Induction of embryonic callus from several Arabica coffee varieties using a combination of plant growth regulator formulations

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Abstract. This study aimed to investigate embryonic callus formation in four Arabica coffee varieties (Andungsari 1, Orange Bourbon, Gayo, and Lini S-795), conducted at Politeknik Negeri Jember in two stages. The first stage is the induction of primary callus from leaf explants of four Arabica varieties in four combination concentratrion of 2,4-D and Kinetin using 1/2 MS+Vitamin B5 (Gamborg) as the base medium. The observed variables included: time of primary callus formation; fresh weight; texture and callus color. The next experiment was to test four Arabica varieties for their regeneration capacity for embryonic callus formation. This study used a non-factorial completely randomized design, with four treatments and five replications. For this test, the primary callus were cultured using medium supplemented with 2,4D and BAP. Observation variables included the time of embryonic callus formation; callus texture; percentage of embryonic callus formation; percentage of proembryo formation. The conclusion of the study was that each variety showed a different response to the added PGRs formulation, in addition, the use of 2,4-D 2 ppm + 1 ppm kinetin was able to stimulate primary callus formation from leaf explants. Gayo and Orange Burbon varieties showed the development of embryonic callus towards the proembryo phase.

1 Introduction

Arabica coffee (*Coffea arabica* L.) is one of the most economically important plantation commodities worldwide, including in Indonesia. Conventional coffee plant propagation has limitations, making in vitro tissue culture techniques an effective alternative method for producing high-quality coffee plants in large quantities. One crucial step in tissue culture is the induction of embryogenic callus, which can open up opportunities for plant regeneration through somatic embryogenesis [1].

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Embryogenic callus induction is influenced by the type and concentration of plant growth regulators (PGRs), particularly auxins and cytokinins. Synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) are often used in certain concentrations to induce callus formation from Arabica coffee leaf explants, while cytokinins such as Kinetin and Benzylaminopurine (BAP) play a role in the differentiation and formation of embryonic callus [2], [3]. The right combination of 2,4-D, Kinetin, and BAP can improve the efficiency of callus induction and the quality of the resulting callus [4].

Several studies have shown that the interaction between Arabica coffee varieties and media formulations with different combinations of plant growth regulators significantly influences the rate of callus emergence, color, area, type, and weight of the callus formed [5]. However, information regarding the most optimal and specific plant growth regulators (PGRs) formulations for sequentially inducing primary and embryogenic callus in these four genetically diverse Arabica coffee varieties remains uncomprehensively tested. The need for variety-specific tissue culture protocols is crucial, given the differing physiological responses of each genotype to auxin and cytokinin concentrations. Specifically, there is no clear data on which combination of PGRs is most effective in shifting primary callus from Andungsari 1, Orange Burbon, Gayo, and Lini S-795 varieties to the embryogenic and proembryo phases.

Therefore, this study aims to examine the effect of various formulations of PGRs combinations on the induction of primary callus and embryogenic callus in several Arabica coffee varieties, namely Andungsari 1, Orange Burbon, Gayo, and Lini S-795. This study is expected to contribute to the development of optimal tissue culture techniques for Arabica coffee with diverse genetic variations, as well as support the mass propagation program of quality coffee through in vitro tissue culture.

2 Materials and Methods

The research was conducted at the Plant Tissue Culture Laboratory of Politeknik Negeri Jember, and was carried out with two experimental stages. The first experiment of primary callus induction from leaf explants for three months. The sterilization and initiation method begins with selecting healthy young leaves, soak the leaves in 2 ml/L liquid detergent for 10 minutes, rinse, and then continue soaking and shaking in a solution of bactericide, fungicide 2 g/L, and erythromycin 4 g/L for 20 minutes. Rinse again and continue soaking in 70% alcohol for 3 seconds, then in 10% and 20% NaOCl solutions for 10 minutes each. Afterward, rinse again and cut the leaves into 1-1.5 cm^2 squares and plant them in the treatment medium. The methods as in previous studies [6].

This study used a factorial completely randomized design, with four Arabica coffee varieties as the first factor and four combinations of plant growth regulators from the auxin and cytokinin groups as the second factor. The treatment used a basic medium of ½ Murashige & Skoog + Vitamin B5 (Gamborg). Thus, there were 16 treatments, each replicated four times. The treatment combinations were as follows:

Table 1. Combination of primary callus induction treatments

Arabica Varieties	**PGRs Formulation**			
	F1=1 ppm 2,4 D + 0,5 ppm Kinetin	**F2 = 2 ppm 2,4 D + 0.5 ppm KIN**	**F3 = 1 ppm 2,4 D + 1 ppm KIN**	**F4 = 2 ppm 2,4 D + 1 ppm KIN**
A1= Gayo	*A1F1*	*A1F2*	*A1F3*	*A1F4*
A2= Andungsari 1	*A2F1*	*A2F2*	*A2F3*	*A2F4*
A3= Lini S-792	*A3F1*	*A3F2*	*A3F3*	*A3F4*
A4= Orange Burbon	*A4F1*	*A4F2*	*A4F3*	*A4F4*

Observations were carried out for 3 months. The variables observed included: 1) Time of primary callus formation (DAP); 2) Fresh weight of primary callus; 3) Callus texture is friable; Intermediate; and compact; 4) Callus color (white; yellowish white; yellowish) [7]. Friable callus is characterized by its brittle texture, easily crumbling or separating, like crumbs. This type of callus leads to the formation of embryonic callus. Intermediate callus has a partially compact and friable texture. Compact callus has a hard, dense, and difficult-to-disintegrate texture (non-friable).

The next experiment was to test four Arabica varieties (Gayo, Andungsari 1, Lini S-795, Orange Burbon) for their regeneration capacity for embryonic callus formation. This study used a non-factorial completely randomized design, with four treatments and five replications. For this test, the four varieties were cultured using Embryonic Callus Induction medium [8] supplemented with 0.22 ppm 2,4D and 6 ppm BAP. The following table shows the treatment combinations in the second stage.

Table 2. Test treatment of four Arabica varieties for embryonic callus regeneration ability

No	Treatment	
	Arabica Varieties	**PGRs Formulation**
1	Gayo	0.22 ppm 2,4D + 6 ppm BAP
2	Andungsari 1	0.22 ppm 2,4D + 6 ppm BAP
3	Lini S-795	0.22 ppm 2,4D + 6 ppm BAP
4	Orange Burbon	0.22 ppm 2,4D + 6 ppm BAP

Observations were carried out for 3 months with observation variables including: 1) Time of embryonic callus formation (DAP); 2) Callus texture; 3) Percentage of embryonic callus formation (%); 4) Percentage of proembryo formation (%). Data were analyzed using ANOVA and if there was significance, a further DMRT (Duncan's Multiple Range Test) was carried out at a 0.05 level.

3 Results and Discussion

3.1 Primary callus formation

Table 3. Results of primary callus formation from combinations of several Arabica coffee varieties with the PGR Kinetin and 2,4-D

Type of Arabica	PGRs Formulation	Primary callus emergence time (DAP)	Fresh weight of primary callus (gr)	Primary callus texture	Primary Callus Color
A1=Gayo	F1 = 1 ppm 2,4 D + 0.5 ppm KIN	15.00 ^{ab}	0.40 ^{abc}	Compact	White
	F2 = 2 ppm 2,4 D + 0.5 ppm KIN	16.80 ^{abc}	0.99 ^{efg}	Friable	White
	F3 = 1 ppm 2,4 D + 1 ppm KIN	20.20 ^{de}	0.28 ^{abc}	Friable	White
	F4 = 2 ppm 2,4 D + 1 ppm KIN	19.40 ^{cd}	1.17 ^{fg}	Friable	Yellowish white
A2=Andungsari 1	F1 = 1 ppm 2,4 D + 0.5 ppm KIN	17.80 ^{bcd}	1.05 ^{efg}	Compact	Yellowish
	F2 = 2 ppm 2,4 D + 0.5 ppm KIN	20.80 ^{de}	1.22 ^{g}	Friable	White

Type of Arabica	PGRs Formulation	Primary callus emergence time (DAP)	Fresh weight of primary callus (gr)	Primary callus texture	Primary Callus Color
	F3 = 1 ppm 2,4 D + 1 ppm KIN	17.80 ^{bcd}	1.16 ^{fg}	Compact	Yellowish white
	F4 = 2 ppm 2,4 D + 1 ppm KIN	14.20 ^{a}	1.27 ^{g}	Friable	Yellowish
A3=Lini S-792	F1 = 1 ppm 2,4 D + 0.5 ppm KIN	20.80 ^{de}	0.43 ^{bc}	Friable	Yellowish
	F2 = 2 ppm 2,4 D + 0.5 ppm KIN	17.60 ^{bcd}	0.84 ^{def}	Compact	Yellowish White
	F3 = 1 ppm 2,4 D + 1 ppm KIN	23.20 ^{ef}	0.52 ^{cd}	Compact	White
	F4 = 2 ppm 2,4 D + 1 ppm KIN	20.60 ^{de}	0.80 ^{de}	Friable	Yellowish
A4=Orange Burbon	F1 = 1 ppm 2,4 D + 0.5 ppm KIN	24.40 ^{f}	0.12 ^{a}	Friable	Yellowish
	F2 = 2 ppm 2,4 D + 0.5 ppm KIN	17.60 ^{bcd}	0.15 ^{ab}	Friable	Yellowish White
	F3 = 1 ppm 2,4 D + 1 ppm KIN	23.00 ^{ef}	0.14 ^{ab}	Friable	Yellowish White
	F4 = 2 ppm 2,4 D + 1 ppm KIN	14.00 ^{a}	0.18 ^{ab}	Friable	White

The number followed by the same letter are not significantly different at ($p<0.05$) level of DMRT test.

Based on observations, all cultured Arabica varieties were able to form primary callus, the initial callus formed from leaf explants. The appearance begins from the area of injury or incision (**Fig 1**). As presented in **Table 3**, the fastest callus emergence, namely at 14 DAP (Days After Planting), was seen in the Orange Burbon and Andungsari 1 varieties. Specifically, the Orange Burbon and Andungsari 1 varieties may have physiological and genetic characteristics that support a positive response to the hormone treatment, thus accelerating callus initiation and growth compared to other varieties.

The fastest callus emergence in these two varieties at 14 DAP indicates that the combination of 2 ppm 2,4-D and 1 ppm kinetin is the optimal concentration to stimulate callus formation in leaf explant tissue. The proper interaction of auxin and cytokinin is crucial for the success and speed of callus induction in various plant varieties, including coffee (*Coffea* spp.) [7]. This is also in line with findings showing that concentrations of around 1-2 ppm 2,4-D combined with 0.5-1 ppm kinetin can stimulate callus formation in a short time and good callus quality, such as color, texture, and optimal water content to support the regeneration of coffee somatic embryos [4], [9], [10].

Fresh callus weight data showed that the formulation of 2 ppm 2,4-D and 1 ppm kinetin produced the heaviest callus, namely 1.27 gr in Andungsari 1, and 1.17 gr in the Gayo variety. Heavier fresh callus weight reflects good callus quality, with actively developing cells and sufficient water storage. The physiological and genetic responses of the Andungsari 1 and Gayo varieties support the performance of the PGR combination in increasing primary callus cell biomass. The use of 2 ppm Kinetin with the addition of 1 ppm BA also appeared to be able to stimulate the fresh weight of coffee callus [7]. Another study also showed that 2 ppm 2,4-D + 1 ppm kinetin produced the greatest fresh callus weight in cinnamon leaf explants [11].

The friable, white or yellowish-white texture of primary callus (**Fig 1**) in most cultured Arabica coffee varieties indicates the onset of embryonic callus formation [4]. This is consistent with previous researchers' statements that embryonic callus in Arabica coffee is

characterized by friable, yellow tissue consisting of small, isodiametric cells with dense cytoplasm and clear nuclei [12], [13]. For further development, this friable primary callus is regenerated on a new medium to be directed into embryonic callus to form a proembryo, the initial phase of the coffee somatic embryo.

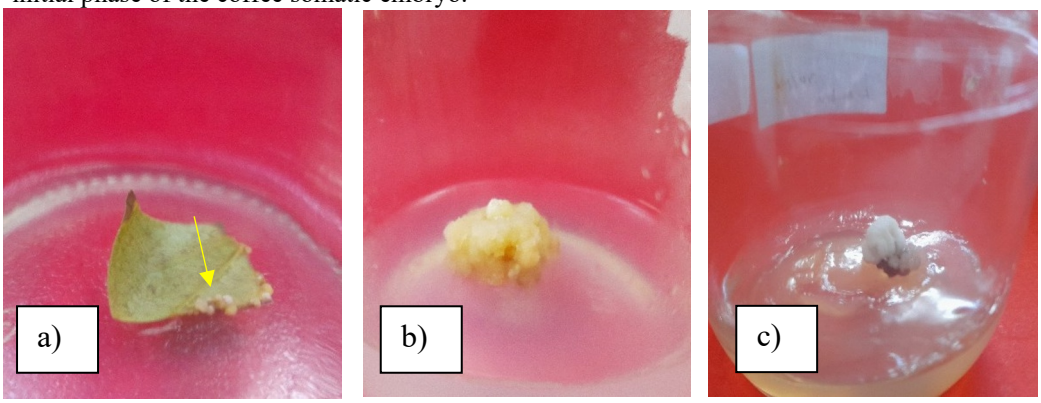

Fig. 1. a) Apearance of Callus from leaf explant; b) Friable callus with yellowish white color; c) Compat callus and white color.

3.2 Regeneration of embryonic callus

Embryonic callus regeneration is the process of forming new callus that grows from primary callus generated in a previous treatment. All primary calli from the four types of Arabica were grown on media with the same PGR, namely 0.22 ppm 2.4D and 6 ppm BAP. This process is crucial in tissue culture because embryonic callus has the potential to develop into whole plants through somatic embryogenesis. Embryonic callus typically has a friable and nodular texture, characteristic of callus with high regenerative capacity [7], [10].

Observations of embryonic callus included the time of embryonic callus emergence, the percentage of embryonic callus formation, its texture, and proembryo formation percentage. ANOVA analysis showed that the Arabica variety tested had a very significant influence on the observed variables. The results of the analysis were further tested using DMRT at the 0.05 level and are presented in the following table.

Table 4. Results of embryonic callus and proembryo formation from single factors of Arabica coffee varieties

Type of Arabica	Embryonic Callus Appearance Time (DAP)	Percentage of Embryonic Callus Formation (%)	Callus Texture	Proembryo formation percentage (%)
Gayo	33.00 [a]	100.0 [c]	friable nodules	87.50 [d]
Orange Burbon	40.00 [a]	100.0 [c]	friable nodules	65.50 [c]
Lini S-795	58.20 [b]	50.0 [a]	compact	27.50 [a]
Andungsari 1	47.25 [b]	75.0 [b]	compact	50.00 [b]

The number followed by the same letter are not significantly different at ($p<0.05$) level of DMRT test.

Based on the data, embryonic callus formation was most rapid in Gayo Arabica coffee, at 33 days after transplanting, and Orange Bourbon coffee, at 40 days after transplanting. Both varieties produced 100% embryonic callus with a friable and nodular texture. Meanwhile,

Lini S-795 and Andungsari 1 varieties produced only 50% and 75% embryonic callus, with a compact texture.

Gayo Arabica coffee and Orange Bourbon coffee varieties produced callus on 33 and 40 days after transplanting, respectively. Both varieties demonstrated high regeneration efficiency, with embryonic callus formation reaching 100%, demonstrating their adaptability and positive response to the culture medium used. The resulting callus texture was friable and nodular, consistent with the characteristics of embryonic callus ideal for embryogenesis induction [14].

In contrast, the Lini S-795 and Andungsari 1 varieties showed lower embryonic callus regeneration rates, at 50% and 75%, respectively, and had a more compact callus texture. This indicates genetic differences between varieties that influence response to tissue culture treatments and the potential of callus to develop into somatic embryos [15], [16]. Differences in callus texture may also indicate variations in the level of cell differentiation and proliferation during callus regeneration, with callus with a compact texture typically exhibiting different meristematic activity than more embryogenic, friable callus [17].

During the observation period, the embryonic callus had developed and begun to enter the globular phase. The Gayo and Orange Burbon varieties formed globules with success rates of 87% and 65%, respectively. Globular callus is characterized by the presence of dense nodules on the surface of the embryonic callus (**Fig 2**). The friable texture, having nodules or spheres with a smooth surface [7] and dense callus tissue indicate the presence of early division and differentiation at the cellular level, which leads to the proembryonic phase as the first stage in the somatic embryogenesis process.

Fig. 2. Nodule formation in embryonic callus

4 Conclusion

The results of this study concluded that each variety showed a different response to the PGR formulation added for primary callus induction and embryonic callus induction. The use of 2 ppm 2,4-D + 1 ppm kinetin was able to stimulate the formation of primary callus from leaf explants. The Gayo and Orange Burbon varieties were more responsive to the use of the PGR formulation, showing the development of embryonic callus towards the proembryo phase, for the next stage of development to form somatic embryos.

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