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Somatic Embryogenesis Induction in *Coffea arabica* L. by 2,4-Dichlorophenoxyacetic acid and 6-Furfurylaminopurine

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Abstract. Coffee plants typically produce for 5 to 20 years before requiring rejuvenation to maintain sustainable production levels. Tissue culture methods offer a way to efficiently produce coffee seedlings with identical genetics on a large scale, while also protecting against pests and diseases. This research focuses on developing suitable culture media for generating coffee through somatic embryogenesis, as an initial stage in creating synthetic seeds. The coffee leaf explant from young leaves. The materials were MS media, alcohol 70%, 2,4-D, Kinetin, vitamin C, Dithane M-45, Agrept, and aquades. The culture used bottles, tweezers, autoclaves, hot plates, and LAF. The treatment was the concentration of 2,4-D and Kinetin. The treatments were: 1) D0K0=0 mL.L^{-1} 2,4-D + 0 mL.L^{-1} Kinetin, 2) D1K0=2 mL.L^{-1} 2,4-D + 0 mL.L^{-1} Kinetin, 3) D2K0=4 mL.L^{-1} 2,4-D + 0 mL.L^{-1} Kinetin, 4) D0K1=0 mL.L^{-1} 2,4-D + 2 mL.L^{-1} Kinetin, 5) D0K2=0 mL.L^{-1} 2,4-D + 4 mL.L^{-1} Kinetin, 6) D1K1= 2 mL.L^{-1} 2,4-D + 2 mL.L^{-1} Kinetin, 7) D2K2=4 mL.L^{-1} 2,4-D + 4 mL.L^{-1} Kinetin, 8) D1K2=2 mL.L^{-1} 2,4-D + 4 mL.L^{-1} Kinetin, and 9) D2K1=4 mL.L^{-1} 2,4-D + 2 mL.L^{-1} Kinetin. Each treatment is replicated three times, so there are 27 experimental units. The treatment D1K1 results showed that the highest percentage of life calli induction was 74%, fastest callus induction times were at 25 days, then yellowish color and a crumbly texture were the most ideal morphological variables.

Keywords: 2,4-D; *coffea arabica*; kinetin; somatic embryogenesis; tissue culture

INTRODUCTION

Indonesia stands among the top five coffee-importing nations globally. However, coffee production in Indonesia has slowed its growth due to limited land area expansion. As per the Ministry of Agriculture's report in 2022, the expansion of coffee plantation areas has seen a modest increase of approximately 1.09%, primarily from small-scale farms. Conversely, the national coffee yield has witnessed an annual decline of 0.62%, alongside another decrease of 1.77% annually. Consequently, the overall coffee production has only seen a marginal uptick of 1.86% per year. This underscores the need for enhanced management and modernization of coffee plantations across Indonesia. According to Kusmiyati and Nursamsiyah (2015); Arifianto & Ismail (2023), consumers are more interested in consuming Arabica coffee species than Robusta or other species of coffee.

The land size positively impacts the plant population since greater land produces more (Ndiwa et al., 2022). However, a decreasing amount of land, old coffee plants, their productivity declines, and increased vulnerability to pests and diseases. According to Puslitkoka, the ideal period of plant life for producing coffee is 10-20 years (Pratamasari & Prajanti, 2022). To combat this, replacing older coffee plants with new ones is essential, ideally when the plants are in their middle age (Meirezaldi et al., 2022). Coffee is a widely cherished commodity, particularly the Arabica variety, which commands higher prices in international markets compared to other subspecies. However, the adoption of tissue culture techniques for coffee plantation management remains limited. Tissue culture, a method of propagation, allows for replicating plants with identical traits to their parent. Enhancing propagation through tissue culture can be facilitated by natural growth regulators. The initial synthetic seed production phase involves somatic embryogenesis through tissue culture, offering an alternative approach to obtaining high-quality seedlings. Synthetic seeds, essentially encapsulated somatic embryos from tissue culture, present a

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promising avenue for seedling procurement (Roostika et al., 2016). The first step in synthetic seed development entails inducing somatic embryo formation by applying growth regulators to the tissue culture medium. This research aims to determine the optimal concentrations of 2,4-dichlorophenoxyacetic acid and 6-Furfurylaminopurine (Kinetin) for inducing somatic embryogenesis in coffee tissue culture.

METHODS

The research was conducted at the Biotechnology Laboratory, part of the Faculty of Agriculture at Universitas Pembangunan Nasional "Veteran" in East Java. The study was conducted from August to November 2023. We used leaf Arabica coffee as explants, specifically young leaves measuring about 1 cm2. Our growth medium of choice was Murashige & Skoog (MS), adding 2,4-dichlorophenoxyacetic acid and 6-Furfurylaminopurine (kinetin) concentration to support plant growth.

Preparation and sterilization of equipment

The equipment used is first sterilized using wet heat sterilization. Sterilization by autoclave. All equipment is wrapped in paper and put into the autoclave, then the autoclave is turned on and set at a temperature of 121^{o} C for 15 minutes.

Media preparation

The treatment involves a media mix with various doses of plant growth regulators (PGR) to stimulate somatic embryogenesis. The treatments include: 1) D0K0=0 mL.L^{-1} 2,4-D + 0 mL.L^{-1} Kinetin, 2) D1K0=2 mL.L^{-1} 2,4-D + 0 mL.L^{-1} Kinetin, 3) D2K0=4 mL.L^{-1} 2,4-D + 0 mL.L^{-1} Kinetin, 4) D0K1=0 mL.L^{-1} 2,4-D + 2 mL.L^{-1} Kinetin, 5) D0K2=0 mL.L^{-1} 2,4-D + 4 mL.L^{-1} Kinetin, 6) D1K1= 2 mL.L^{-1} 2,4-D + 2 mL.L^{-1} Kinetin, 7) D2K2=4 mL.L^{-1} 2,4-D + 4 mL.L^{-1} Kinetin, 8) D1K2=2 mL.L^{-1} 2,4-D + 4 mL.L^{-1} Kinetin, and 9) D2K1=4 mL.L^{-1} 2,4-D + 2 mL.L^{-1} Kinetin. Each treatment is replicated three times, so there are 27 experimental units.

Sample preparation and sterilization

The sample of young Arabica coffee leaf was soaked in a vitamin C solution for 15 minutes, followed by three rinses with distilled water. This treatment prevents the occurrence of oxidation in the leaves. Then, the leaves were briefly immersed in 70% ethanol for 30 seconds and rinsed again with sterile distilled water. Subsequently, we sterilized them with Dithane M-45 fungicide, Agrept bactericide (both at 4g L^{-1}), and Chlorox 10%, mixed with 5 drops of Tween-20 for 20 minutes to minimize contamination risk. Finally, we trimmed the leaf margins and veins, cutting the leaf blades as needed.

Planting explant

Planting is carried out after 2 weeks of storing the media, allowing time to observe any signs of contamination. If the culture media remains sterile, then the planting process proceeds with explants. Each petri dish is used for planting with 4 explants in each dish. Explants are placed on MS media with the adaxial side facing downward in the culture container.

Observation and data analysis

Observations include the percentage of life calli induction in coffee, callus induction times, stages of callus growth, and callus color and formation. The experimental design follows a Completely Randomized Design (CRD), and data analysis using ANOVA, with Tukey's test for further comparison by SAS 9.4.

RESULTS AND DISCUSSION

Percentage of life calli induction of *Coffea arabica*

Somatic embryogenesis consists of two different phases: direct and indirect. The indirect phase involves the formation of the

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callus. The callus growth is indicated to occur and progress into the embryogenesis phase. Morphologically, the callus has not yet formed a specific organ from the explant. It is simply a cluster of unidentified cells that will develop and operate in a particular manner. This stage is crucial for generating new plant material through the callus division.

The percentage of life shows nutritional support in promoting the growth of coffee leaf explants in culture media. The addition of plant growth regulators encourages cell growth. Coffee leaves can divide somatic cells in case to produce callus faster due to the stimulus of the growth regulators auxin and cytokinin. Although not all treatments provide callus, some do not produce callus at all. Only four media are involved in the growth of callus in a relatively high percentage.

Treatment of 2,4-D and Kinetin at a concentration of 2 $mL.L^{-1}$ respectively, and 2 $mL.L^{-1}$ of 2.4-D and 4 $mL.L^{-1}$ of Kinetin result in the highest growth percentage in 70%. This is shown by excellent callus growth. Although the results are not different significantly from other treatments that produce callus.

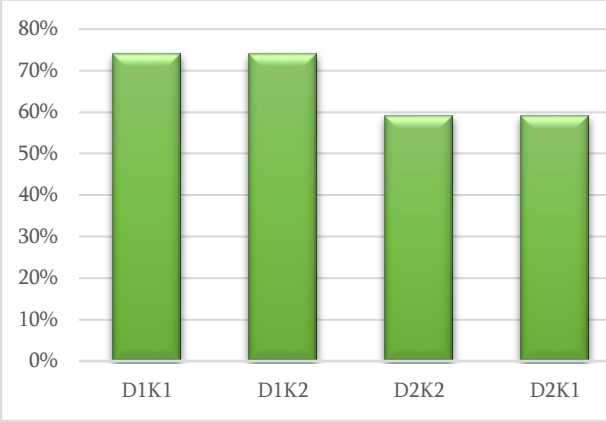

Figure 1. Percentage of life calli induction of *Coffea arabica* (%)

The 2,4-D is a group of synthetic auxin and Kinetin is a synthetic cytokinin used to stimulate cell growth in tissue culture. But, in the phase of indirect somatic embryogenesis, they are more likely to stimulate cell division by producing callus. Callus can appear when auxin and cytokinin are in a balanced amount. Compared to other treatments that can not stimulate cell division at all. This proves that both the concentrations of auxin and cytokinin have a strong influence on the emergence of new cells.

The appearance of callus is caused by the formation of tissue on the leaf wound. Nutrient absorption occurs, leading to cell division and the formation of a callus (Pierik, 1997; (Putri et al., 2020). The character of the callus can be regulated through the concentration of a growing regulator can affect the percentage of growth, texture, and potential regeneration of the callus (Hesami et al., 2018; Homayounvand et al., 2020). The ideal ratio of auxin and cytokinin staining to promote callus growth (Rahman et al., 2019; Bano et al., 2022).

Callus formation requires a balance of auxin and cytokinin. Auxin and cytokinin, either alone or in combination, can cause callus induction, proliferation, and growth acceleration (Ariati, 2012; Kebun Raya et al., 2023). Conversely, the efficiency of the growth regulator depends on the plant type, age, and the availability of endogenous growth regulators. Growth regulators must be present throughout the early phases of callus growth to form the callus.

Other treatments revealed no symptoms of callus formation. The barrier is that coffee leaves contain a high concentration of phenol chemicals, which may lead to constriction and tissue damage when oxidized. According to Hernawan and Setiawan (2003) and Pristiana (2017), coffee leaves contain the largest amount of phenols due to the more intense cytoplasm in the leaves, which synthesizes phenolic chemicals.

Callus induction times

The presence of calluses indicates successful growth in coffee leaf explants in forming new tissues. This phase requires

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varying amounts of time for each individual. Adding exogen plant growth regulators might enhance or inhibit plant growth. Therefore, it needs to be given in the right amount.

High callus regeneration rate, rapid callus growth, and embryonic callus development ability with crumb, globular, and compact callus types. (Sah et al., 2014; Carsono et al., 2021). Higher plant growth regulator concentrations enhance cell responsiveness during callus development, enabling intake of food and differentiation into embryogenic cells (Ikeuchi et al., 2013; Ibrahim et al., 2024).

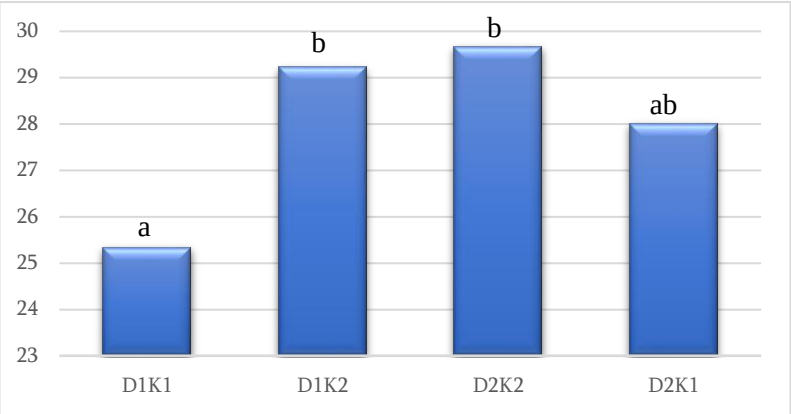

Figure 2. Callus induction times (days)

Note: Mean followed by same letter are not significantly different from each other

The growth rate of the callus is triggered by the addition of the growth regulators auxin and cytokinin. In this study, the fastest growth was obtained from a concentration of 2 $mL.L^{-1}$ 2.4-D + 2 $mL.L^{-1}$ Kinetin, which is about 25 days after planting, but not significantly different from the concentration of 4 $mL.L^{-1}$ 2,4-D + 2 $mL.L^{-1}$ Kinetin. Other treatments did not show any callus appearance at all. This result indicates that arabica leaf explants want a balanced concentration of about 2 $mL.L^{-1}$ of each auxin and cytokinin to accelerate their callus growth.

The optimal callus requires an equal amount of auxin and cytokinin (Alemi et al., 2013; Pramono et al., 2021). The balance of auxin and cytokinin levels is determined by a balanced mix of endogenous and exogenous growth regulators, instead of the amount of hormones lived in (Astutik et al., 2022). Lower concentrations of phytohormones were necessary for callus induction while increasing concentrations resulted in a considerable reduction in callus production (Mutasi et al., 2010; Dar et al., 2021).

The auxins, mostly 2,4-D, were known for their involvement in somatic embryogenesis induction and early embryo proliferation (Pasternak et al., 2002; Sidek et al., 2022). Kinetin encourages cell development and affects its physiology (Taiz al., 1991; Fauziah & Widoretno, 2017). In this study, adding 2,4-D and Kinetin in media at the balanced concentration induced callus better than other concentrations.

Callus formation leads to morphological differences. The influence of the media addition has various morphologies resulting in terms of color and texture. Morphological observation indicates the speed of growth stages towards the somatic embryogenesis phase. The embryonic callus in this study is still in the globular phase. The addition of 2,4-D and kinetin accelerates the initiation into the stages of somatic embryogenesis.

Normal somatic embryogenesis involves various stages, including globular, heart form, torpedoes, and cotyledons (Yang and Zhang, 2010; Sanglard et al., 2019; Yoas et al., 2021). Cotyledon is the most common embryonic phase used for synthetic seed

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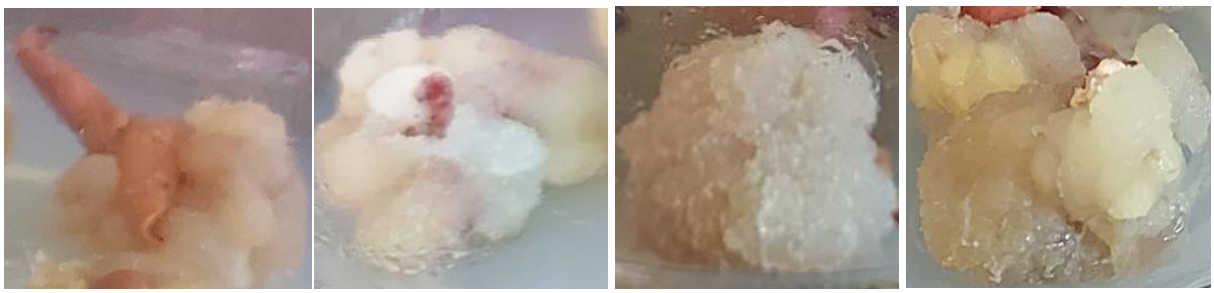
technology. At this stage, new buds have formed, enabling the plant to repopulate itself. According to Simamora and Restanto (2022), the globular phase is an essential early stage of callus, because it indicates the presence of systematic cells that divide continuously.

Callus color and formation stage

The appearance of callus differs with the presence of various growth regulators. The treatment 2 $mL.L^{-1}$ 2.4-D + 2 $mL.L^{-1}$ Kinetin forming callus with the ideal morphology. Callus may generate a wide range of colors and patterns, depending on the plant species and the media utilized. Other treatments generate a white callus because the additional auxin and cytokinin concentrations are inadequate to maintain callus formation.

Yellowish calli are more compact textured and morphogenic. Moreover, nonembriogenic callus aren't regularly shaped, fragile, soft, with a pale yellowish or creamy dull color (Kanani et al., 2020). The white-colored shell shows young cells in the active division phase, while the yellowish callus color then in the adult phase towards active division (Armila et al., 2014; Merthaningsih et al., 2018). Kinetin influences the callus color, hence the presence of yellowish is a reliable indicator of its impact.

2,4-D can stimulate cell elongation by leading cell wall plasticity to dissipate, allowing water to enter the cell wall through osmosis and cells to elongate. Consequently, the friable callus contains a lot of water because the cell wall has not been lignite, and separation of explant source of cells is relatively easy (Nisak et al., 2018; Setiawan et al., 2020). Rasud and Bustaman et al. (2020); Anggraeni et al., (2022) reported that the crushed callus has an intercellular gap that allows for aeration surrounding the cell.



(a) (b) (c) (d)

Figure 3. The appearance of calluses is as follows: (a) D1K1 is a yellowish color and a crumbly texture, (b) D1K2 is a white color with a crumbly texture, (c) D2K2 also shows a white color with a crumbly texture, and (d) D2K1 is a creamy color with a crumbly texture.

Providing growth regulators also influences callus development, color, and texture. High auxin concentrations can enhance callus development, but cytokinin affects callus color. According to George and Sherrington (1984); Sari et al., (2018), the inclusion of kinetin resulted in a yellowish-green and green callus. The color of the callus was caused by a 2,4-D interaction with kinetin, which plays a role in chlorophyll synthesis, and also by environmental conditions, including light exposure. Rahayu and Mardini (2015); Handayani et al., (2019) encourage that cytokinin activity influences chlorophyll synthesis especially.

CONCLUSION

The combination of auxin and cytokinin in the culture medium promotes callus development. The concentration of 2,4-D and Kinetin, each at 2 $mL.L^{-1}$, accelerates callus development and produces the greatest results in terms of callus morphology. The

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future might have examined in various species of coffee.

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