

Establishment of an Efficient Primary Callus Induction for Embryogenic Potential of *Coffea arabica* L.

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Abstract

Coffea arabica L. is a species of coffee that contribute for more than seventy percent of world coffee production. Various attempts have been made to obtain large quantities of planting material through propagation *in vitro* somatic embryogenesis technology. Producing embryo somatic depends on the embryogenic callus formation from the primary callus. The problem is that not all primary calluses meet the embryogenic criteria for developing into an embryo somatic. The objective of this experiment was to evaluate the effect of different plant growth regulators (PGRs) on callus induction (indirect somatic embryogenesis) in AS2K clone of Arabica coffee. Mother plants of Arabica coffee were established in coffee experimental field of Indonesian Coffee and Cocoa Research Institute at Andungsari, Bondowoso, East Java, Indonesia. Leaf explants were cultured on a half-strength Murashige and Skoog (MS) medium supplemented with various concentrations of 1.0, 2.0, 3.0 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1.0, 2.0, 3.0 mg L⁻¹ 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea (thidiazuron) in combination with 1.0 mg L⁻¹ 6-benzylaminopurine (6-BAP). All the experiments were organized in completely randomized design and repeated three times, each experimental unit using minimum seven replicates (a total of 21 explants per combination mediums). The morphology of the different types of callus was observed under a stereo-zoom binocular microscope. Histological analysis on callus formation was observed on the surface of explants by anatomic preparation using the paraffin. The percentage of callus formation was recorded every two weeks until eight weeks. The highest percentage of callus formation (almost 60%) was in medium containing 1 mg L⁻¹ 2,4-D dan 1 mg L⁻¹ BAP. In medium containing TDZ and BAP, the percentage of callus formation was low between 2-10% in all periods of culture and the calli was growing slowly. Morphological and histological studies prove that the callus has a friable and embryogenic texture and begins to develop various stages of somatic embryo formation.

Keywords: callus, *Coffea arabica* L., *in vitro* culture, plant growth regulators, somatic embryo

INTRODUCTION

Plant tissue culture is important in the production of agricultural and ornamental plants, as well as in plant manipulation for enhancing agricultural performance. Plant cell and tissue *in vitro* culture have triggered tremendous interest because it facilitates the investigation of plant physiological and genetic processes, as well as the potential to support the breeding of improved cultivars by increasing genetic variability.

Somatic embryogenesis (SE) is an asexual reproduction approach that grows naturally in many plants species and is widely used for rapid multiplication, crop transformation, and regeneration. Somatic embryogenesis and zygotic embryogenesis share some developmental and physiological similarities because it involves common factors of hormonal, transcriptional, developmental, and epigenetic controls (Salaün *et al.*, 2021). SE also offers an appropriate *in vitro* regeneration procedure as a primary step in plant genetic improvement (Loyola-Vargas, 2016). SE is associated with the formation of haploid or diploid somatic embryos without gamete fusion. SE is influenced by various of factors, such as genotype, explant type, and plant growth regulators. Suitable conditions for SE induction are generally conducted experimentally via trial and error experiments (Loyola-Vargas, 2016). The morphological and histological examination could reveal the somatic embryogenesis mechanism and provide insights for enhancing procedures that influence this regeneration method (Silva *et al.*, 2015).

via friable embryogenic callus formation

(Molina *et al.*, 2002). ISE in coffee comprises a sequence of steps including callus induction and proliferation, and embryo development, as well as germination and conversion into plants (van Boxtel & Berthouly, 1996).

Growth regulators are critical in regulating the formation of somatic embryos in coffee leaf explants. The auxin/cytokinin combination is commonly used for IES induction in *C. arabica*. The most popular auxin 2,4-D was utilized to stimulate callogenesis in *C. arabica* leaf explants. Because auxin inhibits embryo emergence, cytokinins without auxin were used for direct SE induction. The efficiency of the direct SE response, on the other hand, may vary depending on the type and concentration of cytokinins used (Andrea, 2020). A concentration of 5 M of the synthetic cytokinin 6-benzylaminopurine (6-BAP) was sufficient for direct SE induction on *C. arabica* explants (Rojas-Lorz *et al.*, 2019). In leaf explants of the Mundo Novo de *C. arabica* cultivar, 6-BAP at 30 M resulted in higher somatic embryo production than at 10 and 20 M 6-BAP (Almeida & Silvarolla MB, 2009). Despite the high 6-BA concentration, embryo production was reduced, and the process took a long time. The TDZ cytokinin has also been used to induce direct somatic embryo regeneration in *C. arabica* (Kahia *et al.*, 2016; Yi-Chieh *et al.*, 2018), with a concentration of 1M TDZ resulting in a 100% embryogenic percentage. TDZ has a significant impact on embryogenic callus formation and embryo multiplication.

The formation of embryogenic callus

influence of plant growth regulator through

indirect somatic embryogenesis system at various of media compositions of 2,4-D, thidiazuron and BAP to be subsequently

MATERIALS AND METHODS

Planting Material Resource

Mother plants of Arabica coffee were established in coffee experimental field of Indonesian Coffee and Cocoa Research Institute at Andungsari, Bondowoso, East Java, Indonesia. GPS coordinate and elevation of plot were recorded with data showing $S07^{\circ} 55' 42.7''$ and $E113^{\circ} 41' 30.2''$ and 1451 m asl. The second pairs of young leaves (i.e., 100 explants) from the tip on plagiotropic branches were collected from healthy mother plants growing in greenhouse as plant material

Medium

In order to develop an efficient and reliable procedure for somatic embryogenesis, leaf explants were cultured on MS (Murashige and Skoog) medium supplemented with various hormones in different concentrations and combinations for a certain stages of in vitro micropropagation of somatic embryogenesis based on Sanolard *et al*

Surface Sterilization of Explants

The leaves were washed under running tap water and rinsed with sterile distilled water, followed by surface sterilization in 30% sodium hypochlorite solution (5.25%

active chlorine) for 15 min and rinsed thrice using sterile distilled water. The leaf explants were subsequently immersed in 70% (v/v) ethanol for 20-30 s then rinsed thrice with sterile distilled water. The margin and the mid-rib of each leaf were removed and the remaining leaf tissue was cut into 1.0 cm² pieces to be used as explants. The explants were incubated on a half-strength MS medium with the adaxial side down in the culture vessel. These materials were used as source leaf explants for indirect somatic embryogenesis under

Callus Induction

To evaluate the effect of different plant growth regulators (PGRs) on callus induction (indirect somatic embryogenesis), leaf explants were cultured on a half-strength MS medium supplemented with various concentrations of

1.0, 2.0, 3.0 mg L⁻¹ of 2,4-Dichlorophenoxyacetic acid (2,4-D) or 1.0, 2.0, 3.0 mg L⁻¹ 1-phenyl-3-(1, 2, 3-thidiazol-5-yl)-urea (TDZ) in combination with 1.0 mg L⁻¹ N6-benzyladenine (BAP). The pH of all media was adjusted to 5.6 which was

Table 1. Composition of callus inducing medium (CIM) for indirect somatic embryogenesis from leaf explants of *C. arabica* L

Medium components	Callus inducing medium (CIM)					
	CIM 1	CIM 2	CIM 3	CIM 4	CIM 5	CIM 6
MS macro and micro salt	½	½	½	½	½	½
Gamborg's B5 vitamins	+	+	+	+	+	+
BAP, mg L ⁻¹	1	1	1	1	1	1
2,4-D, mg L ⁻¹	1	2	3	-	-	-
Thidiazuron, mg L ⁻¹	-	-	-	1	2	3
L-cysteine, g L ⁻¹	0.08	0.08	0.08	0.08	0.08	0.08
Malt extract, g L ⁻¹	0.4	0.4	0.4	0.4	0.4	0.4
Casein hydrolysate, g L ⁻¹	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose, g L ⁻¹	30	30	30	30	30	30
Gellan gum, g L ⁻¹	2.8	2.8	2.8	2.8	2.8	2.8
pH	5.6	5.6	5.6	5.6	5.6	5.6

growth room at $25\pm 2^{\circ}\text{C}$. The percentage of callus formation was recorded every

Experimental Design and Data Analysis

The treatments is the combination of medium between 2,4-D and BAP or 2,4-D and thidiazuron. All the experiments were organized in a completely randomized design and repeated three times, each experimental unit using seven replicates (total of 21 explants per treatment). All statistical analyses were performed using RStudio software (Version 1.4.1106). One-way analysis of variance (ANOVA) was used to test for statistical significance and followed by Tukey's test

Morphological and Histological Analysis

The morphology of the different types of callus was observed under a stereo-zoom binocular microscope (SZ 4045 TR Olympus® Tokyo, Japan) coupled to the computer running image capture software (DP2-BSW). Histological analysis on callus formation was observed on the surface of explants by anatomic preparation using the paraffin method according to Ruzin (1999). The anatomic samples in the glass slides were observed under a light microscope

RESULTS AND DISCUSSION

Plant Growth Regulators Effects on Callus Formation

The success of AS2K clone Arabica coffee propagation through indirect somatic embryogenesis can be done using leaf explants through callus formation. The advantage of indirect somatic embryogenesis is fast, uniform, and large scales. Moreover,

embryogenic production, the greater the number of embryo will be achieved, and the plantlet will regenerate excessively. The type and concentration of auxin and cytokinin and or their combination differ from each species in producing the best medium (Méndez- Hernández *et al.*, 2019). Auxin is a growth regulator that can trigger cell division, cell growth development and meristem organization for callus formation (Kumar *et al.*, 2016). Auxin is commonly used to stimulate elongation and cell division, as well as to induce the formation of callus, culture suspension, and roots. Cytokinins are compounds that can promote the leaf's cell division, growth, and development. The combination of 2,4-D (auxin) with BAP (cytokinin) will stimulate cell growth and division (Mayerni *et al.*, 2020). Explant type, age, genotype, nutritional status, and interactions between endogenous and exogenous hormones can influence the development of somatic embryogenesis (Sujatha and Visarada, 2013; Singh *et al.*, 2016; Raji *et al.*, 2018).

This study used young leaves because it was more effective than other somatic tissue explants (root, hypocotyl, epicotyl). Callus grew on the explant side of the cut that was in direct contact with the medium. It is suspected that in leaf explants, cytokinins and auxins are not absorbed through the leaf epidermis, but are only absorbed by tissues that are in direct contact with the medium and are not translocated throughout the leaf tissue. The same thing also happened to Robusta coffee culture, where callus only formed on the side of the leaf. However, in some leaf explants, callus can form on the surface or

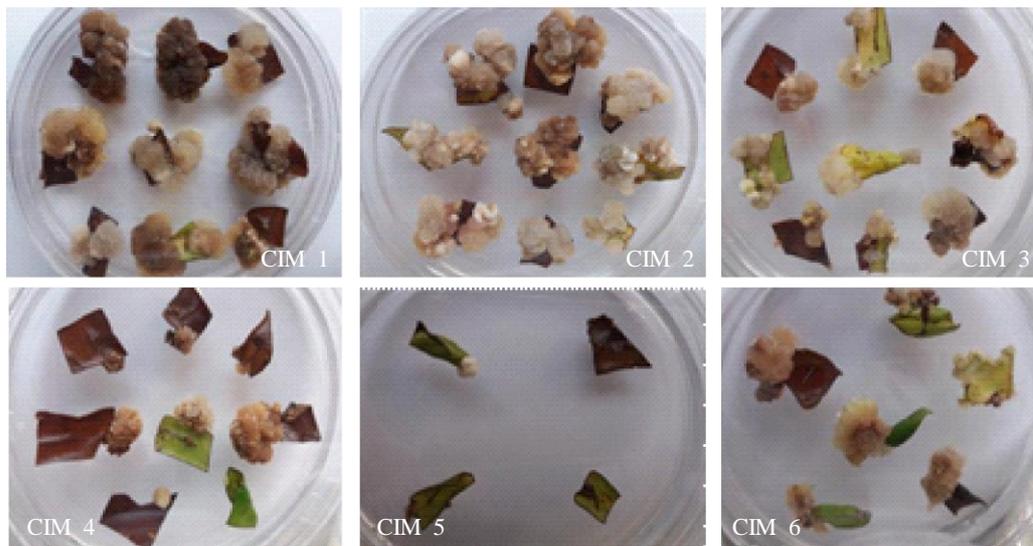


Figure 1. Callus formation through indirect somatic embryogenesis in Arabica coffee AS2K clone on 56 days after culture in CIM (callus inducing medium)

In this study, all concentrations and combinations of growth regulators containing 2,4-D, Thidizuron, and BAP could produce callus formation (indirect somatic embryogenesis) with different percentage responses. The first changes in leaf explant culture after being transferred to MS medium containing 2,4-D, Thidizuron, and BAP were swelling of the leaf explants on the 7th day and then callus formation was started on the 14th day of culture (Figure 2). The highest callus formation was achieved in medium containing 1 mg L⁻¹ 2,4-D and 1 mg L⁻¹ BAP (CIM 1) after 14 days of callus initiation with a percentage of 58%. Furthermore, on the 28th and 42nd days, there was a decrease in the percentage of callus formation on CIM 1 medium because some callus turned brown and died. In addition, the percentage of callus formation increased on the 56th day with a percentage of almost 60% on CIM 1 medium. Callus formation in

the calli was growing slowly. The combination of 2,4-D and BAP was the most effective composition in the callus formation of AS2K clone Arabica coffee. Previous studies have shown that 2,4-D is the best auxin in callus formation (Irene *et al.*, 2019). Among chemical compounds, 2,4-D is the dominant one to induce embryogenic callus in coffee species. This compound can stimulate an increase in endogenous natural auxin levels, can stimulate cell proliferation and produce mass embryogenic callus formation. Callus can form pre-embryoids, which are small white dots that appear on leaf explants. Somatic embryo induction and regeneration is a very sensitive process to various culture conditions. Embryogenic callus will naturally be produced after 60 days of culture on CIM medium with the characteristics of crumb, friable and yellowish color. In addition to CIM 1 medium, CIM 2 medium also produced a high percentage of callus formation. This is presumably due to the interaction and balance between growth regulators added

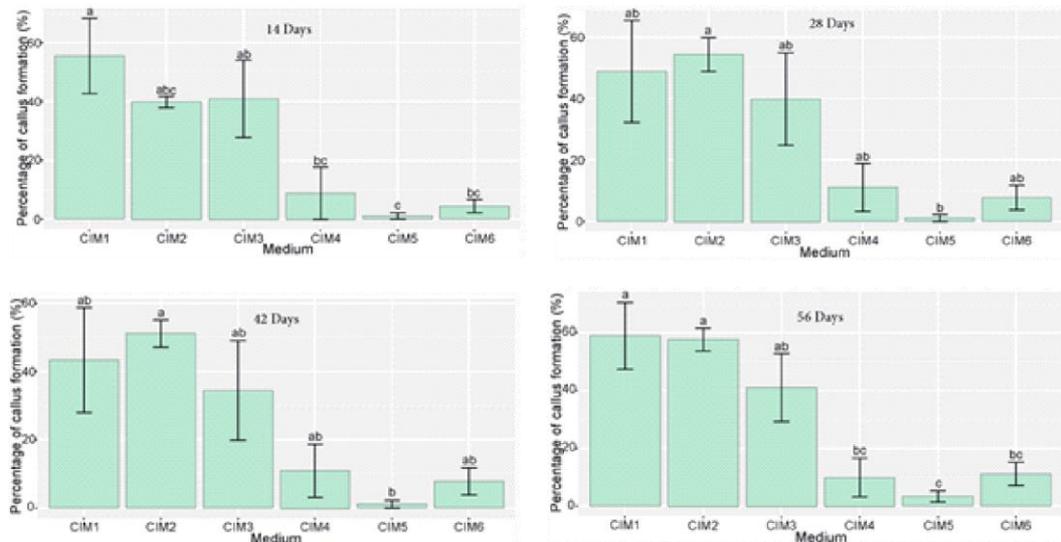


Figure 2. Percentage of callus formation through indirect somatic embryogenesis in Arabica coffee AS2K clone on 14, 28, 42, and 56 days after culture in CIM (callus inducing medium)

will change the ratio of endogenous growth regulators which then becomes a determining factor for the growth process

Growth Type, Structure, and Color of Callus

The effect of different combinations of PGRs and their interaction on callogenesis are presented in Figure 3 and 4. The experimental result clearly showed that the percentages and the characteristic of callus formation of AS2K clone Arabica coffee based on place of callus growth, type of callus growth, structure of callus, and color of callus, differed, based on the extent of the interaction between the combination of PGRs. The interaction between the callus induction percentage, the place where callus formed (whole leaf edge and certain part of the leaf) and the type of callus growth (low, intermediate, and high), varied widely on 14, 28, 42, and 56 days after culture in CIM (Callus Inducing Medium) medium (Figure 3). The highest

42, and 56 days after cultures with the callus growth were low, intermediate, and high, respectively and not significantly different between the period of culture. Furthermore, the interaction between the callus induction percentage, the structure (morphogenic and non- morphogenic) and color of callus (brownish, creamish, and whitish), varied widely on 14, 28, 42, and 56 days after culture in CIM (Callus Inducing Medium) medium (Figure 4). The highest percentage of the morphogenic callus (almost 60%) was reached in CIM 2 medium on 56 days after cultures with creamish color, but it was not a significant difference compared to the CIM 1 on 14 days after cultures. Similarly, the morphogenic callus formation was reached almost 60% in CIM 1 medium on 56 days after culture but the brownish color.

The application of cytokinins affected callogenesis by resulting in decrease of cell wall lignification, facilitating callus initiation and growth in vitro. It has been observed that callus

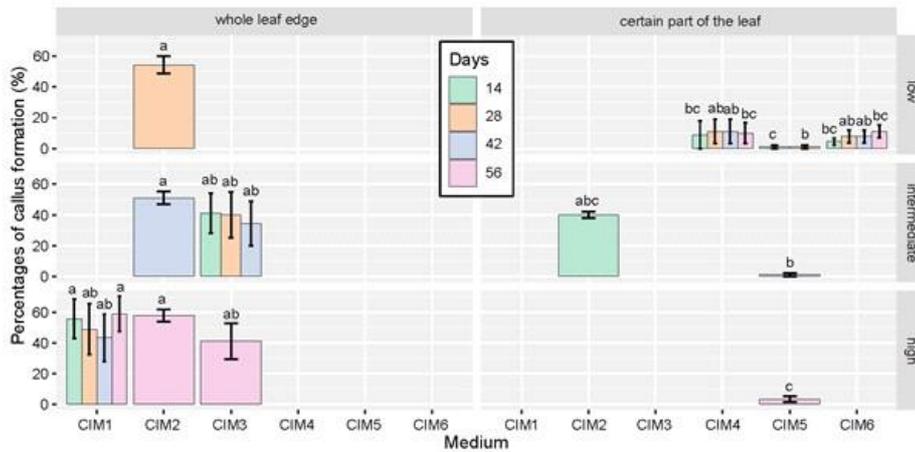


Figure 3. Characteristics of callus formation of Arabica coffee AS2K clone based on location (whole leaf edge and certain part of the leaf) and type of callus growth (low, intermediate, and high) on 14, 28, 42, and 56 days after culture in CIM (callus inducing medium)

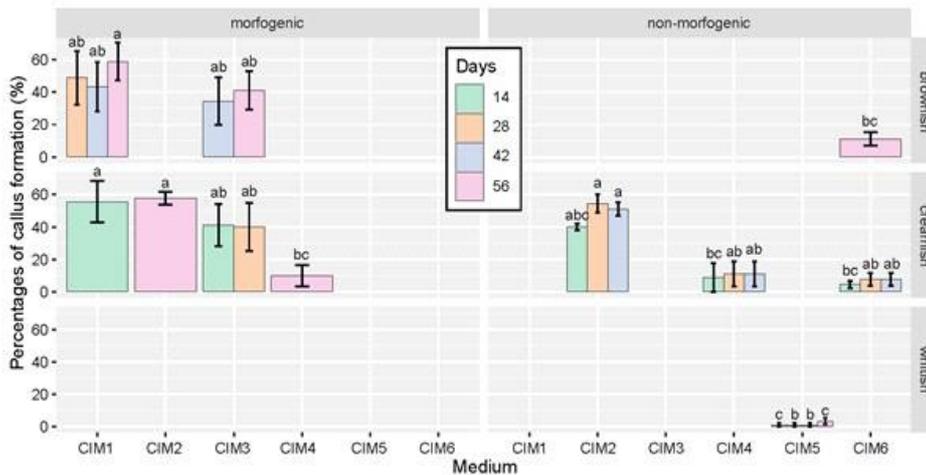


Figure 4. Characteristics of callus formation of Arabica coffee AS2K clone based on the structure (morphogenic and non-morphogenic) and color of callus (brownish, creamish, and whitish) on 14, 28, 42, and 56 days after culture in CIM (callus inducing medium)

were whitish, but some of them are creamish (Figure 5A). At the end of culture on 56 days, some of the callus were still creamish and later turned into brownish (Figure 5B). After 56 days of incubation, the brownish calli grew yellowish friable calli on the surrounding callus (Figure 5C). However, some of the callus are compact with hyperhydric look. Calli with hyperhydric

calli showed high lignification, including of their hard texture, whereas others were embryogenic and separated easily into small fragments (Figure 5C). Similarly colored and structured calli from leaf explants in *C. arabica* have been reported by Bartos *et al.* (2018). It was confirmed that the embryogenic calli were yellowish and

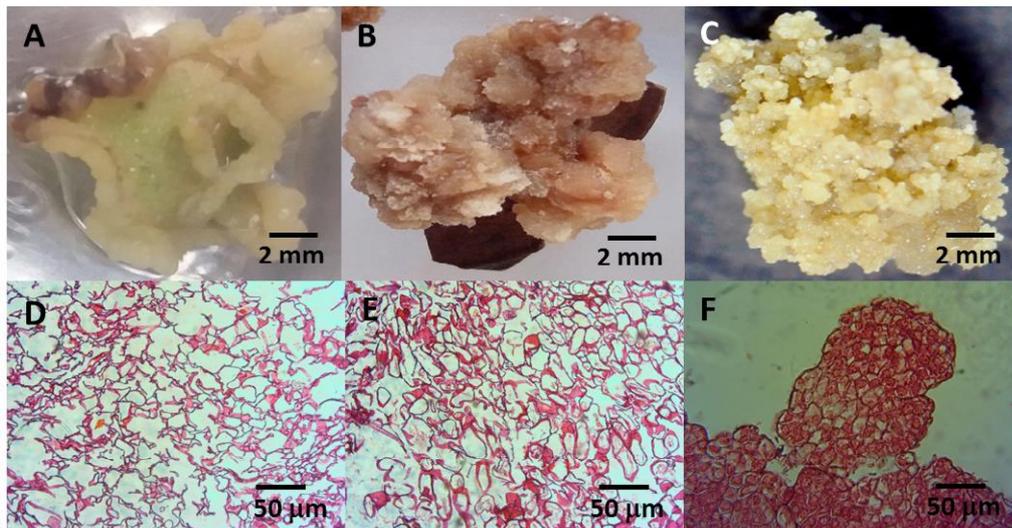


Figure 5. Three types of callus structures induced from leaves of *C. arabica* cultured on callus inducing medium. Morphologically, compact cream-colored, Type I primary calli (A); spongy brown structures, Type II primary calli (B); and friable white-yellowish, Type III embryogenic calli (C). Histological section of cream compact structure calli (D); brown spongy calli (E); and calli with embryogenic structure (F)

Callus Type Based on Morphology and Histology

In our study, explants responded by forming three types of callus which varied in color, textures, and friability (Figure 5). Morphologically, the calli were compact cream-colored (Type I calli; Figure 5A) after 14 days of cultivation in callus inducing medium, spongy brown structures (Type II calli; Figure 5B) after 30 days of cultivation in callus inducing medium, and friable white-yellowish (Type III calli; Figures 5C) after 120 days of cultivation. Both Type I and II calli possessed degenerated contracted cells without a distinct nucleus or a dense cytoplasmic region (Figure 5D and 5E). In contrast, Type III of friable calli were composed of small isodiametric cells with a conspicuous nucleus and a dense cytoplasm (Figure 5F). Similar observations

Type I primary calli (Figure 5A) is mostly made up of large parenchyma cells with developed vacuoles, reduced intercellular spaces, and relatively thick cell walls. Tores *et al.* (2015) revealed the existence of parts characterized by the appearance of large, elongated cells and well-developed vacuoles in histological changes of cell masses with no embryogenic aspects, such as the primary calli presented in this report. Morphological observations also revealed that some of the cells composed of the primary calli already exhibit cell dedifferentiation and strong evidence of mitosis during this stage of embryogenic development (Figure 5D).

The majority of Type II primary calli (Figure 5B) are elongated parenchyma cells with large intercellular spaces, developed vacuoles, and cell walls (Figure 5E). The frequent cell

that most of the cells that make up these propagules are parenchyma cells with lacking nuclei and protoplasm, destroyed cell walls, and a large amount of intercellular spacing while examining the histologic characteristics of non-embryogenic calli of *Coffea liberica* with morphological characteristics.

The Type III embryogenic calli were shown to be entirely composed of meristematic areas surrounded by small cells with isodiametric diameter, dense cytoplasm, visible nucleus, and narrower cell walls (Figure 5C). We even found cells with two nuclei in these propagules, indicating incomplete cytokinesis in the cell division process, along with cells at the onset of linearization, indicating the beginning of the organization involved in the somatic embryo formation process (Figure. 5F). The development of embryogenic calli clusters in this species is composed of small isodiametric cells with dense cytoplasm and prominent nuclei, and that somatic embryos are produced through a series of organized cell divisions. Ribas *et al.* (2011) observed, in histological sections of yellowish embryogenic calli from somatic embryogenesis of *C. arabica* variety Caturra, the proliferation of a homogeneous meristematic region mainly consisting of

These findings support those of Torres *et al.* (2015), who showed in the somatic embryogenesis of young *C. arabica* leaves of variety Catiguá that meristematic cells that form the embryogenic calli, when associated with optimal growth conditions, implement a frequent cell division process, which over period results in the production of embryogenic cell clusters that compose the somatic embryos of the species.

C. arabica leaf segments of approximately 1 cm² were cultures in primary calli induction medium for 30 days to induce somatic embryogenesis. It was confirmed at this stage of cultivation that calli formation began on the seventh day of culture (Figure 6). Histological examinations of leaf segments responsive to callus formation revealed that the development of these calli masses was strongly linked with the vascular bundle in 100% of the evaluated propagules (Figure 6D). It was identified that calli formation primarily happened in the first place near the edge of the excised leaves in these cultures because these areas have stronger contact of the vascular explant tissue with the ingredients of the nutrient medium.

During this stages of *C. arabica* somatic embryogenesis, we confirmed that the cell divisions that resulted in the primary calli

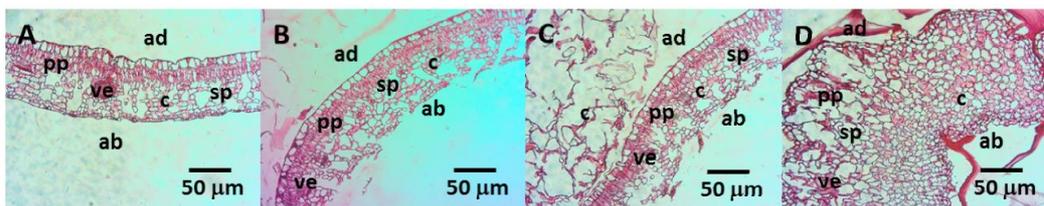


Figure 6. Histology of primary callus formation in *C. arabica* L. leaf segments; A. Explants at 7 days of culture, B. Explants at 14 days of culture C. Explants at 21 days of culture, D. Explants at 30 days of culture; Abbreviations: ab = abaxial face, ad = adaxial face, c = callus, pp = palisade parenchyma, sp = spongy parenchyma, ve = vessel element

i.e., from procambium cell division (Figure 6B-C-D). This finding suggests that using leaves at very advanced stages of development as an explants donor source for the induction of indirect somatic embryogenesis may not be worthwhile.

Cell proliferation resulted in a greater leaf area during cultivation, which was directly caused by the majority of meristematic divisions, which enabled the development during of differentiated cells. Moreover, as callogenesis progressed, we noticed that the effect to which the primary cells were separated from these promeristematic regions started to increase in size (Figure 6C-D).

As a result of their high pressure against the epidermal cells surrounding the leaf, the cells of the palisade parenchyma attached to the primary calli started showing a significant rise in width and a significant decrease in height, demonstrating straightening mechanisms. Nevertheless, it was not recognized in the spongy parenchyma cells adjoining the calli that persisted isodiametric even after cultivation development (Figure B-C). In contrast, we found no evidence of cell division or cellular dedifferentiation in the parenchymal tissue at the start of the development of primary calli. These findings support the theory that procambium cells initiated callogenesis in *C. arabica* leaf explants. The first stages of somatic embryogenesis were studied using leaf explants from eight different varieties of *C. arabica*. According to Futura *et al.* (2014), these findings are primarily due to the procambium possibly being meristematic tissue, which implies that its cells have a higher mitotic potential, enhancing the formation of primary calli. Furthermore, it is probably

tissue of the rib, even if they could have proceeded in the spongy parenchyma as well. With this comprehensive knowledge, morphological markers for each stage of *C. arabica* somatic embryogenesis can be developed. As a consequence, the optimal duration for culture transfer to the next step can be determined more easily and effectively, shortening the time.

CONCLUSIONS

This research concluded that propagation of AS2K clone Arabica coffee through indirect somatic embryogenesis technique was successfully developed using leaf explants inducing the formation of Type I primary callus from procambium cell divisions. The propagation step of coffee through indirect somatic embryogenesis begins with callus induction. The best combination medium for inducing callus formation was 1 mg L⁻¹ 2,4-D and 1 mg L⁻¹ BAP (CIM 1). These propagules differentiate into embryogenic calli, cell masses made entirely of meristematic cells after about 120 days of cultivation. The combination of 2,4-D and BAP was more

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