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RESEARCH ARTICLE

Exploring indirect somatic embryogenesis and somaclonal variation for propagation of three *Coffea arabica* L. cultivars

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ABSTRACT

The propagation of arabica coffee (*Coffea arabica* L.) seedlings through indirect primary embryogenesis has significant potential to produce many healthy seedlings relatively quickly. However, there is a concern that plant growth regulators (PGRs) applied in the culture media could result in somaclonal variations. To address this issue, the appropriate concentration of PGR needs to be identified to increase the success of the somatic embryogenesis process of propagation while preventing somaclonal variations. This study aimed to develop a protocol for the propagation of *C. arabica* through indirect somatic embryogenesis and evaluate somaclonal variation in plantlets derived from somatic embryogenesis, considering the genotype and PGR used. Three cultivars of coffee, i.e., AS2K, S-795, and Sigarar Utang, and two types of PGRs (2,4-D and 2-iP) at different combination concentrations, were used as the factorial experimental design. The concentration of PGRs was 4.52 and 9.04 µM for 2,4 D and 4.93, 9.86, 14.79, 19.72, and 24.65 µM for 2-iP. All combinations were replicated 10 times. The findings revealed that PGRs influenced percentage of callus formation which ranged from 87.02% to 93.81%, while coffee cultivars significantly impacted the development of torpedoes and germination of somatic embryos with the number of torpedoes and germinated somatic embryos were 32.20-94.20 and 21.10-82.20 for 'AS2K', 37.90-62.90 and 27.70-50.20 for 'S-795', and 39.40-72.00 and 29.60-60.60 for 'Sigarar Utang', respectively. Among the PGRs evaluated, the combination of 4.52 µM 2,4-D and 19.72 µM 2-iP demonstrated the highest efficacy with 94.20 torpedoes and 82.20 germinated somatic embryos for 'AS2K', whereas 4.52 µM 2,4-D and 14.79 µM 2-iP were preferred for 'S-795' and 'Sigarar Utang'. Furthermore, molecular analysis using 10 SSR primers of the resulting plantlets indicated no evidence of somaclonal variation.

Key words: Callus induction, molecular analysis, plant growth regulators, somaclonal variation.

INTRODUCTION

Research on somatic embryogenesis in arabica coffee (*Coffea arabica* L.) has yielded an effective plant propagation method through tissue culture. This method is particularly well-suited for coffee production (Avila-Victor et al., 2023; Awada et al., 2023) as it allows for low-cost clonal propagation (Etienne et al., 2018). The primary objective of somatic embryogenesis in *C. arabica* is to achieve rapid and efficient plant propagation (Robledo Paz, 2018; Aguilar et al., 2022) resulting in uniform seedlings with characteristics that identic to the parent plant.

Somatic embryogenesis in *C. arabica* can be achieved either directly or indirectly, as demonstrated by Ibrahim et al. (2013) and Robledo Paz (2018) respectively. Direct somatic embryogenesis involves the production of embryos from pre-embryogenic cells derived from somatic tissue explants. In contrast, indirect somatic embryogenesis begins with callus formation, as described by Ibáñez et al. (2020). The use of indirect somatic embryogenesis is advantageous in generating new varieties of seeds resulting from hybridization, genetic engineering, mutation, or in vitro selection because it allows for the more simultaneous development of embryos, torpedoes, and germination, resulting in a higher number of embryos per explant, as noted by Ibrahim et al. (2013) and Robledo Paz (2018). Additionally, a proposed protocol for indirect somatic embryogenesis in a liquid system has enabled the propagation of "Híbrido de Timor" (HT) 'CIFC 4106' plantlets, overcoming seminal propagation barriers, as reported by de Morais Oliveira et al. (2021).

Although research on the somatic embryogenesis of arabica coffee has made significant progress in the past 30 yr, resulting in the development of various coffee genotypes, several challenges remain, particularly regarding its application in seed propagation. One of the major hurdles is selecting the appropriate Plant growth regulator (PGRs) on a particular genotype. The effect of PGRs on somatic embryogenesis has been described by Campos et al. (2017). In laboratory studies of in vitro culture, the emphasis is often placed on the initial phases of embryonic callus formation in arabica coffee.

Researchers primarily focus on optimizing the production of calli induction during the embryogenic and regeneration processes of arabica coffee by examining the concentration and type of PGRs. Using the right PGRs in the appropriate concentrations and combinations is crucial for achieving the best results. Previous studies have demonstrated that inducing coffee embryogenic callus typically involves a combination of PGRs from the auxin and cytokinin groups. The commonly used auxin is 2,4-dichlorophenoxyacetic acid (2,4-D). At the same time, the cytokinins can vary, such as thidiazuron (Ibrahim et al., 2015; 2017), 6-benzyl amino purine (BAP) (Papanastasiou et al., 2008; Van Boxtel and Berthouly, 1996), or a combination of (2-isopentenyl) adenine (2-iP) and indole-3-butyric acid (IBA) (Etienne, 2005). These combinations of PGRs have shown effective results in inducing coffee embryogenic callus formation in previous studies.

A recent study by Arimarsetiowati et al. (2023a) investigated the use of multiple growth regulators to induce embryogenic calluses, revealing significant differences among the combinations of PGRs. Additionally, research by Sanglard et al. (2019) and Hapsoro et al. (2020) highlighted the impact of factors such as genotype, ploidy level, epigenetic background, and physiological traits on the success rate of callus induction. This study underscores the ongoing need for optimizing PGR concentrations, particularly in developing new coffee genotypes. In some instances, the combination of 2,4-D and thidiazuron has been reported as effective for inducing callus embryogenesis in Indonesian coffee somatic embryogenesis (Ibrahim et al., 2013; 2015). However, it is worth noting that the high cost of thidiazuron poses a challenge for most laboratories in developing countries, limiting its practical use.

It is essential to consider that indirect embryogenesis can lead to the emergence of new genotypes through somaclonal variations, as highlighted by Shahsavand Hassani et al. (2018). Somaclonal variation refers to genetic and epigenetic changes, including gene activation, silencing, mutations, and alterations in epigenetic marks, which can occur during inefficient in vitro culture methods. Plant growth regulators have been identified as one of the factors contributing to somaclonal variation (Garcia et al., 2019). While somaclonal variation can enhance genetic diversity (Ferreira et al., 2023), it can also present challenges in tissue culture micropropagation, particularly from a commercial perspective.

Early detection using molecular markers is crucial to assess the occurrence of somaclonal variation. This non-destructive method can be employed at the plantlet stage and remains unaffected by environmental factors, as emphasized by Thorat et al. (2017). Simple sequence repeats (SSRs) or micro-satellites, is a molecular marker widely used to detect genetic variations in coffee and other crops (Prathibha et al., 2022). This co-dominant marker exhibits a high level of allele variation. It can effectively distinguish between diploid and tetraploid plants, as demonstrated by Hendre and Aggarwal (2014). Additionally, the SDS-PAGE technique on callus cultures has been reported as another method for detection genetic variation (Sahara et al., 2019). Researchers can effectively detect somaclonal variation by employing SSR markers,

enabling them to identify and select plants with desirable traits while minimizing unwanted genetic variability. This approach ensures that genetically stable plants suitable for commercial production are obtained from the experiments.

This research aims to enhance the protocol for arabica coffee propagation using indirect somatic embryogenesis and evaluate the potential somaclonal variation observed in the resulting plantlets. The study focuses on three arabica coffee cultivars: Sigarar Utang, S-795, and AS2K. Each of these cultivars possesses distinct characteristics that set them apart from other cultivars. 'Sigarar Utang' is renowned for its superior production capabilities. 'S-795', on the other hand, demonstrates adaptability to medium altitudes, making it suitable for cultivation in such regions. Lastly, the 'AS2K' exhibits resistance to leaf rust caused by *Hemileia vastatrix*, a common disease affecting coffee plants. These unique attributes make 'Sigarar Utang', 'S-795', and 'AS2K' highly desirable for further study in this research.

MATERIALS AND METHODS

Sterilization and explant preparation

The first fully opened young leaves of three arabica coffee (*Coffea arabica* L.) cultivars, including AS2K, Sigarar Utang, and S-795, collected from the greenhouse were used as explants. The leaves were washed under running water, sterilized in fungicide (based on mancozeb) with a concentration of 0.2% and bactericide solution (based on 15% streptomycin sulfate and 1.5% oxytetracycline) for 1 h, rinsed, and immersed in ethanol 70% for 3 min before being immersed in hypochlorite (10%) for 15 min. The leaves were thoroughly cleaned with distilled water three times. The sterilization process was carried out in a laminar airflow hood.

Primary callus induction and embryogenic callus establishment

Coffee leaves were placed in a sterile petri dish. Each leaf's margin and mid-rib were removed, and the remaining were cut into 1 cm × 1 cm pieces and planted on callus induction media. The modified half-strength Murashige and Skoog (MS) medium was used as a callus induction medium, and in the basal medium, sucrose (30 g L^{-1}), polyvinylpyrrolidone (250 mg L^{-1}), and phytagel (2.5 g L^{-1}) were added. Moreover, the callus induction medium was also supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (4.52 and 9.04 µM) in combination with (2-isopentenyl)adenine (2-iP) (4.93; 9.86; 14.79; 19.72 and 24.65 µM). The pH of all media was adjusted to 5.6, which was consistent throughout all experiments.

After 4 wk, the explants were transferred to new media to induce embryogenic callus formation. The half-strength MS medium was supplemented with sucrose (30 g L^{-1}), Phytagel (Sigma-Aldrich, St. Louis, Missouri, USA; 2.5 g L^{-1}), 2,4-D (4.50 µM), and 6-benzyl amino purine (BAP) (17.75 µM), following the methodology described by Van Boxtel and Berthouly (1996) and Etienne (2005). It is worth noting that the original treatments involving 2,4-D and 2-iP were still recorded to assess their impact on the success of embryogenic callus formation, despite the explants being transferred to fresh media. The cultures were incubated in darkness at approximately 25 °C with 60% relative humidity.

After 4 mo from the subculture, percentage of callus formation and fresh weight of the cultured callus were evaluated. On the other hand, fresh weight of the callus was determined by weighing the callus produced per leaf explant using a digital balance. These evaluations provide meaningful metrics to assess the success and progress of embryogenic callus formation.

A complete factorial design was utilized in this study, incorporating three different coffee plant cultivars as the first factor and 10 combinations of plant growth regulators (PGRs) as the second factor. Each plant cultivar and PGR combination were replicated 10 times, resulting in 300 experimental units (3 cultivars × 10 PGR combinations × 10 replicates).

The study investigated 10 specific PGR combinations, which consisted of two levels of 2,4-D (4.52 and 9.04 µM) and five levels of 2-iP (4.93, 9.86, 14.79, 19.72, and 24.65 µM). These levels of 2,4-D and 2-iP were combined to form the 10 distinct PGR combinations examined in the research.

Regeneration and somatic embryo development

Two hundred milligrams of embryogenic callus from each treatment were weighed and subsequently subcultured in a medium consisting of half MS supplemented with Gamborg medium (B5), sucrose at a concentration of 35 g L^{-1}, Phytagel at 2.5 g L^{-1}, and kinetin at 9.3 M. The medium formulation followed the protocol Van Boxtel and Berthouly (1996) described for somatic embryo generation. The cultures were incubated in a dark room at 25 ± 2 °C, maintaining 60% relative humidity.

The variable of interest in this study was the number of embryos in the torpedo phase. This specific developmental stage of the somatic embryos was observed and recorded as the primary outcome measure for evaluating the success of the treatments.

The torpedo embryos obtained from the MS medium were further cultured in a medium supplemented with 1.33 µM BAP, 40 g L^{-1} sucrose, and 2.5 g L^{-1} Phytagel, following the medium formulation described by Etienne (2005). These additional components were added to promote the germination of the torpedo embryos.

After subculturing, the germination of the embryos was carried out under a light regime, with 16 h light exposure per day. The light intensity was maintained at a range of 1000-1500 lux. The germination process occurred at 25 °C with *ca*. 60% relative humidity.

The variable of interest in this stage was the number of sprouts formed from the germinated embryos. This measure assessed the embryos' successful germination and subsequent growth under the specified conditions.

Throughout the study, the initial combination treatment of plant growth regulators (PGR) and coffee cultivars were consistently applied and followed at each stage of the culture process. The experimental design maintained its structure with two factors and 10 replicates. The first factor corresponded to the different coffee cultivars, while the second factor represented the composition of PGR used in the treatments. This experimental design allowed for the systematic evaluation of the effects of both cultivar and PGR composition on the observed outcomes across the different stages of the study.

Morphological and histological somatic embryogenesis observation

Leaf explants cultured for 8 wk were assessed for their morphology, explicitly examining the developmental stages of the somatic embryos. Various embryo forms were observed and recorded, including globular, oblong, liver, elongated, torpedo, and cotyledonary embryos. Some of these embryos were also selected as histological materials for further analysis.

To prepare the tissue for histological examination, transverse slices were made following a modified paraffin method based on the technique developed by Sass (1958). The tissue samples designated for histological analysis were sectioned using a rotary microtome, producing slices with a thickness of 10 µm. These slices were stained using 1% safranin and 0.5% fast green.

To evaluate the morphological effects and histology of the arabica coffee somatic embryos, a microscope (Axio Vision, Carl Zeiss Microscopy, White Plains, New York, USA) connected to a computer was employed. The Axio Vision software version 4.82 was utilized for image capture and analysis, allowing for detailed examination and assessment of the embryos' morphology and histological features.

Statistical analysis

When appropriate, the quantitative data collected were subjected to ANOVA at P 0.05. Duncan multiple range test (DMRT) was used to compare means. SAS statistical analysis software (SAS Institute, Cary, North Carolina, USA) was used for all statistical analyses.

Early detection for somaclonal variation

Total genomic DNA was extracted from fresh and healthy young leaves of 50 plantlets and the mother plant of 'AS2K', 'S795', and 'Sigarar Utang' *C. arabica* using modified cetyltrimethylammonium bromide (CTAB) methods according to DNA extraction protocol by Orozco-Castillo et al. (1994). The tested plantlets were taken randomly from the best treatment for each cultivar.

The extraction buffer solution was supplemented with fresh mercaptoethanol (0.1%) and polyvinylpolypyrrolidone (PVPP; 1%). The leaf tissue homogenates were incubated for 1 h at 65 °C before

mixing with 500 µL chloroform:isoamyl alcohol (24:1) and centrifuging at 13 000 rpm for 10 min. The supernatant (*ca*. 300 µL) was mixed with 600 µL cold isopropanol and 30 µL sodium acetate (3 M, pH 5.2). The mixture was centrifuged at 13 000 rpm for 15 min, and the dried DNA pellet was dissolved in TE buffer (50 µL), followed by an evaluation of the DNA quality step. The isolated DNA was used as a template in polymerase chain reaction (PCR) analysis. The PCR was performed in a 20 µL reaction mixture containing 1 × MyTaq HS Red Mix PCR Kit (Meridian Bioscience, Cincinnati, Ohio, USA), 10 ng μL^{-1} genomic DNA, and 0.5 µM each forward and reverse primers. The source of a total of 20 SSR primer pairs used in PCR analysis was described by Teressa et al. (2010), Priyono and Sumirat (2012) and De Gaspari-Pezzopane et al. (2012), as presented in Table 1. One cycle of 4-min pre-denaturation at 94 °C, 35 cycles of denaturation for 15 min at 94 °C, annealing for 1 min at the appropriate Tm for each primer pair, primer extension at 72 °C for 2 min, and one cycle of final extension at 72 °C for 7 min comprised the amplification steps. Vertical polyacrylamide gel electrophoresis (PAGE, 8%) was applied to separate the amplicons. The amplicon was stained with ethidium bromide and visualized under UV light using In a Chemi Doc gel system (BIO-RAD, Hercules, California, USA). To detect somaclonal variation early on, the banding pattern of plantlets resulting from somatic embryogenesis and the mother plant of arabica coffee were compared.

Table 1. Primers used in the research, their corresponding nucleotide sequences, and amplicon lengths. [a]Teressa et al., 2010; [b]Priyono and Sumirat, 2012; [c]De Gaspari-Pezzopane et al., 2012.

Nr	Primer	Sequence	Motif
1	ssrR105[a]	F-CACCAATTCCACTGACAATG R-TCCCTGCCAACACACTTC	GA(18)
2	ssrR209[a]	F-CGGGGGTAAAAAGATTGTAA R-TTGGTGGGAGGGGAGTA	GA(16)
3	ssrR268[a]	F-GTATCCCACAATGAAATCAC R-AGTAGAATTTTCAACATATAAG	GA(19)
4	ssrA8847[a]	F-GCACACATGAAAAAGATGCT R-GATGGACAGGAGTTGATGG	GT(18)GA(18)
5	ssrCMA008[a]	F-CATTCTGGTCCTGATGCTCT R-TCATTCACTTATTAACGTCCATC	CT(14)TG(10)
6	ssrCMA059[a]	F-GATGGACAGGAGTTGATGGT R-TTTTAACACTCATTTTGCCAAT	CT(9)CA(8)
7	ssrCMA198[a]	F-AGCAACTCCAGTCCTCAGGT R-TGGAAGCCCGCATATAGTTT	(TG)9(AG)18
8	SSR119699[a]	F-GCCGTGGTGGAAGATGTACT R-CGAGTTCACCAAGAACGTCA	AT(5)
9	SSR123557[a]	F-ATCTCCTCGTTCTTCCCCAT R-GCTTGTAGCAGGCAGGAAAC	CT(4)
10	ssrR175[a]	F-GCAGTGACGCAGCAATG R-AAAAGGAGAGCCAAAGCAGT	GA(20)
11	M428[b]	F-GGGCACCAAAGAAAGTGG R-GATGTGAAATGCAAGCAGGA	AC(9)
12	M329[b]	F-ACTCAGACAAACCCTTCAAC R-GATGTTTTGCATCTATTTGG	GT(10)
13	M363[b]	F-GCATCTTCGTCTTCTTTGG R-GAAACGGGCCACTTGA	AT(6)AC(7)
14	M358[b]	F-CATGCACTATTATGTTTGTGTTTT R-TCTCGTCATATTTACAGGTAGGTT	CA(11)
15	M312[b]	F-TTGCGTAAAGAGAACGAGCA R-GTGTGGAAAATTCACCTGAGC	TG(9)
16	M840[b]	F-GTCAGAGGCAACTGTAGGTTAATG R-CCAAATCCACTATTTCTTGGTTG	AC(19)
17	C.S.[c]	F-GCCGAATGCTCCTTACTTTC R-CAGGATACAGGGGAATGGGATC	EST
18	ICL[c]	F-GGCACAGCATCAAGAACCT R-ATCCAGTATCGCCATCAGC	EST
19	ETR[c]	F-GAGATGGTCCCTGCATGTTA R-TTGCTCGTCTTGAACCATAGC	EST
20	GAL[c]	F-CAGGAACCCAGGATTACA R-AGTTCTGCCCAACAGTCA	EST

RESULT AND DISCUSSION

Primary callus induction and embryogenic callus formation

The study revealed nonsignificant interaction between genotype and plant growth regulators in callus initiation and formation processes. Additionally, the effect of genotype alone was found to be nonsignificant. However, a significant interaction was observed in terms of callus fresh weight. This suggests that the response to the specific media used was consistent among the three Arabica coffee varieties tested.

Furthermore, it was observed that 'AS2K' exhibited superior performance in terms of induced callus fresh weight compared to 'S-795' and 'Sigarar Utang' (Table 2). This indicates distinct differences in the cultivar responses, with 'AS2K' showing more excellent responsiveness to the culture conditions, resulting in higher callus fresh weight.

The results presented in Table 3 indicate that using plant growth regulators (PGRs) in the culture media positively impacted both the callus formation percentage and the callus's fresh weight. Callus induction was observed when the media contained a combination of 2,4-D (at concentrations ranging from 4.52 to 9.04 µM) and 2-iP (at concentrations ranging from 4.93 to 24.65 µM). The concentration of the PGRs played a crucial role in callus formation, with higher concentrations leading to increased formation. It was observed that an increase in the concentration of 2-iP improved callus formation when combined with 4.52 µM 2,4-D. However, this effect was not observed when combined with 2,4-D at 9.04 µM. Interestingly, at higher concentrations of 2,4-D, increasing concentrations of 2-iP no longer affected callus formation.

Based on the data obtained, the most effective combinations for callus formation were found to be 2,4-D at 4.52 µM and 2-iP at 24.65 µM, as well as 2,4-D at 9.04 µM and 2-iP at 4.93 µM (Table 3). These specific combinations resulted in the highest percentages of callus formation and greater fresh weight of the induced callus. The interaction between coffee cultivars and media composition significantly impacted the number of torpedoes formed (Table 4). The highest torpedoes were observed in the 'S-795' and 'Sigarar Utang' cultured in media containing 2,4-D at 9.04 µM and 2-iP at 24.65 µM. However, the difference in torpedoes formed between this combination and the highest concentration was insignificant. Therefore, 2,4-D at a concentration of 4.52 µM and 2-iP at 14.79 µM was considered sufficient.

In the case of 'AS2K', the formation of torpedoes was more prominent when cultured in media containing 2,4-D at a concentration of 4.52 µM and 2-iP at 19.72 µM. These results highlight the specific combinations of PGRs and their concentrations that were most effective in promoting torpedo embryo formation for each coffee cultivar.

Table 2. Effect of Arabica coffee cultivars on the percentage of callus formation and fresh weight 4 mo after culture. Based on DMRT at 5%. Values followed by the same letters in each column are not significantly different.

Cultivars	Callus formation	Callus fresh weight
	%	g
AS 2K	92.57	0.44[a]
S 795	91.23	0.42[ab]
Sigarar Utang	90.11	0.40[b]

Table 3. Effect of plant growth regulators combination on the percentage of callus formation and fresh weight 4 mo after sub-culturing. Based on DMRT at 5%. Values followed by the same letters in each column are not significantly different.

Culture media		Callus formation	Callus fresh weight
2,4 D	2-iP		
µM	µM	%	g
4.52	4.93	87.02^{b}	0.35^{d}
4.52	9.86	87.14^{b}	0.36^{d}
4.52	14.79	89.52^{ab}	0.38^{cd}
4.52	19.72	90.95^{ab}	0.40^{bcd}
4.52	24.65	91.90^{ab}	0.41^{abc}
9.04	4.93	93.33^{a}	0.43^{ab}
9.04	9.86	93.81^{a}	0.44^{ab}
9.04	14.79	92.86^{a}	0.45^{ab}
9.04	19.72	93.81^{a}	0.45^{ab}
9.04	24.65	93.33^{a}	0.47^{a}

Table 4. Interaction effect of arabica coffee cultivars and plant growth regulators combination to number torpedo development in regeneration media 8 mo after sub-culturing. Based on DMRT at 5%. Values in the same column followed by the same lowercase letters or the same row followed by the same uppercase letters are not significantly different.

Culture media		Cultivars		
2,4 D	2-iP	AS2K	S-795	Sigarar Utang
µM	µM			
4.52	4.93	32.20^{eA}	37.90^{cA}	39.40^{dA}
4.52	9.86	44.80^{deA}	43.70^{bcA}	45.80^{bcdA}
4.52	14.79	61.90^{bcdA}	48.60^{abcA}	62.50^{abA}
4.52	19.72	94.20^{aA}	52.10^{abcC}	61.90^{abcB}
4.52	24.65	81.90^{abA}	54.70^{abB}	52.20^{bcdB}
9.04	4.93	66.10^{bcdA}	51.80^{abcB}	43.20^{cdC}
9.04	9.86	56.70^{cdA}	48.70^{abcA}	57.40^{abcA}
9.04	14.79	69.90^{bcA}	53.80^{abcB}	57.90^{abcB}
9.04	19.72	79.40^{abcA}	61.20^{aAB}	55.00^{abcB}
9.04	24.65	83.40^{abA}	62.90^{aC}	72.00^{aAB}

The findings from previous studies provide additional insights into the impact of different concentrations and combinations of PGRs on callus formation. Callus formation was decreased at 5 µM 2,4-D, and callus induction did not occur at concentrations exceeding 10 µM 2-iP (Arimarsetiowati, 2011). In contrast, Mwaniki et al. (2019) reported improved outcomes when combining a low concentration of 0.5 µM 2,4-D with lower concentrations of BAP and kinetin.

More recently, Arimarsetiowati et al. (2023a) conducted studies that demonstrated the effectiveness of using 1 mg L^{-1} 2,4-D in combination with 1 mg L^{-1} BAP, resulting in the highest and heaviest callus compared to other treatments without 2,4-D. It was noted that higher concentrations of PGRs were necessary to stimulate cell responsiveness during the initial stages of callus formation, allowing the cells to absorb nutrients and undergo dedifferentiation into embryogenic cells (Ikeuchi et al., 2013). Interestingly, in the current study, a combination of PGRs at different concentrations resulted in a significantly higher percentage of callus formation (93.81%) compared to the maximum of 50% achieved in the study by Arimarsetiowati et al. (2023b) using a different combination of PGRs. These results suggest that the specific combination and concentration of PGRs used in the current study were particularly effective in inducing embryogenic callus formation. Overall, these findings highlight the importance of optimizing the concentration and

combination of PGRs for achieving successful callus formation in arabica coffee, and the results of the current study demonstrate a higher success rate compared to previous research.

Regeneration and somatic embryo development

The interaction between coffee cultivars and media composition significantly impacted the number of germinating somatic embryos (Table 5). The highest number of germinated somatic embryos was observed in 'S-975' and 'Sigarar Utang' when cultured in media containing 2,4-D at 9.04 µM and 2-iP at 24.65 µM. Similarly, 'AS2K' produced the most germinated somatic embryos when cultured in media containing 2,4-D at a concentration of 4.52 µM and 2-iP at 19.72 µM.

These results demonstrate the genotype-specific responses to media composition, highlighting the importance of selecting the appropriate combination of PGRs and their concentrations to enhance somatic embryo production. These findings are consistent with previous studies by Mwaniki et al. (2019), which also reported significant differences in genotype responses to somatic embryo production.

During the callus induction stage, the composition of the media and the concentration of PGRs played critical roles in explant morphogenesis and the somatic embryogenesis process. The parameters, such as torpedo and germinated somatic embryo numbers, were influenced by the combination of PGRs and their concentrations. Cytokinins and auxins, such as 2,4-D and 2-iP, are commonly used to stimulate cell division and elongation in in vitro culture systems (Ikeuchi et al., 2013; Ogunyale et al., 2014). The appropriate combination of these growth regulators with a basic formulation of the media can enhance the success of the somatic embryogenesis process.

Table 5. Interaction effect of arabica coffee cultivars and plant growth regulators to germinated somatic embryos in maturation medium 2 mo after sub-culturing. Based on DMRT at 5%. Values in the same column followed by the same lowercase letters or in the same row followed by the same uppercase letters are not significantly different.

Culture media		Cultivars		
2,4-D	2-iP	AS2K	S-795	Sigarar Utang
µM	µM			
4.52	4.93	21.10^{eB}	27.70^{bAB}	29.60^{dA}
4.52	9.86	35.20^{deA}	30.70^{bA}	35.90^{bcdA}
4.52	14.79	49.90^{bcdA}	35.60^{abA}	53.40^{abA}
4.52	19.72	82.20^{aA}	39.50^{abB}	50.90^{abcB}
4.52	24.65	69.90^{abA}	41.70^{abB}	41.20^{bcdB}
9.04	4.93	54.10^{bcdA}	38.80^{abAB}	32.20^{cdB}
9.04	9.86	44.70^{cdA}	35.50^{abA}	46.40^{abcA}
9.04	14.79	57.90^{bcA}	41.00^{abA}	46.30^{abcA}
9.04	19.72	67.40^{abcA}	50.20^{aAB}	40.00^{abcB}
9.04	24.65	71.40^{abA}	49.10^{aB}	60.60^{aAB}

Morphological and histological somatic embryogenesis observation

Understanding embryogenesis is crucial to examining the cellular processes underlying these morphological changes. Figure 1 illustrates the stages of indirect somatic embryogenesis induced by 2,4-D and 2-iP treatments, from callus formation to the production of uniform plantlets via the pro-embryonic mass (PEM) and globular embryo stages. Morphological changes in coffee leaf explants treated with plant growth regulators were observed 2 wk after culture. By the third week, the edge of the leaf cut had thickened and initiated the callus formation, with most calli appearing near the contact point with the culture medium (Figure 1A). Embryogenic callus is characterized by a yellowish-white color (Figure 1B). Embryogenic callus, which is meristematic cells, forms a pro-embryogenic mass or PEM (Figure 1C). The PEM has loose bonds between its cells, making them easily detached.

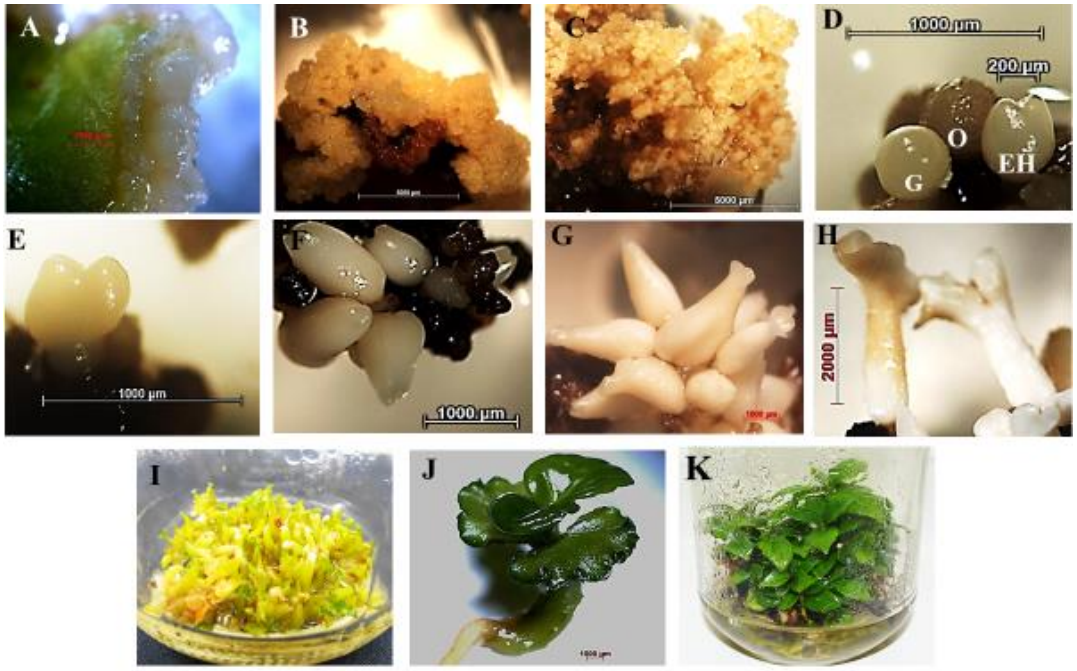

Figure 1. Morphological stage of indirect somatic embryogenesis of arabica coffee. A. Callus in initial induction medium. B. Embryogenic callus in advanced callus induction medium. C. Pro-embryonic mass (PEM). D. Globular. E. Heart. F. Elongated embryo. G. Torpedo. H. Cotyledonary. I. Germinated embryo. J. Plantlet. K. Plantlets ready for acclimatization.

The development of somatic embryos in arabica coffee follows a series of morphological stages. Initially, the proembryo develops into a globular phase embryo, which is small in size, approximately 200 µm in diameter. The globular embryo then progresses into an oblong phase, where the upper part elongates, resulting in an ovoid shape. Subsequently, the embryo enters the liver stage, characterized by a visible indentation at the top and the initiation of cotyledon formation. This bulge expands laterally, causing the embryo to split bilaterally symmetrically. The elongated phase follows the liver stage, and the elongation of the embryo characterizes it. Once the diameter of the elongated embryo reaches around 600 µm with a height of approximately 2000 µm, it enters the torpedo phase.

The cotyledonary phase begins with the opening of the embryo's top, revealing the cotyledon leaf ovary. This stage can be observed after 1 mo in the maturing medium, and the height of the cotyledonary phase exceeds 2000 µm. After 3 mo in the maturing medium, the arabica coffee sprouts exhibit green coloration, indicating the initiation of photosynthesis. The sprouts develop first leaves (cotyledonary leaves) and exhibit embryonic roots (radicle) with a taproot structure. The final stage depicted in Figure 1K shows coffee plantlets ready for acclimatization.

Histological examination of the somatic embryogenesis process reveals the progression of different stages (Figure 2). Wounded leaf cells undergo dedifferentiation and form an embryogenic callus characterized by enlarged nuclei, compact cytoplasm, and thicker cell walls. The nucleus actively divides, and as it approaches the cell wall, it increases in size and becomes centrally positioned. The histological progression from the globular phase to the oblong phase, heart stage, elongated stage, torpedo phase, and initial cotyledonary stage can be observed. The presence of procambium tissue is visible in the oblong phase, and cotyledon formation is seen as a slight bulge at the apical edge of the embryo. As the embryo elongates, the procambium also elongates. In the torpedo phase, both the cotyledon and elongated procambium are visible, with the cotyledon serving as the embryo's preparation for germination. The cotyledonary stage

shows the opening of the cotyledon and the emergence of two cotyledons at the top of the embryo. This aligns with research results of Bartos et al. (2018).

These observations on morphology and histology align with previous studies and confirm the critical role of auxin and cytokinins in initiating somatic embryogenesis in arabica coffee. Although somatic embryos may exhibit irregular shapes and smaller sizes than zygotic embryos, these variations can be attributed to differences in cell elongation and storage components between the two developmental processes.

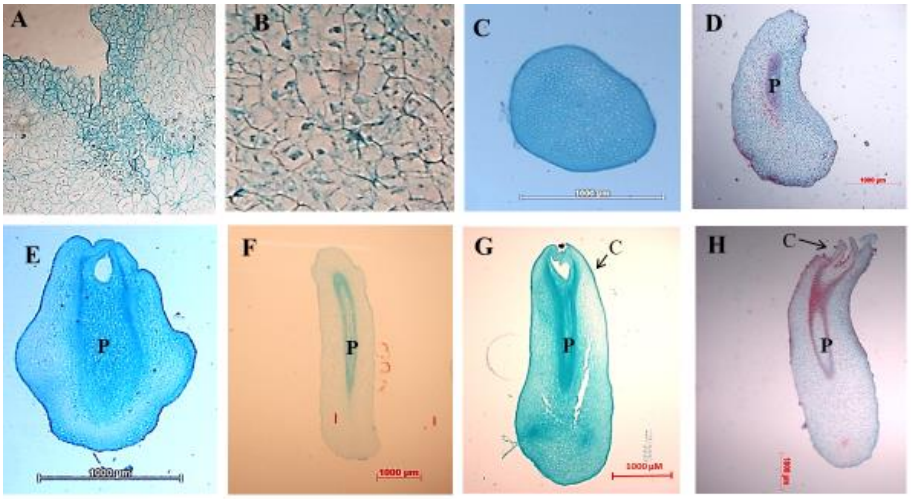

Figure 2. Histological observation of embryogenic callus and development of embryogenesis of arabica coffee. A. Performance of embryogenic callus tissues. B. Performance of active cell tissues (nucleus was multiplied). C. Globular. D. Oblong. E. Heart. F. Elongated stage. G. Torpedo. H. Cotyledonary. P: Procambium; C: cotyledon.

Early detection of somaclonal variation of *C. arabica*

During the primer screening, some primers failed to produce specific amplification products. As a result, 10 primers that generated distinct amplicons were chosen for probing somaclonal variation. Molecular analysis of 50 arabica coffee samples using these 10 SSR primers (ssrR105, ssrR209, ssrR268, ssrA8847, ssrCMA008, ssrCMA059, ssrCMA198, SSR119699, SSR123557, and ssrR175) did not reveal any polymorphic band, indicating the absence of somaclonal variation in the tested samples. Figure 3 shows the amplification results of two of the tested 20 primers.

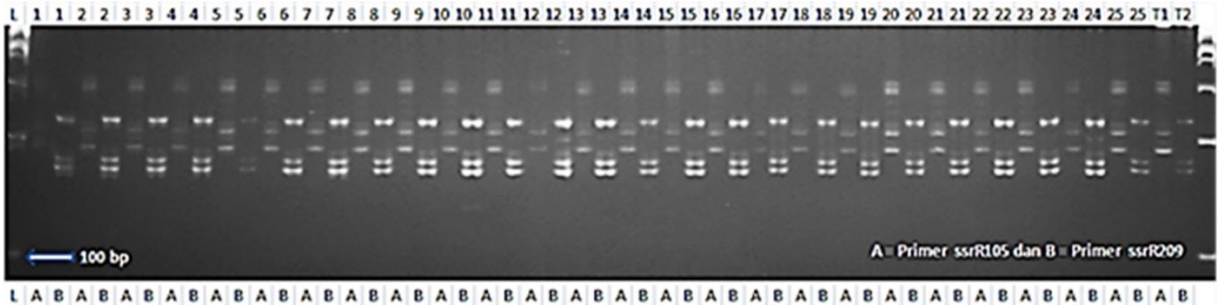

Figure 3. Amplification result of plant DNA from somatic embryogenesis using primers ssrR105 and ssrR209. L: Leader; T1: parent using ssR105 primer; T2: parent using ssR209 primer. Numbers 1-25: Plantlets resulting from somatic embryogenesis using ssrR105 and ssrR209 primers.

The research results are consistent with the findings of Arimarsetiowati et al. (2023b), which also reported no somaclonal variations in arabica coffee. However, it differs from the results of Bobadilla Landey et al. (2013) and Gatica-Arias et al. (2008), who observed somaclonal variations in arabica coffee at different frequencies. Various factors, including the genotype of the plant, source of explants, age of the culture, type and concentration of growth regulators, ploidy levels of plant chromosomes, duration of the callus induction process, and frequency of subcultures can influence the occurrence of somaclonal variations.

The duration of the suspension culture has been shown to affect the occurrence of somaclonal variations in coffee. Etienne and Bertrand (2003) demonstrated that the longer the suspension culture was maintained, the higher the frequency of somaclonal variations. For example, a 3-mo suspension culture resulted in a 1.3% somaclonal variation, while a 12-mo suspension culture led to a 25% increase. Therefore, it is advisable to avoid prolonged callus induction and suspension culture to minimize the occurrence of somaclonal variations.

In this study, no somaclonal variations were detected. The callus induction medium used for arabica coffee somatic embryogenesis included 4.52 µM 2,4-D and 19.72 µM 2-iP for 'AS2K', 4.52 µM 2,4-D and 14.79 µM 2-iP for 'S-795', and 4.52 µM 2,4-D and 19.72 µM 2-iP for 'Sigarar Utang'. Subsequent subculturing to regeneration and ripening media was performed to propagate the somatic embryos. The results of this study, which employed the same cultivars as the study by Ibrahim et al. (2015), were better for all parameters measured, indicating that the application of 2,4-D and 2-iP would be more effective than thidiazuron. Overall, the findings indicate that the chosen medium formulations and culture settings in this study were successful in causing somatic embryogenesis in arabica coffee without causing somaclonal abnormalities.

CONCLUSIONS

The study findings indicated that the optimal medium for in vitro propagation of 'AS2K' was 4.52 µM 2,4-D and 19.72 µM 2-iP. In contrast, for 'S-795' and 'Sigarar Utang' were 4.52 µM 2,4-D and 14.79 µM 2-iP. Importantly, no instances of somaclonal variation were detected in the plantlet samples analyzed. These results have significant implications for advancing in vitro propagation techniques for specific specialty coffee varieties in Indonesia, presenting opportunities for improving the overall process.

Author contribution

Conceptualization: M.S.D.I., R.S.H. Methodology: M.S.D.I., R.S.H., R-Rubiyo, R-Reflinur. Validation: M.S.D.I., R.S.H., SS. Formal analysis: M.S.D.I., R-Reflinur. Writing-original draft: M.S.D.I., R.S.H. Writing-review & editing: M.S.D.I., R.S.H., S.S. Supervision: S.S., A.P., R-Rubiyo. Project administration: M.S.D.I., R.S.H. Funding acquisition: M.S.D.I., R.S.H., R-Rubiyo, R-Reflinur. All authors have read and approved the final version of the manuscript and are considered the main contributors to the study.

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