ORIGINAL ARTICLE

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Histological and ultrastructural analysis of the Banana cv. Prata-Anã embryogenic calluses and cell suspension

Análises histológicas e ultraestruturais de calos embriogênicos e suspensão celular de Banana cv. Prata-Anã

ABSTRACT: The analysis of the developmental stages during somatic embryogenesis has been essential to elucidate the in vitro plants embryogenesis process and can validate the somatic embryogenesis induction and progression. This work aimed to characterize the embryogenic calluses and cell suspension of Banana cv. Prata-Anã, anatomically and ultrastructurally, respectively. For the calluses induction and anatomical analyses, the male flower were disinfested and inoculated on MA1 solid medium. At intervals of 30 days up to 11 months, calluses were collected, fixed in FAA and prepared for analysis in optical microscopy. For the cell suspension and ultrastructural analyses, calluses4 months old calluses, obtained on the first experiment, were transferred to different liquids cultures mediums: MRT, MGM, CNPMF or MA2 and maintained in aggitation. At intervals of 30 days calluses were collected and prepared for analysis in scanning electron microscope. The male flowers showed, after three months on culture medium, heterogeneous calluses formed by clusters of small isodiametric cells that gave rise to globular structure with meristems tissues, clearly differentiated from other cells around the calluses. The globular structure, after ten months on the culture medium, developed to torpedo stage showing the meristematic tissues protoderm, ground meristem and procambium. The cell suspension cultivated on MRT culture medium showed embryos on globular and torpedo stage.

RESUMO: *A análise dos estágios de desenvolvimento durante a embriogênese somática tem sido essencial para elucidar os processos de embriogênese in vitro de muitas plantas e pode ser utilizada para validar a indução e a progressão da embriogênese somática. O objetivo deste trabalho foi caracterizar anatomicamente os calos embriogênicos e ultraestruturalmente as suspensões celulares de Banana cv. Prata-Anã. Para a indução dos calos e análises anatômicas, flores masculinas foram desinfestadas e inoculados em meio de cultura sólido MA1. Em intervalos de 30 dias, até 11 meses, os calos foram coletados, fixados e preparados para análise em microscópio óptico. Para o estabelecimento das suspensões celulares e análise ultraestrutural, calos obtidos no experimento anterior, com 4 meses de cultivo, foram transferidos para diferentes meios de cultura líquidos – MRT, MGM, CNPMF ou MA2, mantidos sob agitação após 30 dias foram coletados e preparados para análise em microscópio eletrônico de varredura. As flores masculinas apresentaram, após 3 meses em meio de cultura, calos heterogêneos formados por um grupo organizado células pequenas de isodiamétricas que deram origem a estruturas globulares com tecidos meristemáticos diferentes das outras células do calo. Depois de 10 meses no meio de cultura, as estruturas globulares evoluíram para o estágio torpedo, no qual foi observada a formação dos tecidos meristemáticos primários, protoderme, procâmbio e meristema fundamental. As suspensões celulares cultivadas em meio de cultura MRT também apresentaram embriões nos estágios globular e torpedo.*

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1 Introduction

The banana stands out as one of the most important fruits in the world market, being mainly source of food and income for thousands of people and, thus, vital for food security in many tropical and subtropical countries from American, African and Asia (Lescot, 2011). However, although some countries have more plantations than others, they produce in less tons per year, their productivity is lower. In the Latim America, the Brazil has 479,614 hectares of Banana plantations producing 6,783,460 t and is overtaken by Ecuador that with half the area planted is able to produce 7,637,320 t (FreshPlaza, 2014).

The Banana crop in Brazil is peculiar in relation to climate diversion and cultivation and commercialization mode. The most diffused cultivars in Brazil are for example: Prata, Pacovan and Prata-Anã, which belong to the AAB group, the Nanica, Nanicão and Grande Naine, which belong to the AAA group. ´Prata-Anã´, also known as 'Enxerto' or 'Prata de Santa Catarina' has hands closer to one another and a bottle-neck tip (Silva et al., 2001).

In face of this, the expressive number of Banana cultivars with agronomic and commercial potential is reduced when consumers preference, productivity, tolerance to pests and diseases, resistance to drought, plant height and resistance to coldness are considered. One of the strategies used to solve problems is to develop new varieties resistant to main diseases, nematodes and pests through breeding programs planned to generate superior genotypes (Silva et al., 2001).

Biotechnological techniques, such as, tissue culture, molecular biology and genetic engineering are used for the plants breeding (Soares et al., 2014), enabling the development of new varieties more adapted to each environment.

Particularly, tissue culture has contributed greatly to plant regeneration of Banana through clonal propagation and micropropagation. Moreover, among the tissue culture techniques the somatic embryogenesis is an important system to produce elite plants on large-scale, including some cultivars of Banana, due to the high multiplication rate and gene transference possible by genetic transformation. For majority of the research, conducted in the world, embryogenic callus are the target tissue used for genetic transformation.

In this technique, the embryos formation occurs directly from the somatic explant tissues (direct embryogenesis) or from the somatic callus (indirect embryogenesis) without gametic fusion (George et al., 2008). Somatic embryogenesis processes follows the same steps of zygotic embryogenesis process, where the globular, heart and torpedo shape are observed. Somatic callus may exhibit morphological characteristics that affect formation, development and germination of embryos. Different morphogenetic expressions reflect the nature and degree of tissues differentiation (Stein et al., 2010) and thus, certain in vitro regenerative events are more easily induced in some tissues than in others.

Somatic embryogenic techniques have been used to facilitate genetic transformation as an avenue to bypass challenges of polyploidy, low fertility, limited genetic variability and long generation time faced during Banana breeding (Sadik et al., 2014) and has been adapted as an alternative method to mass produce disease-free planting materials (Bhojwani & Dantu, 2013).

Therefore, plant regeneration by somatic embryogenesis (SE) is an important system for the biotechnological improvement of plants, in particular for bananas, because of the presumed unicellular origin of embryos (Strosse et al., 2004).

However, in spite of extensive advances in Banana Somatic embryogenesis, low embryo germination and a loss of morphogenetic competence are still the bottlenecks of SE procedures in various bananas (Schoofs et al.,1999). Even though progress has been made in establishing ECS cultures of banana, the system is still hindered by a low frequency of induction of embryogenic callus and the subsequent induction of somatic embryos (Remakanthan et al., 2014).

Somatic embryogenesis in banana is still far from being considered a routine technology, has not been successfully applied to all cultivars, and warrants further improvement for many cultivars that are to be exploited as commercial crops. It is apparent that many morphological and physiological events go hand in hand with plant development during somatic embryogenesis, and the fate of cells in response to different conditions vary. Fundamental studies have not yet been fully pursued on the fate of meristematic cells during developmental stages affected by various physical and chemical factors in the somatic embryogenesis in banana (Jafari et al., 2015).

The understanding of somatic embryogenesis and the success in the application of biotechnological research cannot be achieved if the morphogenesis process is not well comprehended. It is need to identify the cells associated with induction processes and the formation of structures capable of organized growth and eventual development into seedlings.

Histological analysis of somatic embryogenesis development can validate the induction and progression of somatic embryo (Dai et al., 2011). The histological and morphological observation callus can be used for characterizing the somatic embryogenesis process. With the use of this technique, it is possible to evaluate the changes in the explant, the cell proliferation in the beginning of the induction, and the cellular origin of calluses in embryogenesis (Soares et al., 2014).

For plant regeneration from somatic embryogenesis, histological analyses must be carried out to determine the route of regeneration and discover whether the plant originated from somatic embryogenesis or organogenesis. Additionally, it is important to determine whether somatic embryogenesis is a reliable technique for use in the genetic transformation (Alcantara et al., 2014).

The aim of this work was characterize anatomically the embryogenic callus and ultrastructurally the cell suspensions of Banana cv. Prata-Anã.

2 Materials and Methods

Male flower buds of Banana cv. Prata-Anã provided by Pro-Mudas Company, Lavras – BR were used as explants to initiate callus.

Male flower buds, with one and two week's age, were collected from field-grown plants and washed in tap water. Thereafter they were shortened to 6 - 8 cm in length by successively removing the bracts and the hands of the male flowers subtended by them. The male flower buds were disinfected with 70% (v/v) ethanol for five minutes, and then flamed 2 times.

The explants were isolated aseptically from the reduced male flower buds by removing the bracts under a binocular stereomicroscope. The male flower hands that were excised from positions 16 to 7 (1 being the hand closest to the meristematic dome of the male bud) of each bud were inoculated on test tubes containing solid callus induction medium (MA1 medium).

The MA1 medium was composed of MS salts (Murashige & Skoog, 1962), 1.0 mg L–1 biotin, 2.0 mg L–1 glicin, 0.5 mg L–1 nicotinic acid , 0.5 mg L–1 piridoxin.HCl, 0.1 mg L–1 tiamin.HCl, 100 mg L–1 glutamin and supplemented with 30g L–1 sucrose, 100 mg L–1 malt extract and with growth regulators 5.37 µM naphthalene acetic acid- ANA, 18.9 µM dichlorophenoxyacetic acid - 2,4-D, 5.7 µM indole acetic acid – AIA (Kulkarni & Bapat, 2012).

Cultures mediums were and solidified with 8 g L–1 of agar and adjusted to pH 5.8 before autoclaving at 120 °C for 20 min. The explants were maintained at a temperature of 27 ± 2 °C, on the dark for 11 months. The cultures were examined twice a week to record any visible changes in the appearance of the explants in culture.

Callus were collected, every 30 days from 3 to 11 months, and fixed overnight in FAA fixative solution (formalin: acetic acid: alcohol) at 4 ± 1 °C. After this, the FAA fixative was changed to alcohol 70%. Then, calluses were dehydrated by washing in an ethanol/water series that progressed to absolute ethanol. Callus was then, infiltrated on alcohol/resin (50% each) overnight and on resin for 48 h. After the infiltration the samples were embedment in resin Leica according to the manufacturer's protocol, cut with a rotary microtome (5 microns thickness), stained with 0.05% toluidine blue and mounted on slides.

The samples were viewed in photonic microscope Leica DM LS® coupled with Nikon camera Ω.

Callus with four months, obtained on the first experiment, were transferred to Erlenmeyer flasks (125 mL) containing 15 mL of different culture mediums: MRT medium, (½ MS medium, 8.8 µM BA, 1.34 µM de NAA, 554.3 µM inositol, 400 mg L–1 malt, 100 mg L–1 de casein and 1 mL of vitamins solution (1g thiamin, 0.1 g pyridoxine, 0.1 g nicotinic acid and 0.1 mg L–1 glycine), MGM medium (1/2 MS medium, 2.57 µM AIA and 1.11 µM BA), CNPMF medium (MS medium, 4.52 µM 2,4-D, 100 mg L–1 glutamine, 10 mg L–1 ascorbic acid, 44.5 g L–1 sucrose and pH 5.3) (Morais-Lino et al., 2008) or MA2 medium (MS salt medium, vitamins (1.0 mg.L–1 biotin, 2.0 mg L–1 glycine, 0.5 mg L–1 nicotinic acid, 0.5 mg L–1 pyridoxine.HCl, 0.1 mg L–1 thiamin.HCl), 100 mg L–1 inositol, 100 mg L–1 malt extract, 100 mg L–1 glutamine, 45 g L–1 sucrose, 4.52 µM 2,4-D and pH 5.3 (Strosse et al., 2003). The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. The explants were maintained on shaker (100 rpm) at a temperature of 27 ± 2 °C on dark condition. The cultures were examined twice a week to record any visible changes in the appearance of the explants in culture.

The cell suspensions were collected and fixed in modified Karnovisky fixative solution [glutaraldehyde (2.5%) and paraformaldehyde (2.5%) in cacodylate buffer, pH 7.2], for 24 h, at room temperature. After this, the samples were washed in 0.05 M cacodylate buffer (three times every 10 min) and subsequently immersed in a solution of 1% osmium tetroxide and 0.05 M cacodylate buffer for 4 h.

Then, the cell suspension were dehydrated by washing in an acetone/water series (every 10 min) that progressed to absolute acetone, were dried using the critical-point method CPD 030 using liquid CO2 and coated with a thin layer of gold. The samples were observed scanning electron microscope (LEO Evo 040) operating at 20 kV.

3 Results e Discussion

Male flowers showed heterogeneous callus formation after three months on the culture medium MA1 (Figure 1a). Four types of callus were observed: embryogenic callus, yellow nodular callus and dark compact callus. On Musa acuminata Colla, AAA also were observed four types calluses (embryogenic callus, yellow nodular callus, white compact callus and translucent callus) and the percentage of explants that initiated the formation of callus depended more on the developmental stage of the male bud from which the explants were excised than on the cultivar (Youssef et al., 2010).

Callus induction is considered to be of great importance for obtaining well-formed somatic embryos and for the subsequent stages of development, maturation and conversion into plants (Bevitori et at., 2014). Histological studies of callus and cell suspensions can validate successful induction and progression of somatic embryogenesis (Dai et al., 2011). On the histological studies, the heterogeneous callus of Banana cv. Prata-Anã showed regions formed by irregular big cells with large vacuole and clusters formed by isodiametric small cells containing prominent nucleus and dense cytoplasm, strongly stained by toluidine blue (Figure 2a). Embryogenic and non-embryogenic callus differ, not only in morphological structure and embryogenic behavior, as well as in cellular characteristics (Shang et al., 2009). Large vacuole in the callus cells indicates cell degradation, as vacuole plays a critical role in programmed cell death (Lam et al., 2000). Some authors suggest callus subculture screening, to avoid the increase of non-viable cells mass and to select cells mass with potential for regeneration. Thus, the acquisition of embryogenic competence has been attributed to the cells that show meristematic traits during the induction phase (Solís-Ramos et al., 2010).

In this research were observed heterogeneous callus with regions formed by isodiametric small cells containing dense cytoplasm and prominent nucleus. On Cocos nucifera L. (Pérez-Núñez et al., 2006) embryogenic cell had dense cytoplasm and large centralized nuclei with one or two nucleoli. Apple Banana embryogenic calli also showed similar features to Banana cv. Prata-Anã calli. Two types of calli were observed: compact embryogenic calli with yellow appearance and with small cells forming dense cytoplasm and cell clusters and no embryogenic with friable aspect and white, with elongated and dispersed cells. Only the embryogenic callus regenerated plants (Houllou-Kido et al., 2005).

On Banana cv. Prata-Anã. Callus, the isodiametric small cells, after four months on culture medium MA1, formed organized cells cluster surrounded by irregular cells with release of the cluster (Figure 2b). These organized cell clusters, showing constant mitotic division, after five months on culture medium, became more frequent (Figure 2c) and originated nodules

(Figure 1b) composed of isodiametric cells with prominent nuclei and nucleoli and dense cytoplasm (Figure 2c).

Nodules structures represent the pro-embryo phase, which is the first step of sequential embryo formation process (Portillo et al., 2012). Rocha et al. (2012) described embryogenic nodules formation and suggested the possibility their development is associated with embryogenic cell redetermination.

Thereat, at seven months, the embryos arise as globular structure showing meristem tissues, clearly differentiated from other cells of the callus (Figure 1d and 2d). These globular
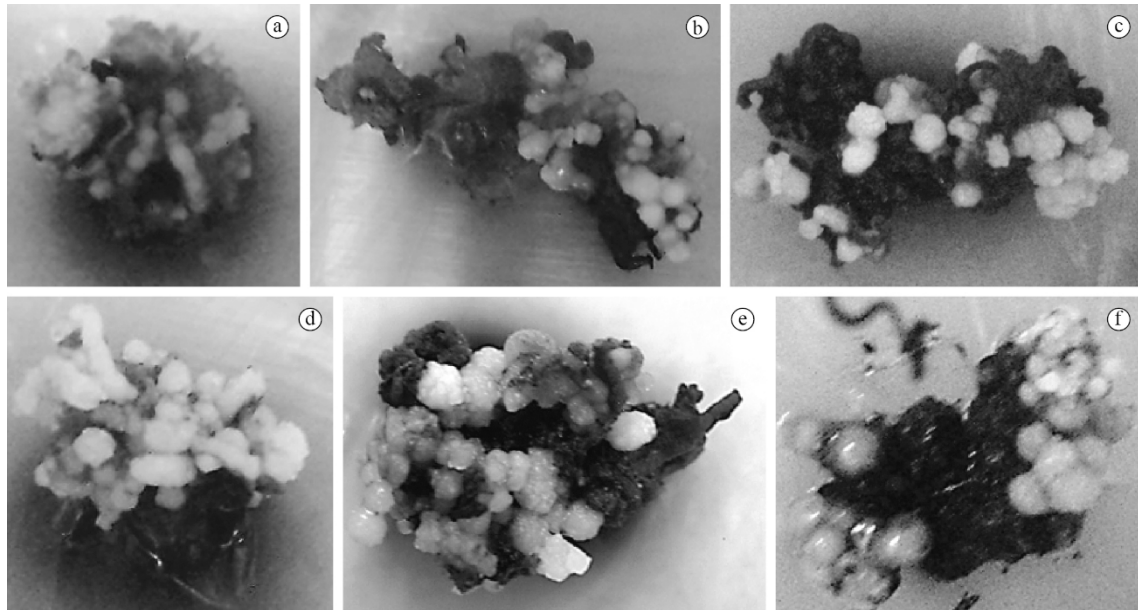
Figure 1. Embryogenic callus development of Banana cv. Prata-Anã. Callus at 3 months on cultivated on MA1 medium (a), 5 months (b), 6 months (c), 7 months (d), 8 months (e) and 10 months (f) showing individual somatic embryos.

Figura 1. Desenvolvimento de calos embriogênicos de Banana cv. Prata-Anã. Calos com 3 meses cultivados em meio de cultura MA1 (a), 5 meses (b), 6 meses (c), 7 meses (d), 8 meses (e) e 10 meses (f) com embriões somáticos individualizados.
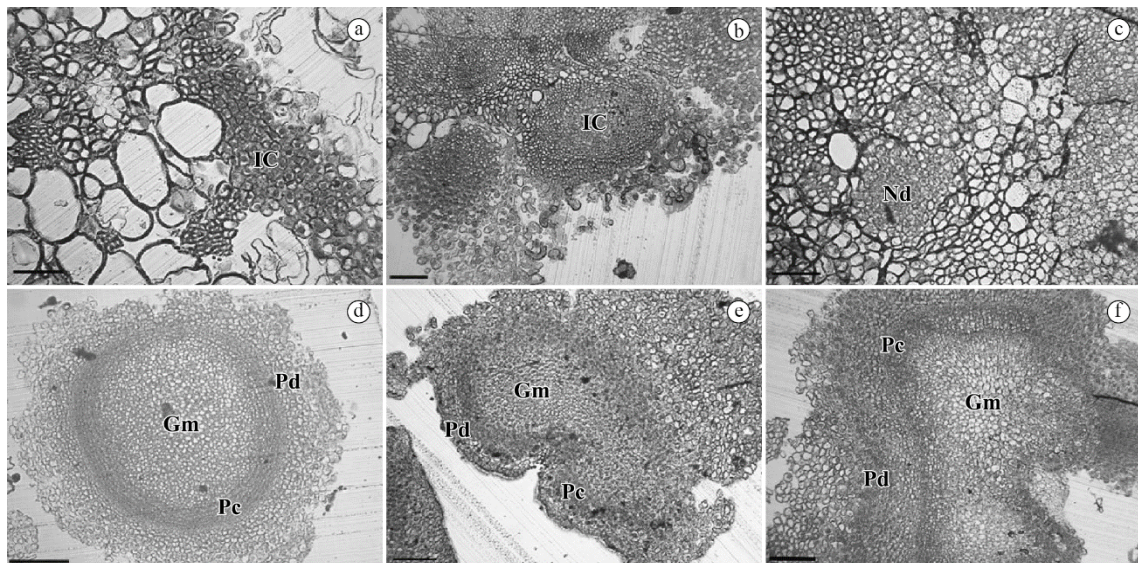
Figure 2. Photomicrography of embryogenic callus development of Banana cv. Prata-Anã. Callus cells after 3 months of cultivation on MA1 medium (a). Globular embryos at 4 months of cultivation (b). Meristematic cells at 5 months of cultivation (c). Individual somatic embryos at 7 months of cultivation (d). Somatic embryo at 10 months of cultivation (e). Somatic embryo at 10 months of cultivation (f). IC = Isodiametric cells; Nd = Nodules; Pd = Protodermis; Gm = Ground meristem; Pc = Procambial. Bar = 100µm.

Figura 2. Fotomicrografia do desenvolvimento de calos embriogênicos de Banana cv. Prata-Anã. Células de calos após 3 meses de cultivo em meio de cultura MA1 (a). Embriões globulares aos 4 meses de cultivo (b). Células meristemáticas aos 5 meses de cultivo (c). Embriões somáticos aos 7 meses de cultivo (d). Embriões somáticos aos 10 meses de cultivo (e). Embriões somáticos aos 10 meses de cultivo (f). IC = Células isodiamétricas; Nd = Nódulos; Pd = Protoderme; Gm = Meristema fundamental; Pc = Procâmbio. Barras = 100µm.

structures developed to torpedo stage, after ten months (Figure 1f), in which was possible observe the meristematic tissues: protodermis, ground meristem and procambium (Figure 2d), as well as bipolar organization (Figure 2f).

Protodermis, ground meristem and procambial could be identified in embryogenic structure by their cells shape, size and position. Procambial cells were long, narrow and intensely stained by toluidine blue. The procambial strands were related to proximal region and ramify in the median region toward the distal region, becoming peripheral, closer to the protodermis. Ground meristem cells were isodiametric, and are bigger on the medular than in the cortical cotyledon region. Protodermis cells were tabular in outline, prominent in the distal region and form a distinct layer that covers the entire embryo (Figure 2d, f). The occurrence of protodermis procambial like tissues may indicate differentiation and are connected by an organized vascular system. An epidermis-like layer has been observed in callus protuberances during the induction of organogenesis (Popielarska et al. 2006).

Histology and scanning electron microscopy (SEM) are effective tools in determining the cell composition and structures of different callus types with respect to the potential for regeneration (Narciso & Hattori, 2010). Embryogenic calli with nodular aspect are also observed in oil palm, which have meristematic regions that developed pro-embryos, and later globular embryos (Angelo et al., 2009). Moreover, in this work, only the embryogenic regions with small isodiametric cell organized in clusters developed somatic embryos and no meristematic regions with large and irregular cells not formed embryos (Figure 2a). This fact emphasizes, the need of callus screening during subcultures, promoting the development of cells with embryogenic potential.

The presence of meristematic region in early development of globular structures indicated that cells are actively dividing. The reprogramming of cell division is required for dedifferentiation and for the establishment of embryogenic competence. Corroborating with this, globular structure of the Banana cv. Prata-Anã, developed to the torpedo stage. Several studies with different species (Angelo et al., 2009; Demeter et al., 2010; Elviana et al., 2011; Steinmacher et al., 2011) reported that protoderm was the first tissue identified somatic embryogenesis development and presence of protoderm is one of the unique features of somatic embryo development. Somatic embryos of Musa acuminata cv. 'Berangan' (AAA) consisting of normal cells with nuclei, regular protoderm, and distinct procambium were able to regenerate into plants better than embryos with highly vacuolated cells and indistinct procambium (Jafari et al., 2015).

Filippi et al. (2001) working with Banana cv. Nanicão Jangada observed development of two somatic embryos types with distinct morphologial charateristics, the first type showed mushroom structure like, similar to zygotic embryos from Musa acuminata Colla and the second type showed elongated morphology presenting protoderm, fudamental meristem and procambium, similar to monocots zygotic embryos and to Banana cv. Prata-Anã somatic embryos of this work. Steinmacher et al. (2011) related that, the histological analyses of Bactris gasipaes Kunth. callus revealed the presence of a specific zone with small cells an epidermis-like layer. On Garcinia mangostana L. callus,

the meristematic region and protoderm were observed in the early formation of the globular structure (Elviana et al., 2011).

On the other hand, callus with four months transferred to a liquid medium showed different behavior according with the culture medium. Callus cultivated on CNPMF medium (Figure 3d-f) and MA2 medium (Figure 3g, h) had cluster of organized small cells forming globular structures while the callus cultivated on MRT medium (Figure 3a, b) showed globular and torpedo embryos. Callus cultivated on MGM medium showed only aggregated of disorganized cells (Figure 3c).

Cells suspensions were largely influenced by the culture conditions, especially by medium composition and the plant growth regulator. The CNPMF and MA2 medium contains auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) (452.48 μM and 4.52 μM), MRT medium contains auxin α-Naphthalenacetic acid (NAA) (1.34 μM) and citocinin 6-Benzylaminopurine (BA) (8.88 μM) and MGM contains indol acetic acid (IAA) (2.57 μM) and also BA (1.11 μM). The callus cultivated on CNPMF medium and MA2 medium had cluster of organized small cells forming globular structures while the callus cultivated on MRT medium showed globular and torpedo embryos.

Auxins have been shown to act like molecular glue binding to its TIR1 receptor and promoting ubiquitin-dependent degradation of Aux/IAA repressor proteins, activating the auxin response elements (Guilfoyle, 2007). The 2,4-D is synthesized auxin and have a dual role during the induction of somatic embryogenesis, one related to auxin signalling and the other to a stress component, that also changes the endogenous content of auxins (Fehér et al., 2003). Auxin 2,4-D (CNPMF and MA2 medium) induced, on cells suspension of Banana cv. Prata-Anã, the formation of organized small cells forming globular structures.

The increased proportion of cytokinin in hormonal balance is thought to promote the expression of somatic embryogenesis and the later development of the embryos and the BA-enriched medium (MRT medium), promoted the growth of proembryos which then developed from the globular stage to torpedo stage. Cells callus conversion process in somatic embryos occurs with repetitive cycles suspension of cell division, associated with physiological and biochemical conditions and environmental stimuli necessary for cell differentiation and maturation of somatic embryos (Guerra et al., 1999).

During the process of somatic embryos formation are known two cycles: on the first cycles occurs induction and development of embryogenic cells and high concentrations of auxin are required, and on the second cycle the auxin concentration decreases or is eliminated for the development of pro-embryos and subsequent somatic embryos formation (Fehér et al., 2003). As verified in this study, the conversion of callus on somatic embryos occurred on MRT culture medium with reduced amount of NAA and the auxin absence IAA and 2,4 D used only for callus induction.

In addition to growth regulators were added amino acids on the culture medium, such as thiamine, pyridoxine, nicotinic acid and glycine. These amino acids are mentioned to have great effect during the somatic embryos conversion as increase the regeneration rate (Asad et al., 2009). In studies of callus conversion in somatic embryos of sugar cane, the amino acids glycine, arginine and cysteine showed significant effect on somatic embryogenesis (Asad et al., 2009).

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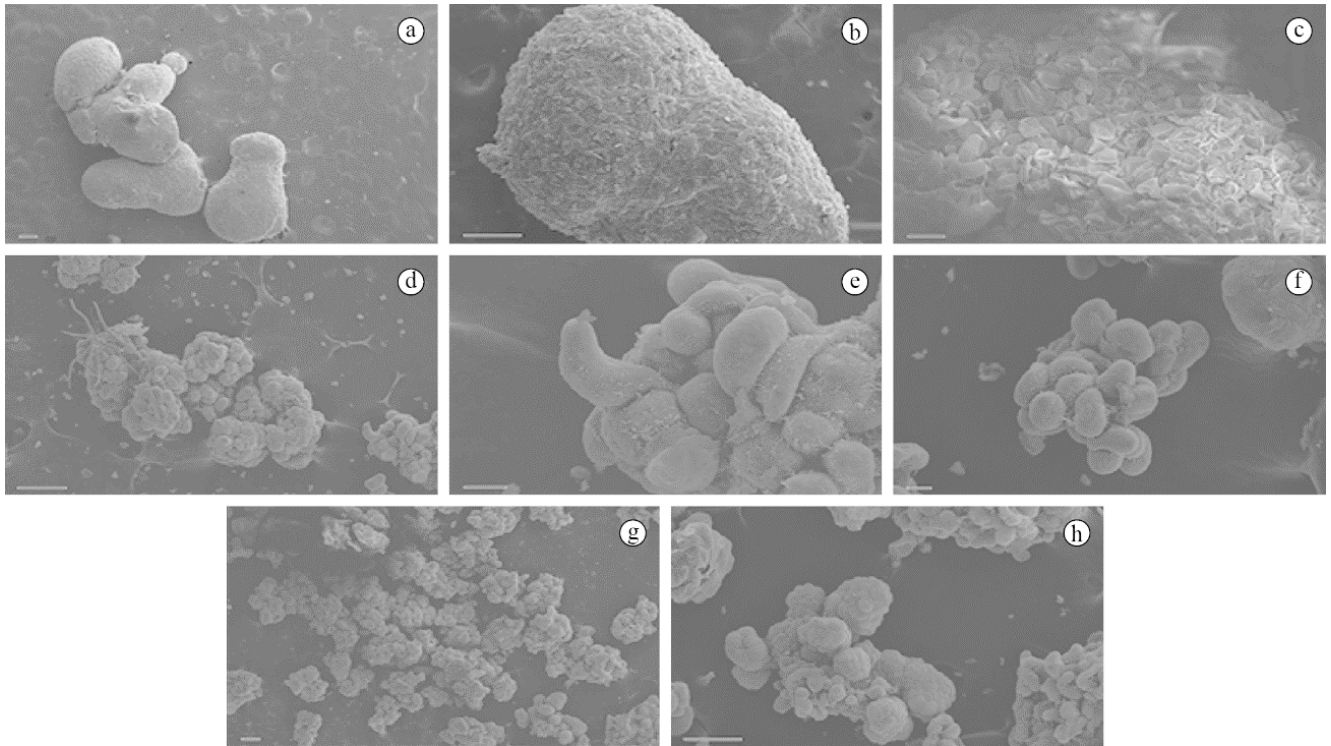
Figure 3. Scanning electron micrograph of Banana cv. Prata-Anã cell suspension. Somatic embryo at globular and torpedo stage cultivated on MRT medium (a,b). Cell suspension cultivated on MGM medium (c). Cluster of isodiametric cells cultivated on CNPMF medium (d-f). Cluster of isodiametric cells cultivated on MA2 medium (g,h). Bar = 100μm (a-h), Bar= 20 μm (e-f).

Figura 3. Eletromicrografia de células em suspensão de bananeira cv. Prata-Anã. Embriões somáticos nos estádios globular e torpedo cultivados em meio de cultura MRT (a,b). Suspensões celulares cultivadas em meio de cultura MGM (c). Aglomerados de células isodiamétricas cultivadas em meio de cultura MA2 (g,h). Barras = 100 μm (a-h), Barras = 20 μm (e,f).

4 Conclusions

Anatomically male flowers form heterogeneous callus that, after three months on the culture medium presents isodiametric small cells on organized cells cluster. After ten months on the culture medium, the globular structure, develop to the torpedo stage in which is possible observe the meristematic tissues: protoderm, ground meristem and procambium.

For the cell suspension, MRT medium provide the embryo development and the CNPMF and MA2 medium induce the formation of globular structures. The liquid culture medium MRT was better for obtaining embryos from suspension cells for Banana cv. Silver dwarf compared to the other tested culture medium.

Thus, we reported the development of embryogenic callus of Banana cv. Prata- Anã that showed important histological changes typical of embryogenic structures. Additionally, we showed that Banana cv. Prata-Anã are suitable somatic embryogenesis and these results could lead to new ways of manipulating callus by selectin the embryogenic parts.

This can improve the acquisition of a large number of embryogenic calli from a smaller number of calli while maintaining the embryogenic features and the consequent transformation ability. The understanding the background of this process will be useful for genome manipulation in this plant group.

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