

Callus Formation of *Coffea canephora* Induced with 2,4-Dichlorophenoxyacetic Acid and Tomato Extract Supplements

Andi Ilham Latunra¹⁾, Eva Johannes¹⁾, and Nurul Rifqah Fahira^{1*)}

¹⁾Department of Biology, Faculty of Mathematics and Natural Science, Hasanuddin University, Makassar, Indonesia

*Corresponding author: fahiranr19h@student.unhas.ac.id

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Abstract

Somatic embryogenesis is an effective approach for enhancing the production of *Coffea canephora* embryos that can grow into new plants. Plant growth regulators (PGRs) are necessary for initiating somatic embryogenesis. Due to its stability, 2,4 dichlorophenoxyacetic acid has become a common synthetic PGRs. PGRs can be obtained from natural ingredients, such as tomatoes. This study aimed to analyze the effect of 2,4-dichlorophenoxyacetic acid and tomato extract addition and its appropriate concentration for callus induction of Robusta coffee in vitro. This research was conducted at the Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Hasanuddin University. This study used a Complete Randomized Design with two factors. The first factor was the concentration of tomato extract (0%; 7.5%; 10%; and 12.5%). Meanwhile, the second factor was the concentration of 2,4 dichlorophenoxyacetic acid (0 ppm; 1 ppm; 2 ppm; and 3 ppm). Observation parameters include the percentage of callus formation, callus growing time, callus fresh weight, callus color, and callus texture. The quantitative data were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney Test to compare the effect of each treatment. The results showed that several treatments had best effect in Robusta callus induction, such as the application of 2,4 D with a concentration of 2 ppm and 3 ppm, as well as the addition of a combination of 2 ppm and 3 ppm 2,4-D with 10% tomato extract. Therefore, tomato extract is considered not to have a significant effect on the formation of Robusta coffee callus.

Keywords: Callus induction, Robusta coffee, organic, tomato extract

INTRODUCTION

Coffee is the most traded plantation commodity in the world. Coffee is one of the plantation commodities with the highest export value in Indonesia. Coffee marketing opportunities are high domestically and abroad (Sulistiyo *et al.*, 2023). The level of regional coffee consumption in Indonesia is also relatively high. During 2016-2021, Indonesia was predicted to experience an average increase in coffee consumption of 8.22% per year (Dewi *et al.*, 2020).

In Asia and Oceania, Indonesia is the largest coffee exporter after Vietnam. In the 2021/2022 period, Indonesia became the third country in the world Robusta coffee producer that contributed to the production of around 10,000 bags of 60 kg of coffee (ICO, 2023). The Indonesian annual coffee report recorded an increase in demand for Robusta coffee exports which was 2% higher than Arabica coffee types (USDA, 2023). Due to the advantageous geographic conditions of its cultivation, Indonesian Robusta coffee can be improved as a specialty coffee with distinct taste characteris-

tics (Kasim *et al.*, 2020; Ibnu & Rosanti, 2022). Robusta coffee is preferred with its stronger and bitter taste with high chlorogenic acid and caffeine content (Velásquez & Banchón, 2022)

However, Indonesia's Robusta coffee production decreased by 1.1% in the 2021/22 period compared to the previous period, due to strong winds and rains that occurred during the coffee flowering period (ICO, 2023). Generally minimal maintenance inputs such as the selection of planting material with poor quality, as well as limited fertilizers and plant protection materials also contribute to the decline in Robusta coffee production (USDA, 2023). It has been observed that the utilization of random seeds by the community for coffee cultivation tends to impact productivity levels negatively (Rismayanti & Nafi'ah, 2021). Meanwhile, Robusta coffee has self-incompatible traits that prevent its pollen from fertilizing the ovum (De Souza *et al.*, 2021). Consequently, cross-pollination becomes necessary for Robusta, resulting in significantly higher genetic diversity (Simon-Gruita *et al.*, 2019). In addition to the heterogeneous seeds, conventionally grown trees of this variety tend to yield lower, and it may take up to 20 years to develop a new cultivar (Campos *et al.*, 2017; Etienne *et al.*, 2018). Furthermore, several plant pest pathogens such as *Hypothenemus hampei*, *Zeuzera* sp., *Xylosandrus* sp., *Coccus viridis*, *Ferrisia virgata*, *Cercospora*, *Psychidae*, and *Pratylenchus coffeae* may interfere Robusta coffee production (Yulitaasary *et al.*, 2022; Faizin & Maghfiroh, 2023; Langkai *et al.*, 2023).

Somatic embryogenesis is the alternative of coffee micropropagation by using the totipotent properties of plant somatic cells to produce new plants without involving gamete fusion. This process can occur either directly or indirectly (Ardiyani & Pancaningtyas, 2017). Direct somatic embryogenesis involves the

use of explants with young tissue that contains more stem cells, resulting in up to 10 embryos per explant after approximately 70 days (Campos *et al.*, 2017). Indirect somatic embryogenesis, on the other hand, starts with the formation of a group of cells in the form of a primary callus on the explant, which is followed by the formation of an embryogenic callus, pro-embryonic mass, globular embryo, heart-shaped embryo, torpedo-shaped embryo, cotyledons, and plantlets (Ardiyani & Pancaningtyas, 2017; Etienne *et al.*, 2018). This process takes around 9–10 months and can produce hundreds of embryos per gram of callus (Campos *et al.*, 2017).

In plant propagation through in vitro cultivation methods, somatic embryogenesis has emerged as a highly successful technique. Somatic embryogenesis has revealed exceptional efficacy in increasing large-scale seed production of superior genotypes in crops with substantial economic significance (Islam, 2015; Yan *et al.*, 2020). Somatic embryogenesis is also a major prerequisite in the propagation of woody plant clones, as it can facilitate the development of individual cells into bipolar structures of roots and meristem buds simultaneously (Islam, 2015; Deb & Gangmei, 2017). The application of somatic embryogenesis in Robusta coffee allows the production of genetically identical plants from small amounts of leaf planting material, thus becoming an alternative to accelerate the production of superior seeds (Oetami, 2017; Fehér, 2019; Méndez-Hernández *et al.*, 2023).

Despite its various benefits, somatic embryogenesis faces general challenge related to the possibility of somaclonal variation, which causes changes in genotype and has the potential to change plant phenotype (Ibrahim *et al.*, 2018; Arimarsetiowati *et al.*, 2023). In somatic embryogenesis of coffee plants, the risk of somaclonal variation is influenced by the explant source, culture

period, plant genotype, and the use of growth regulators (Campos *et al.*, 2017). Previous research by Ibrahim *et al.* (2018), proved the existence of somaclonal variations due to massive cell division triggered by excessive administration of the growth regulator substance cytokinin. The doses of exogenous growth regulators also affect the development of callus. Therefore, plant growth regulators (PGRs) are necessary in determining the success of in vitro plant propagation. (Fitroh *et al.*, 2018).

The practice of somatic embryogenesis frequently involves the use of synthetic growth regulators. However, these chemical substances can pose potential environmental and human health risks. Improperly regulated concentrations of synthetic PGRs may result in cytotoxicity, unwarranted genetic variations, and morphological abnormalities that may inhibit in vitro multiplication (Kocaman & Güven, 2016; Özkul *et al.*, 2016; Dewir *et al.*, 2018). Natural-based PGRs have more advantages than synthetic PGRs. These advantages include more affordable material prices, easier to obtain materials, and effects that are not much different from synthetic plant growth regulators (PGRs) (Sari *et al.*, 2019).

Plant cultivation through tissue culture requires the availability of adequate nutrition in the media because the metabolic capabilities of the plant differ from those of field plants. Furthermore, when indirect somatic embryogenesis is applied, callus growth can be a lengthy process, and inadequate nutrition within the media may cause browning or other abnormalities (Abdalla *et al.*, 2022). Tomatoes are one form of the natural PGRs that can provide nutrients for culture plants. Tomatoes have a high nutritional value with the content of various important compounds such as carbohydrates (glucose, sucrose, fructose, raffinose, arabinose, xylose, galactose, and myoinositol), vitamin A, vitamin C, folate,

potassium, carotenoids (lycopene, β -carotene, α -carotene, γ carotene, δ -carotene, ζ -carotene, phytoene, phytofluene, and neurosporene), flavonoids (rutin, naringenin chalcone, quercetin, myricetin, and kaempferol), and phenolic acid (chlorogenic acid, caffeic acid, p-coumaric acid, and ferulic acid) (Wu *et al.*, 2022). Furthermore, tomato extract addition also can produce similar effects to the use of PGRs and natural anti-browning agents because of auxin, cytokinin, and antioxidant content (Helena *et al.*, 2022). Several previous studies have proven the positive effect of adding tomato extract on plant growth in vitro (Oktaviana *et al.*, 2016; Sari *et al.*, 2019; Serliana *et al.*, 2019; Agustin *et al.*, 2020; Dewi *et al.*, 2021; Maulidia *et al.*, 2022). Therefore, this research was conducted to find out appropriate concentration of 2,4-dichlorophenoxyacetic acid and tomato extract for in vitro callus induction of Robusta coffee.

MATERIALS AND METHODS

Explant and Media Preparation

The research was carried out in the tissue culture laboratory of Hasanuddin University from February 2023 to May 2023. The explants in this research came from the second to third young leaves of Toraja local Robusta coffee shoots. The explants were cultured on Murashige and Skoog (MS) media with 2,4 Dichlorophenoxyacetic acid (2,4-D) and tomato extract with different concentrations.

Red raw tomatoes thoroughly washed and cut into pieces. Then, 100 g tomatoes were blended with 100 ml of distilled water with 1:1 ratio. The obtained tomato extract was filtered using filter paper so that a tomato extract stock solution with a concentration of 100% was obtained. The concentration

Table 1. Treatment combination of 2,4-D and tomato extract

Tomato Extract (T)	2,4 Dichlorophenoxyacetic acid (D)			
	D ₀ 0 ppm	D ₁ 1 ppm	D ₂ 2 ppm	D ₃ 3 ppm
T ₀ 0%	0 ppm 2,4-D + 0% tomato extract	1 ppm 2,4-D + 0% tomato extract	2 ppm 2,4-D + 0% tomato extract	3 ppm 2,4-D + 0% tomato extract
T ₁ 7.5%	0 ppm 2,4-D + 7.5% tomato extract	1 ppm 2,4-D + 7.5% tomato extract	2 ppm 2,4-D + 7.5% tomato extract	3 ppm 2,4-D + 7.5% tomato extract
T ₂ 10%	0 ppm 2,4-D + 10% tomato extract	1 ppm 2,4-D + 10% tomato extract	2 ppm 2,4-D + 10% tomato extract	3 ppm 2,4-D + 10% tomato extract
T ₃ 12.5% (13,75 mL)	0 ppm 2,4-D + 12.5% tomato extract	1 ppm 2,4-D + 12.5% tomato extract	2 ppm 2,4-D + 12.5% tomato extract	3 ppm 2,4-D + 12.5% tomato extract

of each tomato extract in the treatment can be obtained by dilution as shown in the table.

Tomato extract had four different concentration levels (0%, 7.5%, 10%, and 12.5%). Meanwhile, 2,4-D was used with four levels of concentration (0 ppm, 1 ppm, 2 ppm, and 3 ppm). Combination concentrations of plant growth regulators (2,4-D and tomato extract) added to the culture medium are coded as shown in Table 1.

The combination of these two factors resulted in 16 treatment combinations that were repeated 3 times, then the total experimental data was 48 data. Media sterilization was done by autoclaving at 17.5 psi and at a temperature of 121 °C for 15 minutes, then stored in the culture room at 25 °C before being used to keep the media aseptic.

Explant Sterilization

Robusta coffee leaves were cleaned with 2 mL of liquid detergent and rinsed with running water. The leaves were sterilized with 2 g.l-1Dithane M-45 solution for 30 minutes, then rinsed with distilled water. In the LAF, the leaves were soaked in 70% alcohol for 3-5 minutes, then rinsed with distilled water. Next, the leaves were soaked in 20% calcium hypochlorite solution for 15 minutes. Next, the leaves were rinsed thoroughly using distilled water.

Callus Induction

The sterilized explants were cut into $\pm 1 \text{ cm}^2$ with a scalpel. Then, the explants were planted in bottles containing MS media containing 2,4-D and tomato extract with the leaf facing down, each culture bottle containing one explant. Bottles containing explants were stored in the incubation room at $\pm 25 \text{ }^\circ\text{C}$ with 60% relative humidity for two months in a dark room.

Observation Parameters

The parameters observed in this research include the percentage of callus formation, callus growing time, callus fresh weight, callus color, and callus texture. Explant ability in callus formation is calculated by the following formula:

$$\text{Callus formation percentage} = \frac{\text{total callus formed}}{\text{total explant of entire treatment}} \times 100\%$$

The callus growth time is calculated from the first day of callus appearance in the form of swelling or discoloration of the explant. The wet weight of the callus is obtained by weighing the callus that has been cleaned from the culture medium after 60 days of culture. The quantitative data, such as the percentage of callus formation, callus growing time, and callus fresh weight, were analyzed by the Kruskal-Wallis test, followed by the Mann-Whitney Test to compare the effectiveness of each treatment.

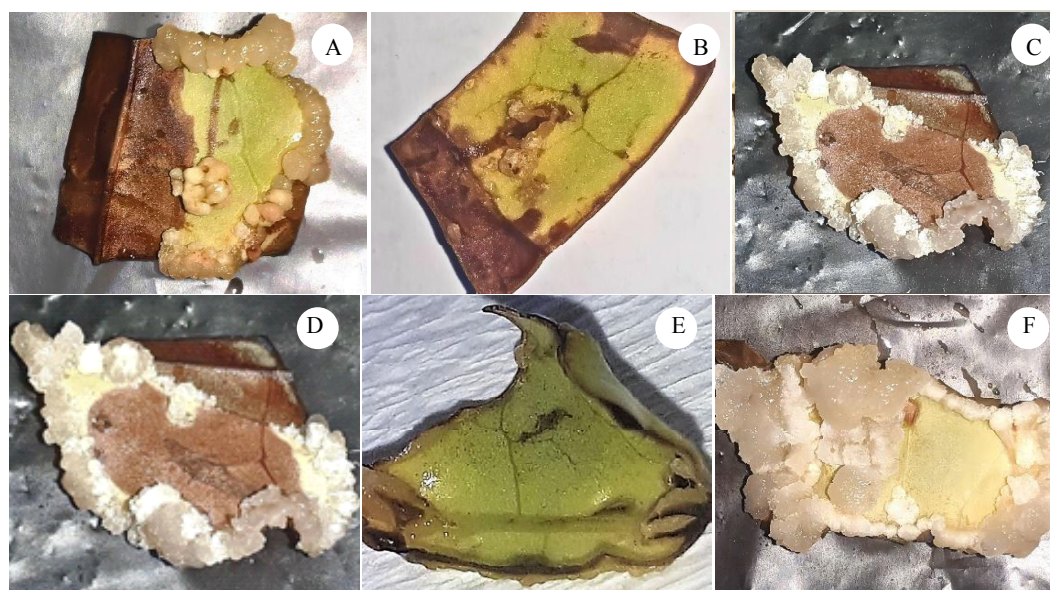


Figure 1. Callus color and texture of Robusta coffee callus 60 days after culture: Yellowish-white (A), brownish-yellow (B), brown (C), friable (D), compact (E), and intermediates (F)

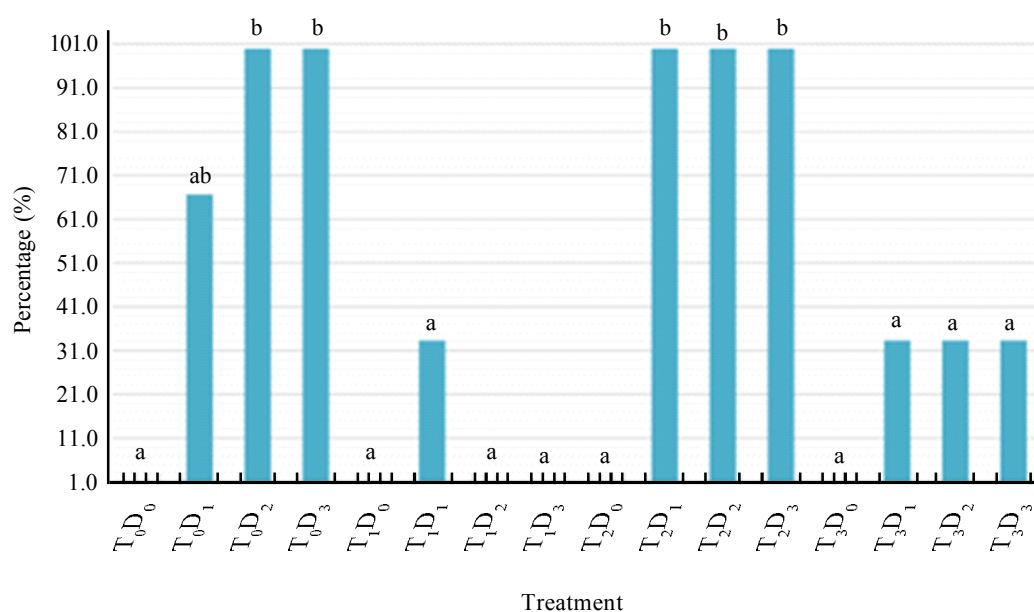
Meanwhile, qualitative data such as callus color and texture were obtained through visual observation with a magnifying glass at the end of the observation (60 days after planting). The color and texture of callus are indicator of the differentiation of embryogenic and non-embryogenic callus. Previous studies have revealed several differences in the morphological features between the two types of callus (Ardiyani, 2015; Ardiyani & Pancaningtyas, 2017; Campos *et al.*, 2017). Embryogenic callus, which exhibits efficient growth and optimal functionality, is characterized by a shiny or yellowish appearance and is composed of small, loosely arranged cells that resemble friable. Conversely, non-embryogenic callus that has undergone functional degradation typically manifests as brown and is composed of elongated cells that are clustered together, forming a dense and compact sponge-like configuration. The morphological characteristics of Robusta coffee callus are shown in Figure 1.

RESULTS AND DISCUSSION

Percentage of Callus Formation

During a 60-day observation period, we monitored the development of callus tissue from Robusta coffee explants. Our findings indicate that six treatments were unable to induce callus formation, four treatments had 33% callus formation, one treatment had 66% callus formation, and five treatments showed 100% capacity of callus formation. The average percentage of callus formation for each treatment is shown in Figure 2.

The results showed that treatments without any added plant growth regulators and those with independent addition of tomato extract (T_1D_0 , T_2D_0 , and T_3D_0) were unable to induce callus formation. This inability is assumed due to the unfulfilled need for auxin to induce callus. This is consistent with previous research on the Robusta coffee clone BP 308 conducted by Ibrahim & Hartati (2017), revealed the inability of explants to



* Values followed by the same letter in the same column show no significant difference according to the Mann-Whitney test at 5%.

Figure 2. Percentage of callus formation in Robusta coffee 60 days after culture

forming callus without the addition of exogenous auxin, even in the presence of cytokinins with intense activity, such as thidiazuron.

The application of tomato extracts with different concentrations (T_1D_1 , T_1D_2 , T_1D_3) is unable to induce callus formation, indicating the inefficacy of low concentrations of endogenous tomato hormones. Navarro & Munné-Bosch (2022) have researched the endogenous hormone content in ripe tomato fruit, revealing the presence of trans-zeatin type cytokinin hormones (5-10 ng/g dw), trans-zeatin riboside (60 ng/g dw), which is a direct precursor of trans-zeatin, and auxin IAA (20 ng/g dw). The low IAA content may decrease the effectiveness of callus induction, as it is generally carried out on media with high auxin concentrations (Asghar *et al.*, 2022). Furthermore, Takato *et al.* (2017) have reported that the natural auxin IAA tends to be thermolabile. Therefore, the

sterilization process at high temperatures can cause the degradation of IAA levels. Several previous studies have also indicated that the administration of endogenous auxin, such as 2,4-D can modify the IAA balance by either decreasing or increasing IAA levels (Agila *et al.*, 2015; De Castro Marcato *et al.*, 2017; Xu *et al.*, 2022). The decrease in IAA levels is assumed to be related to the ability of 2,4-D to stimulate the conjugation of endogenous IAA with aspartate, as well as a decrease in the YUC gene, which is significant in determining IAA levels (Islam, 2015; Takato *et al.*, 2017). The low level of auxin might cause problem as the appropriate ratio of auxin and cytokinin hormones is necessary for promoting callus induction and somatic cell differentiation (Baday, 2018; Junairiah *et al.*, 2018; Rismayanti & Nafi'ah, 2021; Arimarsetiowati *et al.*, 2022).

Meanwhile, the low callus formation in the treatment with a combination of 12.5% tomato extract and auxin 2,4-D (T_3D_1 , T_3D_2 ,

and T_3D_3) could be due to the supplemented tomato extract, and may increase the susceptibility to contamination. Prior research by Yulianti *et al.* (2016), reported the potential for contamination from bacteria or fungal spores which may still be found in organic extracts even though they have undergone sterilization. This explanation is corroborated by the findings of Heriansyah & Indrawanis (2020), who found a correlation between the high concentration of tomato extract and the increased potential for contamination in explants cultured in vitro. The outcome is primarily due to the vulnerability of leaf explants to microbial invasion via the epidermis and stomata.

The most successful callus formation achieved in the treatment with independently 2,4-D addition (T_0D_1 , T_0D_2 , T_0D_3) and treatment with 10% tomato extract combined with auxin type 2,4-D (T_2D_1 , T_2D_2 , and T_2D_3). Similar results were obtained by Lizawati *et al.* (2020), which demonstrated a 100% success rate for callus formation across all treatments, including a single treatment of 2,4-D without cytokinin. According to Oliveira *et al.* (2022), the auxin 2,4-D has been widely recognized for its effectiveness in initiating the callus induction stage in the somatic embryogenesis of coffee plants. The inducing ability of 2,4-D is related to its involvement in cell expansion and cell division during the S phase as well as the G2 to M phase transition.

Contrary to other treatment combinations using tomato extract, the concentration of 10% showed excellent callus induction ability. Tomato extract in appropriate concentration could be a promising supplement in promoting callus formation. Apart from the low dose of endogenous auxin content in tomatoes, tomatoes contain various compounds that are adequate for plant growth in vitro. Ascorbic acid found in tomatoes can act as an antioxidant

and a cofactor for biologically important enzymes, thereby preventing browning, regulating cell division and expansion, and stimulating somatic embryogenesis in various plants (Dwiyani *et al.*, 2015; Hapsoro *et al.*, 2018; Darmawati *et al.*, 2021; Helena *et al.*, 2022). This free radical inhibitory activity is also shown in lycopene (Dwiyani *et al.*, 2015; Mose *et al.*, 2020). Other compounds, such as carotenoids in tomatoes, contribute to preventing photooxidation. Meanwhile, the sugar content of tomatoes is necessary as an energy source for in vitro culture (Mose *et al.*, 2020).

Callus Growing Time

Figure 3 provides information on how each treatment affected the duration of callus growth in Robusta coffee. The results of the Mann-Whitney test in Table 2 show that the treatment with 2 ppm 2,4-D and 10% tomato extract (T_2D_2) offered the fastest average callus growth time (13,3 days after culture). The T_2D_2 treatment was not significantly different from the T_2D_3 treatment but significantly different from the other treatments. Moreover, the T_3D_3 treatment had the longest average callus growth time, but it did not show any significant difference from the T_1D_1 , T_3D_1 , and T_3D_2 treatments.

Callus is a group of actively dividing cells that form an irregular arrangement. Callus formation begins with an explant incision, which activates the immune mechanism by releasing secondary metabolites to cover the wound. The interaction between metabolites from plants and the exogenous PGRs causes cell elongation, which contributes to the swelling and curvature of the affected tissue. The resulting cell proliferation and differentiation will eventually develop into a callus. In indirect somatic embryogenesis, the callus is a material source for developing new plants. The efficacy of this technique is dependent on the growth regulators introduced into the culture media.

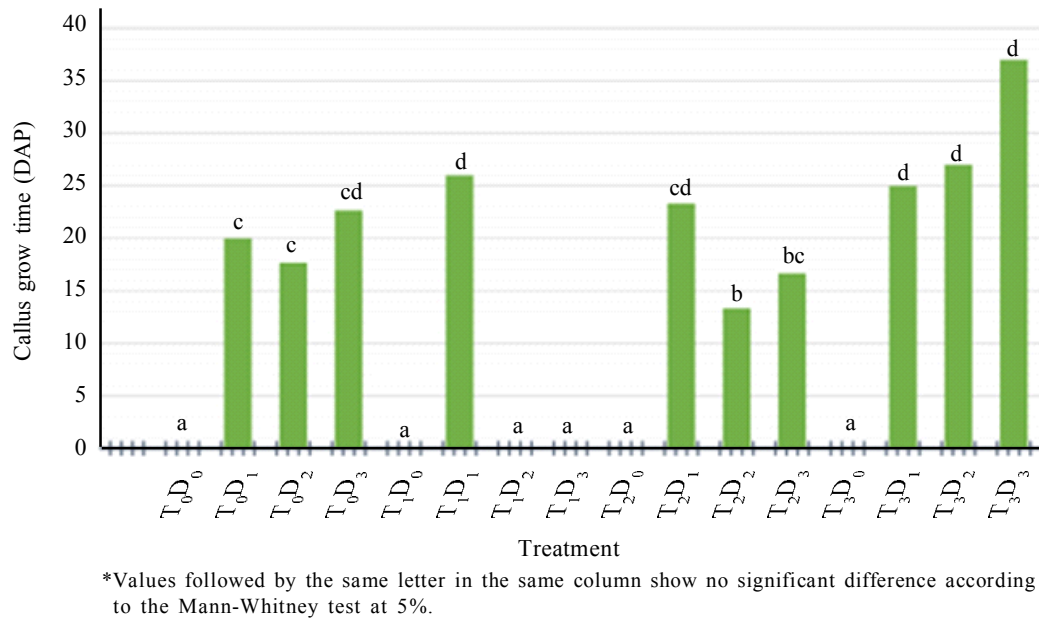


Figure 3. Average callus grow time in Robusta coffee 60 days after culture

In this research, the fastest average callus growth time was obtained at 13.3 days after planting when implementing a 2 ppm 2,4-D and 10% tomato extract (T₂D₂) treatment. This observation aligns with Awada *et al.* (2019) research, which discovered the formation of callus on wounded coffee explants two weeks after planting due to advanced cell division. In addition, Hapsoro *et al.* (2019) demonstrated the fastest response at 12 days after culture, characterized by swelling of the incision scar.

According to the research conducted by Awada *et al.* (2019), the development of callus in treatments T₂D₂ and T₂D₃ was significantly impacted by the availability of specific metabolites. The study proved that each developmental phase in somatic embryogenesis requires the availability of certain compounds. During the initial phase of explants until primary callus formation, the concentration of carbohydrates, such as glucose, sucrose, fructose, and ribose decreased, as these carbohydrates were utilized in sugar metabolism.

Other metabolites such as leucine, lysine, fumaric acid, malic acid, galactonic acid, and threonic acid concentration also decrease below average as a result of the citric acid cycle, amino acid metabolism, ascorbate, and alderate metabolism.

Exogenous metabolites are necessary to support the continuity of callus development phases. Metabolites such as glucose, fructose, fumaric acid, malic acid, and galactonic acid, which are needed in various callus metabolisms, are contained in tomatoes and used as natural PGRs (Agius *et al.*, 2018). Essential amino acids such as leucine and lysine are also abundant in tomatoes (Nour *et al.*, 2018).

The T₃D₃ treatment had the most prolonged effect on callus formation than the other treatments (37 days after culture). This result is one week longer than Ibrahim *et al.* (2019), which induced BP 436 Robusta coffee callus on media with 2-iP. Different callus induction times may occur due to different effectivity of the growth regulators and the

coffee varieties. Additionally, differences in callus induction time can be caused by dissimilarities in cell sensitivity to exogenous PGRs and different cell division times due to different cell cycles (Junairiah *et al.*, 2018).

Callus Fresh Weight

The quantities of callus fresh weight can determine the success of callus formation. The increase in callus fresh weight is associated with the cell's absorption of water and nutrients, which leads to elongation. The ratio of auxin and cytokinin is a crucial factor in regulating cell enlargement (Fitriana *et al.*, 2019).

Figure 4 shows the effect of different treatments on robusta coffee callus fresh weight. Treatment with 10% tomato extract and 2 ppm 2,4-D (T_2D_2) had the highest weight, similar to T_0D_2 , T_0D_3 , and T_2D_3 . Meanwhile, the lowest average callus wet weight results were shown in the T_1D_1 , T_3D_2 , and T_3D_3 treatments which were similar to T_0D_1 and T_2D_1 treatments.

In this study, treatments T_1D_1 , T_3D_2 , and T_3D_3 produced the lightest callus fresh weight (0.06 grams). The results are relevant to Baday (2018) research indicating a correlation between PGRs accumulation and callus fresh weight proliferation. However, excessive use of PGRs will reduce the average fresh weight of the callus. Similarly, excessive use of tomato extract can interfere with callus development, as Sari *et al.* (2019) demonstrated in a study that revealed high concentrations of coumarin acid in tomatoes may inhibit callus development. In addition, coumarin acid is reported to be an allelopathic compound that can interfere with photosynthesis, nutrient absorption, and plant metabolism (Dahiya *et al.*, 2017).

Meanwhile, the treatment that was able to encourage the highest increase in fresh weight, was shown in media enriched with 10% tomato extract and 2 ppm 2,4-D. This treatment has a similar effect to T_0D_2 , T_0D_3 , and T_2D_3 . The potency of the tomato extract might be related to the appropriate ratio

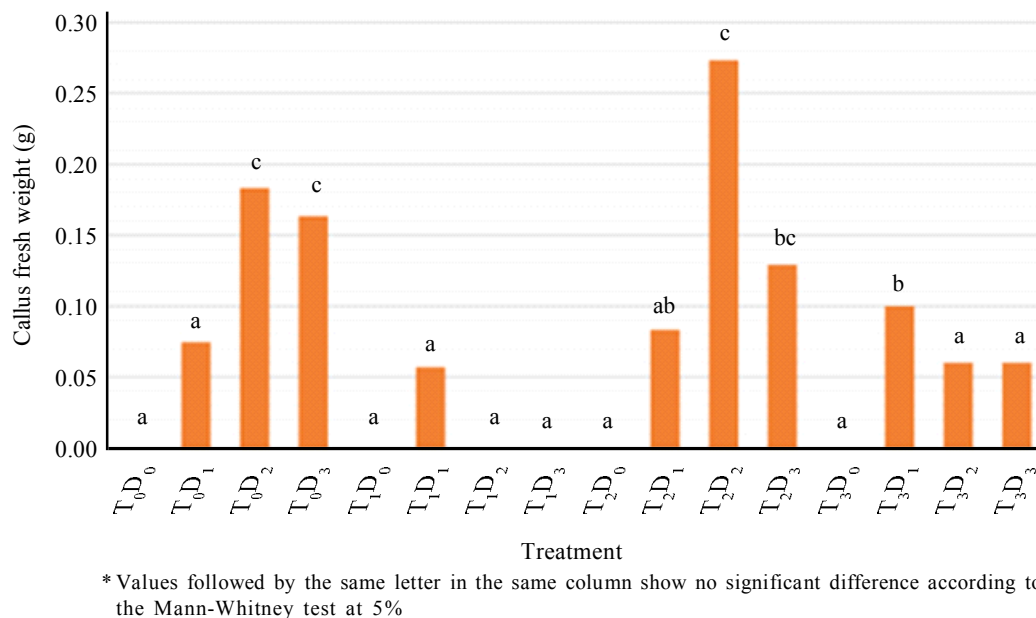


Figure 4. Average of callus fresh weight of Robusta coffee 60 days after culture

between endogenous and exogenous PGRs. Bogdanoviæ *et al.* (2021) research demonstrated the efficacy of 2,4-D in the induction and proliferation of embryogenic callus, thus affirming the potential of synthetic auxin.

Auxin is a crucial hormone in initiating the stages of indirect somatic embryogenesis by influencing the elasticity of the cell wall and encouraging the pumping of H^+ ions into the cell wall by proteins in the plasma membrane. Enzyme activation by H^+ ions causes the breaking of the cellulose hydrogen cross-links that comprise the cell wall, leading to cell elongation and water absorption (Ulva *et al.*, 2019). The intact cell wall matrix will be rearranged during cell growth, affecting cell weight (Junairiah *et al.*, 2018). Meanwhile, cytokinin is vital in conducting dedifferentiation and morphogenetic development (Asghar *et al.*, 2022). These hormones facilitate an increase in cell fresh weight by promoting meristematic division and cell growth through their interactions with enzymes that regulate cell division (Ulva *et al.*, 2019).

Aside from the appropriate PGRs role, it is assumed that ferulic acid and potassium (K) in tomatoes are crucial in enhancing the fresh weight of callus. Ferulic acid in

tomatoes has the ability to promote cell elongation (Vondráková *et al.*, 2016). On the other hand, the K element can initiate embryo swelling through the formation of micelles without cell walls, which facilitates water absorption (Setiari *et al.*, 2017).

Callus Color

The color of the Robusta coffee callus was recorded and presented in Table 2. At the end of the observation, a yellowish-white, brown, and brownish-yellow callus was obtained, as shown in Figure 1.

In previous studies, callus color was used to distinguish between embryogenic and non-embryogenic callus based on their regeneration ability. Embryogenic callus typically appears as yellowish-white, yellow, or brownish-yellow, while brown callus is an indication of non-embryogenic callus that has lost its cell capability to proliferate and regenerate (Ibrahim & Hartati, 2017; Livramento *et al.*, 2018; Ibrahim *et al.*, 2019; Rismayanti & Nafi'ah, 2021).

Brown callus formed from the T_0D_2 , T_0D_3 , and T_1D_1 treatments indicated the loss of callus proliferation ability. Abdalla *et al.*

Table 2. Callus color of Robusta coffee 60 days after culture as affected by combinations of tomato extract and 2,4-D treatments

Treatments		Repetition		
Tomatoe (%)	2,4-D (ppm)	I	II	III
0	0	-	-	-
7.5	1	Yellowish White	Yellowish White	-
10	2	Yellowish White	Brown	Yellowish White
12.5	3	Yellowish White	Yellowish White	Brown
0	0	-	-	-
7.5	1	Brown	-	-
10	2	-	-	-
12.5	3	-	-	-
0	0	-	-	-
7.5	1	Yellowish White	Yellowish White	Yellowish White
10	2	Yellowish White	Yellowish White	Yellowish White
12.5	3	Yellowish White	Yellowish White	Yellowish White
0	0	-	-	-
7.5	1	Yellowish White	-	-
10	2	Yellowish White	-	-
12.5	3	Brownish Yellow	-	-

Note: Calluses that do not grow with each treatment are marked with a hyphen (-)

(2022) revealed that browning was assumed to be a response to callus stress due to incision wounds which release phenols. Polyphenol oxidase (PPO) and Polyphenol Peroxidase (POD) oxidize phenols to quinones, causing browning. When quinones form chemical bonds with cellular proteins, it can disrupt cell metabolism, inhibit growth, and lead to explant death.

The combination treatment between 10% and 12.5% tomato extract with auxin 2,4-D (T_0D_1 , T_0D_2 , T_0D_3 , T_2D_1 , T_2D_2 , T_2D_3 , T_3D_1 , T_3D_2 , and T_3D_3) offered the capability to avoid browning and has the potential to become embryogenic callus. These findings are supported by research by Helena *et al.* (2022), that revealed the efficacy of tomato extract in reducing bamboo browning during the initiation phase. The anti-browning ability of tomato extract is related to the presence of lycopene and ascorbic acid, which can reduce quinone production. Lycopene has the ability to protect cells from oxidative damage through its inhibitory activity against the quinone reductase 2 (QR_2) enzyme which is involved in quinone production (McNerney & Styczynski, 2017). Similarly, ascorbic acid

is capable of reducing quinones to their original substrate through a process of hydrogen atom donation. Furthermore, it is believed that ascorbic acid serves as a competitive inhibitor of polyphenol oxidase (Ali *et al.*, 2015; Santos *et al.*, 2022).

Callus Texture

The texture of a callus can indicate whether it has the potential to become an embryonic or non-embryogenic callus. A friable callus has small, granule-shaped cells, while a compact callus is made up of larger cells that form a spongy structure or a thickening on the edge of the robusta coffee explants. Explants with both callus type (compact callus and friable callus) are classified as intermediate callus. The results of the observation of callus texture are shown in Table 3.

At the end of the observation, callus with friable, compact, and intermediate texture was obtained, as shown in Figure 1. The friable callus has greater potential to become an embryogenic callus. Friable calluses have loose connections between cells, so they are easily separated from one

Table 3. Texture of Robusta coffee callus 60 days after culture as affected by combinations of tomato extract and 2,4-D treatment.

Treatments		Repetition		
Tomato (%)	2,4-D (ppm)	I	II	III
0	0	-	-	-
7.5	1	Compact	Compact	-
10	2	Friable	Intermediate	Friable
12.5	3	Friable	Intermediate	Friable
0	0	-	-	-
7.5	1	Friable	-	-
10	2	-	-	-
12.5	3	-	-	-
0	0	-	-	-
7.5	1	Compact	Friable	Compact
10	2	Intermediate	Friable	Friable
12.5	3	Friable	Friable	Intermediate
0	0	-	-	-
7.5	1	Compact	-	-
10	2	Compact	-	-
12.5	3	Friable	-	-

Note: Calluses that do not grow with each treatment are marked with a hyphen (-).

another. Callus friability is influenced by the auxin hormone used. Auxin can induce somatic embryogenesis by causing cells to isolate. Cell isolation is essential in inhibiting competition for nutrients between cells and supporting the metabolism of embryogenic cells (Arimarsetiowati & Ardiyani, 2016).

Callus with a compact texture is an indication of non-embryogenic callus. The compact callus is composed of dense cells that are difficult to separate. The denseness of the cells in a compact callus reduces its ability to absorb the nutrients required for its growth (Junairiah *et al.*, 2018). Although unsuitable for plant propagation, a callus with a compact texture is reported to be more capable of accumulating secondary metabolites than a friable callus (Ulva *et al.*, 2019).

The research conducted by Livramento *et al.* (2018) has found that Arabica coffee explants with a lower concentration of 2,4-D auxin exhibit the highest potential for inducing embryogenic callus, in contrast with the findings presented in Table 3. Different effectivity of these plant growth regulators may vary depending on the types of plants and the mother plants used as explant sources. Explants derived from mother plants cultivated in a sterile environment have a greater potential of becoming embryogenic callus than those grown in a greenhouse or external environment because mother plants grown outside the lab are more susceptible to pests and growth disturbances, making them unsuitable for use as explant sources for callus formation in indirect somatic embryogenesis.

CONCLUSIONS

The addition of tomato extract was unable to give a significant result in the growth of Robusta coffee callus, compared to treatment with synthetic auxin (2,4-D). It is important

to note that utilizing tomatoes as a substitute for plant growth regulators carries a certain level of risk, as their hormone levels can be unstable, making it difficult to attain the balance ratios for callus formation. However, the essential compounds in tomatoes have advantageous potential as supplements for in vitro, with an average percentage of callus formation of 100%, the shortest average callus growth time (13.3 days after culture), the highest average wet weight of callus (0.27 grams), as well as the ability to form callus which has the potential to become embryogenic callus an intermediate to a friable texture without browning indication.

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