Morphological and ultrastructural analysis of various types of banana callus, cv. Prata anã

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ABSTRACT. This work was carried out to characterise morphologically and ultrastructurally the banana callus, obtained from the scalp method. Genotypes of banana, cv. Prata anã, cultivated *in vitro* were used to induce meristems at the leaf base; subsequently, structures known as *scalps* were formed. For ultrastructural analysis, five samples of callus were collected, fixed in Karnovsky solution and analysed by scanning electron microscope (SEM) and transmission electron microscope (TEM). The formation of three types of callus was observed: Type 1 - transparent watery callus, Type 2 - yellow callus with small clusters, Type 3 - yellow callus with large clusters. SEM analysis showed that Type 1 callus cells were elongated and that Type 2 and Type 3 callus cells were isodiametric, which is a characteristic of embryogenic cells. The TEM analysis showed that Type 1 callus cells had thin walls, a large number of small vacuoles and dispersed cytoplasm. The Type 2 callus cells showed dense cytoplasm, large vacuoles and a large amount of mitochondria. The Type 3 callus cells had thick and intercellular spaces. Thus, the Type 2 callus cells had characteristics consistent with embryogenic callus cells.

Keywords: scanning electron microscopy, transmission electron microscopy, *Musa* sp., cytology, embryogenesis.

Análise morfológica e ultra-estrutural de diferentes calos de bananeira, cv. Prata anã

RESUMO. Este trabalho foi realizado com o objetivo de caracterizar morfologicamente e ultra-estuturalmente calos (*scalp method*) de bananeira. Genótipos de bananeira, cv. Prata anã, cultivadas *in vitro*, foram utilizados para indução de meristemas na base das folhas e formação de estruturas conhecidas como *scalp*, que foram transferidas para meio ZZs. Os calos obtidos foram caracterizados morfológica e ultra-estruturalmente. Para as análises ultra-estruturais, foram coletadas, fixadas em Karnovsky e preparadas cinco amostras, que foram analisadas em microscópio eletrônico de varredura e de transmissão. Foi verificado formação de 3 tipos de calos (Tipo 1- calo transparente aquoso; Tipo 2- calo com glomérulos amarelos menores; Tipo 3- calo com glomérulos amarelos maiores). A análise por MEV mostrou que os calos Tipo1 apresentaram células alongadas; os calos Tipo 2 e Tipo 3 apresentaram células com formato isodiamétrico que condizem com características de calos embriogênicos. A análise por MET mostrou que os calos Tipo1 apresentaram parede delgada, grande quantidade de pequenos vacúolos e citoplasma disperso. Os calos Tipo 2 apresentaram citoplasma denso, vacúolos grandes e presença de mitocôndrias. Os calos Tipo 3 apresentaram parede espessa e espaços intercelulares. Assim, os calos Tipo 2 evidenciam características de calos embriogênicos.

Palavras-chave: microscopia eletrônica de varredura, microscopia eletrônica de transmissão, *Musa* sp., citologia, embriogênese.

Introduction

The *Musa* sp., which belongs to the Musaceae family, is an herbaceous plant of great economic and social importance. The *Musa* sp. is cultivated in approximately 120 countries and is a source of income and food for millions of people, especially in developing countries (FAO, 2011). The main objective of the banana breeding programs is to search for materials having characteristics of agronomic interest such as disease resistance, reduced plant size and organoleptic fruit quality (MADAIL et al., 2011). The biotechnology techniques allied to conventional breeding have contributed to the sustainability of agriculture by providing materials compatible with environmental changes, especially in underdeveloped countries, where there is a specific need for new technologies (ADENLE, 2011).

In this context, the tissue culture of plants associated with genetic engineering techniques has been widely used to obtain agronomically improved

cultivars. Among these tissue culture techniques, somatic embryogenesis has been presented as a great option for both gene transfer by genetic transformation and mass propagation of elite plants, providing a high rate of multiplication, easier production scheduling, and easier maintenance of embryos in culture medium (ASLAM et al., 2011; BARROS, 1999; GUPTA et al., 1993).

The embryos can be formed *in vitro* from two basic patterns of embryogenesis. For direct somatic embryogenesis, the formation of somatic embryos comes directly from the explant, where the cells are programmed for differentiation. For indirect somatic embryogenesis, dedifferentiation occurs from the explants and consequently leads to callus formation. Such cell groups are able to respond to the stimulatory effects from the culture medium and later develop into embryos (KERBAUY, 1999).

Pescador et al. (2000) reported that the *in vitro* plant manipulation system used to obtain embryogenic and morphogenic responses depends on morphologic, genetic, biochemical, cytologic and physiologic factors. Understanding the factors that regulate somatic embryogenesis and control synchronicity of embryo development will enable genetic transformation as well as mass propagation of elite plants (PIHAKASHI-MAUNSBACH et al., 1993).

Ultrastructural analysis of the callus can determine if the cytological and morphological characteristics are associated with embryogenic capacity provided by manipulating the culture media. Once the culture conditions are optimised, it will be possible to increase production of embryogenic callus and, later, embryogenic cell suspensions, which could develop into large-scale embryogenic cell propagation. This could also allow for the early selection of embryogenic callus, thereby reducing the cost, time and labour involved in this process (NOGUEIRA et al., 2007).

The embryogenic cells, regardless of the direct or indirect process, present fast mitotic division, small size, dense cytoplasm, large nuclei with prominent nucleoli, small vacuoles and the presence of starch grains. These characteristics can be analysed by morphological and ultrastructural techniques and suggest an intense synthesis of RNA and extensive metabolic activity (ASLAM et al., 2011). Therefore, the purpose of this study was to characterise callus obtained from banana meristems through the scalp method with scanning electron microscopy and transmission electron microscopy.

Material and methods

The experiments were performed in 2010 at the Central Laboratory of Molecular Biology (LCBM)

and the Laboratory of Electron Microscopy and Ultrastructural Analysis (LME), at the Federal University of Lavras, Lavras (UFLA) Minas Gerais State, Brazil.

For the experiments, we used banana genotypes, cv. Prata anã, from the experimental area of the UFLA, which were cultured *in vitro* for 3-4 months on B2 medium. To induce meristem growth, known as scalps, the leaf base explants were inoculated in P5 and P4 media. To induce callus formation, the "scalps" having grown approximately 3 mm x 3 mm x 3 mm in size were transferred to medium ZZss (Table 1). The growing conditions consisted of a 16 hour photoperiod at a temperature of 25 ± 1°C, as proposed by Strosse et al. (2003).

Table 1. Composition of culture media used in somatic embryogenesis of *Musa* sp.

	B2	P5	P4	ZZss
Macro-elements	MS	MS	MS	½ MS
Micro-elements	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS
Ascorbic acid (mg L^{-1})	-	10	10	10
Myo-inositol (mg L^{-1})	50	-	-	-
IAA (mg L^{-1})	-	0.175	0.175	-
BAP (mg L^{-1})	4	2.273	22.73	-
2,4-D (mg L^{-1})	-	-	-	1
Zeatin (mg L^{-1})	-	-	-	0.219
Sucrose (g L^{-1})	30	30	30	30
Gelling agent (Agar) (g L^{-1})	6	6	6	6
pH	5.8	5.8	5.8	5.8

The callus were further morphologically characterised into three types (Type 1 - transparent watery callus, Type 2 - yellow callus with small clusters, Type 3 - yellow callus with large clusters) by five reviewers using a colour and texture questionnaire. The callus were photographed in a stereomicroscope with a camera attached.

Five samples of homogeneous regions of callus according to each Type were collected and fixed in Karnovsky solution (2.5% glutaraldehyde and 2.5% paraformaldehyde) in cacodylate buffer 0.05 M, pH 7.0, for 24 hours at 4°C. Subsequently, the callus were placed in 30% glycerol for 30 minutes and then immersed in liquid nitrogen to be cut with a scalpel. The fragments were washed three times (10 minutes) in 0.05 M cacodylate buffer and post-fixed in 1% osmium tetroxide for two hours. Later, the fragments were dehydrated in an acetone gradient (25, 50, 75 and 90%) for 10 minutes each and 100% acetone twice for 10 minutes. The samples were then taken to the critical point apparatus using liquid CO_2 to

complete drying. Finally, the samples were mounted on aluminium supports (stubs), coated with gold using a gold evaporator SDC 050 and observed in a scanning electron microscope LEO EVO 40XVP.

Five samples of homogenous regions classified as Type 1, Type 2 and Type 3 were analysed by transmission electron microscopy (TEM). The samples were placed in 30% glycerol three consecutive times. The fragments were then washed in cacodylate buffer (0.05 M, pH 7.2) and post-fixed in OsO_4 solution (1%) for one hour. Next, dehydration was performed using an acetone gradient (25, 50, 75 and 90%) for 10 minutes and 100% acetone twice for 10 minutes. Resin embedding was performed initially to 30% for 8h and then to 70%. After 12h, the samples were submitted twice with 100% resin for 24h. The material was then placed in a mould suitable for polymerisation and placed in an oven at 70°C for 48h. Afterwards, the blocks were shaped as trapezes, having 1 mm on each side and then sliced into sections using an ultramicrotome. Ultrathin sections were subjected to contrast in uranyl acetate and lead citrate (BOSSOLA; RUSSELL, 1998 adapted by ALVES, 2004). The observation of the specimens was performed using a Transmission Electron Microscope (Zeiss EM 109).

Results and discussion

The three types of *Musa* sp. cv. Prata anã callus used in this study were the following: transparent watery callus (Figure 1A), yellow callus with small cell aggregates (Figure 1B) and yellow callus with large cell aggregates (Figure 1C).

It was observed through scanning electron microscopy (SEM) that cells from transparent aqueous callus showed an elongated shape (Figure 2A). According to Grando et al. (1993), this type of callus is formed by a spongy, translucent, white, mushy tissue. Figueiredo et al. (2007) also observed non-embryogenic translucent callus cells that were elongated in shape and poorly organised. Chaudhury and Qu (2000) noticed that non-embryogenic callus cultures obtained from young inflorescences of *Cynodon dactylon* (Bermudagrass) had a disorganised aspect with uncompressed, elongated and tubular cells. Nogueira et al. (2007) also observed during the

first non-embryogenic callus culture of *Byrsonima intermediate* (murici-pequeno) cells having 140 x 30μm dimensions and an elongated shape. Steiner et al. (2005), working with *Araucaria angustifolia* (Paraná pine) callus, observed some elongated vacuolated cells, with vacuolation defined as an early marker of cell death (FILONOVA et al., 2000).
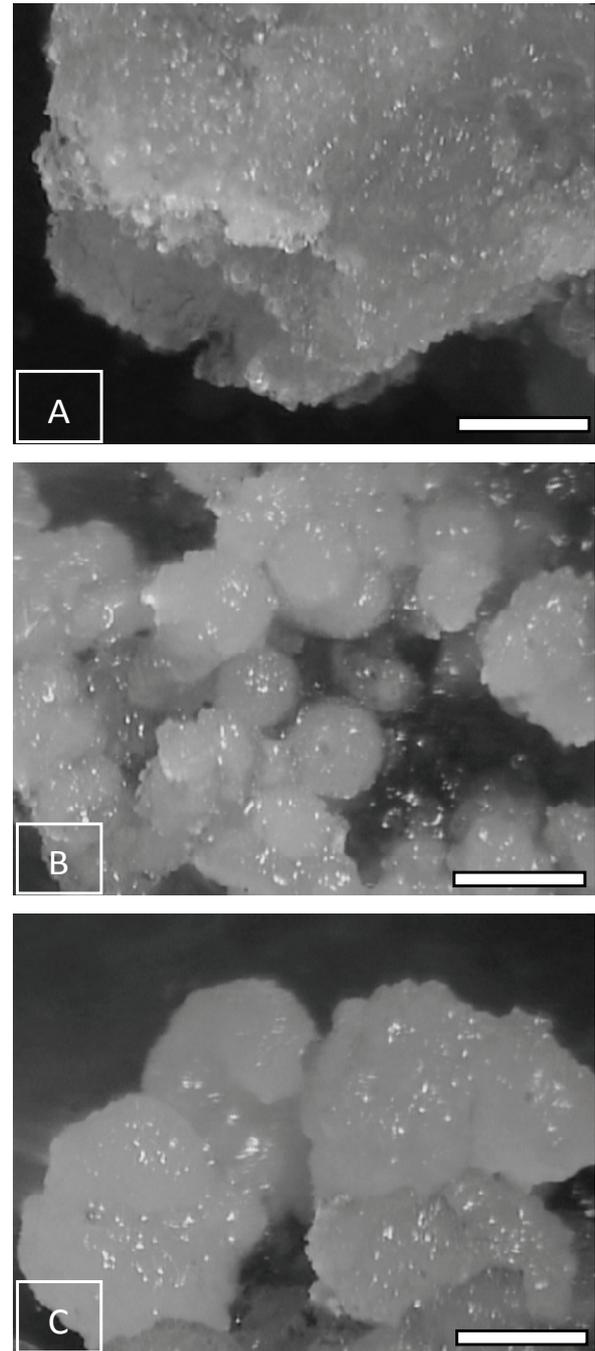

Figure 1. Callus of *Musa* sp., cv. Prata anã, cultured *in vitro* on ZZss medium. A) Type 1 - Transparent aqueous callus; B) Type 2 - Yellow callus with small clusters; C) Type 3 - Callus with large yellow clusters. Bar 5 mm. Lavras, Minas Gerais State, 2010.

The yellow callus showing small clusters (Figure 2B) had isodiametric cells, and those having large yellow clusters were isodiametric in shape (Figure 2C). Strosse et al. (2003) also observed this common formation of callus in *Musa* sp. (banana), which generally characterises callus as compact or embryogenic with individual embryos (few embryos). According to Hossain et al. (2009) embryogenic suspensions of *Musa* present cells with two distinct morphologies: elongated and isodiametric. These cells undergo transverse cell divisions, resulting in the formation of embryogenic cell clusters that are considered proembryos. Figueiredo et al. (2007) also found in *Passiflora* spp. (passion fruit) dark yellow callus with a predominant isodiametric cell shape, a characteristic of embryogenic callus. In *Phoenix dactylifera* (date-palm), globular callus exhibited small isodiametric meristematic cells, varying from 80 to 20 micrometres in diameter (SANÉ et al., 2006). Studies with *Coffea arabica* (coffee) have shown small and isodiametric cells, with diameters between 15 to 20 micrometres (QUIROZ-FIGUEROA et al., 2002).

According to Strosse et al. (2003), the formation of callus with yellow heterogeneous glomeruli in *Musa sp*. acquire two patterns, friable compact callus or embryogenic callus with individual embryos (few embryos).

The TEM analysis showed that the Type 1 callus presented some cells with thin walls, small vacuoles and scattered cytoplasm (Figure 3A) and also non-viable cells (Figure 3B). Stein et al. (2010) also observed in non-embryogenic callus of *Inga vera* (guaba) non-viable cells that had the same morphology as that of Type 1 callus of *Musa* sp.

On the other hand, the Type 2 callus showed cells with dense cytoplasm, large vacuoles and large amounts of mitochondria (Figure 3C and D). According to Shang et al. (2009), embryogenic and non-embryogenic callus differ not only in relation to their morphological structure and embryogenic behaviour but also in their cellular characteristics.

With respect to the morphology, as seen in Types 2 and 3 of banana callus, the embryogenic cells appear to be similar to meristematic cells with isodiametric shape. Regarding the cells' embryogenic characteristics, such cells generally present dense cytoplasm (SHANG et al., 2009), vacuole clusters, starch grains (SCHWENDIMAN et al., 1988) and isodiametric mitochondria (BOBÁK et al., 2004).

In this work, the presence of large vacuoles in cells of Type 2 callus was also observed. According Canhoto et al. (1996), the cell pro-embryos may be covered by vacuolated cytoplasm that can exist only in a small space between the plasma membrane and the tonoplast. Stein et al. (2010) observed that in embryogenic callus, organelles such as mitochondria and rough endoplasmic reticulum occupied a peripheral position because they were affected by large vacuoles.
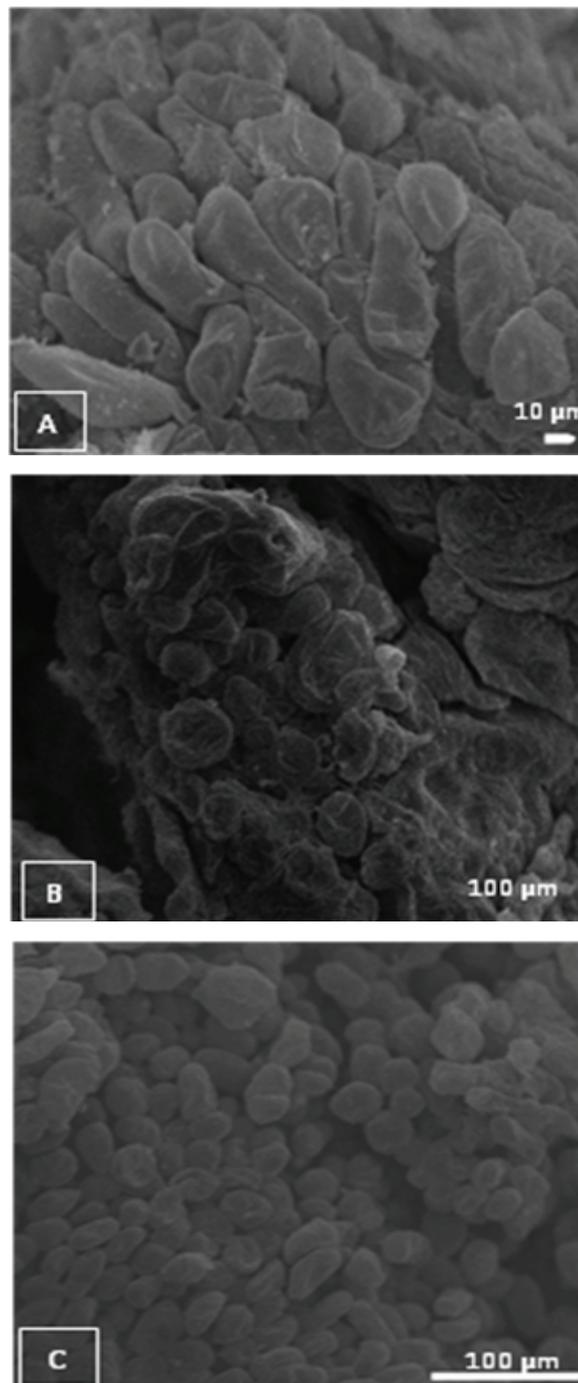

Figure 2. Scanning electron micrographs of cells from callus of *Musa* sp., cv. Prata anã. A) Callus Type 1 - Elongated cells, B) Callus Type 2 - Groups of cells, C) Callus Type 3 - Isodiametric cells. Lavras, Minas Gerais State, 2010.
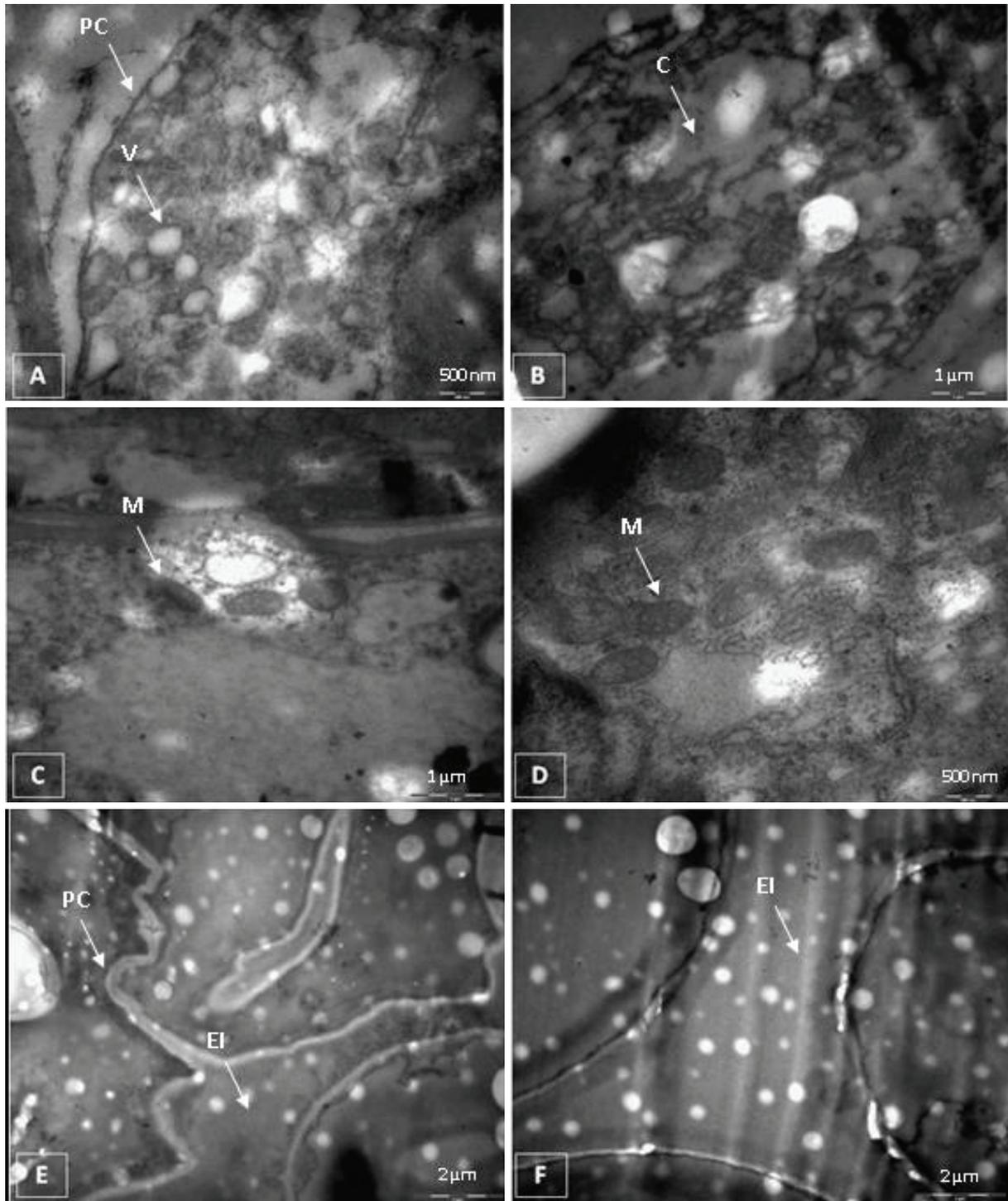
Figure 3. Electron transmission microscopy from *Musa* sp. cv. Prata anã callus. A) Callus Type 1 – Thin cell walls, small vacuoles, and dispersed cytoplasm; B) Non-viable callus cells; C) and D) Callus Type 2 - dense cytoplasm, large vacuoles and mitochondria; E and F) Callus Type 3 – Thick walls and intercellular spaces. Lavras, Minas Gerais State, 2010.

No starch grains were observed in the cells of callus Types 1, 2 and 3. In embryogenic tissues of *Feijoa sellowiana* (guava), the same was observed (CANGAHUALA-INOCENTE et al., 2009). Nevertheless, cells of the Type 2 callus showed an abundant presence of mitochondria in the electron matrix, with a developed crest and a circular format. This circular mitochondria shape is related to embryogenic cells, whereas the elongated mitochondria shape is related to non-embryogenic cells. This characteristic has been widely associated with the embryogenic system, indicating high

metabolic activity related to the high respiration rate (CANHOTO et al., 1996).

The cells of Type 3 callus showed thickened walls and intercellular spaces (Figure 3E and F). These results were also found by Figueiredo et al. (2007) and Nogueira et al. (2007) when they analysed non-embryogenic callus. Furthermore, these calluses exhibited disorganised nuclei, few mitochondria with few crests and small endoplasmic reticulum and Golgi complexes. According Steinmacher et al. (2011), embryogenic cells have no cell wall thickening and show large amounts of plasmodesmata connecting the cells.

Therefore, electron microscopy can be applied to assess the changes and the activity of cellular organelles and cell types and to characterise regions of potentially embryogenic explants.

Conclusion

The analysis system described here, which uses external morphology combined with scanning and transmission electron microscopy, can significantly contribute to our understanding of the formation of an organ or embryo from a single cell.

The transparent aqueous showed no morphological or cytological embryogenic callus. The calluses with large yellow clusters had embryogenic morphological aspects; however, the cytological features did not indicate embryogenic capacity.

The yellow calluses, showing small clusters, presented morphological and cytology characteristics of embryogenic callus.

Acknowledgements

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