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Induction and Regeneration of Ginger Callus (*Zingiber officinale*) in Malaysia by Administration of 2,4 D and NAA

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Abstract

Ginger (*Zingiber officinale*) is a valuable medicinal plant widely used in food and health industries. This study, conducted at the Malaysian Agricultural Research and Development Institute (MARDI) in September 2024, aimed to assess the impact of hormone concentrations on ginger callus yield and regeneration. The study employed a factorial design with two factors and three replications. The first factor involved MS 24-D media at doses of 0.5 mg/L, 1.0 mg/L, 3.0 mg/L, and 5.0 mg/L. The second factor included Naphthaleneacetic Acid (NAA) at doses of 0.5 mg/L, 1.0 mg/L, 3.0 mg/L, and 5.0 mg/L. Thidiazuron (TDZ) was also used as an additional growth regulator hormone to influence callus formation. The results indicated that growth regulator hormones such as 2,4-dichlorophenoxyacetic acid (24-D), NAA, and TDZ significantly enhanced callus formation efficiency, increasing callus weight. NAA treatment was particularly effective in increasing callus weight. The phenolic content analysis revealed that brown callus exhibited strong defense mechanisms due to a high accumulation of phenolic compounds. Phenolic compounds, known for their antioxidant and antibacterial properties, enhance plant resilience to environmental stress and hold promise for pharmacological applications. Incorporating TDZ in ginger culture demonstrates the potential for improving callus quality with high phenolic content, offering opportunities for agricultural applications.

Keywords: 24-D Hormone, Ginger Callus, NAA, Phenolic, Regeneration

1. Introduction

The ginger plant (*Zingiber officinale*) is a valuable commodity from the Zingiberaceae family, commonly known as ginger. Believed to have originated in India, ginger has gained popularity as a spice, particularly in Europe, where it has been used for centuries. Apart from being an antioxidant-rich herb, ginger is often a hot beverage to provide warmth during cold weather. Ginger possesses various beneficial properties. Its gingerol content offers protection against cell damage caused by free radicals, reduces inflammation, and is highly effective in alleviating nausea, particularly in pregnant women. It also helps reduce muscle and joint pain, manage osteoarthritis, and maintain stable blood sugar and cholesterol levels (Helmalia et al., 2019). In addition to traditional propagation methods, ginger can be propagated vegetatively through tissue culture techniques. This approach offers several advantages, including shorter seedling time, large-scale seedling production, and disease-free seedlings with identical properties to the parent plant. Therefore, tissue culture efficiently produces high-quality ginger seeds (Marlina et al., 2019).

Tissue culture is an efficient method for propagating plants, allowing mass production in a short time without disturbing the existence of plants in their natural habitat by utilizing small parts of plants such as cells, organs, or tissues. Tissue culture can accelerate the spread of superior ginger varieties resistant to diseases and pests and produce high-quality tubers. These advantages contribute to local food security and reduce dependence on external supplies. In addition, this technique can also eliminate pathogens, ensuring the health of the resulting plants. Plants produced through tissue culture are known to be uniform, disease-free, and can be available in the long term. In addition, tissue culture can produce secondary metabolites from medicinal plants through the callus initiation process, a collection of amorphous cells that have not been differentiated (Anggraeni et al., 2022).

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One effective strategy to increase ginger production is propagating plants in vitro through callus culture techniques. In vitro culture methods allow aseptic plant growth and development regulation in culture media. Callus, a collection of undifferentiated cells, can be induced to form various plant organ structures such as roots, shoots, and rhizomes. This technique has many advantages, such as producing uniform seedlings free from pests and diseases and allowing for continuous production throughout the year. In addition, calluses can be initiated from all parts of the plant, with meristematic parts having a higher potential to form calluses (Purba et al., 2017).

Without Plant Growth Regulators (PGRs), ginger plants can face various problems that inhibit their growth process. PGRs are important in stimulating the formation of roots, shoots, and calluses and maintaining hormonal balance in plants. Without PGRs, ginger growth will be inhibited, roots will not develop properly, and callus formation in tissue culture will be complex. In addition, hormonal imbalance can cause abnormal growth and reduce ginger yields. Therefore, PGRs are necessary to support optimal growth and increase ginger plant yields. Plant hormones, especially auxins and cytokinins, play an important role in callus formation and regeneration. Auxin 2,4-D and cytokinin kinetin work synergistically in cell division by regulating the activity of CDK (Cyclin Dependent Kinase), an enzyme that plays a role in this process. The right combination of auxins and cytokinins can affect the amount of callus produced and the ability of the callus to root and sprout. Several studies have shown that optimal hormone ratios can increase regeneration efficiency, but there is still significant variability in plant response to hormone treatment. Plant cell division, growth and development can be enhanced by administering cytokinins hormones. Cell division will stimulate callus growth and formation, determining whether the connection process becomes faster or slower.

In Malaysia, the tropical climate supports the growth of ginger (*Zingiber officinale*), making the country a major spice producer. Research on ginger plant propagation techniques is crucial to enhance production quality and quantity. Currently, propagation is done through dividing rhizomes, which can spread diseases like bacterial wilt (Pseudomonas solanacearum), leaf spots (Phyllosticta zingiberi and Pyricularia zingiberi), downy mildew (Pythium aphanidematum), and leaf yellowing (Fusarium oxysporum) (Abed-Ashtiani et al., 2016) (Zahid et al., 2021). Previous studies on Plant Growth Regulators (PGRs) application to ginger plants have shown that a combination of auxin (2,4-D), cytokinin (BAP), and Naphthaleneacetic Acid (NAA) can accelerate callus formation and plant propagation in vitro. Thidiazuron (TDZ) has also been found to stimulate calluses and shoot formation in ginger. This study differs by testing a combination of three PGRs (2,4-D, NAA, and TDZ) with varying doses for optimal results. Additionally, it evaluates phenolic content in callus, which can enhance plant resistance to stress and offer pharmacological benefits. Unlike previous studies in different environments, this study in Malaysia utilizes tissue culture techniques in tropical conditions. In vitro culture technology can be an effective solution to increase the production of high-quality ginger seeds rapidly and efficiently.

Given the rising global demand for ginger and challenges in traditional cultivation, understanding the impact of hormones on callus formation and regeneration is essential. This study aims to determine the hormone concentrations for callus production and regeneration of ginger (*Zingiber officinale*) in Malaysia.

2. Material and Methods

2.1. Place and Time

This research was conducted at the Malaysian Agricultural Research and Development Institute (MARDI), located in Serdang, Malaysia, with coordinates 2°58'52.3"N 101°41'55.0"E, at an altitude of 1,400 meters above sea level. The research took place in September 2024.

2.2. Materials and Tools

The main material used in this study is Bentong variety ginger rhizome, known for its quality and excellence. The media used are MS media (Murashige and Skoog), 24D, NAA, agar sugar, fungicide, detergent, decon (5%), 10-20% Clorox solution, tween 20, BAP, TZD, sucrose HCL, distilled water, folin-ciocalteu reagent, sodium carbonate, tissue water, polybags and soil. The tools used in this study were culture bottles, bunsen lamps, Laminar Air Flow Cabinet (LAFC), Petri dishes, dissection equipment, namely large tweezers, small tweezers, scalpel knives, analytical scales, hand sprayers, magnetic stirrers, Erlenmeyer flasks, pH meters, autoclaves, measuring pipettes, mortals, centrifuges, ovens, refrigerators, culture racks, cameras and stationery. The ideal temperature for tissue culture is 20°C up to 25°C. Higher or lower temperatures may affect the growth or survival of the culture. The room's relative humidity is between 60% and 80% to avoid dehydration in tissue culture.

2.3. Research Methods

This research method used a factorial Completely Randomized Design (CRD) consisting of two treatments simultaneously with three repetitions for each treatment. Factor I: Dose of 24-D, consisting of four levels, namely: D 1 = 0.5 mg / L; D 2 = 1.0 mg / L; D 3 = 3.0 mg / L; D 4 = 5.0 mg / L. Factor II: NAA dose, consisting of four levels, namely: N 1 = 0.5 mg / L; N 2 = 1.0 mg / L; N 3 = 3.0 mg / L; N 4 = 5.0 mg / L.

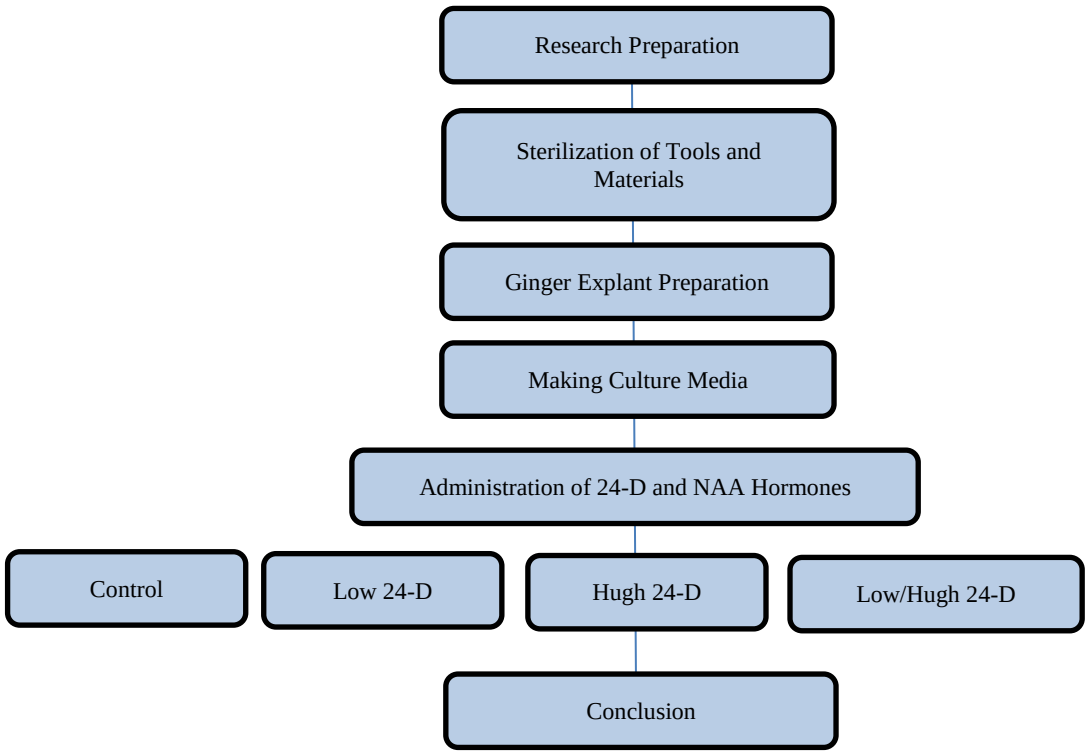

Figure 1. Research flow diagram

2.4. Callus Induction

Ginger was cultivated in a screen house at the Malaysian Agriculture Research and Development Institute (MARDI) in Serdang for initial callus formation. Meristematic shoots of germinated Bentong ginger were collected and cleaned under running tap water for one hour. The explants were cleaned using a commercial laboratory detergent, decon 5% (v/v), where the explants were rinsed thoroughly. Thereafter, the explants were soaked in 1% (v/v) fungicide for one hour, followed by washing under running tap water for five minutes to ensure optimal cleanliness.

Next, the explants underwent surface sterilization using 10–20% Clorox solution mixed with a few drops of Tween 20 in a sterile flask. The explants were then rinsed with sterile distilled water several times to ensure thorough cleaning. The sterilized explants were then inoculated into a basal medium enriched with 3% sucrose and 3.0 mg/L BAP. The resulting plantlets (Figure-2 c, d) were used for in vitro meristem isolation (Figure-1e), which were then cultured on MS basal medium with various concentrations of 24-D (0.5; 1.0; 3.0; and 5.0 mg/L) and NAA (0.5; 1.0; 3.0; and 5.0 mg/L). The 24-D and NAA doses were also tested (0.5 D + 0.5 N, 1 D + 0.5 N, 3 D + 0.5 N, 5 D + 0.5 N, and 1 N + 1 D) for callus initiation. The pH of the medium was adjusted to 5.8 before autoclaving for 15 min at 121°C.

The culture flasks were incubated in a culture chamber with white fluorescence light with an intensity of 3000 lux and a photoperiod of 16 hours at a temperature of 25 ± 2°C. Callus growth was observed and subcultured every month, with callus weight recorded after 3 months of culture. The callus obtained was transferred to the same fresh medium (subculture), and measurements of fresh weight, number of roots, and morphology were recorded.

2.5. Somatic Embryogenesis Proliferation and Plant Regeneration

The callus from the previous experiment was used in proliferation and regeneration activities. The callus formed was friable and white in color, and after this initial phase, the callus was subcultured on the same medium for 6 weeks to support its further growth.

In the proliferation experiment stage, the resulting callus was cultured on media with variations in carbon sources, particularly sucrose at 30, 60, and 90 g/L concentrations. In addition, the concentration of growth regulators also varied, using TDZ and BAP in the range of 0.5–3.0 mg/L to 0.5–5.0 mg/L. This arrangement aims to evaluate the effect of carbon sources and hormone variations on callus weight.

After 6 weeks of culture, data on callus weight were recorded to assess callus growth and proliferation. All proliferating calli were subcultured back on the same medium for the next two months to support further growth. The somatic embryos formed initially appeared green but then browned in the observation, and their morphology was carefully observed and recorded.

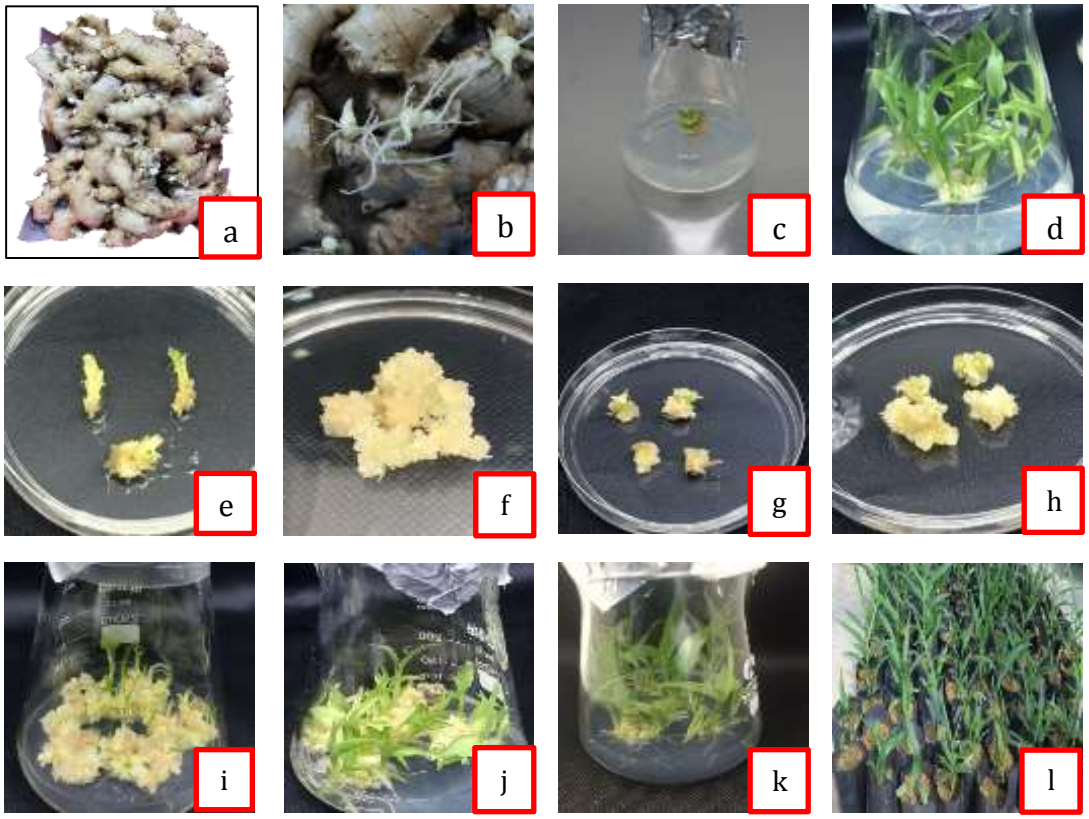

Figure 2. In vitro regeneration of ginger betong plants. Raw material (a), Meristematic shoots of ginger betong (b), In vitro plants (c, d), Isolated Meristem (e), Ginger betong embryo (fgh), regeneration of young plants in regeneration media (i), Young plants with roots ready to be transferred to the greenhouse (jk), 6-week-old young plants maintained in a greenhouse with 75% shade (l)

2.6. Phenolic Analysis

Phenolic analysis began with determining total phenolic content (TPC) in callus extract samples, aiming to measure the concentration of phenolic compounds. This procedure begins with a pipette of 0.5 ml of Folin-Ciocalteu reagent in each test tube, then adding 8 ml of distilled water to dilute the reagent. Next, 0.5 ml of callus extract was added to each test tube, except for an empty test tube that served as a control. To neutralize the reaction, 1 ml of sodium carbonate was added, which plays an important role in forming a measurable complex. After that, 3 ml of the reaction mixture was transferred into a cuvette for measurement. The samples were then analyzed using a spectrophotometer at a wavelength of 725 nm.

2.7. ANOVA Statistical Analysis

Statistical analysis using one-way ANOVA (Analysis of Variance) was applied to evaluate the differences between treatments in this study. Using the Statistical Package for the Social Sciences (SPSS version 20.0) software, data from 25 replications for each treatment were analyzed to determine whether there were significant differences between the group means. Significance between means was tested using Duncan's Multiple Range Test, with a significance limit of $p \leq 0.05$. This analysis assesses the effect of variations in hormone concentrations (NAA, BAP, TDZ, and 24-D) and carbon sources (sucrose) on callus formation and plant regeneration.

3. Results and Discussion

3.1. Callus Induction and Morphology

Based on the findings of the study investigating the effects of 2.4-D and NAA on callus induction in ginger plants, it can be concluded that plant growth regulators play a significant role in enhancing the efficiency of callus formation. The experiment conducted on MS media with various concentrations of 2.4-D (0.5, 1.0, 3.0, and 5.0 mg/L) and NAA (0.5, 1.0, 3.0, and 5.0 mg/L) demonstrated that the combination of these hormones elicited diverse responses.

Table 1 illustrates that in the absence of growth regulators, the weight of the callus produced is significantly low, with only 1.52 grams in the control medium. This aligns with the findings of Waryastuti et al. (2017), which describe callus formation as characterized by swelling and the emergence of white tissue. The low weight of the callus is accompanied by a decline in quality, evident through color change to brown (browning) and a watery texture. Callus without hormones exhibits an increase in polyphenol oxidase enzyme activity and the accumulation of phenolic compounds, indicating oxidative stress. The lack of growth

regulators diminishes callus weight and impacts cell viability and regeneration capacity. Media devoid of hormones yield callus weights 40-60% lower than those with hormones, leading to increased cell necrosis and decreased protein and carbohydrate levels. Hence, optimizing the use of growth regulators is crucial for enhancing callus production and secondary metabolite accumulation. The balance between auxin and cytokinin concentrations is pivotal in regulating plant growth. Higher auxin levels promote callus formation, a mass of undifferentiated cells, while an abundance of cytokinins shifts growth towards shoot formation, initiating new organ growth (Tarigan et al., 2022). Maintaining a balanced composition of media components, particularly growth hormones, is vital for supporting sustainable callus growth. This study underscores the significance of growth regulators in inducing and maintaining high-quality callus.

Table 1. Effect of 24-D and NAA on Ginger Callus Induction

Treatment	Concentration	Callus Weight (grams)	Number of Roots
MSO	0	1.52±1.1	0
NAA	0.5	4.24±0.9	0
	1	3.40±1.2	5
	3	4.27±0.5	6
	5	4.28±0.5	6
2.4-D	0.5	4.30±0.5	0
	1	5.80±0.5	0
	3	5.70±1.7	0
	5	5.70±1.7	0
2.4-D + NAA	0.5 D + 0.5 N	5.54±2.0	4
	1 D + 0.5 N	4.37±0.7	1
	3 D + 0.5 N	4.41±0.8	1
	5 D + 0.5 N	5.20±1,	0
	1 N + 1 D	6.51±2.1	0

Testing with NAA showed that higher concentrations tended to produce callus with greater weight. At a concentration of 0.5 mg/L, the callus weight reached 4.24 grams and was stable at 4.28 grams at 5 mg/L. NAA in the range of 0.5-5.0 mg/L effectively induced callus formation with optimal weight, thanks to its ability to increase cell division and expansion by regulating Cyclin-dependent kinase (CDK) proteins. This was stated by (Wahyuni et al., 2020) that the higher the concentration of NAA, the higher the callus explants. However, although NAA is effective, high concentrations can inhibit the formation of adventitious roots, as seen from the low number of roots formed (only 6). To optimize root regeneration, it is important to consider using NAA and other hormones, such as IBA or IAA. This statement follows research (Arifin et al., 2022), which states that administering ZPT IBA (Rootone-F) to snake plant cuttings with the origin of the middle cuttings gives the best results in the number of roots. The two-stage culture technique, which separates callus induction and root regeneration in media with different hormone compositions, can solve the trade-off between the two processes.

Combining 2,4-D and NAA hormones showed significant results in callus induction, where the combination of 1 mg/L NAA and 1 mg/L 2,4-D produced the highest callus weight of 6.51 grams. This shows that 1 mg/L NAA and 1 mg/L 2,4-D showed the best callus weight. The results of this study also follow the results of the study (Alqamari et al., 2020), namely that treatment with a combination of 1.00 ppm NAA and 1.00 ppm BAP was proven to be the most effective in inducing callus formation on explants. This shows that the right proportion between auxin and cytokinin is significant in callus tissue formation. The balanced interaction between 2,4-D and NAA in a 1:1 ratio activates the dual auxin signalling pathway, which optimizes cell division and protein biosynthesis. However, it is important to note that no roots are formed in this combination because the concentration of 2,4-D equivalent to NAA can inhibit cell differentiation required for root formation. The combination's high total auxin concentration (2 mg/L) can suppress gene expression in adventitious root initiation. A two-stage culture approach, where the first stage focuses on callus induction using 2,4-D and NAA, followed by a second stage with hormones more suitable for root regeneration, such as IBA or IAA, can be an effective solution. In addition to plant growth regulators, sucrose composition plays an important role in callus formation and development. Sucrose is broken down into glucose and fructose as a carbon source in the culture medium. Glucose is an energy source for callus formation, while fructose is an antioxidant in cell development (Bhojwani & Dantu, 2013).

3.2. Somatic Embryogenesis Proliferation and Plant Regeneration

The study showed significant and complex results on the effect of different carbon sources combined with plant growth regulators (TZD and BAP) on callus proliferation,

somatic embryo initiation, and callus browning in ginger plants.

Table 2 shows that the callus produced on media with 30 g/L sucrose carbon source and a combination of 1 mg/L TZD and 0.5 mg/L BAP hormones showed the highest weight, which was 9.5 grams. Sucrose at a concentration of 30 g/L functions optimally as a carbon source and osmotic pressure regulator, supporting callus proliferation. The synergistic interaction between TZD and BAP in a ratio of 2:1 activates the auxin-cytokinin signal pathway, effectively triggering cell division and callus differentiation. However, the low efficiency of somatic embryo formation (0.5%) is a concern. This is per the results of research (Sitinjak et al., 2015) that the callus formed begins in part in contact with the media with morphological characteristics in the form of swelling of the explant, indicating the presence of cell proliferation activity in the explant.

Table 2. Effect of Callus Proliferation, Green Somatic Embryo Initiation and Percentage of Browning Occurring in Cultivation of Different Carbon Sources Combined with Plant Growth Regulators (TZD and BAP)

Carbon source (g/L)	TZD (mg/ liter)	BAP (mg/ liter)	Callus Weight (Proliferation) (grams)	Efficiency of Callus Developing Green Somatic Embryo (%)	Percentage of Browning (%)	Callus Morphology
Sucrose	0.5	0.5	6.2±2.2	1.2±0.5	0	Green patch on the callus, friable and white
30		1.0	7.3±1.2	2.4±0.6	0	Green patch on callus, friable and white
		3.0	8.1±3.1	5.3±0.8	0	Green patch on callus, friable and white
		5.0	8.3±2.1	0	0	Green patch on callus, friable and white
	1.0	0.5	9.50±2.4	1.2±0.7	0	Friable and yellowish
		1.0	7.3±2.2	1.3±0.1	0	Friable and yellowish
		3.0	4.1±1.2	0.9±0.2	0	Friable and yellowish
		5.0	3.7±0.8	0	0	Friable and yellowish
	3.0	0.5	2.4±0.3	0	21±5.1	Bwon and friable
		1.0	3.6±0.5	0	10±3.3	Bwon and friable
		3.0	3.5±0.3	0	10±5.0	Bwon and friable
		5.0	1.1 ± 1.2	0	10±1.5	Bwon and friable
Sucrose	0.5	0.5	9.1±3.1	0.5±0.1	0	Bwon and friable
60		1.0	8.2±3.0	0.6±0.1	0	Bwon and friable
		3.0	4.2±1.4	1.3±0.1	0	Bwon and friable
		5.0	3.3±1.1	0	0	Bwon and friable
	1.0	0.5	3.8±0.4	0	10±0.9	Brown and watery
		1.0	4.0±2.1	0	11±2.2	Brown and watery
		3.0	5.1±2.0	0	21±5.5	Brown and watery
		5.0	4.3±3.0	0	31±1.7	Brown and watery
	3.0	0.5	3.3±1.2	0	40±7.9	Brown and watery
		1.0	3.5±1.2	0	42±4.1	Brown and watery
		3.0	3.1±0.4	0	31±5.7	Brown and watery
		5.0	3.1±0.5	0	40±11.8	Brown and watery
Sucrose	0.5	0.5	2.1±0.3	0	81±7.9	Brown and water
90		1.0	1.1±0.1	0	70±5.5	Brown and water
		3.0	2.4±0.4	0	80±8.0	Brown and water
		5.0	1.0±0.7	0	82±12.7	Brown and water
	1.0	0.5	2.6±1.1	0	100±21.2	Brown and water
		1.0	2.0±0.5	0	100±18	Brown and water
		3.0	2.4±1.0	0	100±12.5	Brown and water
		5.0	2.2±0.5	0	100±11.3	Brown and water
	3.0	0.5	2.5±0.3	0	100±8.9	Brown and water
		1.0	1.3±0.5	0	100±5.6	Brown and water
		3.0	1.5±0.3	0	100±4.2	Brown and water
		5.0	1.4±01	0	100±2.9	Brown and water

The emergence of callus on explants in vitro is a response to the addition of the type and concentration of exogenous ZPT, light, and the presence of injury to the explant so that it stimulates the tissue in the explant to produce a callus formation reaction as a response to wound closure (Indah & Ermavitalini, 2013) (Ikeuchi et al., 2013). ZPT BAP (Benzyl Amino Purin) is a synthetic cytokinin group often used in in vitro plant propagation. However, it has the same basic structure as kinetin. However, its effectiveness is better when compared to kinetin because

BAP has a benzyl group.

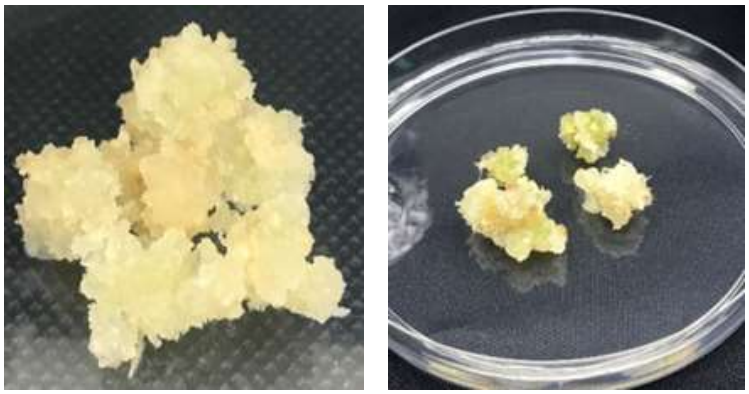

Figure 3. Callus results from 30 g/L sucrose carbon source and a combination of 1 mg/L TZD and 0.5 mg/L BAP hormones.

In contrast, the use of sucrose at high concentrations (60 g/L) had a negative impact on callus growth, with callus weight only reaching 3.3-5.1 grams and a browning rate of 40-42%. Adding sucrose at lower concentrations was not enough to increase callus growth, while high concentrations can disrupt the culture medium's osmotic pressure, affecting the absorption of nutrients by the callus. This disturbance can inhibit cell growth and cause cell death due to cell wall rupture. High sucrose concentrations also trigger the accumulation of phenolic compounds and the activation of polyphenol oxidase enzymes, contributing to tissue browning. In addition, osmotic stress caused by high sucrose concentrations can induce the production of reactive oxygen species (ROS), which leads to cell damage and decreased callus growth.

Using sucrose at very high concentrations (90 g/L) significantly negatively impacted callus growth, as seen from the very low callus weight (1.0-2.6 grams) and the browning level reaching 100%. Increasing the sucrose concentration caused the callus to turn brownish, while at a concentration of 90 g/L, the callus showed a yellowish-green color indicating the presence of chlorophyll. Brown callus indicates that the constituent cells are aging and accumulating oxidized phenolic compounds. Excess sucrose causes fatal hyperosmotic stress to cells, resulting in plasmolysis and mass cell death. Extreme sucrose levels disrupt the water potential balance in cells, causing dehydration and permanent damage to cell membranes. The accumulation of phenolic compounds under these stress conditions contributes to total tissue browning, indicating failure of the cellular antioxidant defense system. At the right concentration, kinetin can play a role in the development of chlorophyll in callus. Various studies on the effect of sucrose administration at various concentrations on the production of secondary metabolite compounds through in vitro culture have been conducted (Irmawati et al., 2007), showing that the optimal sucrose concentration of 30 g/L in the formation of callus biomass. High osmotic pressure can reduce cell performance in growth and development so that cells experience plasmolysis.

3.3. Phenolic Analysis of Ginger Callus

The results of phenolic analysis of various types of ginger callus, as shown in Table 3, showed significant variations in the concentration of phenolic compounds among the samples tested. Phenolic compounds are important components in plants that function as antioxidants and have a role in resistance to biotic and abiotic stress.

Table 3. Ginger callus phenolics

No	Sample	Conc (µg/mL)
1	Brown Callus	80,116
2	Callus On Tree	24,565
3	Rooted Callus	35,704
4	Embryo Callus	22,891
5	Transparent Callus	2,733
6	Green Callus	8,363

Table 3 shows that brown callus contains very high phenolic compounds, reaching 80,116 µg/mL. The high concentration of phenolic compounds indicates a strong defense mechanism in the callus. Kinetin, at the right concentration, contributes to the development of chlorophyll in callus. Browning of callus can be caused by loss of water content due to high osmotic pressure in the media, which can also cause browning. The brown color of callus reflects the accumulation of phenolic compounds and increased activity of the polyphenol oxidase enzyme in

response to stress. High osmotic pressure can reduce cell performance, leading to plasmolysis. In addition, external factors such as light exposure also affect callus pigmentation; white callus can produce protochlorophyll when exposed to light. Browning in callus culture is often caused by oxidizing phenolic compounds to quinones, which are then polymerized into brown pigments. Light can increase the oxidation of phenolic compounds induced by the polyphenol oxidase enzyme, causing browning in the callus.

Green callus indicates the presence of chlorophyll, but low phenolic levels indicate that secondary metabolic activity is very minimal, even though callus growth is going well. This can be a weakness in the natural defense system against biotic and abiotic stress. Flavonoids, which are included in the category of phenolic compounds, are often produced as a plant defense mechanism. In tissue culture, resistance to pathogens and environmental stress is highly dependent on accumulating these defense compounds. Callus with low phenolic content requires optimization of culture conditions to increase secondary metabolite production and, thus, strengthen plant resistance. In addition, sucrose plays a role in protein synthesis, the higher the sucrose concentration, the better the protein synthesis process. Increasing sucrose concentration also triggers increased cellular respiration activity, while lower concentrations can decrease the respiration rate and inhibit protein synthesis.

According to, adding various sucrose concentrations to media containing 2 ppm of 2,4-D hormone cannot induce callus formation and can even cause necrosis in explants. This moderate phenolic level reflects a good balance between plant growth and defense. A callus with moderate phenolic content generally has better regeneration potential because secondary metabolism does not dominate and does not inhibit cell differentiation. Balanced phenolic levels in rooted and embryonic callus support the development of more complex structures while maintaining defense mechanisms against environmental stress. In addition, light can increase the oxidation of phenolic compounds through the polyphenol oxidase enzyme, which can result in the browning of the callus.

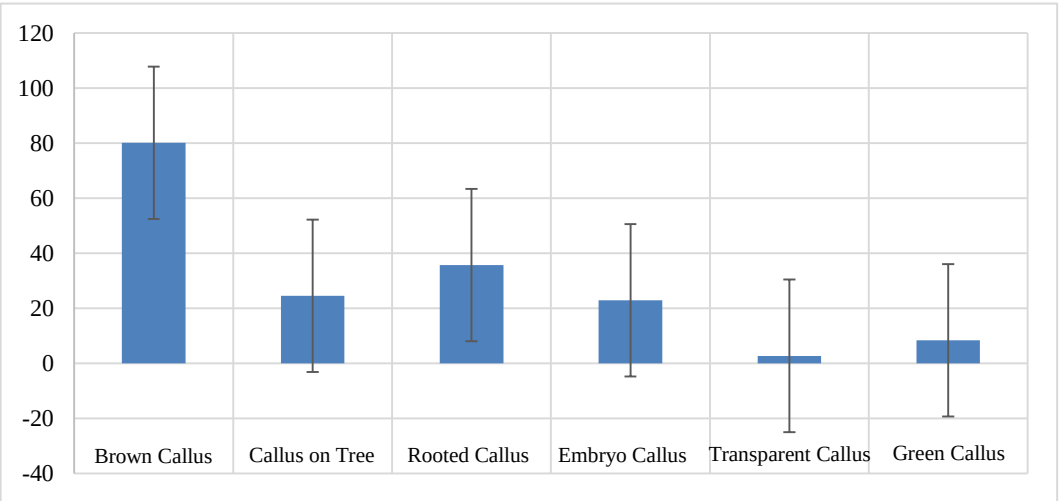

Figure 4. Phenolic Analysis Diagram

The analysis results showed significant variations in phenolic concentrations in various types of callus. Embryo callus had the highest content, 22,891 µg/mL, green callus with 8,363 mg/mL, and transparent callus with the lowest, 2,733 µg/mL. High phenolic levels in embryo callus are closely related to the somatic embryogenesis stage which requires secondary metabolites for cell differentiation. Conversely, low phenolic concentrations in transparent and green callus indicate an active vegetative growth phase, where the main focus of cells is division, not the production of secondary metabolites. Kinetin plays a role in the development of chlorophyll in callus, while browning can occur due to loss of water content due to high osmotic pressure, which can cause plasmolysis.

The callus sample analysis results showed significant callus formation variations under various treatment conditions. Brown callus showed the highest percentage (around 80%) compared to other callus types, indicating an intensive phenolic oxidation process in the callus tissue. Slimy callus and root callus occupied the middle position with percentages of around 35% and 25%, respectively, indicating a moderate growth response. Meanwhile, embryonic callus reached around 22%, indicating a fairly good regeneration potential. Transparent callus had the lowest percentage (<5%), followed by green callus (around 8%), indicating varying levels of cell differentiation.

4. Conclusion

Growth regulator hormones such as 24-D and NAA have been demonstrated to influence callus formation efficiency substantially. In the absence of hormones, the weight of callus is recorded at 1.52 grams. Conversely, when the appropriate hormonal combination is employed, the callus weight can reach 6.51 grams. While NAA

treatment has been demonstrated to enhance callus weight, excessive concentrations can impede root formation, underscoring the need for optimal hormone dosage and concentration modulation to ensure efficient callus development. The carbon source sucrose (30 g/L) has been demonstrated to yield optimal outcomes for callus proliferation. However, it should be noted that higher concentrations can cause harmful osmotic stress. Phenolic analysis indicates that brown callus exhibits a robust defensive response, attributable to its high accumulation of phenolic compounds, which renders it a promising candidate for pharmacological applications.

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References

Abed-Ashtiani, F., Kadir, J., Nasehi, A., Hashemian-Rahaghi, S. R., & Golkhandan, E. (2016). Occurrence of leaf spot or blast on ginger (*Zingiber officinale*) caused by *Pyricularia zingiberi* in Malaysia. *Plant Disease, 100*(7), 1505. https://doi.org/10.1094/PDIS-01-16-0122-PDN

Alqamari, M., Thalib, B., & Fitra, S. (2020). Kajian media MS dengan penambahan auksin dan sitokinin terhadap pertumbuhan dan perkembangan kultur tunas aren (*Arenga pinnata* (Wurmb) Merr). *Jurnal Pertanian Tropik, 7*(1), 109–115. https://doi.org/10.32734/jpt.v7i1.April.3790

Anggraeni, D., Ismaini, L., Surya, M. I., Rahmi, H., & Saputro, N. W. (2022). Inisiasi kalus daun *Talinum triangulare* (Jacq.) Willd pada beberapa kombinasi konsentrasi zat pengatur tumbuh 2,4-Dichlorophenoxyatic Acid dan Benzyl Adenine. *Agrikultura, 33*(3), 276–288. https://doi.org/10.24198/agrikultura.v33i3.40540

Anggraito, Y. U., Susanti, R., Iswari, R. S., Yuniastuti, A., Lisdiana, L., WH, N., Habibah, N. A., & Bintari, S. H. (2018). *Metabolit Sekunder dari Tanaman: Aplikasi dan Produksi*. Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Negeri Semarang.

Arifin, A. Z., Hidayanto, F., & Mahfud, R. I. (2022). The effectiveness of substances in growth regulators on growth of root cutting of mother-in-law's tongue leaves (*Sansevieria trifasciata*). *Jurnal Agronomi Tanaman Tropika (JUATIKA), 4*(1), 139–146. https://doi.org/10.36378/juatika.v4i1.1780

Bhojwani, S. S., & Dantu, P. K. (2013). *Plant tissue culture: An introductory text*. https://doi.org/10.1007/978-81-322-1026-9

Chen, Y. M., Huang, J. Z., Hou, T. W., & Pan, I. C. (2019). Effects of light intensity and plant growth regulators on callus proliferation and shoot regeneration in the ornamental succulent *Haworthia*. *Botanical Studies, 60*(10), 1–8. https://doi.org/10.1186/s40529-019-0257-y

Durroh, B., & Winarti, Y. (2020). Pemanfaatan air kelapa dan aplikasi pupuk organik untuk merangsang pertumbuhan bibit tebu G3 hasil kultur jaringan. *Agro Bali: Agricultural Journal, 3*(1), 21–27. https://doi.org/10.37637/ab.v3i1.415

Helmalia, A. W., Putrid, P., & Dirpan, A. (2019). Potensi rempah-rempah tradisional sebagai sumber antioksidan alami untuk bahan baku pangan fungsional. *Canrea Journal: Food Technology, Nutrition, and Culinary Journal, 2*(1), 26–31. https://doi.org/10.20956/canrea.v2i1.113

Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant callus: Mechanisms of induction and repression. *The Plant Cell, 25*(9), 3159–3173. https://doi.org/10.1105/tpc.113.116053

Inayah, T. (2015). Pengaruh konsentrasi sukrosa pada induksi embrio somatik dua kultivar kacang tanah (*Arachis hypogaea* L.) secara in vitro. *Agribusiness Journal, 9*(1), 61–70. https://doi.org/10.15408/aj.v9i1.5086

Indah, P. N., & Ermavitalini, D. (2013). Induksi kalus daun nyamplung (*Calophyllum inophyllum* Linn.) pada beberapa kombinasi konsentrasi 6-Benzylaminopurine (BAP) dan 2,4-Dichlorophenoxyacetic Acid (2,4-D). *Jurnal Sains dan Seni Pomits, 2*(1), 1–6.

Irmawati, I., Solichatun, S., & Anggarwulan, E. (2007). Pertumbuhan dan kandungan reserpin kultur kalus *Rauvolfia verticillata* pada variasi konsentrasi sukrosa dalam media MS. *Biofarmasi Journal of Natural Product Biochemistry, 5*(1), 38–46. https://doi.org/10.13057/biofar/f050105

Julianti, R. F., Nurchayati, Y., & Setiara, N. (2021). Pengaruh konsentrasi sukrosa dalam medium MS terhadap kandungan flavonoid kalus tomat (*Solanum lycopersicum* syn. *Lycopersicum esculentum*). *Jurnal Metamorfosa, 8*(1), 141–149. https://doi.org/10.2307/2257356

Khaerasani, I., Prihastanti, E., & Haryanti, S. (2017). Pertumbuhan kalus eksplan rimpang jahe merah (*Zingiber officinale* Rosc.) pada berbagai konsentrasi sukrosa secara in vitro. *Buletin Anatomi dan Fisiologi, 2*(1), 43–49. https://doi.org/10.14710/baf.2.1.2017.43-49

Maninggolang, A., Polii-Mandang, J. S., & Tilaar, W. (2018). Pengaruh BAP (Benzyl Amino Purine) dan air kelapa terhadap pertumbuhan tunas pucuk dan kandungan sulforafan brokoli (*Brassica oleracea* L. var. italica Plenck) secara in vitro. *Agri-Sosioekonomi Unsrat, 14*(1), 439–450. https://doi.org/10.35791/agrsosek.14.1.2018.19730

Marlina, A., Nopsagiarti, T., & Jamalludin, J. (2019). Penggunaan berbagai konsentrasi ragi terhadap pertumbuhan subkultur jaringan manggis (*Garcinia mangostana* L) secara in vitro. *Jurnal Agronomi Tanaman Tropika (JUATIKA), 1*(2), 12–18. https://doi.org/10.36378/juatika.v1i1.46

Marthani, Q. K., Anggraito, Y. U., & Rahayu, E. S. (2016). Kalogenesis eksplan setengah biji korobenguk (*Mucuna pruriens* L.) secara in vitro menggunakan BAP dan NAA. *Life Science, 5*(1), 72–78.

Matheka, J. M., Magiri, E., Rasha, A. O., & Machuka, J. (2008). In vitro selection and characterization of drought-tolerant somaclones of tropical maize (*Zea mays* L.). *Biotechnology, 7*(4), 641–650. https://doi.org/10.3923/biotech.2008.641.650

Purba, R. V., Yuswanti, H., & Astawa, I. N. G. (2017). Induksi kalus eksplan daun tanaman anggur (*Vitis vinifera* L.) dengan aplikasi 2,4-D secara in vitro. *E-Jurnal Agroekoteknologi Tropika, 6*(2), 218–228. http://ojs.unud.ac.id/index.php/JAT

Rasud, Y., & Bustaman, B. (2020). Induksi kalus secara in vitro dari daun cengkeh (*Syzygium aromaticum* L.) dalam media dengan berbagai konsentrasi auksin. *Jurnal Ilmu Pertanian Indonesia, 25*(1), 67–72. https://doi.org/10.18343/jipi.25.1.67

Schaller, G. E., Bishopp, A., & Kieber, J. J. (2015). The yin-yang of hormones: Cytokinin and auxin interactions in plant development. *The Plant Cell, 27*(1), 44–63. https://doi.org/10.1105/tpc.114.133595

Shofiyani, A., & Purnawanto, M. (2017). Pertumbuhan kalus kencur (*Kaempferia galanga* L.) pada komposisi media dengan perlakuan sukrosa dan zat pengatur tumbuh (2,4-D dan Benzil Aminopurin). *AGRITECH, 19*(1), 55–64.

Sitinjak, M. A., Isda, M. N., & Fatonah, S. (2015). Induksi kalus dari eksplan daun in vitro keladi tikus (*Typhonium* sp.) dengan perlakuan 2,4-D dan kinetin. *Jurnal Biologi, 8*(1), 32–39.

Sitorus, E. N., Hastuti, E. D., & Setiari, N. (2011). Induksi kalus binahong (*Basella rubra* L.) secara in vitro pada media Murashige & Skoog dengan konsentrasi sukrosa yang berbeda. *Bioma: Berkala Ilmiah Biologi, 13*(1), 1–7.

Sudrajad, H., Suharto, D., & Wijaya, N. R. (2016). Inisiasi kalus sanrego (*Lunasia amara* Blanco.) dalam kultur jaringan. *Proceeding Biology Education Conference, 13*(1), 619–623. https://simdos.unud.ac.id/uploads/file_pendidikan_1_dir/ddeec13c19c352d21ccca286966a08ec.pdf

Taranto, F., Pasqualone, A., Mangini, G., Tripodi, P., Miazzi, M. M., Pavan, S., & Montemurro, C. (2017). Polyphenol oxidases in crops: Biochemical, physiological, and genetic aspects. *International Journal of Molecular Sciences, 18*(377), 1–16. https://doi.org/10.3390/ijms18020377

Tarigan, D. M., Barus, W. A., Nasution, F. Y. H., & Lestami, A. (2022). Utilization of shallot extract and administration of indole acetic acid to *Chrysanthemum* sp. in vitro culture. *Jurnal Natural*, *22*(2), 102–107. https://doi.org/10.24815/jn.v22i2.25778

Wahyuni, D. K., Huda, A., Faizah, S., Purnobasuki, H., & Wardojo, B. P. E. (2020). Effects of light, sucrose concentration, and repetitive subculture on callus growth and medically important production in *Justicia gendarussa* Burm.f. *Biotechnology Reports*, *27*(May), 1–9. https://doi.org/10.1016/j.btre.2020.e00473

Waryastuti, D. E., Setyobudi, L., & Wardiyati, T. (2017). Pengaruh tingkat konsentrasi 2,4-D dan BAP pada media MS terhadap induksi kalus embriogenik (*Curcuma xanthorrhiza* Roxb.). *Jurnal Produksi Tanaman*, *5*(1), 140–149.

Zahid, N. A., Jaafar, H. Z. E., & Hakiman, M. (2021). Micropropagation of ginger (*Zingiber officinale* Roscoe) 'Bentong' and evaluation of its secondary metabolites and antioxidant activities compared with the conventionally propagated plant. *MDPI*, *10*(Maret), 1–17.