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Effects of Combined 2,4-D and BAP Treatments on the Formation of Leaf-Derived Callus in *Pogostemon cablin* (Blanco) Benth.

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Abstract

Pogostemon cablin (Blanco) Benth., a key source of patchouli oil (2.5 to 5% content), is widely used in the perfume and cosmetic industries but faces propagation challenges due to its time-consuming nature and susceptibility to pathogens. To overcome these limitations, *in vitro* techniques have been adopted to produce healthy and uniform seedlings efficiently. This study evaluated the effects of varying concentrations of 2,4-D (0 to 1.5 mg l^{-1}) and BAP (0 to 1.5 mg l^{-1}) on the *in vitro* induction of patchouli calli using axenic leaf explants cultured on Murashige and Skoog (MS) medium for 60 days. Growth parameters assessed included callus emergence time, morphological traits (texture and color), callogenesis percentage, organogenesis, and fresh callus weight. The results indicated that the combination of 2,4-D and BAP significantly influenced callogenesis, organogenesis, and callus morphology. The optimal combination (A0B3: 2,4-D 0 mg l^{-1} + BAP 1.5 mg l^{-1}) achieved 100% callogenesis and organogenesis, with an average callus fresh weight of 0.25 g and an initiation time of 40.5 days. Calli exhibited a crumbly texture with a yellowish-white color, highlighting the role of auxin and cytokinin interactions in patchouli callus induction. These findings provide a foundation for scaling up *in vitro* patchouli oil production.

Keywords: auxin; callus; cytokinin; *in vitro* culture; patchouli

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INTRODUCTION

Patchouli (*Pogostemon cablin* (Blanco) Benth.), a prominent essential oil-producing plant, is the source of patchouli essential oil (PEO), which is highly sought after across various industrial sectors. Indonesia has been known to supply nearly 90% of the global demand for patchouli oil, cementing its position as the world's largest producer (Jadid et al., 2024b). Among the different Indonesian patchouli accessions, Aceh-derived patchouli is particularly valued due to its high essential oil content, ranging from 2.5 to 5%, making it a preferred choice in the market (Astuti et al., 2022). Aceh patchouli thrives at altitudes up to 1,200 m above sea level, with humidity levels of 70 to 90%, annual rainfall of 1,600 to 3,000 mm, and temperatures between 24 to 28 °C (Nisa et al., 2024). Different patchouli accession plants probably possess different quality and quantity of patchouli oils that might be further processed in perfume, cosmetics, and other types of pharmaceutical industries due to their strong fixative properties, antiseptic, insecticide, and aromatherapy properties (Jadid et al., 2024b). Therefore, providing good quality patchouli plants should be considered to ensure the global market demand.

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Traditionally, patchouli propagation relies on vegetative methods such as stem cuttings. However, this approach is hindered by several limitations, including a high mortality rate, inconsistent growth, and a lengthy cultivation period of approximately 2 to 4 months. To address these challenges, *in vitro* culture techniques might offer an alternative propagation method capable of producing healthy and uniform seedlings in large quantities with greater efficiency (Swamy and Sinniah, 2016). This technique involves the propagation of plants under controlled environmental conditions using sterilized explants in either solid or liquid media (Ramadani and Jadid, 2024). However, the success of *in vitro* propagation is significantly influenced by the use of plant growth regulators (PGRs), which play a crucial role in enhancing growth and development during the tissue culture process (Agarwal, 2015).

In vitro callus induction using PGRs plays a vital role in accelerating the good quality of plants. Therefore, it is also promoting sustainable agriculture in developing countries. This method is crucial for advancing both scientific research and industrial applications. Callus culture facilitates the rapid and efficient propagation of plants, which is essential for preserving genetic diversity and reducing reliance on conventional seeds that may have limited availability, especially in medicinal crops (Benjamin et al., 2019; Efferth, 2019; Muthi'ah et al., 2023). Furthermore, callus tissues facilitate genetic transformation, enabling the development of medicinal plants with improved traits such as higher metabolite content or enhanced stress resistance. Numerous studies have explored callus induction techniques to enhance the biosynthesis of targeted phytochemical compounds such as alkaloids, flavonoids, and essential oils, which are extensively used in pharmaceuticals and cosmetics. Supplementary approaches such as precursor feeding and elicitation using PGRs can be more efficiently implemented through callus culture systems (Ramadani and Jadid, 2024). The presence of auxin and cytokinin in the culture media plays an important role in regulating cell division and differentiation, with their balanced interaction promoting efficient callus formation (Aziz et al., 2014; Hardjo et al., 2019; Rasud et al., 2020; Haring et al., 2024; Jadid et al., 2024a). Among the auxins, 2,4-D (2,4-Dichlorophenoxyacetic acid) is widely recognized for its efficacy in promoting callus induction (Muthi'ah et al., 2023). Meanwhile, cytokinin-based PGRs, such as BAP (6-Benzylaminopurine), also play a critical role in cell differentiation during somatic embryogenesis (Skoog and Miller, 1957; Ikeuchi et al., 2013; Wardani, 2020).

Despite the extensive use of *in vitro* culture techniques for callus induction in various plant species, limited research has focused on optimizing the combination of 2,4-D and BAP for callus induction in Aceh-derived patchouli, a high-value essential oil plant. This study is the first to systematically explore the effects of different concentrations of 2,4-D and BAP on callus induction in *Pogostemon cablin* (Blanco) Benth. to enhance the propagation efficiency, improve callus quality, and potentially increase essential oil yields in patchouli. By focusing on Aceh patchouli, known for its superior oil content, this research seeks to contribute novel insights that could benefit both sustainable agricultural practices and the global patchouli oil market.

The callus form-induced callogenesis exhibits varying characteristics in texture and color. Textural categories include friable, intermediate, and compact (non-friable) forms. The texture of the callus reflects its quality and shows whether cells are actively dividing or have entered a quiescent state (Haring et al., 2024). High-quality calluses are characterized by a friable texture, which facilitates separation into single cells in suspension cultures and enhances oxygen aeration between cells (Marisa et al., 2021). Another callus characteristic is represented by its color, ranging from white to green, which offers further insights into their condition. Visual assessment of callus color can also help determine whether the cells remain active or have ceased differentiation, with white or green hues generally indicating active, viable cells, and darker or translucent shades often signifying senescence or cell death (Wardani, 2020). To date, research on callus induction in patchouli plants remains scarce. Furthermore, studies investigating the optimal concentration of the 2,4-D and BAP combination for patchouli *in vitro* culture, particularly regarding callus formation, are also limited. Therefore, the present study investigates the effects of various 2,4-D and BAP concentration combinations on the callus induction of Aceh patchouli (*Pogostemon cablin* (Blanco) Benth.) using axenic leaf explants under *in vitro* conditions.

MATERIALS AND METHOD

Media preparation and axenic leaf segment inoculation

The solid Murashige and Skoog (MS) medium was prepared according to the method done by Jadid et al. (2024b). These MS media were supplemented by 16 combinations of 2,4-D (A) and BAP (B) concentrations (Table 1).

Explants used in this study were obtained from *P. cablin* sterile plantlet collection of the Laboratory of Plant Bioscience and Technology, Department of Biology, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia. Leaf explants were obtained from axenic patchouli plants cultured in MS0 medium for 3 months of culture. The explants from *P. cablin* planlets were 1 cm^2 cut and placed into the previously prepared MS medium with combined 2,4-D and BAP concentrations. The medium contained sucrose (30 mg ml^{-1}) and agar (8 mg ml^{-1}). The culture incubation period was carried out for 60 days (Khairan et al., 2021). The cultures were maintained in cultivation jars at 23 °C under white light with a photon flux intensity of 40 to 52 μmol m^{-2} s^{-1}. A total of 16 explants were used in each experimental repeat, with two independent repeats conducted. Additional maintenance during the incubation period was carried out by spraying 70% alcohol on the surfaces of the incubation jars twice a day to minimize exogenous contamination.

Table 1. Detailed combination of 2,4-D and BAP concentrations on callus induction of patchouli

Treatments	2,4-D (mg l^{-1})	BAP (mg l^{-1})
A0B0 (Control)	0.0	0.0
A0B1	0.0	0.5
A0B2	0.0	1.0
A0B3	0.0	1.5
A1B0	0.5	0.0
A1B1	0.5	0.5
A1B2	0.5	1.0
A1B3	0.5	1.5
A2B0	1.0	0.0
A2B1	1.0	0.5
A2B2	1.0	1.0
A2B3	1.0	1.5
A3B0	1.5	0.0
A3B1	1.5	0.5
A3B2	1.5	1.0
A3B3	1.5	1.5

Callus emergence time record and fresh weight measurement

The observation of callus emergence was conducted daily, according to Anjalani et al. (2024). The parameter for callus emergence time refers to the specific day on which the callus formed on the explant, calculated in days after planting (DAP). This event is characterized by swelling of the explant and the appearance of small white spots. The fresh weight of the callus formed in this study was measured using an analytical balance (PCB 1000-2) after 60 DAP.

Frequency of explant forming callus, organogenesis responses, and callus morphology

The frequency of callogenesis and organogenesis was determined by calculating the percentage of explants that formed callus, assessed on the 60th DAP according to the formula reported by Jadid et al. (2024a). The photographic records were taken using a digital camera directly on the petri dishes under the laminar airflow cabinet. Observations and image analysis conducted with the camera software were used to evaluate the callus morphology. The morphological parameters of the callus were assessed by observing the color and texture of the formed callus (friable or compact). Callus color parameters include variations such as white (W), white-greenish (WG), white-yellowish (WY), yellowish (Y), green (G), green-yellowish (GY), yellow-brownish (YB), brownish (B), dark brown (DB), and black or dead (D). Callus texture parameters were evaluated based on the apparent structure of the callus, categorized as compact (C), intermediate (I), or friable (F) textures (Bojko et al., 2024).

Data analysis

This experiment was conducted using a completely randomized design. The frequency of explant forming callus and organ, as well as callus characteristics, were analyzed descriptively. Meanwhile, the callus fresh weight parameter was statistically analyzed using one-way ANOVA followed by the Tukey test (Minitab 19 software).

RESULTS AND DISCUSSION

Percentage of callogenesis, organogenesis, and callus morphology

The explants responded to the combination of 2,4-D and BAP, as evidenced by callus formation

in twelve treatments, achieving a 100% success rate in treatments A0B1, A0B2, A0B3, A1B1, A1B2, A1B3, A2B1, A2B2, A2B3, A3B0, A3B1, and A3B2 (Table 2). In contrast, callus formation was absent in four treatments—A0B0 (Control), A1B0, A2B0, and A3B3. Explants exhibiting organogenesis at a 100% rate were observed in treatments A0B1, A0B2, and A0B3, while the remaining 13 treatments did not show organogenesis. BAP, a cytokinin, plays a critical role in stimulating cell division. Cytokinin, as PGR, synergizes with auxins to induce callus formation (Hemmati et al., 2020). The application of BAP and 2,4-D as growth regulators positively influences callus development by promoting cell division and elongation through antagonistic, additive, and synergistic interactions, ultimately enhancing cell growth (Castro et al., 2016; Zarbakhsh et al., 2024).

All explants treated with BAP alone at 0.5, 1, and 1.5 mg l^{-1} (BAP A0B1, A0B2, and A0B3) showed 100% of callogenesis and organogenesis responses (Table 2). The callus of these treatments exhibited a friable texture and a yellowish-white color. The organogenesis presented in treatment A0B1 occurred indirectly (Figure 1), as evidenced by the formation of plant organs such as shoots, leaves, and roots after the explants had regenerated into the callus. Conversely, treatments A0B2 and A0B3 demonstrated direct organogenesis, where the explants formed shoots directly at the base of the leaves. The development of shoots in *in vitro* culture is a critical factor in the successful production of numerous, uniform seedlings within a relatively short timeframe. A greater number of shoots results in increased seedling production through tissue culture techniques (Zarbakhsh et al., 2024).

In this study, most shoots emerged from the cut base of the leaves, an area containing an abscission zone. This zone is characterized by thinner cell walls compared to those in the leaf blade and petiole. Consequently, cells at the leaf base are more responsive to nutrient and PGR uptake from the callus induction medium, facilitating the formation of callus and shoots at the base more readily than in other parts of the explant (Bhat et al., 2024). For propagation purposes, indirect organogenesis offers the advantage of generating a large mass of callus, which can be subdivided into multiple culture containers. However, this method requires additional effort, as the callus must be subcultured onto various media, such as root initiation and shoot induction media, demanding greater maintenance. In contrast, direct organogenesis enables the direct emergence of plant organs (shoots, roots, and leaves) from the explants without passing through a callus formation phase or requiring sub-culturing onto specific media, allowing for direct acclimatization. However, the number of shoots produced via direct organogenesis is limited and requires more time to develop (Bansal et al., 2024).

In addition to plant genotypes, physiological factors such as meristematic growth capacity and the developmental status of cells or tissues significantly influence the success of bud regeneration. These factors are closely associated with cellular metabolism, the availability of endogenous PGRs, and the activity of genes regulating growth and development (Remakanthan et al., 2014). Nevertheless, not all plant cells showed positive responses to PGRs. In some cases, cellular responsiveness is restricted to specific stages within the plant growth cycle (Gurav et al., 2020). The exogenous application of BAP and 2,4-D, particularly with a higher concentration ratio of BAP, resulted in a 100% shoot formation rate. This outcome can be attributed to the role of BAP in cell differentiation, promoting shoot growth and axillary bud proliferation. In addition, BAP also inhibits root formation. Other reports showed that an appropriate concentration of BAP is highly effective in stimulating natural shoot

Table 2. Effect of combined 2,4-D and BAP on callogenesis and organogenesis frequencies of patchouli *in vitro* culture after 60 DAP

Treatment	Callogenesis percentage (%)	Organogenesis percentage (%)
A0B0	0	0
A0B1	100	100
A0B2	100	100
A0B3	100	100
A1B0	0	0
A1B1	100	0
A1B2	100	0
A1B3	100	0
A2B0	0	0
A2B1	100	0
A2B2	100	0
A2B3	100	0
A3B0	100	0
A3B1	100	0
A3B2	100	0
A3B3	0	0

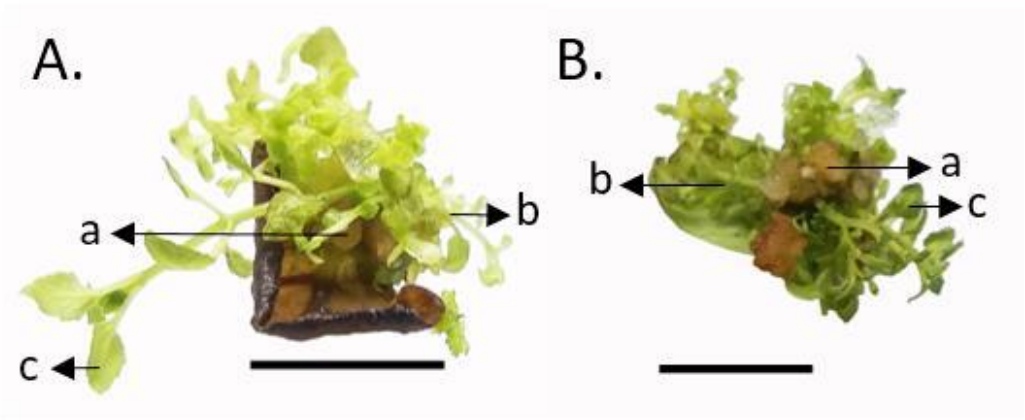

Figure 1. Indirect and direct organogenesis of patchouli *in vitro* culture after 60 DAP, A = A0B1 (indirect organogenesis), B = A0B3 (direct organogenesis)
Note: a = Callus; b = Shoot; c = Leaf. Bar scale = 1 cm

multiplication and organogenesis (Sagai et al., 2016; Suminar et al., 2017). It is also noteworthy that treatments involving the application of BAP without 2,4-D achieved a 100% shoot formation rate. This finding demonstrates that plant organs and tissues might possess endogenous hormones capable of driving growth and development to completion, even in the absence of externally applied growth regulators (Marković et al., 2023).

The results of this study demonstrated that single BAP treatments at concentrations of 0.5, 1, and 1.5 mg l^{-1} effectively induced shoot formation. Similar findings were reported in a study on *Curcuma* explants, where the absence of 2,4-D and the addition of BAP successfully stimulated shoot formation (Waryastuti, 2017). BAP has been widely recognized for its efficacy in promoting *in vitro* shoot proliferation in various plant species, including mangosteen (*Garcinia mangostana*), citrus (*Citrus* spp.), papaya (*Carica papaya*) (Litz and Jaiswal, 1991), and banana (*Musa acuminata* × *balbisiana*) (Imelda, 2008; Muthi'ah et al., 2023).

The calluses produced in the A0B1 treatment exhibited a friable texture and yellowish-white coloration and were predominantly regenerated into shoots (Figure 2). In contrast, treatments A0B2 and A0B3 showed callus formation after bud emergence, with similar friable texture and yellowish-white coloration (Table 3).

Callus texture serves as a critical indicator of callus quality, reflecting whether the cells remain actively dividing or have stagnated in division. High-quality callus typically has a friable texture, which is soft and composed of loosely connected cells with ample intercellular space. This structure facilitates the separation of cells into single-cell suspensions in culture and improves oxygen aeration among cells. Conversely, a compact callus, with tightly connected cells, is challenging to separate (Muthi'ah et al., 2023). Several factors influence callus texture, including the plant species used as explants, the composition of the culture medium, growth regulator concentrations, and environmental conditions. The friable texture observed in callus is primarily attributed to the action of the 2,4-D, which promotes cell elongation by increasing cell wall plasticity. This process allows water to enter the cell walls via osmosis, leading to cell elongation. Friable

Table 3. Callus morphology observed in patchouli *in vitro* culture using combined 2,4-D and BAP concentration after 60 DAP

BAP (mg l^{-1})	2,4-D (mg l^{-1})			
	0	0.5	1	1.5
0.0	-	-	-	Compact (Brownish)
0.5	Friable (Yellowish white)	Compact (Brownish)	Compact (Brownish)	Compact (Brownish)
1.0	Friable (Yellowish white)	Compact (Brownish black)	Compact (Brownish black)	Compact (Brownish)
1.5	Friable (Yellowish white)	Compact (Black)	Compact (Brownish black)	-

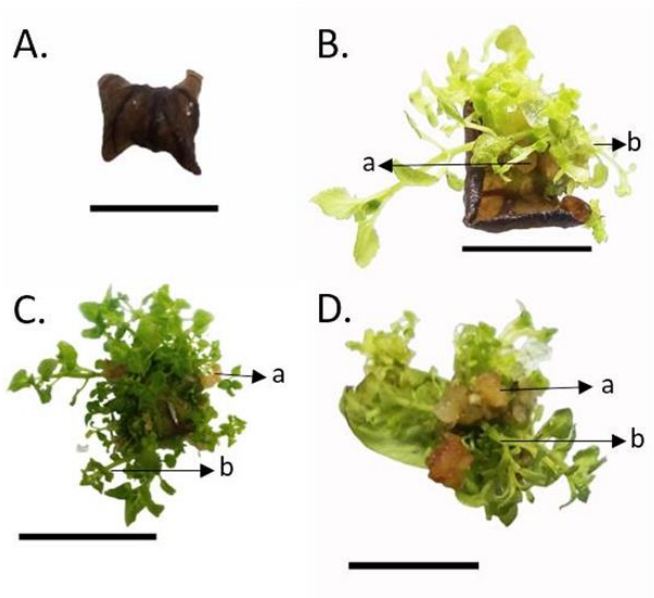

Figure 2. Callogenesis and organogenesis responses of patchouli in vitro culture after 60 DAP, A = A0B0 (Control), B = A0B1, C = A0B2, D = A0B3
Note: a = Callus; b = Shoots. Bar scale = 1 cm

callus typically contains a high water content due to the absence of lignified cell walls, enabling easy separation of individual cells within the cell cluster (Muzika et al., 2024).

In treatments A0B1, A0B2, A0B3, A1B1, A1B2, A1B3, A2B1, A2B2, A2B3, A3B0, A3B1, and A3B2, organogenesis was absent, while callogenesis reached 100%, producing calluses with a compact texture and brown coloration. The high callogenesis percentage observed in these treatments is likely attributed to the interaction between endogenous and exogenously applied hormones, which promoted callus growth. Callus formation in plant *in vitro* cultures depends on the balance of PGRs within the explants, as well as the type of explants and plant species used. The supplementation of auxin and cytokinin in the culture medium influences the endogenous hormone concentrations, with a balanced combination of these PGRs yielding favorable results for callus formation through their roles in cell enlargement and division (Suminar et al., 2017; Sosnowski et al., 2023). Callus induction is often initiated by mechanical wounding, such as cutting or slicing explants, which triggers a physiological response to both exogenous and endogenous growth regulators (Bhat et al., 2024).

In treatments A1B1, A2B1, A3B0, A3B1, and A3B2, calluses exhibited a brown coloration, whereas treatments A1B2, A2B2, and A2B3 produced calluses with a blackish-brown morphology (Figure 3). In treatment A1B3, the calluses displayed a distinct black coloration, which is influenced by the concentration of 2,4-D. At low concentrations, 2,4-D may promote the hormesis effect. This phenomenon occurs when a substance like 2,4-D stimulates biological activity at low concentrations but exhibits toxic effects at higher levels (Bondy, 2023). At low 2,4-D concentrations, the auxin hormone stimulates growth and metabolic processes in plant cells. Conversely, high concentrations of 2,4-D have been shown to have an adverse impact on callus induction when derived from root, internodal, and leaf explants, its toxic effects dominate, inhibiting cell growth and differentiation which lead to a decline in callus development (Teoh et al., 2023). Moreover, an excessive amount of 2,4-D can decrease the rate of callus development, as *in vitro* totipotency is influenced by genes that regulate hormone levels in the cells and their sensitivity to those hormones (Carsono et al., 2021).

The accumulation of ethylene, a natural plant hormone, due to excessive auxin-like (2,4-D) in the culture media often leads to culture browning (Rahayu et al., 2016). Additionally, factors such as the presence of secondary metabolites, prolonged incubation without subculturing, and callus age contribute to browning and necrosis in cultured tissues (Budisantoso et al., 2017). Under the growth inhibition mechanism, high concentrations of 2,4-D not only directly impede callus or tissue growth but also induce oxidative stress in plant tissues, leading to the excessive

accumulation of phenolic compounds. These compounds may be produced as a defensive response to the stress caused by 2,4-D. This can result in the discoloration of the callus, often observed as a blackish-brown coloration, which could indicate tissue damage or senescence (Budisantoso et al., 2017). Consequently, calluses grown in high 2,4-D concentrations exhibit more brownish coloration compared to those exposed to low concentrations. The effects of 2,4-D on phenolic compound accumulation vary depending on factors such as plant species, tissue type, and specific culture conditions. Additional variables, including plant cultivar, culture medium composition, and culture duration, also significantly influence phenolic compound accumulation in callus tissues (Sukamto, 2011).

Callus emergence time

Callogenesis primarily occurs at the exposed edges of the explant after planting. This process is triggered by mechanical injury to the plant tissue, leading to callus formation as the wounded area undergoes healing. The development of callus at the injury site serves as a key indicator of plant growth in *in vitro* culture system. The ANOVA test revealed that the combination of 2,4-D and BAP concentrations significantly influenced the time of callus appearance in patchouli leaf explants ($p \leq 0.05$) (Figure 4). Different concentration combinations resulted in varying callus initiation times. Further analysis using the Tukey test showed that treatments A1B1 and A0B2 had significantly faster callus appearance times compared to other combinations, with an average of 24 DAP. In contrast, the A0B3 treatment exhibited the longest callus appearance time, averaging 40.5 DAP. No callus formation was observed in treatments A0B0 (Control), A1B0, A2B0, and A3B3.

The A1B1 treatment, with an average callus appearance time of 24 DAP, likely benefited from a balanced ratio of auxins and cytokinins conducive to *in vitro* culture. Auxins, such as 2,4-D, are known to enhance cell division, which accelerates callus formation. Conversely, the delayed callus formation observed in the A0B3 treatment (40.5 DAP) may be attributed to the explants prioritizing bud formation. Studies suggest that higher concentrations of BAP often promote bud development at the expense of callus formation in some tissue cultures (Yin et al., 2025).

This phenomenon can be influenced by the higher concentration of BAP in the culture medium, which tends to prioritize bud growth and directly stimulate organ formation. At lower hormone concentrations, callus formation may be more prevalent. The interaction of BAP with auxins, such as 2,4-D, can profoundly affect the overall culture response, with certain hormonal combinations favoring bud growth over callus formation. Additionally, the hormonal response may vary depending on the plant species and tissue type used in the culture system.

Callus fresh weight

The statistical analysis indicated that the combination of 2,4-D and BAP concentrations significantly influenced the callus fresh weight (Figure 5). In addition, the results also revealed that callus fresh weight obtained from A1B1, A1B2, A0B3, and A2B2 treatments were not statistically different. Similarly, treatment A1B3 showed no significant difference from A2B3, while A2B1 was comparable to A3B0 and A0B2.

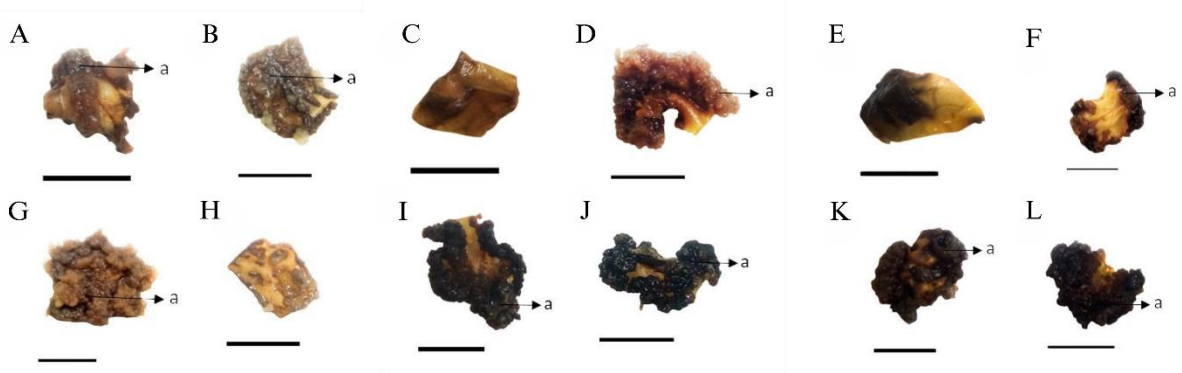

Figure 3. Callogenesis and organogenesis responses of patchouli in vitro culture after 60 DAP, A = A1B0, B = A1B1, C = A2B0, D = A2B1, E = A3B0, F = A3B1, G = A1B2, H = A0B3, I = A2B2, J = A1B3, K = A3B2, L = A3B3
Note: a = Callus. Bar scale = 1 cm

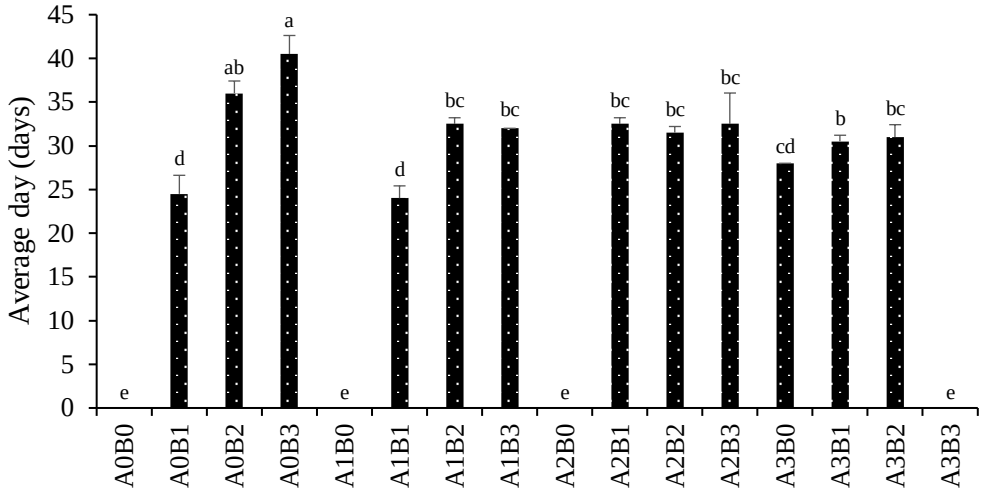

Figure 4. Callus appearance time at various combinations of 2,4-D and BAP
Note: The means indicated with the same letters are not statistically significant according to Tukey's HSD test at $p < 0.05$

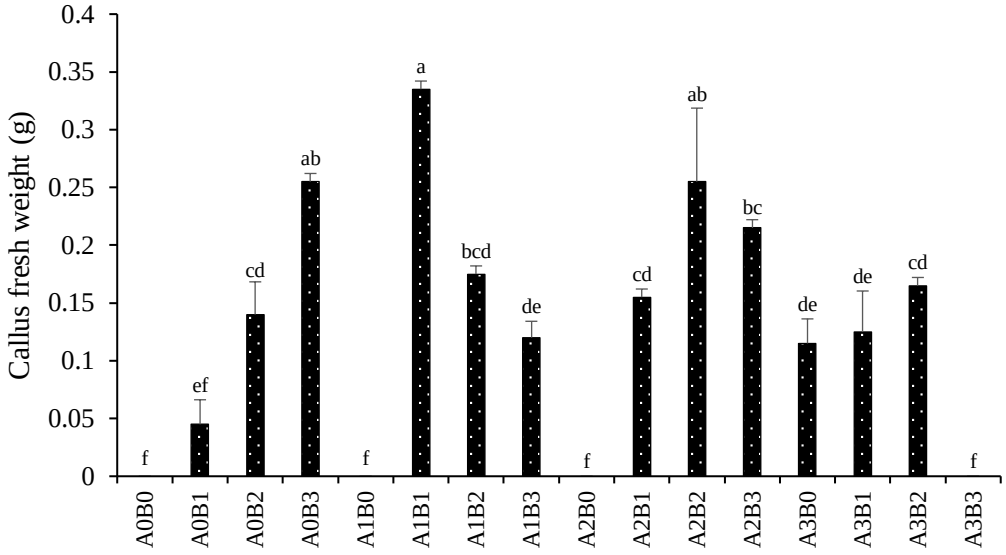

Figure 5. Effect of 2,4-D and BAP combination on callus fresh weight
Note: The means indicated with the same letters are not statistically significant according to Tukey's HSD test at $p < 0.05$

Moreover, no significant difference was observed between treatments A2B0 and A3B3. The highest fresh weight was recorded in treatment A1B1, with an average of 0.335 g, indicating high water content. The increase in callus fresh weight is also presumed to result from the optimal concentrations of 2,4-D and BAP provided. The addition of BAP in combination with auxin facilitates continuous cell division and development. When the concentrations of these two hormones are nearly balanced, cell mass continues to increase (Jadid et al., 2024a). Optimal cell division leads to the robust growth of the callus, consequently increasing its fresh weight (Kruglova and Zinatullina, 2024). Furthermore, it is worth noting that the increase in callus weight is closely linked to water content, the rate of cell division, and cell enlargement. The processes of cell division and enlargement result in an increase in both the number and size of cells, thereby enhancing callus weight.

In contrast, treatment A0B0 (Control) exhibited no callus formation. It is likely because no exogenous growth regulators were applied. As a result, the endogenous auxin and cytokinin concentrations were insufficient to stimulate cell division (Jadid et al., 2024a). The minimal fresh weight in this treatment is attributed to the lack of explant development and the occurrence of browning. Physiological death and

browning in explants lead to tissue mortality. Browning is caused by the metabolism of phenolic compounds, which are toxic and can inhibit growth or even cause tissue death. This phenomenon signifies the physiological deterioration of the explants (Liu et al., 2024).

The variation in callus weights reflects differences in the water absorption capacity of plant cells. Fresh weight is also influenced by callus texture; friable callus promotes rapid cell division, leading to increased callus mass and weight (Liu et al., 2024). Differences in callus fresh weight may arise from varying conditions experienced by individual calluses during growth. The increase in callus weight is primarily attributed to cell division, which increases the number of cells (Zhai et al., 2025). Growth rates also depend on the tissue's ability to absorb available nutrients, which is significantly influenced by aeration and callus texture. Dense and compact calluses exhibit lower nutrient absorption capacity compared to those with less compact textures (Rybin et al., 2024).

Cytokinins accelerate cell division by activating CDKs (*cdc2, cdk4, cdk6*) through interactions with specific cyclins. They also enhance *cdc2* and *CyD3* transcription, promoting cell proliferation (Ariani et al., 2016). Meanwhile, auxins induce cell elongation and increase fresh weight by enhancing water absorption (Rusnak et al., 2024). These hormonal effects on cell proliferation and elongation are consistent with findings on callus fresh weight per explant in half-seed cultures of velvet beans, which varied across treatments. This variation may be attributed to differences in the responsiveness of individual explants in forming callus, as well as their physiological state. Some explants released dark water droplets during culture, leading to medium discoloration from brown to black. Permadi et al. (2024) reported that certain plant species release phenolic compounds *in vitro*, which may undergo oxidation and cause browning. This phenomenon suggests that individual explants have varying tolerance thresholds to oxidative stress (Ariani et al., 2016).

CONCLUSIONS

The combination of 2,4-D and BAP significantly affects callus induction and growth parameters, including callogenesis, organogenesis, callus morphology, and fresh weight. The optimal treatment, A0B3 (0 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BAP), successfully achieved 100% callogenesis and organogenesis, producing calluses with desirable friable texture and yellowish-white coloration. These results provide valuable insights into the synergistic roles of cytokinin and auxin in patchouli callus induction and their implications for *in vitro* propagation. This study also provides an optimal callus-inducing culture medium for further scale-up exploration of patchouli oil production using *in vitro* culture approaches. Additionally, investigations into the biochemical composition of callus-derived oils, optimization of culture conditions for large-scale production, and the genetic stability of regenerated plants would further enhance the commercial viability of this approach.

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