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Morphological characteristics and cell viability of coffee plants calli

Características morfológicas e viabilidade celular de calos de cafeeiro

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ABSTRACT

*The aim of this research was to characterize and compare two types of calli from leaf explants of **Coffea arabica** (cultivar Catiguá). Cells of different types of callus were successfully characterized regarding viability and internal and external morphological characteristics. It was obtained two morphologically distinct types of callus: (i) yellow friable and (ii) transparent watery. The yellow friable calli showed higher cell viability and embryogenic characteristics. Scanning and transmission electron microscopy showed embryogenic characteristics in cells of the yellow friable calli evidenced by the presence of small and isodiametric cells, while transparent watery calli showed elongated cells and large cytoplasm vacuolization.*

Key words: ***Coffea arabica**, electron microscopy, tetrazolium test.*

RESUMO

*O objetivo deste trabalho foi caracterizar e comparar dois tipos de calos de explantes foliares de **Coffea arabica** (cultivar Catiguá). Células de diferentes tipos de calos foram caracterizadas quanto a viabilidade e características morfológicas externas e internas. Foram obtidos dois tipos de calos morfologicamente distintos: (a) amarelo friável e (b) transparente aquoso. Os calos amarelos friáveis apresentaram maior viabilidade celular e características embriogênicas. Microscopia eletrônica de varredura e transmissão mostraram características embriogênicas em calos amarelos friáveis evidenciadas pela presença de células pequenas e isodiamétricas. Os calos transparentes aquosos apresentaram células alongadas e vacuolizadas.*

Palavras-chave: ***Coffea arabica**, microscopia eletrônica, teste do tetrazólio.*

INTRODUCTION

Coffee is one of the world's major commodities, and Brazil ranks first in production, consumption, and export (AGRIANUAL, 2013). However, ***Coffea arabica*** L. has propagation problems by conventional cutting methods concerning long life cycle, phenological characteristics of the species, and environmental influences (ROSA et al., 2007).

The somatic embryogenesis can be employed to promote rapid *in vitro* multiplication of coffee plants recommended for planting, as hybrids from breeding programs. According RIBAS et al. (2011), currently, embryogenic callus derived from leaf tissues is the most widely used target tissue for the genetic transformation of ***C. arabica*** and different callus stages can affect plant transformation. However, the induction of embryogenic tissues in ***C. arabica*** takes longer and is more difficult than in the others cultivated species. The limited vailability of embryogenic tissues, together with the low transformation efficiency of this type of tissue is one of the main limitations to genetic transformation in coffee.

Distinction between embryogenic and non-embryogenic callus is important to improve protocols for induction and regeneration of somatic embryos *in vitro* so, procedures to estimate cell viability have become necessary. SILVA & MENENDÉZ-YUFFÁ

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(2006) reported that the methods used to evaluate the presence of viable and non-viable cells can be classified in two groups: those that stain only the dead cells, and those in which only the living cells are coloured, because the colour is a product of cell metabolic activity. Acetocarmine enables inferring chromosomal integrity and characterization of viable cells (STEINER et al., 2005; MUNHOZ et al., 2008), while Evans Blue enters damaged cell walls in apoptosis process, allowing for identification of nonviable cells (BHARGAVA et al., 2007). Viability can also be quantified with activity assays, using for example the 2, 3, 5-triphenyltetrazolium chloride (TTC). In the mitochondrial electron transport chain, TTC is reduced to triphenylformazan (TPF) and acts as final electron acceptor in place of oxygen. This reaction corresponds to cellular respiration and indicates cell metabolic activity and viability (DIAS & ALVES, 2008).

Morphological and ultrastructural features of cells can be visualized by electron microscopy, which allows inferring characteristics of embryogenic cells, such as isodiametric shape, small size, large nucleus, visibly evident nucleus, and organelles related to increased cell metabolism. In addition, electron microscopy can also identify features of nonviable cells, such as elongated and vacuolated cells (NOGUEIRA et al., 2007).

Taken together these analysis may identify calli with embryogenesis characteristics in early stages of calli culture. In this study it was characterized and compared two types of calli from leaf explants of coffee plants for cellular shape, viability and ultrastructural features, enabling the improvement of protocols for *in vitro* plant regeneration by indirect embryogenesis.

MATERIAL AND METHODS

The experiments were performed in the Central Laboratory of Molecular Biology and Laboratory of Electron Microscopy and Ultrastructural Analysis of the Federal University of Lavras (UFLA), Lavras, MG.

Callus induction

For callus induction it was used plant leaf explants of ***Coffea arabica*** cv. 'Catiguá MG2' maintained in a greenhouse. Leaves were washed in running water for 10 minutes and in the laminar air flow, they were decontaminated in NaOCl (40g L^{-1} dissolved in water) for 20 minutes, and washed in autoclaved distilled water three times for 10 minutes. The explants were inoculated in a BERTHOULY & MICHAUX-FERRIÈRRE medium (1996) modified for calli induction, supplemented with 2.26µM 2,4-D; 5µM IBA; 10µM 2iP; 200mg L^{-1} inositol; 400mg L^{-1} malt extract; 100mg L^{-1} casein; 10mg L^{-1} thiamine; 1mg L^{-1} nicotinic acid; 1mg L^{-1} pyridoxine; 1mg L^{-1} glycine; L^{-1} 30g sucrose; 6g L^{-1} agar; and pH adjusted to 5.7 or 5.8. The explants were maintained in a growth chamber at 25°C in the dark. After three weeks, they were transferred to a BERTHOULY & MICHAUX-FERRIÈRRE medium (1996) modified for embryogenic calli induction, supplemented with 17µM BAP and 4.52µM 2,4-D; 60mg L^{-1} adenine; 200mg L^{-1} inositol; 200mg L^{-1} malt extract; 100mg L^{-1} casein; 20mg L^{-1} thiamine; 20mg L^{-1} glycine; 40mg L^{-1} L-cysteine; 30g L^{-1} sucrose; 6g L^{-1} agar; pH adjusted to 5.7 or 5.8. After five months, two types of callus were characterized: (i) a yellow friable and (ii) a transparent watery, and used in subsequent experiments.

Cell viability

Analysis was performed using tetrazolium test (BENSON, 1994) with five replications of 50mg callus collected from five dishes, and the level of cell viability was calculated according to the amount of triphenylformazan produced by living cells. To assess pro-embryogenic characteristics by double staining with acetocarmine and Evans Blue, 25mg callus were collected for each replication, amounting to five replications. Samples were prepared according to DURZAN (1988). Callus cells were visualized in 10x and 20x objective lens in photonic microscope (Leica DM LS® microscope with Nikon® camera attached). The areas stained with acetocarmine (AC) and Evans Blue (EB) were measured with ImageTool software. AC and EB areas were added to provide total area and percentage of each area was obtained for statistical analysis. Data was analyzed in a completely randomized design (CRD) with 25 replications for each type of callus. Sisvar 5.0 software (FERREIRA, 2008) provided statistical analysis, and the means were analyzed by Scott-Knott test at 5% nominal level of significance.

Electron microscopy

For scanning electron microscope analysis, samples of both types of callus were immersed in fixative (modified Karnovsky, 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.05M cacodylate buffer, pH 7.2) for 24h, and prepared according to the BOSSOLA & RUSSELL (1998) protocol. Samples were observed through the LEO Evo 40 scanning

electron microscope, and cell dimensions (width and length) were assessed using the Smart User Interface (Zeiss) program. The experiment used a completely randomized design (CRD) with 50 replications per treatment. For statistical analysis it was used Sisvar 5.0 software (FERREIRA, 2008), and the means were analyzed by Scott-Knott test at 5% significance level. For analysis in Zeiss EM 109 transmission electron microscope, samples were prepared according to the BOSSOLA & RUSSELL (1998) protocol.

RESULTS AND DISCUSSION

It was observed two morphologically distinct types of callus: (i) yellow friable and (ii) transparent watery. Tetrazolium viability test showed the mean absorbance values 0.4340 and 0.0643 for the yellow friable and the transparent watery callus, respectively. According to DIAS & ALVES (2008), TTC chloride (2,3,5 - triphenyl tetrazolium chloride) is colorless in the oxidized form and red when reduced to TFF (triphenylformazan). The viability of plant tissue is proportional to the amount of TFF produced by living cells (SILVA & MENENDÈZ-YUFFÁ, 2006), and is also related to red color intensity.

Results of double staining with acetocarmine (AC) and Evan Blues (EB) showed that AC reacted in 84.79% cells in the yellow friable callus, and in 28.55% cells in the transparent watery. Conversely, the yellow and friable reacted less to EB (15.21% of cells), whereas the transparent watery reacted in 71.45%. Cells in the yellow friable callus were small, round (isodiametric), arranged in clusters, and AC reactive; cells in the watery transparent callus were elongated and EB reactive (Figure 1). The results showed that the yellow friable callus has higher cell viability, wich may contribute to a higher potential for embryogenesis, as strongly AC reactive cells have embryonic characteristics, according to CANGAHUALA-INOCENTE et al. (2007).

Our data corroborate the findings of STEINER et al. (2005), who emphasized the AC affinity for small isodiametric cells and EB affinity for elongated cells in callus of ***Araucaria angustifolia***. EB penetrates membrane ruptures, turning cell interior blue early indicating cell deth (BHARGAVA et al., 2007). GATICA-ARIAS et al. (2008) evaluated the embryogenic viability of cells suspensions of coffee plants using EB stain. The results showed that nonviable cells turned intensely blue, whereas viable cells did not react and showed isodiametric shape, dense cytoplasm, and nucleus with prominent nucleoli. According to MUNHOZ et al. (2008), positive reaction to AC demonstrates chromosomal integrity.

Scanning electron microscopy showed that round cells arranged in clusters, with diameters 24x21µm, characterizing a spherical shape predominated in the yellow friable callus and elongated cells, with diameters 268x36µm and showed

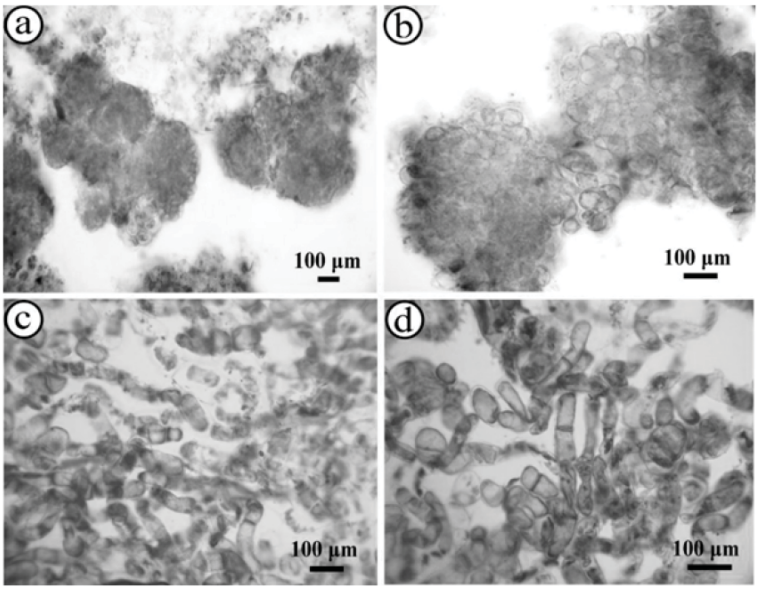

Figure 1 - Double staining with Acetocarmine and Evans Blue. (a) and (b) Cell mass of yellow friable callus. (a) Deep red stained cells; (b) Red stained isodiametric cells; (c) and (d) Cell mass of transparent watery callus: blue stained elongated cells.

dispersed disposition in the transparent watery callus (Figure 2). These results are in agreement with those described by SONDAHL et al. (1979) that obtained from leaf explants two distinct cell populations of the callus tissue: one consisting of elongated cells and the other of small spherical embryogenic cells. The small spherical cells became separated from one another and subsequently, differentiation of the embryogenic cells into globular forms of coffee embryos occurred. NAKAMURA et al. (1992) described by scanning electron microscopy that callus derived from mature leaves of ***C. arabica*** has shown the presence of small spherical cells, characteristic of embryogenic tissues. The presence of small meristematic cells with diameter range of 15 to 20µm were observed in globular callus of coffee plants and showed small isodiametric cells (QUIROZ-FIGUEROA et al., 2002). BOBÁK et al. (2004) reported that in ***Drosera spathulata*** Labill, callus showing grouping of small, irregular and isodiametric cells originated embryos which passed through heart, torpedo and cotyledonary stages, and finally regenerated seedlings.

Transmission electron microscopy showed that cells of the yellow friable callus had dense cytoplasm, nuclei with prominent nucleoli, a large number of amyloplasts, and absence of vacuoles. Cells of the transparent watery callus, however, showed an autophagic vacuole occupying the whole cytoplasmic space, vesicles, increased intercellular space, and absence of other cytoplasmic organelles (Figure 3). These results confirm the findings of NOGUEIRA et al. (2007), who reported disorganized nuclei and few cytoplasmic organelles in cells of ***Byrsonima intermedia*** callus in the first subculture.

According to FUKUDA (2000), autophagy may be involved in degradation of cell contents, and the vacuole plays a central role in the process of programmed cell death. Our results are also similar to those achieved by ASLAM et al. (2011), who analyzed ***Phoenix dactylifera*** L. and for STEINMACHER et al. (2011) in callus cells of ***Bactris gasipaes***. The authors found embryogenic cells with well-developed nucleus, prominent nucleoli, and starch granules. NAKAMURA et al. (1994) studied callus cells of somatic embryos of ***Coffea*** cvs. 'Mundo Novo' and 'Catuaí Amarelo' and reported this biochemical pattern as the primary source of energy needed for intense cell division and embryo development. The consumption of starch grains provide energy for developing somatic embryos, which suggests active regulation of the amount of starch in callus.

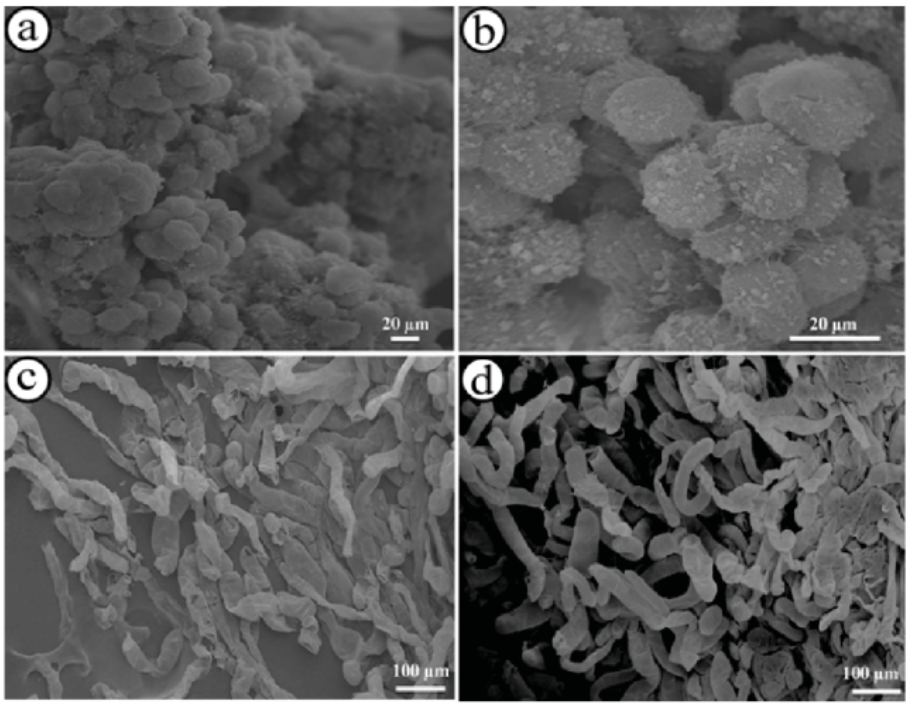

Figure 2 - Scanning Electron Microscopy of callus cells. (a) Cells in the yellow friable callus arranged in clusters; (b) Round cells in the yellow friable callus; (c) and (d) Transparent watery callus with elongated cells.

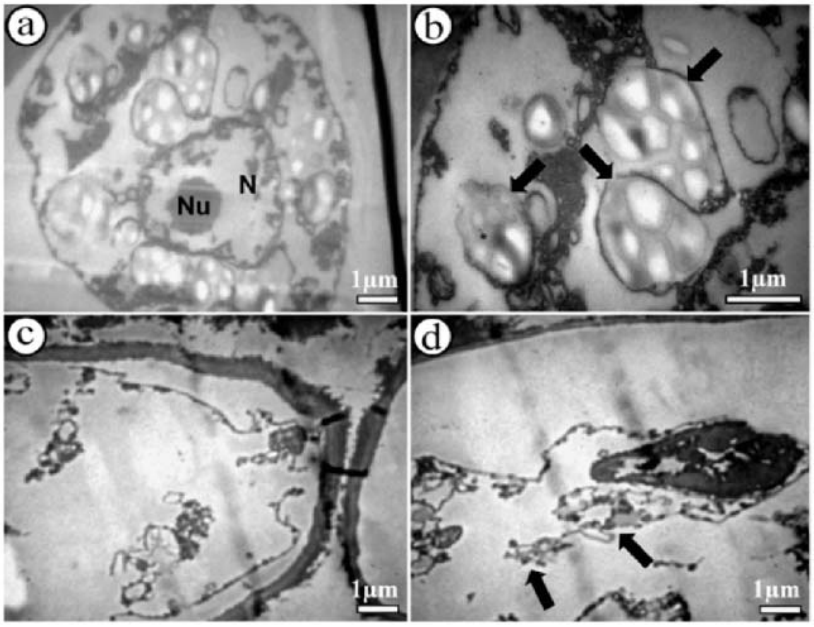

Figure 3: Transmission electron microscopy displaying cells in callus of coffee. (a) Yellow friable callus: nucleus (N) and nucleolus (Nu.); (b) Yellow friable callus: highlighted presence of amyloplasts (black arrows); (c) Transparent watery callus: vacuole occupying the whole cytoplasmic space; (d) Transparent watery callus: presence of vesicles (black arrows).

CONCLUSION

In ***Coffea arabica*** cv. 'Catiguá' yellow friable callus showed embriogenic characteristics and higher cell viability.

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