization was required. All subsequent handling and subculturing were conducted aseptically inside a Laminar Air Flow (LAF) cabinet. Propagules were transferred to ½ MS (Murashige & Skoog, 1962) medium according to each treatment. All cultures were incubated in a controlled room at a temperature of 24–27 °C, relative humidity of 50–60%, and under dark conditions for 8 weeks. Observations were carried out twice a week for 8 weeks after planting (WAP). Observed variables included the percentage of surviving propagules, the percentage of callus formation, callus initiation time, callus color, proportion of the propagul that forms a callus, callus texture, and fresh weight of callus.

Research Variables
Percentage of Survival Propagules

The percentage of survival propagules was calculated using the formula (Ardyansah et al., 2024):

$$\text{Survival propagules (\%)} = \frac{\text{number of survival propagules}}{\text{number of propagules planted}} \times 100 \quad (1)$$

Percentage of Callus Propagules

The percentage of propagules with callus was calculated using the formula (Saripah et al., 2024):

$$\text{Callus propagules (\%)} = \frac{\text{number of callus propagules}}{\text{number of propagules planted}} \times 100\% \quad (2)$$

Callus Initiation Time

Callus initiation time was observed twice a week; from the first time the propagules were planted until callus appeared. Identification was performed visually by recording the time of callus initiation.

Callus Color

Callus color was observed at the end of the observation period, with a score assigned based on the color of the callus. The callus color score reflects the visual appearance of the callus cells, allowing for the identification of the level of cell division activity. The higher the callus score, the better the callus. Callus color was observed using a scoring system based on Hidayat et al. (2022) with a modified score. A score of 7 = white, a score of 6 = yellowish white, a score of 5 = greenish

white, a score of 4 = green, a score of 3 = brownish yellow, and a score of 2 = blackish brown.

Proportion of the Propagul that Forms a Callus

The proportion of the propagules that form callus was determined based on the extent of the propagul's surface area covered by callus. Assessment was conducted visually using a scoring system based on Sekar et al. (2023) with a modified score. Observations of the proportion of propagules that formed callus can be distinguished as listed in Table 1.

Table 1. Scoring Values of the Proportion of Propagules that from Callus

Propagul proportion which shaping callus (%)	Score	Information
0-25	1	Very few propagul parts are overgrown with callus
26-50	2	A small part of the propagul is covered with callus
51-75	3	Quite a lot of propagul parts are covered with callus
76-100	4	Propagules are covered with callus

Callus Texture

Callus texture was classified into three types: friable, intermediate, and compact. Friable callus texture has large intercellular spaces and loose intercellular bonds, making it easy to separate (Wahyuni et al., 2020). Intermediate callus texture is a callus mass consisting of groups of cells that are partly compact and partly friable (Rasud et al., 2020). Compact callus texture has a dense and hard structure with tight intercellular bonds, making it difficult to separate (Tarigan et al., 2023).

Fresh Weight of Callus

The callus was measured by weighing it using a scale. The fresh weight of the callus (mg) was calculated by weighing the callus at the end of the observation.

Data Analysis

Data analysis was conducted using two methods: qualitative and quantitative. The data obtained from the observation result were analyzed using the Analysis of Variance (ANOVA) or the Scheirer-Ray-Hare Test if the assumptions of the ANOVA were not met. Data analysis used the IBM Statistics SPSS 27 and RStudio version 4.5.1 programs to determine whether there was an effect of the treatment on the observation variables. If significant differences between treatments were detected, they were analyzed using the Duncan Multiple Range Test (DMRT) or Dunn's post hoc test with Bonferroni correction at a 5% significance level.

Result and Discussion

Percentage of Survival Propagules

Based on the results of statistical tests, the type of propagules and media formulation each had a significant influence as a single factor on the percentage of viable propagules 8 weeks after sowing. However, the interaction between the two factors did not have a significant influence. Viable propagules were characterized by fresh, dry, and browning propagules.
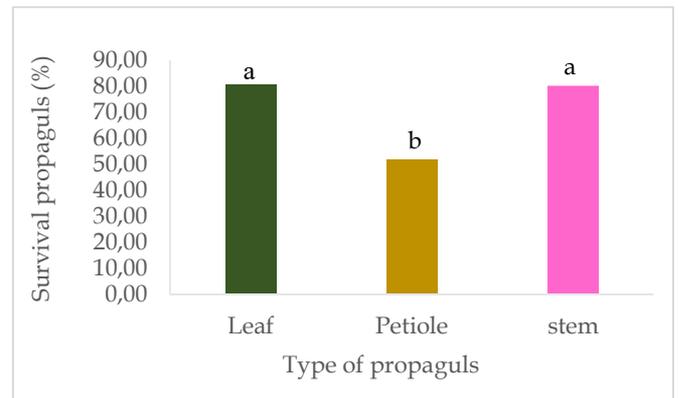

Figure 1. Percentage survival of kluwih teak propagules planted in ½ MS culture media at 8 weeks on various types of propagules
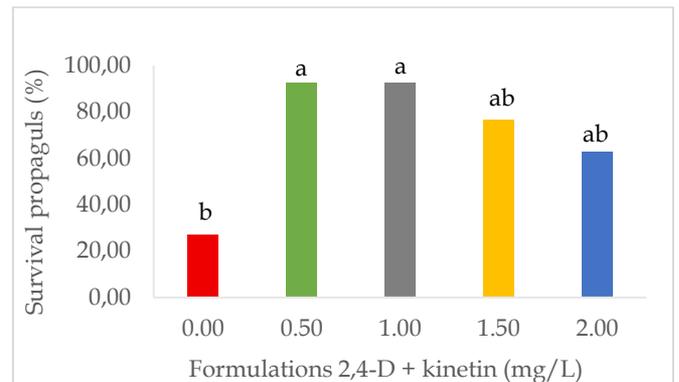

Figure 2. Percentage survival of kluwih teak propagules planted in ½ MS culture media at 8 weeks in various media formulations

The use of leaf and stem propagules both resulted in a high percentage of survival propagules at 8 weeks after planting (WAP), while more periole propagules died (Figure 1). The low percentage of survival propagules in petiole propagules was largely due to browning. Browning causes propagul death due to the accumulation of phenolic compounds produced in response to injury or stress, producing brown pigments that can damage propagul tissue and inhibit growth (Hany et al., 2023). The lower survival rate of petiole

propagules in this study is likely due to the softer and thinner structure of the petiole tissue. This makes cell damage more likely during cutting, triggering the release of phenolic compounds and increasing the risk of browning (Zhao et al., 2021). The selection of propagules, including their developmental stage, tissue type, and physiological condition, plays a critical role in browning (Liu et al., 2024).

A high percentage of survival propagules was obtained in the PGRs 0.50 mg/L + kinetin 0.50 mg/L and PGRs 2,4-D 1.00 mg/L + kinetin 1.00 mg/L media formulations (Figure 2). This indicates that the 2,4-D + kinetin media formulation at levels of 0.50 mg/L to 1.00 mg/L supports the growth and survival of kluwih teak propagules in tissue culture. This is in accordance with reports that the combination of auxin and cytokinin can stimulate callus proliferation and growth (Puspitasari & Habibah, 2021). The percentage of survival propagules of kluwih teak decreased when the 2,4-D + kinetin media formulation was increased. This indicates that the use of 2,4-D + kinetin media formulation at certain concentrations can damage tissue and cause cell death, as a result of oxidation of phenolic compounds and physiological stress at given concentrations (Guo &

Jeong, 2021). The low percentage of survival propagules in media without PGR is due to the absence of growth stimulation, so that the propagules remain inactive and then experience stress, browning, and death. In addition, propagules may have low amounts of endogenous hormones so that cells do not divide and die gradually. Propagule cutting causes wounds that trigger oxidative stress; the role of PGR is to help suppress browning reactions or increase cell resistance (Putri et al., 2023).

Percentage of Callus Formation

Based on the results of statistical tests, the interaction between the type of propagules and media formulation has a significant effect on the percentage of callus formation in kluwih teak. Leaf propagules with 2,4-D 0,50 mg/L + kinetin 0.50 mg/L kinetin medium formulation performed better than other treatments in terms of callus formation percentage. The callus og kluwih teak formed on leaf propagules with a concentration of 2,4-D 0.50 mg/L + kinetin 0.50 mg/L was 100%, while no callus was produced on leaf stalk propagules from culture media without media formulation (Figure 3).
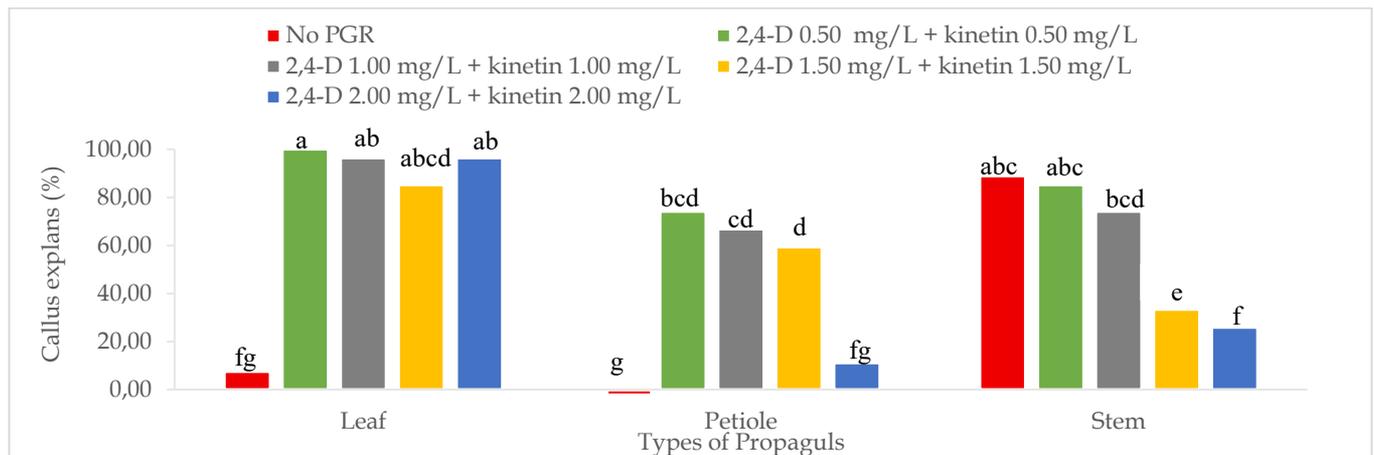

Figure 3. Percentage of callus formation produced from kluwih teak propagules planted in ½ MS culture media with 2,4-D and kinetin treatment at 8 weeks

Kluwih teak leaf petiole propagules grown in ½ MS media without PGR formulation (control) did not form callus, and leaf propagules without PGR (control) largely did not form callus. This condition occurs because media without exogenous PGR are unable to stimulate callus formation. This condition shows that physiologically, the content of endogenous auxin and cytokinin in the propagul is very low. Callus formation is triggered when auxin and cytokinin are in the right ratio and balance. Various studies have shown that media without PGR often fail to induce callus induction. For example, in *Coffea arabica* leaf cultures, no callus formation was observed in PGR-free medium (Irene et

al., 2019). The balance between auxin and cytokinin is crucial in directing the fate of cultured cells. Auxin promotes callus induction and embryogenic potential, while cytokinin regulates cell division and differentiation (Arli & Noli, 2024; Kumar et al., 2025). In kluwih teak stem propagules without media formulation (control), the percentage of callus formation reached 88.9%, presumably due to higher endogenous auxin and cytokinin content compared to leaves and petioles. Furthermore, callus growth accompanied by root formation was also observed in leaf propagules without media (Figure 4).

Figure 4. Kluwih teak propagules experienced callus growth accompanied by rooty callus growth (= 2 mm)

Callus Initiation Time

Based on the results of statistical tests, the interaction between the type of propagules and media formulation has a significant effect on the callus initiation time in kluwih teak. Leaf propagules with a 2,4-D 0.50 mg/L + kinetin 0.50 mg/l medium

formulation produced the fastest callus formation at 20 days after planting, while the slowest callus formation occurred in stem propagules with 2,4-D 2.00 mg/L + kinetin 2.00 mg/L medium formulation at 47 days after planting (Figure 5).

Callus initiation time is influenced by several factors. These factors include the plant part used, the concentration, and the type of PGR used. The rate of callus formation is determined by the effectiveness of the PGR administered and the endogenous PGR present in the propagul (Rasud & Bustaman, 2020). Callus formation begins with swelling in the propagul, indicating cell activity (Merthaningsih et al., 2018). This swelling occurs due to the effect of 2,4-D, which stimulates cell division and multiplication due to the absorption of water, nutrients, and PGRs from the media (Dwipayana et al., 2016). The administration of kinetin can increase cell proliferation (Noli et al., 2024).
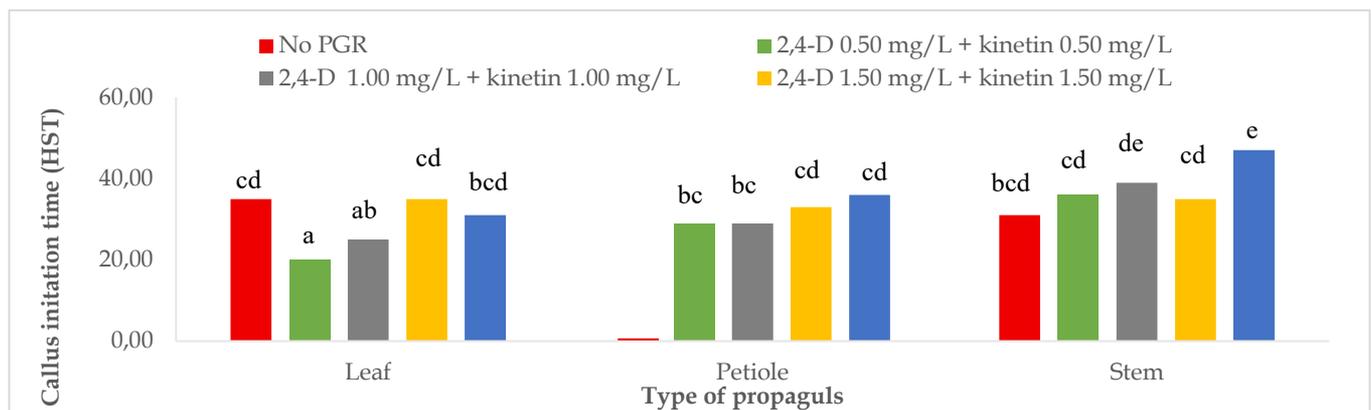

Figure 5. The interaction effect of propagul type and 2.4 + kinetin media formulation on callus initiation time

The results showed that callus initiation time in leaf propagules was faster than in petiole and stem propagules. This is because leaves are thin, allowing their cells to more easily absorb nutrients from the medium. These absorbed nutrients and energy are then used by the cells to grow and divide, forming callus (Wahyuni et al., 2020). The results of this study indicate that propagules derived from stems require longer callus formation times than leaf propagules. Research by (Verma et al., 2016) also showed that in some *Crocus species*, leaf propagules initiated callus formation faster than stem propagules under the culture conditions tested.

Proportion of Propagules Forming Callus

Based on the results of statistical tests, only the single factor of 2,4-D + kinetin media formulation had a significant effect on the proportion of propagules that formed callus. In contrast, the propagule type factor and the interaction between propagule type and media formulation did not have a significant effect. The PGRs

medium formulation of 2,4-D 0.50 mg/L + kinetin 0.50 mg/L had the highest score for the proportion of propagules forming callus, with a score of 3.16, followed by the medium with 2,4-D 1.00 mg/L + kinetin 1 mg/L, with a score of 3.14. A higher score indicates a greater proportion of propagules forming callus, making these two treatments superior to the other treatments. Meanwhile, the control treatment without PGR addition showed the lowest score 0.67, which was significantly different from the other treatments (Figure 6).

The results showed that 1 2,4-D + kinetin media formulation of 0.50 mg/L – 1.00 mg/L could increase the proportion of propagules that formed callus in kluwih teak. This is thought to be because the combination can stimulate optimal cell division and growth. Furthermore, this concentration is within a range that does not cause physiological stress in the propagules, thus supporting more even callus growth. A high proportion of propagules that formed callus indicates that a large proportion of the propagul surface area was able to respond well to hormonal treatment. Conversely,

when treatment with media formulations that are too low or too high, the proportion of propagules that formed callus tends to decrease. Media formulations that are too low do not provide sufficient hormonal stimulus to trigger dedifferentiation and cell division, while concentrations that are too high can cause phenolic oxidation and metabolic disorders, thus inhibiting callus formation (Dar et al., 2021).
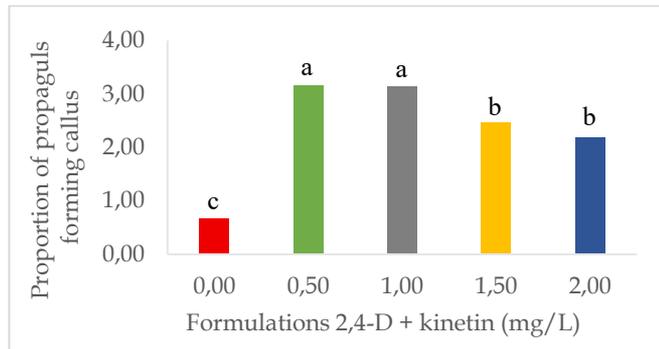

Figure 6. Proportion of propagules that formed callus of kluwih teak planted at various concentrations of PGR 2,4-D + kinetin used at 8 weeks

Callus Color

Based on the results of statistical tests, the interaction between the type of propagules and media formulation has a significant effect on the callus color in kluwih teak. Leaf propagules formulated with 2,4-D 0.50 mg/L + kinetin 0.50 mg/L showed the highest callus color score of 6. This score was followed by stem propagules without PGR addition, with a score of 5.83. A higher callus color score reflects better callus color quality, so the 2,4-D 0.50 mg/L + kinetin 0.50 mg/L medium produces the most optimal callus color in leaf propagules and the medium without PGR produces the most optimal callus color in stem propagules (Figure 7).

Callus color is an important indicator of whether its constituent cells are still actively dividing or have decreased activity (Afiyah et al., 2022). Callus color variations are influenced by the type and concentration of growth regulators added to the culture medium. In this study, the most common color of callus was brownish-yellow, indicating a decrease in tissue quality and physiological stress in the callus (Hariyati et al., 2016; Kuswandi & Husna, 2023). Meanwhile, white to yellowish-white callus was obtained in the treatment of 0.50 mg/L 2,4-D + 0.50 mg/L kinetin medium for leaf propagules and in the media without PGR for stem propagules. The white color of the callus indicates young cells that are actively dividing, whereas the yellowish-white color indicates that mature cells are entering the active division phase (Royani et al., 2015).
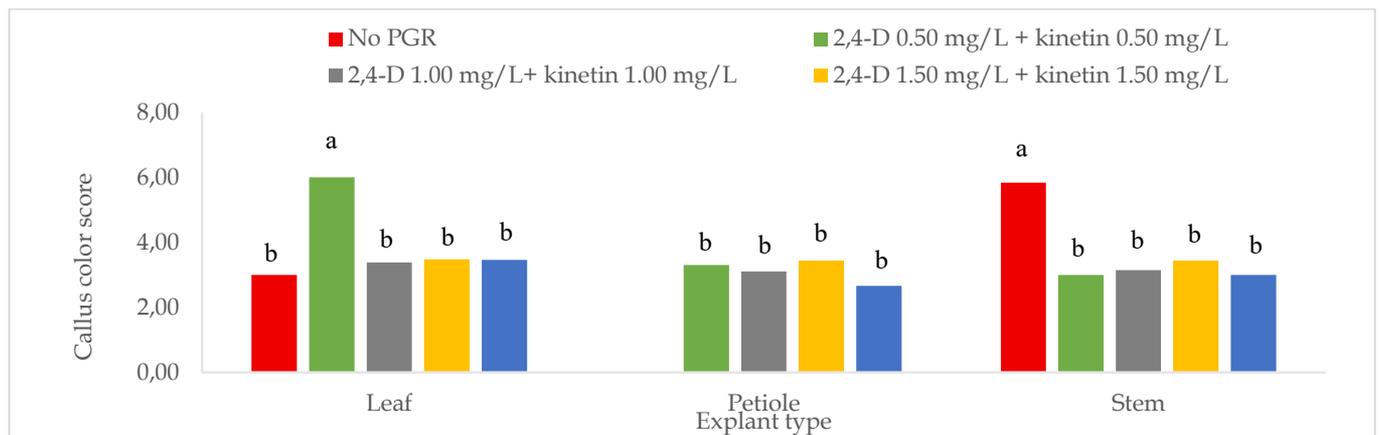

Figure 7. Callus color score produced from kluwih teak propagules planted in ½ MS culture media with 2,4-D and kinetin treatment at 8 weeks

Callus Texture

Observations after 8 weeks showed that the 2,4-D 1 mg/L + kientin 1 mg/L media formulation for leaf, petiole, and stem propagules was dominated by a friable texture. Furthermore, the friable texture was also dominant in leaf propagules with the 2,4-D 0.50 mg/L + kinetin 0.50 mg/L media formulation (Table 2).

The callus texture obtained from leaf propagul treatment with the addition of 2,4-D 0.50 mg/L + kinetin 0.50 mg/L, and treatment with the addition of 2,4-D 1.00 mg/L + kinetin 1.00 mg/L from both leaf propagules, leaf petioles, and stems was dominated by a friable texture (Figure 8). The friable texture is characterized by a loose and easily disintegrated structure and has many intercellular spaces. The friable texture has the potential to be developed into plantlets as a good callus quality because the callus can be separated into individual cells (Yanti & Wardana, 2023). Friable callus generally differentiates more easily and develops into somatic embryos. Meanwhile, compact callus is dense and difficult to separate, and has a higher potential for organogenesis, such as forming roots or shoots (Toharah et al., 2017).

Table 2. Texture of Kluwih Teak Callus Formed on Various Types of Propagules 8 Weeks

Types of propagules	Formulations media 2,4-D and kinetin (mg/L)	Compact (%)	Intermediate (%)	Friable (%)
Leaf	0.00	100.00	0.00	0.00
Leaf	0.50	7.41	44.44	48.15
Leaf	1.00	23.08	23.08	53.85
Leaf	1.50	100.00	0.00	0.00
Leaf	2.00	100.00	0.00	0.00
Petiole	0.50	60.00	5.00	35.00
Petiole	1.00	11.11	0.00	88.89
Petiole	1.50	100.00	0.00	0.00
Petiole	2.00	100.00	0.00	0.00
Stem	0.00	100.00	0.00	0.00
Stem	0.50	73.91	4.35	21.74
Stem	1.00	35.00	15.00	50.00
Stem	1.50	55.56	11.11	33.33
Stem	2.00	71.43	0.00	28.57

Figure 8. Callus from leaf propagules (A,B), leaf stalks (C,D), and kluwih teak stems (E,F) with compact callus texture (A,C,E) and crumbly callus texture (B,D,F) (— = 2 mm)

Fresh Weight of Callus

Based on the results of statistical tests, the type of propagules and media formulation each had a significant influence as a single factor on the fresh weight of callus at 8 weeks after sowing. However, the interaction between the two factors did not have a significant influence. The fresh weight of callus from leaf propagules was higher than that from leaf petiole and stem propagules (Figure 9). Tissue sensitivity to auxin differs between plant parts (Amanda et al., 2024; Kaňuková et al., 2024). This difference in sensitivity also explains the higher fresh weight of callus in leaf propagules compared to other organ parts.

The media formulation without PGR produced the lowest fresh weight of callus of 10 mg. The highest fresh weight of callus was obtained in the media formulation of PGRs 2,4-D 0.50 mg/L + kinetin 0.50 mg/L, which was significantly different from the media formulation

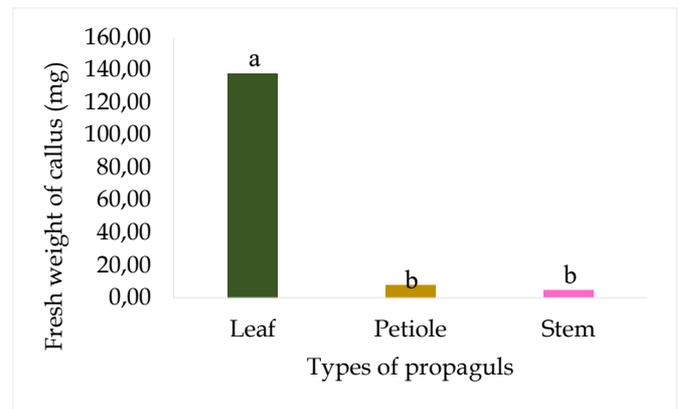without PGR and the media formulation of 1.50 to 2.00 mg/L (Figure 10).

Figure 9. Fresh weight of kluwih teak callus planted in ½ MS culture media at 8 weeks on various types of propagules
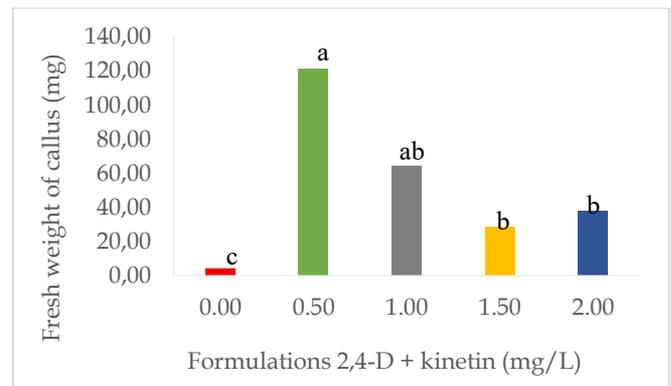

Figure 10. Fresh weight of kluwih teak callus planted in ½ MS culture media at 8 weeks in various media formulations

Physiologically, the fresh weight of callus consists of water and carbohydrate content. According to Ulva et al. (2019), callus experiences an increase in fresh weight due to an increase in cell number and the entry of water from the media into the cells, resulting in cell enlargement. Water enters the cells because the cells' permeability to water increases, while pressure on the

cell walls decreases due to the influence of axin, increasing cell volume. Callus growth itself occurs due to increased cell division triggered by the hormones auxin and cytokinin (Schaller et al., 2014).

In this study, increasing the concentration of the media formulation caused a decrease in the fresh weight of the kluwih teak callus. This condition is suspected to occur because the hormone concentration in the media is not suitable for the physiological needs of the kluwih teak callus, thus inhibiting cell division and triggering physiological stress. This stress can reduce the callus' ability to absorb water and undergo cell expansion, thus inhibiting callus growth (Deng et al., 2023).

Conclusion

The interaction between propagule type and medium formulation had a significant effect on the percentage of callus formation, initiation time, and callus color. In contrast, propagule survival, the proportion of propagules forming callus, and fresh weight of callus were each influenced by single factors. Leaf propagules cultured in medium containing 0.50 mg/L 2,4-D combined with 0.50 mg/L kinetin produced the highest callus formation rate, the shortest initiation time, and yielded friable callus with a yellowish-white appearance. A friable texture was also observed across all propagule types in the 1.00 mg/L 2,4-D + 1.00 mg/L kinetin treatment. Overall, the combination of 0.50 mg/L 2,4-D and 0.50 mg/L kinetin applied to leaf propagules proved to be the most effective treatment for callus induction in kluwih teak wood. In general, the balance of auxin and cytokinin concentrations plays a crucial role in determining the success of callus induction in kluwih teak. These findings can be used as a basis for developing efficient callus induction protocols to support clonal propagation and in vitro germplasm conservation of kluwih teak.

Author Contributions
Conceptualization, S.N.A., A.S.W., and I.R.; methodology S.N.A., A.S.W., and I.R.; software, S.N.A.; validation, S.N.A., A.S.W., and I.R.; formal analysis , S.N.A., A.S.W., and I.R; investigation, S.N.A.; resources; Y.S.A.F., and I.R.; data curation, S.N.A., writing—original draft preparation, S.N.A.; writing—review and editing, S.N.A., A.S.W., and I.R.; visualization, S.N.A.; supervision A.S.W., and I.R.; supervision (technical), Y.S.A.F.

Conflict of Interest
The author declares no conflict of interest.

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