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Effect of BAP and 2,4-D Combination on the Callus Induction of Robusta Coffee (*Coffea canephora* Pierre ex A.Froehner) Leaf Explants

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Abstract

Background: *Sustainable production of Robusta coffee (Coffea canephora) is constrained by the limited availability of high-quality planting materials, while information regarding the optimal auxin–cytokinin combinations for initiating callus cultures remains insufficient. Accordingly, this study was conducted to examine the effects of combined applications of benzyl amino purine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction and subsequent growth derived from young leaf explants of Robusta coffee.* **Methodology:** *Explant cultures were established on Murashige and Skoog (MS) medium enriched with different concentrations of 2,4-D (1, 1.5, and 2 ppm) in combination with BAP (1.5, 2, and 2.5 ppm), arranged in a completely randomized design with three replications. Data collection was conducted throughout a 30-day incubation period and included measurements of callus initiation time (days), callus formation percentage, callus growth intensity, and callus morphological features. The resulting data were analyzed statistically using analysis of variance (ANOVA), followed by the Kruskal–Wallis test.* **Findings:** *Result describe that the treatment combining 2,4-D at 1 ppm with BAP at 1.5 ppm produced the most favorable response, as indicated by the shortest callus initiation period (7.33 DAP). In contrast, the application of 2,4-D at 1 ppm in combination with BAP at 2.5 ppm achieved a 100% callus induction rate and the highest callus growth intensity (2 on a 5-point scale), along with a compact callus exhibiting a yellowish-green coloration. Overall, these results highlight the critical influence of auxin–cytokinin equilibrium in enhancing callus induction in Robusta coffee and underscore its significance as a foundational reference for establishing an efficient in vitro propagation strategy.* **Contribution:** *In general, this study contributes to providing important technical information supporting the large-scale and sustainable production of high-quality Robusta coffee seedlings.*

Keywords: *2,4-D; Benzyl Amino Purine; Callus Induction; In Vitro Culture; Coffeea canephora*

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INTRODUCTION

As a major plantation commodities worldwide, including in Indonesia, coffee (*Coffea* sp.) plays a substantial role in supporting national economic growth and international trade activities. Robusta coffee (*Coffea canephora*) accounts for more than 80% of Indonesia's coffee production due to its higher yield potential and resistance to leaf rust compared to Arabica. However, there are still challenges in terms of providing consistently high-quality, disease-free seeds which are more adaptive to environmental changes. Conventional propagation methods, such as seed germination and vegetative cuttings, are often limited by low efficiency, high variability, susceptibility to disease, and long production times (Sun et al., 2024). Consequently, these constraints impede both the development of scientific knowledge on propagation and the practical implementation of efficient mass propagation system for sustainable Robusta coffee seedlings production.

In response to these constraints, in vitro culture has been recognized as an effective approach for the large-scale propagation of coffee plants. This method provides several benefits, including uniform plantlet production, minimized risk of pathogen dissemination, and the ability to regulate growth conditions under controlled environments conditions (Hasnain et al., 2022; Méndez-Hernández et al., 2023;Restiani et al., 2022). During the regenerative phases of in vitro culture, callus induction is considered a pivotal step, as callus represents an aggregate of de-differentiated, totipotent cells capable of developing into complete plants. The success of callus formation is strongly influenced by the type, concentration, and combination of plant growth regulators (PGRs), with auxins and cytokinins playing a particularly important role (Avilez-Montalvo et al., 2022; Habibah et al., 2023; Kaban et al., 2024).

Auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), are known to promote dedifferentiation, expansion, and proliferation of cells, thereby triggering callus formation (Robles-Martínez et al., 2016; Rybin et al., 2024). Meanwhile, cytokinins, such as Benzyl Amino Purine (BAP), stimulate cell division, support proliferation, and prevent early differentiation of callus tissue (Pasternak, 2024; Qiu et al., 2019). An appropriate balance between auxin and cytokinin is considered essential, as disproportionate levels may lead to direct organogenesis rather than callus proliferation (Saleem et al., 2022). Evidence from earlier investigations involving both Arabica and Robusta coffee indicates that an optimal combination of 2,4-D and BAP enhances the effectiveness and quality of callus induction (Guo et al., 2023; Rasud & Bustaman, 2020). However, the optimal concentration for Robusta leaf explants has not been explored.

Accordingly, The effects of different combinations of 2,4-D and BAP concentrations on callus induction from Robusta coffee leaf explants were investigated in this study. Specifically, assessments were conducted on callus initiation time, the percentage of explants that developed callus, the level of callus growth intensity, and the morphological characteristics of the resulting callus. These findings are expected to provide insights into optimizing Robusta coffee propagation techniques *in vitro*, supporting sustainable seedling production and long-term productivity in coffee cultivation.

METHOD

This present study used a complete randomized factorial design to evaluate the combination effects of BAP and 2,4-D on callus initiation time (DAP), percentage of callus initiation (%), the intensity of callus growth, and callus morphology of *in vitro* leaf explants of robusta coffee (*Coffea canephora*) plants with three replicates.

Research Variables

Independent Variables: Concentration of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Benzyl Amino Purine (BAP). Control variables was Environmental conditions and growth media, and Dependent Variables: Callus growth (%), Callus initiation time (DAP), and callus growth intensity.

Treatments,

Control = MS medium + ascorbic acid, without the addition of 2,4-D and BAP.

D (2,4-D concentration)

D1 = 2,4-D concentration 1 ppm

D1.5 = 2,4-D concentration 1.5 ppm

D2 = 2,4-D concentration 2 ppm

B (BAP concentration)

B1.5 = BAP concentration 1.5 ppm

B2 = BAP concentration 2 ppm

B2.5 = BAP concentration 2.5 ppm

Table 1. Variations in PGRs Concentration

	Treatment			
	Control (B0)	**B1.5**	**B2**	**B2.5**
Control (D0)	Control (D0 B0)			
D1		D1 B1.5	D1 B2	D1 B2.5
D1.5		D1.5B1.5	D1.5B2	D1.5B2.5
D2		D2 B1.5	D2 B2	D2 B2.5

Number of repetitions (n) for each treatments and control : 3 times

Materials

The equipment used for this research included an autoclave, aluminum foil, culture bottles, bunsen burners, petri dishes, erlenmeyer flasks, beakers glass, measuring cups, stoves, lighters, Laminar Air Flow (LAF), pipettes, magnetic stirrers, stirrers, tweezers, droppers, measuring pipettes, pH sticks, scalpels, analytical scales, and ovens. The materials, including plant and chemical materials used in this study included young *C. canephora* leaf explants (PASTY) 2,4-Dichlorophenoxyacetic acid (2,4-D), agar, 70% alcohol, ascorbic acid, distilled water, Benzyl Amino Purine (BAP), liquid detergent, H_2O_2,

HCl, rubber, parchment paper, filter paper, labels, instant MS medium, 1N NaCl, plastic wrap, sucrose, tissue, Tween 80.

Plant Material

The plant material used in this study was obtained from Robusta coffee (*C. canephora*) plants collected at Pasar Satwa dan Tanaman Hias Yogyakarta (PASTY). The sampling process was carried out by selecting healthy plants that showed normal morphological growth without visible symptoms of pest or disease infection. From these plants, young leaves located at the upper part of the shoots were chosen because young tissues generally have higher meristematic cells with morphogenic potential and are more responsive to callus culture induction compared to older, fully expanded leaves.

Culture Media and Conditions

For in vitro culture, Murashige and Skoog (MS) basal medium was used to maintain the explants (Murashige & Skoog, 1962), which is formulated to supply essential macro- and micronutrients, vitamins, and a carbon source required for tissue growth. The basal medium was enriched with various combinations of plant growth regulators, consisting of 2,4-D applied at 1, 1.5, and 2 ppm and BAP at 1.5, 2, and 2.5 ppm. To minimize explant browning resulting from phenolic oxidation, a frequent limitation in coffee tissue culture, ascorbic acid was also incorporated into the culture medium. The cultures were maintained under controlled laboratory condition throughout the experiment with room temperature of 25 ± 2 ℃, 24-hour lighting and light intensity of 3000 lux.

Data collection

The observations were conducted during 30 days after planting. The observed parameters including: the time required for callus initiation, expressed in days after planting (DAP), and the proportion of callus initiation (%), which was assessed by determining the percentage of explants that were able to form callus successfully. Callus growth intensity was also evaluated using a scoring system ranging from 1 (very low growth) to 5 (very high growth), based on the extent of tissue proliferation. In addition, qualitative characteristics such as callus texture, color, and compactness were recorded to provide a more comprehensive description of callus morphology. These parameters were selected to obtain both quantitative and qualitative information on callus induction under different hormonal treatments.

Preparation of Media Using Ascorbic Acid

The MS media used for making 200 ml is 0.886 grams of Caisson instant MS media, which is weighed and then placed in an Erlenmeyer flask. After that, 6 grams of sucrose is added. Initially, 100 mL of distilled water was transferred into an Erlenmeyer flask and mixed using a magnetic stirrer until complete dissolution was achieved. The

required concentrations of BAP and 2,4-D were then incorporated into the solution, after which distilled water was added to obtain a final volume of 200 mL, followed by gentle agitation to ensure homogeneity. Finally, the pH of the medium was adjusted to approximately 5.7–5.8. Subsequently, 1.6 grams of agar is added to each Erlenmeyer flask and heated until the agar is completely dissolved. The homogenized medium is then sterilized using an autoclave for 15 minutes at a temperature of 121 °C with a pressure of 1 atm.

Place in a LAF chamber for 15 minutes, then aseptically weigh 0.05 grams of ascorbic acid using a 50 ml beaker, add 50 ml of sterile distilled water little by little until dissolved. Prepare culture bottles, micropipettes, stirrers, ascorbic acid solution, parchment paper, aluminum foil, rubber, and media. All tools must be sterile, then UV the LAF chamber again for 10 minutes. After the sterilization and UV irradiation processes are complete, add the ascorbic acid solution to the medium using a measuring pipette, then stir until thoroughly mixed. The prepared medium is then poured into each culture bottle in 20 ml portions, covered with aluminum foil and parchment paper, and secured with rubber bands. The prepared medium is incubated for 3–7 days.

Sterilization of Equipment

The equipment to be sterilized is prepared in advance, then sealed tightly with parchment paper. Next, the equipment is sterilized in an oven at 150 °C for 1 hour.

Media Sterilization

Materials such as media and distilled water are also prepared for sterilization. These materials are sealed tightly with aluminum foil and parchment paper, then sterilized using an autoclave at a temperature of 121 °C with a pressure of 1 atm.

Laminar Air Flow (LAF) Sterilization

The Laminar Air Flow (LAF) is first sterilized using 70% alcohol to ensure that the surface is free of contaminants. The sterilization process is carried out by wiping the entire work area, including the inner walls, tables, and areas around the filter, using a sterile cloth moistened with alcohol. Once completed, the UV lamp on the LAF is turned on to kill any microorganisms that may still remain in the work area. This process is allowed to run for 1 hour to ensure maximum sterilization before the LAF is used.

Preparation and Pre-Sterilization of *Coffea canephora* Explants

The sterilization process of *C. canephora* leaf explants begin with the preparation of the explants. The leaves are selected in fresh condition, without physical damage, and free from infection. The leaves are then cut into 1 cm x 1 cm pieces to facilitate handling during the sterilization process. The sterilization process begins by soaking the explants in running water for 20 minutes to clean any dirt attached to the leaf surface. Subsequently,

the explants were cleansed using a liquid detergent solution consisting of 3 mL detergent mixed with 27 mL distilled water and supplemented with three drops of Tween 80 for a duration of 5 minutes to eliminate residual debris and microorganisms. The explants were then rinsed with non-sterile distilled water for 1 minute, a procedure that was repeated three times. As a final step, a 1-minute rinse with sterile distilled water was carried out to ensure complete cleanliness of the explants prior to proceeding to the subsequent tissue culture stage.

Table 2. Chemical Pre-sterilization Method for *Coffea canephora* Leaf Explants

Plant Type	Explant Type	Pre-Sterilization Type	Concentration	Soaking Time (minute)
Robusta Coffee (*C. canephora*)	Young leaves explant of Robusta coffee (*C. canephora*)	Water flow		20
		Liquid detergent + *tween* 80	3ml/L	5
		Rinse using distilled water 3 times		1
		Rinse using distilled water 3 times		1

Sterilization of *Coffea canephora* explants in LAF

The pre-sterilized *C. canephora* explants are transferred to a sterile beaker for further sterilization. In a sterilized Laminar Air Flow (LAF), the explants were submerged in 70% ethanol for a duration of 2 minutes to inactivate any microorganisms remaining on their surfaces. Upon completion of the immersion period, the ethanol was carefully decanted into a separate Erlenmeyer flask to prevent contamination of the working area. Subsequently, the explants were gently rinsed three times with sterile distilled water to eliminate residual traces of alcohol.

Following surface sterilization with 70% alcohol, the explants were subjected to a second sterilization step using a 5% H_2O_2 solution for 10 minutes to eliminate residual surface microorganisms, as the oxidative action of hydrogen peroxide effectively inactivates bacterial and fungal contaminants without causing damage to plant tissues. The explants were subsequently rinsed slowly three times with sterile distilled water to remove any remaining H_2O_2 and to prepare them for the inoculation stage. The 5% H_2O_2 solution was prepared outside the laminar airflow (LAF) cabinet by adding 10 mL of H_2O_2 to 90 mL of distilled water in a 100 mL Erlenmeyer flask, with the procedure carried out

using gloves and a mask due to the corrosive properties of the solution, followed by thorough mixing to ensure homogeneity.

Inoculation of *Coffea canephora* explants

The inoculation process begins by preparing sterile Petri dishes lined with two sheets of filter paper to maintain the cleanliness of the explants. *C. canephora* explants that have undergone the rinsing stage are taken using sterile tweezers and transferred to the Petri dishes. Next, the explants are cut with a sterile scalpel into pieces measuring approximately 1 cm. The explant pieces are inoculated into the culture medium provided in the culture bottle. After all the explants are placed, the culture bottle is tightly sealed using aluminum foil and covered with plastic wrap to maintain sterile conditions.

Observation

Observation begins on the second day after inoculation. Explants are observed regularly every two days to evaluate various growth parameters. The observed variables comprised the duration of callus initiation expressed in days after planting (DAP), the proportion of callus formation expressed as a percentage, and the level of callus growth intensity as determined according to the scoring criteria presented in Table 3. In addition, morphological observations were described in detail to record changes in the shape, color, and texture of the explants during the observation process. All observation data were recorded in detail on a pre-prepared worksheet, accompanied by photographic documentation to facilitate further analysis and evaluation.

Data analysis

The influence of combined BAP and 2,4-D treatments on callus growth was evaluated using both descriptive and inferential statistical approaches. Callus initiation time data were processed with *IBM SPSS* Statistics version 25, beginning with a normality assessment at a significance threshold of 0.05. When the resulting significance value exceeded 0.05, the data were regarded as normally distributed and subsequently subjected to analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

In contrast, datasets yielding significance values below 0.05 were considered non-normally distributed and were therefore analyzed using the non-parametric *Kruskal–Wallis* test, with *Dunn's* test applied as a post hoc procedure to identify differences among treatments. At the conclusion of the experiment, the percentage of callus formation was determined by calculating the proportion of explants that successfully produced callus using the specified formula:

$$Percentage\ of\ callus\ (\%) = \frac{Number\ of\ explants\ forming\ callus}{Number\ of\ explants\ per\ treartment}\ X\ 100$$

Table 3. Scoring of callus growth intensity refers to Kaban et al., (2024)

Score	Description
1	The explant does not form callus
2	≤ 25% of the callus covers the explant
3	> 25% - 50% of the callus covers the explant
4	> 50% - 75% of the callus covers the explant
5	> 75% - 100% of the callus covers the explant

RESULT AND DISCUSSION

The Growth of *Coffea canephora* Callus

This study was performed to investigate the effect of plant growth regulators (PGRs), combination of cytokinin Benzyl Amino Purine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on the induction and growth of callus in *C. canephora* leaf explants. The explants were cultured on MS medium with several combinations of 2,4-D concentrations (1 ppm; 1.5 ppm; 2 ppm) and BAP concentrations (1.5 ppm; 1 ppm; 2.5 ppm), then observed for 30 days after planting. The results described that the treatment combining 1 ppm 2,4-D with 2.5 ppm BAP resulted in the most favorable callus growth response in the explants. Figure 1 illustrates leaf explants of *C. canephora* subjected to one of the 2,4-D and BAP combinations, serving as a representative depiction of morphological changes observed throughout the incubation period, from the initial state to the emergence of callus formation.

The growth of *C. canephora* callus on MS medium with the addition of 2.4-D 1 ppm and BAP 2.5 ppm showed clear stages of development during the 30 days of observation. In the early phase, from 2 DAP (Figure 1a) to 16 DAP (Figure 1h), the explants remained in the same form without significant changes, indicating that the cell dedifferentiation process had not yet occurred optimally.

Entering 18 DAP (Figure 1i), slight changes began to appear in the form of tissue thickening and texture changes on the edge of the leaf explant surface, which was an early indication of callus formation. The initiation of callus development in the explants was influenced by the application of plant growth regulators (PGRs), particularly those belonging to the auxin and cytokinin classes. Thickening of the explant at the cut surface (Figure 1i) that has undergone wounding indicates callus growth (Ikeuchi et al., 2013; Rasud & Bustaman, 2020; Wijaya et al., 2020). In general, callus formation in *in vitro* culture is largely determined by the relationship or balance between auxin and cytokinin.

Auxins such as 2,4-D are involved in inducing cellular dedifferentiation, a process through which somatic cells acquire increased plasticity and meristematic characteristics, thereby promoting active cell division (Habibah et al., 2018; Habibah et al., 2023;

Restiani et al., 2022). This phase represents an early step in the development of an undifferentiated callus mass.

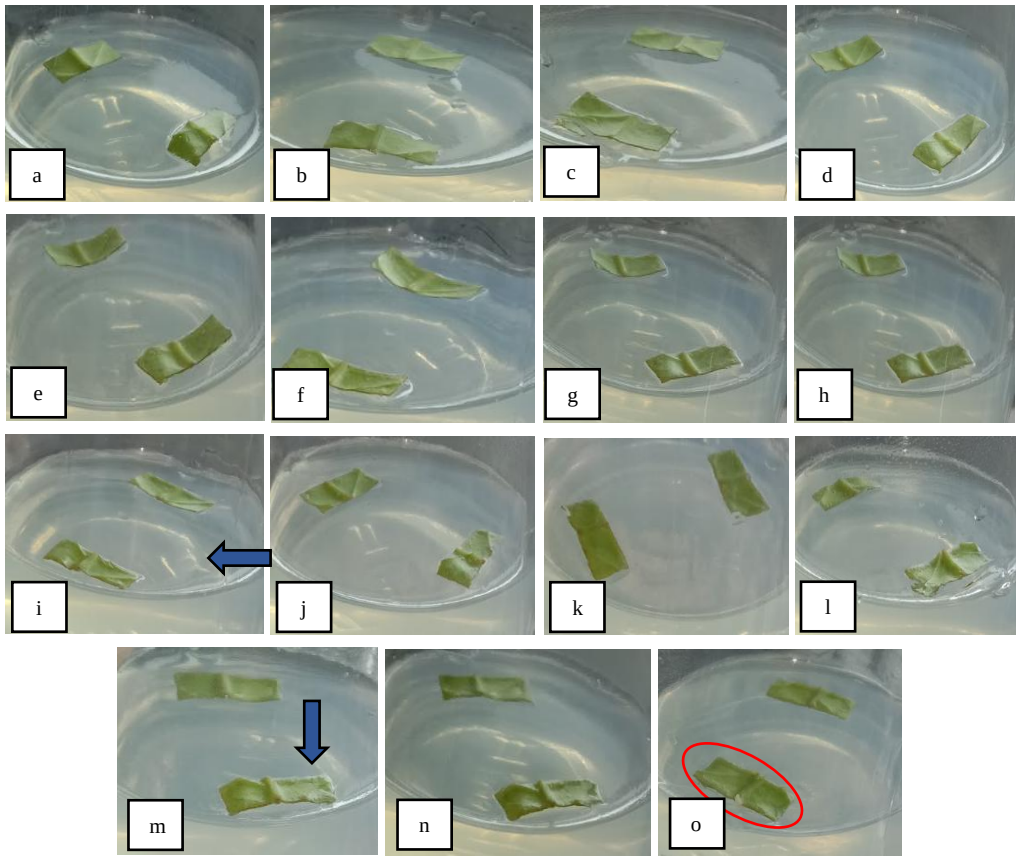


Figure 1. Growth of *C. canephora* callus on MS medium supplemented with 1 ppm 2,4-D and 2.5 ppm BAP. The explant condition was documented at sequential observation times: (a) 2 DAP, (b) 4 DAP, (c) 6 DAP, (d) 8 DAP, (e) 10 DAP, (f) 12 DAP, (g) 14 DAP, (h) 16 DAP, (i) 18 DAP, (j) 20 DAP, (k) 22 DAP, (l) 24 DAP, (m) 26 DAP, (n) 28 DAP, and (o) 30 DAP.
Notes: Blue arrows indicate callus formation along the margins of the leaf explants; Red circles denote callus formation at the midrib region of the leaf explants

In contrast, cytokinins, including BAP, function to support subsequent cell proliferation and division, sustain tissue growth, and regulate auxin dominance to prevent premature differentiation of callus into specific organs, such as roots or shoots (Yulia et al., 2020). The combined application of auxins and cytokinins has been shown to be essential in controlling explant development, as a relatively balanced hormonal ratio generally results in more optimal and stable callus proliferation (Saleem et al., 2022). In addition, gradual callus expansion was observed on both the right and left margins of the leaf explants between 20 and 26 days after planting (DAP), as illustrated in (Figure 1m),

but the color of the explants remained green and did not change. According to Avila-Victor et al., (2023), this phase reflects the active proliferation period in *C. canephora*, with callus growth generally occurring within 15 - 30 days depending on the PGR combination used. At the end of the observation period, from 28 DAP to 30 DAP, the formed callus appeared denser and more homogeneous, indicating that the cell division rate began to decrease and the tissue entered a stable phase (Guo et al., 2023; Purba et al., 2017).

Cultivation of *C. canephora* on MS medium supplemented with 1 ppm 2,4-D and 2.5 ppm BAP resulted in relatively optimal growth performance, as evidenced by the onset of callus formation at 18 days after planting (DAP), followed by vigorous proliferation during the 20–26 DAP interval and the development of a more compact and uniform callus toward the final observation phase (28–30 DAP). At these concentrations, the combined application of auxin and cytokinin was demonstrated to effectively induce cellular dedifferentiation, enhance proliferative activity, and sustain stable callus development.

Callus Initiation and Percentage of Formation

Callus formation on Robusta coffee leaf explants was initiated between 7.33 - 30 days after planting (DAP), and this variation depended on the concentration of plant growth regulators in the medium. The fastest callus formation (7.33 ± 12.70) was observed in the treatment combining 2,4-D at a concentration of 1 ppm with BAP at a concentration of 1,5 ppm (D1B1.5) and the highest percentage of callus (100%) and callus growth intensity (2 out of 5) was recorded under the treatment combining 1 ppm 2,4-D with 2.5 ppm BAP (D1B2.5). Conversely, callus induction was not achieved in the control treatment lacking plant growth regulators, thereby underscoring the essential contribution of externally supplied hormones in promoting cellular dedifferentiation and subsequent proliferation.

Table 4 The Effect of 2,4-D and BAP Concentrations on Growth Percentage and Callus Initiation Time During 30 Days Observation Period

Treatment	Percentage of Callus Formation (%)	Callus Initiation Time (DAP)
Control	0	-
D1 B1.5	33.33	7.33 ± 12.70[a]
D1 B2	100	27.33 ± 8.32[a]
D1 B2.5	100	30 ± 0[a]
D1.5B1.5	00.00	-
D1.5B2	66.67	10.66 ± 9.23[a]
D1.5B2.5	66.67	20 ± 17.32[a]
D2 B1.5	66.67	13.33 ± 15.27[a]

D2	B2	66.67	20 ± 17.32[a]
D2	B2.5	33.33	8.66 ± 15.01[a]

Note: Notations marked with the same letter indicate no significant difference between treatments in the Kruskal-Wallis test (p>0.05)

Prior to comparing treatment effects, callus growth intensity data were subjected to a Shapiro–Wilk normality test. The analysis indicated a non-normal distribution of the data ($p < 0.05$); therefore, subsequent evaluation was performed using the non-parametric Kruskal–Wallis test. The results revealed no statistically significant effect of the treatments on DAP values ($p = 0.068 > 0.05$), indicating that callus growth intensity did not differ markedly among the various combinations of 2,4-D and BAP.

From a physiological perspective, variation in callus initiation timing is governed by the interaction between auxin (2,4-D) and cytokinin (BAP) in promoting cell division. Treatments D1B2 and D1B2.5 demonstrate that a moderate level of 2,4-D combined with a higher concentration of BAP provides adequate hormonal stimulation to accelerate cell division, which is reflected in increased DAP values. In contrast, concentrations that are too low or unbalanced tend to inhibit the response because the auxin–cytokinin ratio is not optimal.

The results of the observation of callus growth percentage showed variation between treatments. The control did not form callus (0%), in line with Sari et al., (2018), who reported that media without PGRs was unable to induce cell dedifferentiation. The highest percentages (100 %) were achieved in D1B2 and D1B2.5, with average growth rates of 27.33 ± 8.32 and 30 ± 0.00, respectively. This results supported previous reports by Blinstrubiene et al., (2020) and Chitphet et al., (2025) that the combination of a balanced auxin–cytokinin ratio resulting in the optimal proliferation of callus.

In contrast, treatments such as D1B1.5 and D2B2.5 only reached 33.33 %, while D1.5B2, D2B1.5, and D2B2 reached 66.67 %, and D1.5B1.5 showed no growth (0 %). In treatments with low responses, there was no swelling or new tissue formation until day 30, presumably due to an imbalance in the auxin-cytokinin ratio. High concentrations of 2,4-D can increase the production of ABA as a growth inhibitor, while low BAP reduces cell division. This is in line with Clapa et al., (2025), who stated that an inappropriate auxin–cytokinin ratio can inhibit callus formation and development.

Growth Intensity of *Coffea canephora* Callus

Figure 2 presents the mean intensity of *C. canephora* callus growth under different combinations of the growth regulators 2,4-D and BAP. The observational data indicate that callus growth intensity varied markedly across the various treatments involving 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyl amino purine (BAP). No callus development was detected in the control treatment lacking growth regulators or in the D1.5B1.5 combination. The D1B1.5, D2B1.5, D2B2, and D2B2.5 treatments had low values of 0.67, followed by the D1.5DB2.5 treatment at 1. The D1B2.5 treatment produced

the highest callus growth intensity of 2, followed by the D1B2 and D1.5B2 treatments at 1.33.

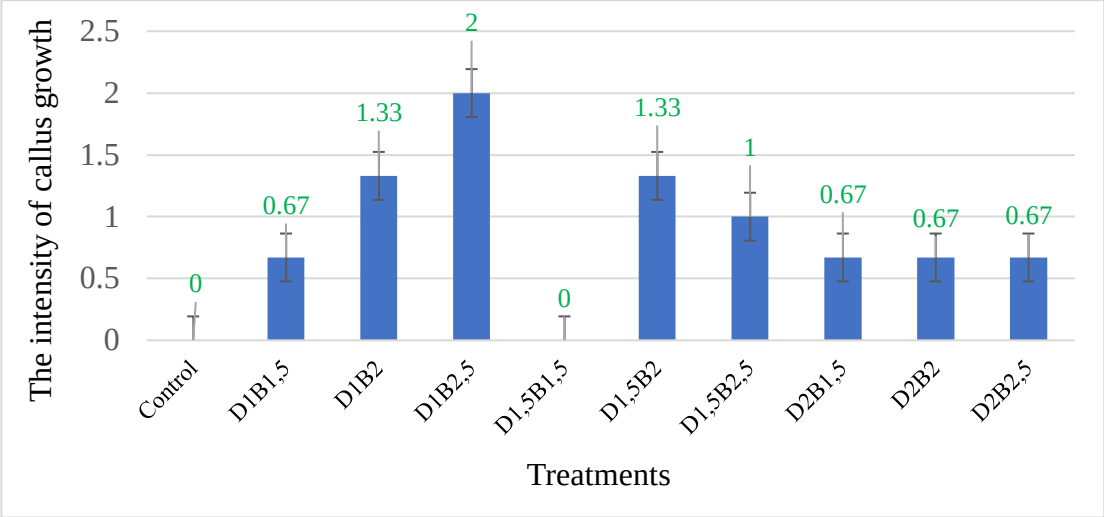


Figure 2. The Effect of 2,4-D and BAP Concentrations on the Growth of *C. canephora* Callus

Increased callus growth intensity in treatments with low concentrations of 2,4-D (1 ppm) combined with high doses of BAP (2.5 ppm) resulted in the highest performance in *C. canephora*, indicating an effective combination of auxin and cytokinin in stimulating cell division and callus tissue formation, thereby optimizing callus growth. 2,4-D (auxin) acts as an inducer of cell division and callus mass formation by increasing meristematic division activity, while BAP (cytokinin) plays a role in stimulating cell division and regulating tissue differentiation (Eoh, 2021; Habibah et al., 2023). This is in line with the research by Avila-Victor et al., (2023), where a higher proportion of cytokinin compared to auxin tends to increase callus and embryogenic responses as well as regeneration output.

In contrast, an increase in 2,4-D concentration is not always followed by an increase in response, as there is an optimum point; too high a concentration can actually slow down initiation and reduce callus quality, for example, by becoming watery or less compact. This condition occurs because excessive 2,4-D levels can inhibit cell division and cause hormonal signal imbalances, including interactions with ethylene, which results in a decrease in regeneration competence (Ikeuchi et al., 2013; Wójcik et al., 2020). The lack of callus formation in the control treatment indicates that culture media lacking plant growth regulators (PGRs) are incapable of inducing cellular differentiation in *C. canephora* explants, thereby emphasizing the crucial role of PGRs, particularly auxins, in initiating callus development under in vitro conditions (Carsono et al., 2021; Sari et al., 2018). Moreover, the combined application of 2,4-D and BAP was shown to significantly influence the intensity of callus growth in *C. canephora*, with the most

favorable response observed in the D1B2.5 treatment. Thus, Selecting the right concentration of these two PGs is key to obtaining maximum callus formation results.

Observational data revealed that varying combinations of 2,4-D and BAP exerted a significant influence on the growth intensity of *C. canephora* callus, with the D1B2.5 treatment (1 ppm 2,4-D combined with 2.5 ppm BAP) producing the highest growth score (2). This was followed by the D1B2 and D1.5B2 treatments, each yielding a growth intensity of 1.33. In contrast, no callus development was detected in the control treatment or in several other combinations, including D1.5B1.5. These findings indicate that an appropriate balance between auxin and cytokinin is essential for enabling explants to undergo effective cell division and differentiation. Accordingly, the D1B2.5 combination may be regarded as the most effective treatment for maximizing callus growth, whereas treatments with disproportionate levels of 2,4-D or BAP warrant careful consideration due to their limited capacity to induce callus formation.

Morphology of *Coffea canephora* Callus

The growth of callus was observed by assessing two parameters, namely the growth intensity and morphology of the *C. canephora* callus that was formed. The effect of treatment on these two parameters was observed to determine the intensity of the callus and the characteristics and morphology of the callus produced, including its texture and color. The assessment of callus intensity was carried out using a scoring method (Figure 3 and Table 3), while callus morphology was observed based on texture and color. Both parameters were determined on the 30th day after planting.

(3a) (3b)

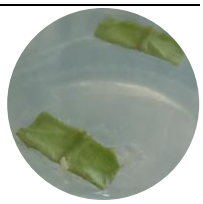
Figure 3. Growth Scoring of *C. canephora* Callus in Treatments D1B2 (3a), D1B2.5 (3b). Notes figure: bar scale 1 mm; Red circles denote callus formation

Figure 3 shows the best treatment from the variation in 2,4-D and BAP concentrations. On day 30, the explants showed callus formation with an intensity score of 2, covering most of the surface of the *C. canephora* leaves, yellowish-green in color, and compact in texture. In the 2.4-D 1 ppm treatment with BAP 2 ppm and 2.5 ppm, callus formed with low to moderate intensity, characterized as compact with colors varying from green to yellowish. This indicates that the combination is effective in inducing callus, although growth was not yet optimal on day 30. The difference in the concentration of

2,4-D and BAP in each treatment not only affected the growth rate of the callus, but also influenced the morphological characteristics of the callus formed. These morphological characteristics, such as the texture and color of the callus, can be seen in Table 5.

Observations show that *C. canephora* callus generally has a compact texture with colors ranging from yellowish green to greenish yellow. In treatments D1B1.5, D1B2, D1B2.5, and D2B2.5, compact yellowish-green callus was formed, indicating low photosynthetic activity. The green color indicates chlorophyll content, while the yellowish color indicates pigment degradation (Arimarsetiowati et al., 2023). In treatments D1.5B2, D1.5B2.5, D2B1.5, and D2B2, the callus was greenish-yellow in color with a compact texture. This color variation is related to hormonal balance that promotes cell proliferation with limited differentiation. Green callus generally has higher regeneration potential than yellow callus (Ashokhan et al., 2020; Kaban et al., 2024).

Table 5. Morphology of *Coffea canephora* Callus in Each Treatment for 30 DAP

Concentration of 2,4-D (ppm)	**Concentration of BAP (ppm)**		
	1,5	**2**	**2,5**
1			
	Compact, yellowish green	Compact, yellowish green	Compact, yellowish green
1,5	-		
	-	Compact, greenish yellow	Compact, greenish yellow
2			
	Compact, greenish yellow	Compact, greenish yellow	Compact, yellowish green

Compact callus is characterized by a dense texture with tightly packed nodular cells (Avila-Victor et al., 2023a). Its formation is influenced by 2,4-D as an auxin that triggers dedifferentiation and BAP as a cytokinin that maintains compact morphology (Habibah et al., 2023; Sari et al., 2018). A balanced auxin-cytokinin ratio produces compact callus with high embryogenesis potential (Di Bonaventura et al., 2024). On day 30, compact callus shows high stability because cell growth is in the exponential phase, resulting in dense tissue that does not easily turn into friable callus. This morphological stability is also supported by culture media with appropriate nutrients, PGRs concentrations, and solid texture (Avila-Victor et al., 2023b).

No callus development was observed in either the control or the D1.5B1.5 treatment, thereby emphasizing the essential role of plant growth regulators in initiating callus formation. A disproportionate hormonal balance, such as a relatively higher concentration of 2,4-D (1.5 ppm) combined with a lower level of BAP (1.5 ppm), is presumed to suppress cellular division, ultimately inhibiting callus induction. Consequently, an appropriate auxin–cytokinin ratio is critical for successful callus formation, with the D1B2.5 treatment identified as the most favorable combination for promoting *C. canephora* callus growth.

CONCLUSION

This study shows that the combination of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Benzyl Amino Purine (BAP) affects the initiation time, formation percentage, and growth intensity of callus in *Coffea canephora* leaf explants. The combination of these two plant growth regulators (PGRs) promotes callus formation and supports its growth in culture media, thereby playing an important role in the success of *in vitro* culture and serving as a reference for further research on the propagation of *C. canephora*. Analysis of the experimental data demonstrated that the combination of 1 ppm 2,4-D with 1.5 ppm BAP was the most effective in achieving the shortest callus initiation period (7.33 DAP). In contrast, optimal callus development in *C. canephora* was obtained with the application of 1 ppm 2,4-D combined with 2.5 ppm BAP, as evidenced by a 100% callus formation rate and the highest callus growth intensity score (2 on a 5 point scale). This treatment also produced compact, yellowish-green callus morphology that was stable at the 30 days of observation, indicating conditions that support optimal tissue growth.

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