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Histological analyzes reveal that isodiametric cells with cell division and starch reserve are promising in the culture of cell suspensions of coffee.

ABSTRACT

Biotechnological techniques have been extensively studied in order to provide practical results for coffee improvement. Among these techniques, somatic embryogenesis is one used in this crop improvement.

Aims: to describe and correlate the morphology of *Coffea arabica* cell suspensions with their growth curve through electron microscopy and photonic analysis.

Methodology: during 28 days and with collections every four days, the growth curve of the cell suspensions was performed by calculating the growth and the growth rate of the same. At each collection, cell suspensions samples were used to perform the histological and ultrastructural analyzes.

Place and Duration of Study: The experiments were conducted in the Laboratório Central de Biologia Molecular (LCBM) and the Laboratório de Microscopia Eletrônica e Análise Ultraestrutural (LME) at Federal University of Lavras, (Brazil), in the year 2017.

Results: In the period evaluated (28 days of culture), the growth curve of the cell suspensions exhibited sigmoid standard with four distinct phases: lag, exponential, linear and deceleration, represented by the regression equation $y = -0{,}084x^2 + 3{,}9399x + 2{,}85$, significant at 5% probability. Histological and ultrastructural analyzes allowed visualization of embryogenic characteristics in all the sampling and with greater frequency in the sampling that corresponded to time zero up to 20 days of culture.

Conclusion: The importance of establishing the growth curve of cell suspensions is in the identification of the phases in which the fundamental kinetic processes occur, allowing the correct manipulation of the same. Associated with this data, the identification of distinct types of cells allowed identification of the ideal cell suspension to obtain an efficient propagation system.

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Keywords: Cells suspension, Clonal propagation, Embryogenic potential, Growth curve, Microscopic analyses.

ABBREVIATIONS

SE somatic embryogenesis; **CVS** sedimented cell volume; **GR** growth; **GRR** growth rate; **TEM** Transmission Electron Microscopy; **SEM** Scanning Electron Microscopy

1. INTRODUCTION

One of the most important crops cultivated around the world is coffee. There are two main cultivated species, *Coffea arabica* and *Coffea canephora*. Both species are difficult to improve through conventional breeding, taking at least 20 years to produce a new cultivar. Biotechnological tools such as genetic transformation, micropropagation and somatic embryogenesis (SE) have been extensively studied in order to provide practical results for coffee improvement [1].

Micropropagation techniques can be applicable to mass production of selected *C. arabica* clones. Among these techniques, somatic embryogenesis is one powerful biotechnological tool used in crop improvement. Embryogenic cells show two important characteristics; they are able to multiply or to proliferate, which makes SE suitable for mass production of elite cultivars, and the fact that plants can be regenerated from one single cell. SE can also be used to conserve interesting genotypes and/or the ones that are threatened with extinction [2]. There are several methods for inducing SE in the coffee tree which are genotype-dependent and leads to an almost empirical development of specific protocols for each species [3, 4, 5].

Among these methods, cell culture in suspension is a useful tool for micropropagation, genetic transformation, isolation of protoplasts, and cryopreservation, with high biotechnological value, because by means of this technique, a large number of cells can be obtained and, consequently, a larger number of plants, aiming at propagation on a commercial scale [6]. The establishment of plant cell culture in suspension is a difficult process, even when the species/genotype in question is able to adapt itself to this manner of culture. Populations of homogeneous cells with rapid multiplication are quite useful for studies of physiological processes in plants. Therefore, cell culture in suspension is a

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focus of study in many plant species. Through it, automation and reduction in operational costs are possible [7, 8].

Particularly, the use of embryogenic cell suspensions has frequently been associated with an increased likelihood of genetic instability and somaclonal variation in the regenerated plants [9]. The loss of genetic fidelity is detrimental for commercial purposes when the main objective is strictly the clonal propagation of elite plant material to ensure the maintenance of selected traits. Although embryogenic cell suspensions have been developed for some major crops, they have not been widely applied for commercial purposes. However, research made by [6] showed that SE based on embryogenic cell suspensions is efficient and reliable for true-to-type propagation of selected *C. arabica* varieties. The authors report that over 99% of coffee trees regenerated fully conform to the mother plant morphologically - they grow, flower and produce normally.

In spite of the advances achieved in establishment of embryogenic cell culture in suspension for the coffee plant, this system is still limited because it contains cell types in various stages of development. The lack of uniformity of the cell suspensions is related to low quality of the culture for biotechnological purposes. In addition, another challenge of this process is determining adequate conditions for establishing repetitive cycles of cell division and restricted control of differentiation processes in such a way that the culture is made up of pre-embryonic cells or somatic embryos in initial globular stages of development. Thus, it is important to monitor the life cycle of cells in suspension through a growth curve to identify the stages of cell development, which is specific for each culture and also for the cultivars, so as to establish an efficient protocol [10].

Responses obtained from the growth curve are enriched when associated with other knowledge tools, among them, microscopic analyses, which allow characteristics of embryogenic cells that differ from non-embryogenic cells to be visualized. Scanning electron microscopy allows observation of the external shape of the cells and calli, through which cell agglomerates characteristic of embryogenic cells may be observed. Through transmission electron microscopy, the size of the nucleus, the presence of nucleolus, the cytoplasmatic organelles, and the vacuoles can be observed in the cells [11, 12].

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Within this context, the aim of this study was to characterize the growth of cell suspensions of *C. arabica* during the growth curve through microscopic analyses, identifying the best stage of the curve for maintaining the cell suspensions with highest embryogenic potential, relating this to the culture time of the cell suspensions. Identification of distinct types of cells allowed identification of the ideal cell suspension to obtain an efficient propagation system, open the way for the use of the embryogenic cell suspension technique to other plant species: a revolution in the world of plant micropropagation on an industrial scale.

2. MATERIALS AND METHODS

2.1 Callus induction

Leaf explants of *C. arabica* cv. Bourbon Amarelo plants maintained in a greenhouse were used for callus induction. Leaves were washed in running water for 10 minutes and in a laminar flow cabinet they were decontaminated in NaCl (40 g L^{-1} dissolved in water) for 20 minutes and washed in autoclaved distilled water three times for 10 minutes. The explants were inoculated in MS/2 [13] culture medium supplemented with 20 µM of 2,4-D, 4.92 µM of IBA, 9. 84 µM of 2iP, 30 g L^{-1} of sucrose, 6 g L^{-1} of agar) for induction of primary calli. After a month, the explants were transferred to the embryogenic callus induction medium MS/2 supplemented with 10 µM of 2,4-D, 9.84 µM of 2iP, 4.92 of µM IBA, 20 g L^{-1} of sucrose, and 6 g L^{-1} of agar. The explants remained in this medium until the appearance of embryogenic calli [14].

2.2 Obtaining and multiplying cell suspensions

Embryogenic cell suspensions were obtained by transferring calli with embryogenic features to Erlenmeyer flasks containing 3 mL liquid multiplication medium consisting of MS/2 culture medium supplemented with 5 µM of 2,4-D, 4.92 µM of IBA, 9.84 µM of 2-iP, 0.5 g L^{-1} of citric acid and 20 g L^{-1} of sucrose [14] at an inoculum density of 10 g calli L^{-1} [15]. As the cell suspensions multiplied, they were transferred to larger volume Erlenmeyer flasks (25, 50 and 125 mL). The flasks were maintained in the dark under constant agitation at 100 rpm in a growth room at 25 °C, and the culture medium was renewed by 90 % every 15 days. The renewals of the culture medium occurred on the same day of collected of cell suspensions for analyses microscopics.

2.3 Growth curve

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The growth curve was determined from cells in suspension after 10 months of culture. The cell suspensions were inoculated in sedimented cell volume (CVS) of 1 mL of suspension per 15 mL of multiplication medium and kept in the dark in an orbital shaker at 100 rpm rotation and temperature of 25ºC. Sampling were made every four days over 28 days, for a total of 7 sampling. Growth (GR) and growth rate (GRR) of the suspensions were calculated by means of the respective equations: GR (%) = [(CVS *final* – CVS *initial*) x 100] ÷ CVS *final,* and GRR (mean) = (InCVS *final* – InCVS *initial*) ÷ time [14]. Collection time was closed at 28 days based on the study of [16], in which the author reported stabilization of the growth curve at 30 days of culture for suspensions of *C. arabica* cv. Catiguá.

The experiment was conducted in a completely randomized design containing four replications per collection. The data were subjected to polynomial regression analysis, with a 5% level of significance, using the software SISVAR 4.2 [17].

2.4 Photonic microscopy

For photonic analysis, 50 µL samples of cell suspension were collected during the growth curve and identified as sampling: T0 (before the Growth Curve), T1 (4 days), T2 (8 days), T3 (12 days), T4 (16 days), T5 (20 days), T6 (24 days), and T7 (28 days). The samples were fixed in FAA (formaldehyde, acetic acid, and alcohol) for 72 hours and transferred to 70% ethanol for conservation. After fixing, they were dehydrated in ethyl series, and subsequently infiltrated in a 50% alcohol plus resin solution overnight and then transferred to pure resin for 48 hours. Finally, they were embedded in Leica resin according to the manufacturer's protocol. The samples were sectioned with a thickness of 5 mm using a rotary microtome and stained with 0.12% toluidine blue solution and Lugol's solution. The stained sections were then mounted on slides and observed with a photonic microscope (Zeiss Axio Scope.A1, Oberkochen, Germany). Images were observed using AxioVision 4.8 capture system.

2.5 Transmission Electron Microscopy (TEM)

For analyses in TEM, the samples were collected at 4 and 28 days (T1 and T7) after the beginning of the growth curve. The samples were immersed in fixative (modified Karnovsky, 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.05 M cacodylate buffer, pH 7.2) for 24 hours and prepared

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according to the protocol described by [18]. Visualization of the samples was performed in a transmission electron microscope Zeiss EM 109, Oberkochen, Germany.

2.6 Scanning Electron Microscopy (SEM)

For analyses in Scanning Electron Microscopy (SEM), the samples were collected at 4 and 28 days (T1 and T7) after the beginning of the growth curve. They were immersed in fixative (modified Karnovsky, 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.05 M cacodylate buffer, pH 7.2) for 24h, and prepared according to the protocol of [18]. Samples were observed through the LEO Evo 40 (Oberkochen, Germany) scanning electron microscope.

3. RESULTS

3.1 Growth curve

The data of growth and growth rate were used to construct a growth curve as a function of culture time. In the period evaluated (28 days of culture), the growth curve of the cell suspensions exhibited sigmoid standard with four distinct phases: lag (0 to 4 days), exponential (4 to 20 days), linear (20 to 24 days), and deceleration (24 to 28 days) represented by the regression equation $y = -0{,}084x^2 + 3{,}9399x + 2{,}85$. The importance of establishing the growth curve of cell suspensions is in the identification of the phases in which the fundamental kinetic processes occur, allowing the correct manipulation of the same. Therefore, the subculture of the cellular suspensions of *C. arabica*, var. Borbon Amarelo, may occur up to 28 days, when there is a deceleration in its growth (Fig. 1).

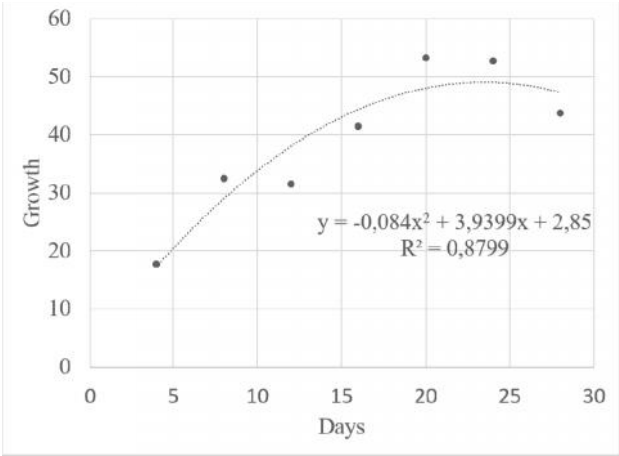

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Figure 1 – Growth curve analyzed through growth in cell volume after sedimentation (CVS) of cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo, beginning at 10 months of culture, significant at 5% probability.

The cell suspensions exhibited a constant growth rate percentage up to the 8th day. The GRR followed the GR at all points of the growth curve. The GRR began its decline on the 8th day, following the decline of the GR (Fig. 2). It is suggested that the beginning of the linear phase and later deceleration at 24 days occurred through depletion of nutrients in the culture medium, thus indicating that renewal of the medium should occur in the linear or stationary phase.

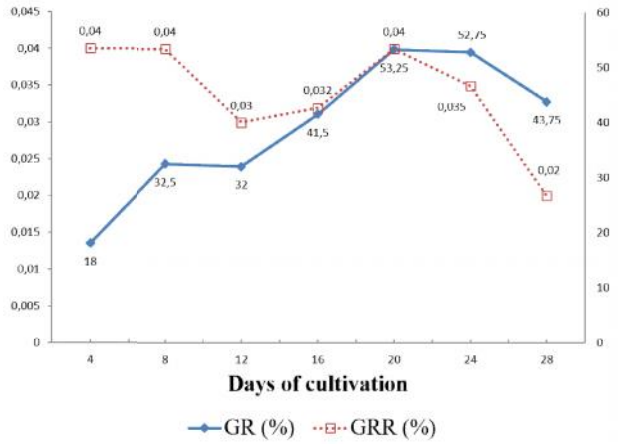

Figure 2 – Graphical representation of analysis of growth and growth rate of cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo in relation to sedimented cell volume (CVS) over 28 days at 4-day collection intervals.

In this study, this lag phase occurred from the 1st to the 4th day of culture, which is considered short, and exhibited growth of 18% and growth rate of 0.04%. The phase of exponential growth, the period in which maximum cell division occurs, was from the 4th to the 20th day of culture, exhibiting the highest GR of the embryogenic cell suspensions: 35.25% (mean obtained among the five collection times); for GRR, the value was 0.04%. The linear phase, in which cell division decreases and cell expansion occurs, was observed from the 20th to the 24th day of culture. The deceleration phase was observed from the 24th to the 28th day of culture.

3.2 Microscopic analysis

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In the present study, embryogenic characteristics were observed in all the sampling and with greater frequency in the sampling that corresponded to time zero up to 20 days of culture. For 20 days, there was predominance of large agglomerates of small isodiametric cells with apparent cell division, large nuclei, and evident nucleoli. From 20 days of culture on, non-embryogenic cells were observed with greater frequency, characterized by large, elongated, and vacuolated cells at the edge of the embryogenic agglomerates, probably in the process of cell death (Fig. 3). In relation to cell and nuclear diameter, embryogenic cells had from 8 to 24 µm of cell diameter and from 7 to 10 µm of nuclear diameter, reaffirming the morphology of large nuclei. For the non-embryogenic cells, cell diameter from 36 to 97 µm was observed. The cell suspensions collected at 28 days after the beginning of the growth curve had mostly large, elongated, and vacuolated cells, probably in the process of cell death, characteristics of material without embryogenic potential (Fig. 3 H).

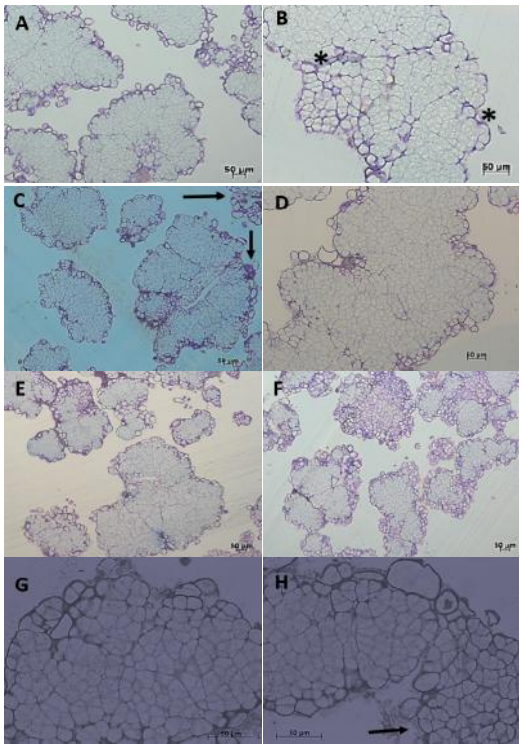

Figure 3 – Photomicrography of embryogenic characteristics of cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo collected during the growth curve. A) T0 B) T1 large nuclei and visible cell division* C) T2 increase in the frequency of large vacuolized cells at the edge of the agglomerates (arrows) D) T3 E) T4 F) T5 G) T6 H) T7 dead cells in asymmetric shape (arrows).

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The occurrence and storage of starch grains mainly occurred in the cells with embryogenic characteristics, corresponding to sampling T0 and T1 (Fig. 4 A, B). At the end of the sampling, more specifically at 24 and 28 days of culture, the starch grains were larger, however with a smaller number of cells (Fig. 4 G, H).

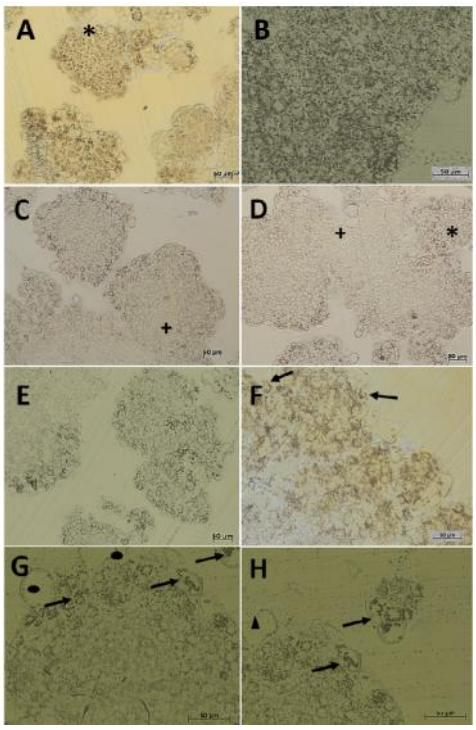

Figure 4 – Photomicrography of the presence of starch grains in the cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo collected during the growth curve. A) T0 agglomerates of small cells containing starch* B) T1 C) T2 increase in the frequency of non-embryogenic cells, without starch^{+} D) T3 non-embryogenic cells^{+} and embryogenic cells* in the same histological section E) T4 F) T5 cells containing starch grains (arrows) G) T6 large cells without visible nucleus at the edge of the agglomerate (represented by the black circle) and large starch grains in the cells (arrows) H) T7 Dead cells with irregular shape at the edge (represented by the triangle).

In the present study, these cell types varied in relation to predominance during the growth curve. Cells with distinct morphologies were observed over the entire growth curve, and these morphologies were identified as Type 1 – embryogenic cells, small cells with dense cytoplasm, absence of large vacuoles, with large nucleus, prominent nucleoli, and the presence of starch grains (Fig. 3, 4). The other cell type was denominated Type 2 – non-embryogenic cells, large, dispersed, vacuolated cells

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without nuclei, and little or no presence of starch grains. This cell type was consigned to high vacuolization of the peripheral cells, which little by little detach from the embryogenic aggregate and enter in the process of apoptosis (Fig. 4 H).

Analyses TEM allowed visualization of cells in suspension in the first collection of the suspensions at 4th day of the growth curve, containing an organized cell system with prominent nucleus and nucleolus, high nucleus/cytoplasm ratio, abundant presence of amyloplasts, and cell wall with small intercellular spaces (Fig. 5A; 5B). In the 28th day, during the growth curve of the suspensions, analyses by TEM identified the presence of vacuoles, some occupying nearly the whole of the cytoplasm, large intercellular spaces, and absence of cell content (Fig. 5C; 5D). Adding Fig. 3H to these results, which shows the presence of elongated cells (shape shown by the toluidine blue stain), without cell content, it is clear that most of the cells at 28th day had signals of low metabolic activity and/or the process of cell death, through all the characteristics cited here.

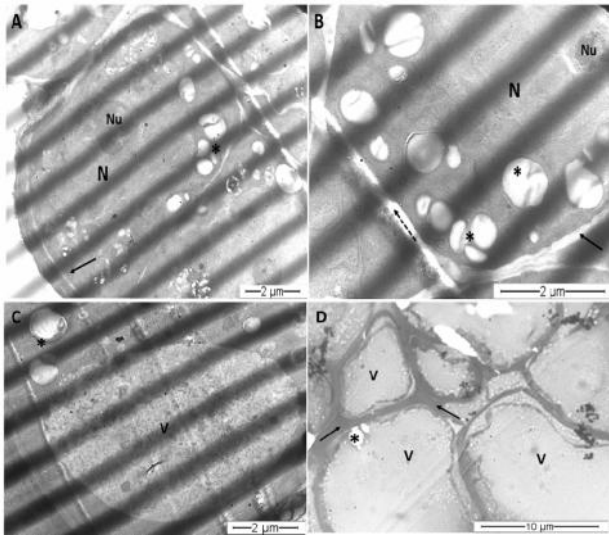

Figure 5 – Transmission electron micrography of cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo in reference to the 4th (A and B) and 28th day (C and D) of culture on the growth curve. (A) Cell with large nucleus (N) and prominent nucleolus (Nu), cell wall (arrow). Presence of amyloplasts (*); (B) presence of numerous large amyloplasts (*). Small intercellular spaces (dotted arrow). Cell wall (arrow); (C) Cell with large vacuole (V) occupying a large part of the cytoplasm. Few amyloplasts (*); (D) Large intercellular spaces (arrows). Large vacuoles (V). Absence of cell content.

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Analyses SEM at 4th day of the growth curve (T1), we noted the presence of rounded and isodiametric cells, forming cell agglomerates and a lower frequency of shriveled and ruptured cells together with the agglomerates, as well as the presence of cells in division (Fig. 6).

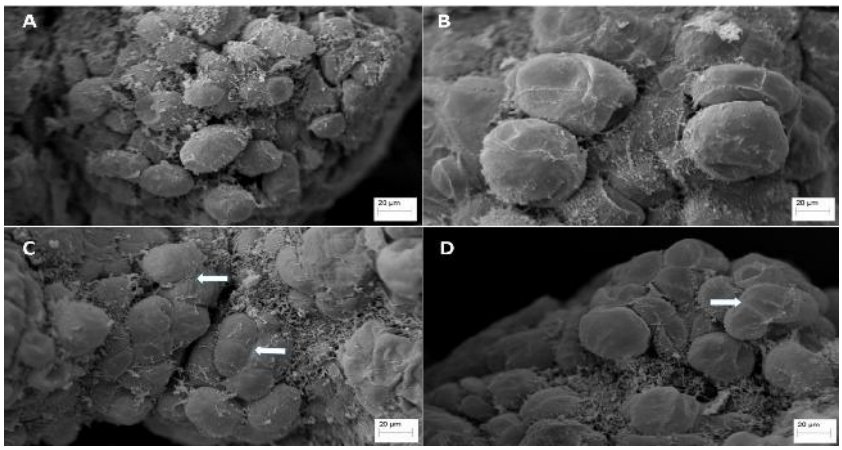

Figure 6 – Scanning electron micrography of cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo in reference to the 4th day of culture on the growth curve. Presence of cell agglomerates and isodiametric cells. (C and D) cells in division represented by the arrow.

In the period of 28th day of the growth curve, cells were found with shortage of nutrients and in a deceleration phase, in which the presence of asymmetric, shriveled, and ruptured cells was observed, probably due to cell death, that is, apoptosis (Fig. 7).

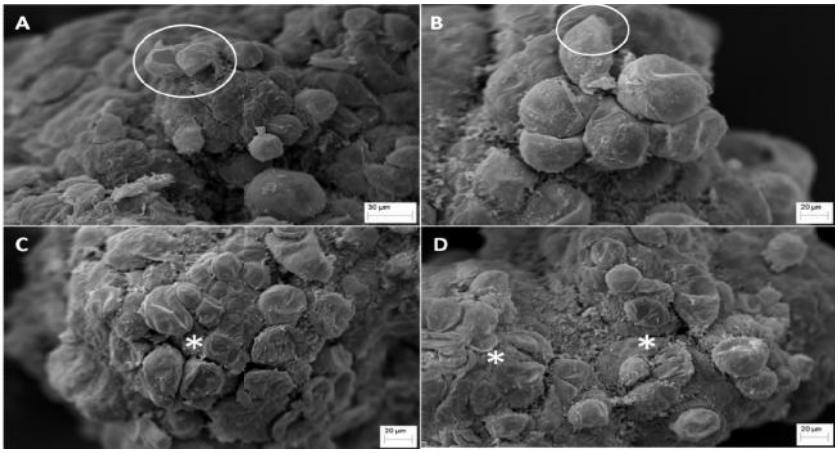

Figure 7 – Scanning electron micrography of cell suspensions of *Coffea arabica* cultivar Bourbon Amarelo in reference to the 28th day of culture on the growth curve. Presence of asymmetric cells. (A and B) ruptured cells, identified by the circle. (C and D) region with high frequency of shriveled cells, represented by the *.

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4. DISCUSSION

In this study did not identify all the phases of the callus curve in the coffee plant, as well as in other work already done with this same species. [19] observed only three distinct phases [lag (0 - 42 days after inoculation), exponential (42 – 77 days), and linear (77 – 84 days)], and according to the authors, due to the low growth speed that this species has. The growth curve may be affected by the species under study, the explant used, and the culture conditions, among other factors. In the linear phase, the cells are no longer in cell division; thus, the growth stabilizes. Therefore, if the objective is multiplication of the explant, sub-culturing and transfer of the material should not go beyond this phase because multiplication requires high cell division [20].

The lag phase, which comes before cell division, is characterized by accumulation of biomass by cells. This phase was of short duration probably because the suspensions were already in growth with 10 months of culture when the growth curve began. A lag phase of short duration was also observed by [21]. The authors reported that although the curve obtained is similar to the curve model observed in suspensions of plant cells, the lag phase was practically non-existent. This was due to the methodology used for standardizing the standard culture, because the suspensions were already in exponential growth when they were used. Various studies have shown that the period of the lag phase varies according to the species and the initial explant used, and, in the case of this study, the determining factor was the time of culture before carrying out the growth curve. [22] found in calli obtained from leaf segments of the pequi tree (*Caryocar brasiliense* Camb) that the lag phase occurred up to the 7th day of culture. However, [23] observed a long lag phase up to the 30th day in kiwi (*Actinidia deliciosa).* [24] analyzing embryogenic cell suspension obtained from nodal and leaf explants of *C. canephora* cv. Apoatã, identified the lag phase up to the 28th day of culture, equivalent to 39% growth. [25] identified the lag phase up to the 40th day of culture of calli originating from leaf explants of the inga tree.

The linear phase, in which cell division decreases and cell expansion occurs, has a fast period due to the more homogeneous characteristic of the plant material. For the deceleration phase, there are reports in the literature of the short period of this phase in species such as Brazil nut (*Bertholletia excelsa*), which lasted from 60 to 67 days [20], and in *Cordia verbenacea*, in which deceleration

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occurred from 18 to 21 days [26]. This phase as ideal for sub-culturing and transferring the plant material, due to reduction of nutrients and accumulation of toxic substances [24].

Cell growth in suspension of *C. arabica* cv. Catiguá was analyzed by [16] through the growth curve. Over 60 days of culture, with collection intervals every 10 days and renewal of 1/6 of the nutrient medium, the authors report that from the 30th day to the 40th day of culture, there was stabilization of the GRR, followed by decline at 50 days, suggesting that renewal of 1/6 of the volume of the nutrient medium was not sufficient to supply the nutritional needs of the cells in suspension based on constant GR. Although the cultivar in this study had a different response, the results obtained by [16] confirmed the need for renewal of the nutrient medium as of 20 days of culture or renewal of the culture medium greater than 1/6 of the volume in order to maintain the viability of cell suspensions of *C. arabica*.

For species that take a long time to reach the deceleration phase, contrary to what occurred with suspensions of *C. arabica*, sub-culturing and transfer is recommended at the beginning of the deceleration phase. However, the suspensions of *C. arabica* began deceleration at 24 days, probably due to reduction in nutrients, and the sub-culturing and transfer of the medium is recommended up to the end of the exponential phase, that is, at 20 days of culture. It is also important to note that at the end of the exponential phase, the cells have already carried out maximum cell division, using up much of their energy reserves (starch), which leads us to infer that the cells need to be supplied with new nutrients.

The importance of establishing the growth curve of cell suspensions under these conditions is in identification of the phases in which the fundamental kinetic processes occur, visualized even using suspensions with 10 months of culture. Although the cell suspensions did not exhibit continuous growth, it was possible to analyze the embryogenic potential and deduce the best handling of these cell suspensions.

In relation to cellular and nuclear diameter, embryogenic cells showed 8 to 24 µm cell diameter and 7 to 10 µm nuclear diameter, reaffirms the morphology of large nuclei. For non-embryogenic cells, it was observed cell diameter of 36 to 97 µm. Was observed results similar to those of the present study analyzing histological sections of *C. arabica* cv. Catuaí vermelho. The authors found embryogenic

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cells of 15 to 25 µm cell diameter and for the non-embryogenic cells, 39 to 48 µm diameter [27]. Studies on *C. arabica* cv. Caturra Rojo showed embryogenic cells with diameters from 15 to 20 µm [28].

Typical morphology of meristematic cells include: small, isodiametric, and homogeneous cells with only thin primary cell wall, dense cytoplasm, and small vacuole. The authors analyze the morphology/function relationship, inferring that small size allows a greater number of cells per tissue volume and faster cell divisions; isodiametric cells optimize the divisions because direction does not affect their efficiency since one cell axis is not larger than the other; the thin cell wall is primordial because if cell walls are thick and lignified, they can hinder cell divisions - therefore, the thin cell wall facilitates divisions and, furthermore, greater inflow of nutrients, without which cells would die of exhaustion; and the small vacuole allows greater space for the organelles, which are structures to be replicated during divisions, as well as the fact that large vacuoles are characteristic of old cells in processes of apoptosis [29].

Embryogenic characteristics of coffee plant cells similar to the characteristics in this study were also observed by [30] in *C. arabica* and *C. canephora* in a culture of embryogenic calli of seven months of age. The authors identified cell aggregates similar to small proembryogenic masses, with high tissue homogeneity, dense cytoplasm rich in soluble and reserve proteins, voluminous nucleus located in the central region of the cells, and numerous small starch grains around the nucleus. These characteristics corroborate those observed in the cell suspensions of 10 months of age of this study. In other study, embryogenic cell suspensions of *C. arabica* cultivar Catiguá MG2 showed embryogenic characteristics as described in the present work with embryogenic cell suspensions cultivar Bourbon Amarelo [31].

As observed in the present study, starch grains were identified in embryogenic material of coffee [28, 31] and macauba palm (*Acrocomia aculeata)* [32]. The storage of starch, in embryogenic cells or in adjacent cells, is a phenomenon that commonly indicates the acquisition of embryogenic competence [30]. However, the probable function of starch in the process of somatic embryogenesis is still unclear. It is speculated that the storage of starch may be related to the poor mitotic activity of

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cells that stored it, since embryogenic cells, usually under higher mitotic activity, had less starch storage [32].

Results similar to those of the present study were observed in calli obtained from anthers of the inga tree, with the presence of starch grains and large nucleus with prominent nucleolus [33]. Starch grains were identified by [28] in embryos of *C. arabica* cv. Caturra Rojo and by [34] in embryos of *Eucalyptus globulus,* which confirms the relationship of the presence of starch grains with cells with embryogenic potential.

In the present study, the large number of amyloplasts found in collection 1 were probably consumed, no longer being detected in collection 7. The starch grains supply energy for the formation and development of the somatic embryo [32], however, for formation of these embryos, an adequate medium that stimulates their development is necessary.

Organized cell proliferation on the surface of the agglomerates indicates competence for formation of somatic proembryos through the presence of cells in division [33]. It is suggested that calli have different capacities for somatic embryogenesis to the extent that their cells are in different conditions and have different characteristics. Embryogenic and non-embryogenic calli exhibit differences, not only in morphological structures and embryogenic responses, but also in their cell characteristics.

The application of TEM and SEM to identify the embryogenic and non-embryogenic materials was reported in coffee [12], banana [11] and of inga trees [25]. In calli coming from leaves of inga trees, cells with rounded morphology and also elongated cells were observed, the latter not being considered as embryogenic [25]. [11] identified three cell types in *Musa* sp., cv. Prata anã, namely, elongated cells, group of cells, and isodiametric cells, these last being defined as meristematic.

The importance of the growth curve established for in vitro culture is the identification of phases which occur in the fundamental processes the kinetics of growth of the plant material. Aware of the importance of the understanding of the mechanisms that involve the induction of cellular competence to embryogenesis, histological and ultrastructural analyzes become necessary for optimization and validation of its use in somatic embryogenesis protocols.

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5. CONCLUSIONS

Histological variations were observed in the cell suspensions of *C. arabica* cultivar Bourbon Amarelo during the growth curve. The two cells types found differed in cell morphology. Type 1, characterizing the embryogenic cells, maintains its embryogenic characteristics until differentiating in Type 2, when the cells are no longer found in embryogenic activity. Cells with embryogenic characteristics were related to the exponential phase of the growth curve, whereas non-embryogenic cells predominated in the deceleration phase. The importance of establishing the growth curve of cell suspensions is in the identification of the phases in which the fundamental kinetic processes occur, allowing the correct manipulation of the same. Associated with this data, the identification of distinct types of cells allowed identification of the ideal cell suspension to obtain an efficient propagation system.

REFERENCES

1. Campos NA, Panis B, Carpentier SC. Somatic Embryogenesis in Coffee: The Evolution of Biotechnology and the Integration of Omics Technologies Offer Great Opportunities. Front Plant Sci. 2017;21(8):1460.
2. Yang X, Zhang X. Regulation of somatic embryogenesis in higher plants. Crit Rev Plant Sci. 2010; 29(1):36–57.
3. Santana-Buzzy N, Rojas-Herrera R, Galaz-Ávalos RM, Ku-Cauich JR, Mijangos-Cortés J, Gutiérrez-Pacheco LC. et al. Advances in coffee tissue culture and its practical applications. In Vitro Cell Dev Biol Plant. 2007;43(6):507–520.
4. Lubabali AH, Alakonya AE, Gichuru EK, Kahia JW, Mayoli RN. In vitro propagation of the new disease resistant *Coffea arabica* variety, Batian. Afr. **J**. Biotechnol. 2014;13(24):2414-2419.
5. Aga E, Khillare Y. *In vitro* multiplication of *Coffea arabica* L. from leaf explants through indirect somatic embryogenesis. Int. j. botany stud. 2017;2(1):17-22.
6. Etienne H, Bertrand B, Dechamp E, Maurel P, Georget F, Guyot R. et al. Are Genetic and Epigenetic Instabilities of Plant Embryogenic Cells a Fatality? The Experience of Coffee Somatic Embryogenesis. Human Genet Embryol. 2016;6:136.

UNDER PEER REVIEW

7. Mazarei M, Al-Ahmad H, Rudis MR, Joyce BL, Stewart CN Jr. Switchgrass (*Panicum virgatum* L.) cell suspension cultures: Establishment, characterization, and application. Plant Sci. 2011; 181(6):712– 715.
8. Silva AT, Barduche D, Livramento KG, Ligterink W, Paiva LV. Characterization of a Putative Serk-Like Ortholog in Embryogenic Cell Suspension Cultures of *Coffea arabica* L. Plant Mol Biol Report. 2013;32(1):176–184.
9. Lu S, Wang Z, Peng X, Guo Z, Zhang G, Han L. An efficient callus suspension culture system for triploid bermudagrass (*Cynodon transvaalensis* x *C. dactylon*) and somaclonal variations. Plant Cell Tissue Organ Cult. 2006;87(1):77-84.
10. Santos DN dos, Nunes CF, Soares JDR, Valente TCT, Alves E, Labory CRG, et al. Cytological characterization of *Jatropha curcas* callus in different periods of cultivation. Crop Breed Appl Biotechnol. 2013;13(4):228-233.
11. Ribeiro LO, Paiva LV, Pádua MS, Santos BR, Alves E, Stein VC. Morphological and ultrastructural analysis of various types of banana callus, cv. Prata anã. Acta Sci Agron. 2012; 34(4):423-429.
12. Pádua MS, Paiva LV, Silva LC, Livramento KG, Alves E, Castro AHF. Características morfológicas e viabilidade celular de calos de cafeeiro. Cienc Rural. 2014;44(4):660-665.
13. Murashige T, Skoog F. A revised médium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15:473-497.
14. Teixeira JB, Junqueira CS, Costa Pereira AJC, Mello RIS, Silva APD, Mundim DA. Multiplicação clonal de café (*Coffea arabica* L.) via embriogênese somática. Documentos 121. Brasília, Embrapa. 2004.
15. Zamarripa A, Ducos JP, Bollon H, Dufour M. Production of somatic embryos of coffee in liquid medium: effects of inoculation density and renewal of the medium. Café Cacao Thé. 1991;35:233-244.
16. Silva AT. 2011. Análise da expressão dos genes baby boom (*BBM*) e somatic embryogenesis receptor kinase (*SERK*) envolvidos na embriogênese somática do cafeeiro. 129 p. PhD Thesis. Universidade Federal de Lavras, Lavras, Minas Gerias, Brasil.

UNDER PEER REVIEW

17. Ferreira DF. Sisvar: um programa para análises e ensino de estatística. Revista Symposium. 2008; 6:36-41.
18. Bossola JJ, Russell LD. Electron Microscopy. 2nd. ed. Boston Jones and Bartlett Publishers. 1998.
19. Santos CG, Paiva R, Paiva PDO, Paiva E. Indução e análise bioquímica de calos obtidos de segmentos foliares de *Coffea arabica* L., cultivar RUBI. Ciênc Agrotec. 2003; 27(3):571-577.
20. Serra AGP, Paiva R, Paiva PDO. Análises bioquímicas de calos formados de explantes foliares de castanha do Brasil (*Bertholletia excelsa* H. B. K.). Ciênc agrotec. 2000;24(4):833-840.
21. Celedón PAF, Kobayashi AK, Vieira LGE. Utilização de nutrientes por células de algodoeiro cultivadas em suspensão. Sci Agric. 2000;57(3):431-437.
22. Landa FSL, Paiva R, Paiva PDO, Filho JSSB. Indução *in vitro* de calos explantes foliares de pequizeiro (*Caryocar brasiliense* Camb.). Ciênc Agrotec. 2000;24(ed. especial):56-63.
23. Mezzetti B, Conte LS, Rosati P. *Actinidia deliciosa in vitro*: II., growth and exogenous carbohydrates utilization by explants. Plant Cell Tissue Organ Cult. 1991;26(3):153-160.
24. Santos CG, Paiva R, Paiva PDO, Paiva E. Indução e análise bioquímica de calos em segmentos foliares e nodais de C*offea canephora* L. cv. Apoatã. Magistra. 2008;20(1):22-29.
25. Stein VC, Paiva R, Vargas DP, Soares FP, Alves E, Nogueira GF. Ultrastructural calli analysis of *Inga vera* Willd. Rev Árvore. 2010b;34(5):789-796.
26. Lameira AO, Pinto JEBP, Cardoso MG, Arrigoni-Blank MF. Estabelecimento de cultura de células em suspensão e identificação de flavonóides em *Cordia verbenacea* DC. Rev Bras Plant Med. 2009;11(1):7-11.
27. Bartos PMC, Gomes HT, Laid A, Gomes SM, Teixeira JB. Histologia de calos provenientes da embriogênese somática de *Coffea arabica* L. Abstract. In 7th SIMPÓSIO DE PESQUISA DOS CAFÉS DO BRASIL, Araxá, Minas Gerais, Brasil. August. 2011.

UNDER PEER REVIEW

28. Quiroz-Figueroa FR, Fuentes-Cerda CFJ, Rojas-Herrera R, Loyola-Vargas V. Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Rep. 2002;20(12):1141–1149.

29. Castro EM, Pereira FJ, Paiva R. Histologia vegetal: estrutura e função de órgãos vegetativos, Lavras. 2009.

30. Ribas AF, Dechamp E, Champion A, Bertrand B, Combes M-C, Verdeil J-C, et al. Agrobacterium-mediated genetic transformation of *Coffea arabica* (L.) is greatly enhanced by using established embryogenic callus cultures. BMC Plant Biology. 2011;11:92.

31. Torres LF, Diniz LE, Livramento KG, Freire LL, Paiva LV. Gene expression and morphological characterization of cell suspensions of *Coffea arabica* L. cv. Catiguá MG2 in different culture stages. Acta Physiol Plant. 2015;37(9):175.

32. Moura EF, Ventrella MC, Motoike SY, Júnior AQS, Carvalho M, Manfio CE. Histological study of somatic embryogenesis induction on zygotic embryos of macaw palm (*Acrocomia aculeata* (Jacq.) Lodd. ex Martius). Plant Cell Tissue Organ Cult. 2008;95(2):175-184.

33. Stein VC, Paiva R, Nogueira RC, Vargas DP. Curva de crescimento e índice de divisão celular de calos de Ingazeiro. Agrária. 2010a;53(2):159-163.

34. Pinto G, Silva S, Neves L, Araújo C, Santos C. Histocytological changes and reserves accumulation during somatic embryogenesis in *Eucalyptus globulus*. Trees. 2010;24(4):763-769.