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The use of histological analysis for the detection of somatic embryos in sugarcane

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The aim of this study was to establish an *in vitro* system for the induction, maturation and regeneration of somatic embryo in sugarcane from buds of cultivar RB 867515. Embryogenic calluses were obtained on semi-solid MS medium supplemented with 4.42 mg L^{-1} 2,4-D. After four weeks of culture of explants on the callus induction medium, globular structures were obtained. At the end of 20 days in maturation medium, somatic embryos were observed. Histological analysis showed somatic embryos with caulinar and root apex, protodermal tissue, and the vascular system, which apparently has no connection with the vascular tissue explant that gave rise to it confirming the presence of the somatic embryo. The embryos were transferred to regeneration medium containing 1 mg L^{-1} GA_3 and BAP, and after 1 to 2 weeks of culture, green points were observed, indicating the beginning of the formation of shoots.

Key words: *Saccharum* spp, bud culture, 2.4-D, morphogenetic pathway, embryogenesis, plant regeneration.

INTRODUCTION

Brazil is the largest sugarcane and ethanol producer in the world. The country contributes more than 50% of the trades in the international market. The cultivar RB867515 has been the most commonly planted cultivar in Brazil in the last two years. It has been planted in places where the soil has low fertility, a sandy texture and a low quantity of water (RIDESA, 2011). It reached 22.1% of the area cultivated with sugarcane in 2011 (Barbosa et al., 2012). The conventional breeding of sugarcane is a long process and can take up to 12 years for a new variety to become commercialised. Biotechnology, particularly transgenesis, is an important alternative in variety development. For majority of the research conducted in the world, embryogenic callus is the target tissue used for sugarcane transformation (Lakshmanan, 2006). Callus formation and plant regeneration vary with type of the explant, sugarcane genotype, culture conditions and others factors (Gandonou et al., 2005; Snyman et al., 2006), so it is important to characterize the plant regeneration process in important genotypes such as RB867515.

In vitro regeneration of sugarcane through somatic embryogenesis has been related to different explants such as young leaves (Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983; Chen et al., 1988; Brisibe et al., 1994; Falco et al., 1996a, 1996b; Chengalrayan and Galo-Meagher, 2001; Gandonou et al., 2005; Nieves et al., 2008; Asad et al., 2009; Watt et al., 2009), immature inflorescence (Liu, 1993; Blanco et al., 1997) and apical meristems (Ahloowalia and Maretzki, 1983; Rodríguez et al., 1997; Chengalrayan and Galo-Meagher, 2001). The histological and morphological observation of vegetative material can be used for characterising the somatic embryogenesis process. With the use of this technique, it is possible to evaluate the changes in the explant, the cell

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proliferation in the beginning of the induction, and the cellular origin of calluses in embryogenesis. The understanding of somatic embryogenesis has intensified biotechnological sugarcane research (Lakshmanan et al., 2005) and the success in the application of biotechnological research cannot be achieved if the morphogenesis process is not well comprehended. Molina et al. (2005) developed an efficient and reproducible protocol for regeneration of plantlets in sugarcane buds via organogenesis pathway. However, there is little information about the potential of somatic embryogenesis through sugarcane buds.

For this reason, this study aimed to analyse the main events that take place during somatic embryogenesis in sugarcane, cultivar RB867515, using buds as explants. The histological analysis was used to investigate the somatic embryogenesis stages of sugarcane.

MATERIALS AND METHODS

This study was carried out in the Plant Cell and Tissue Culture Laboratory at the Federal University of Viçosa (UFV), MG, Brazil. The RB867515 cultivar, which was approximately 10 months old, was collected at the Sugarcane Research and Improvement Centre (CECA), located in Oratorios, MG (latitude 20° 25 'S, longitude 42° 48' W, at 494 m of altitude). Six nodal segments were collected in the median region of the stalk. The stalks were cut into segments of approximately 6.0 cm and stored in a BOD incubator (Diurnal Growth Chamber, Forma Scientific, USA) at 25 ± 2°C, with 16 h photoperiod. After 20 days of incubation, the most vigorous and developed shoots were selected (Figure 1A). Two outer leaves were removed from the plants (explants) and these explants were disinfested in 70% alcohol (v/v) for 60 s; and commercial bleach (2.5% v/v) and Tween-20® for 20 min. After each step of disinfestations, the explants were rinsed three times with autoclaved distilled water.

The explants were sectioned into 0.5 cm length segments and inoculated in sterile Petri dishes (60 × 15 mm; JProlab) containing 30 ml of semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 3 mg L^{-1} 2.4-D. We used five Petri dishes with five explants were placed per Petri dish. The Petri dishes were incubated in the dark at 27 ± 2°C for 30 days. After this period, the callus obtained was sub-cultured on new medium containing the same concentration of growth regulators used previously. After another 30 days, the most developed embryogenic callus were transferred to culture medium with a low concentration of 2.4-D (0.5 mg L^{-1}) for the induction of somatic embryos. Twenty days after induction in medium with low concentrations of 2.4-D, the embryogenic calluses were transferred to the maturation medium. This medium was composed of MS solution, with 60 g L^{-1} sucrose and 1.32 mg L^{-1} of ABA. After inoculation, the Petri dishes were kept in a dark room for two days and subsequently transferred to light conditions for 20 days. The mature somatic embryos were transferred to MS medium supplemented with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} GA_3. The Petri dishes were kept in the light until development of the green structure. The embryos that formed plants were transferred to MS medium containing 1 mg L^{-1} BAP, 1 mg L^{-1} GA3 and 0.1 mg L^{-1} NAA.

For histological analyses, explants were sampled at 0, 5, 10, 15, 30, and 60 days of the induction stage, and 20 days of the maturation stage. The samples were fixed in FAA_{50} (formaldehydel: acetic acid: $alcohol_{50\%}$ on 5:5:90 ratio) and stored in 70% ethanol (Johansen, 1940). The samples were dehydrated in ascending ethanol series and embedded in glycol methacrylate (Historesin, Leica) according to the manufacturer's recommendations. The material was sectioned transversally and longitudinally in 5 µm slices with a rotary microtome (Leica RM 2155-UK). The sections were stained with toluidine blue (O'Brien et al., 1964) for metachromasy, and the slides were mounted in synthetic resin Permount®). Images were also obtained with a photomicroscope (Olympus AX70) equipped with the photo-U system. The blue Evans and carmine acetic acid test were performed on callus for confirmation of embryogenic cells.

RESULTS

Explants were inoculated in the longitudinal direction, in induction medium supplemented with 4.42 mg L^{-1} of 2.4-D (Figure 1B). After 10 days of induction, clusters of callus were observed in the peripheral regions of the explants (Figure 1C). These calluses were compact and whitish in colour. An intense division of parenchymal cells near the phloem was also observed (Figure 1D). At 15 days after induction, the formation of organised and individualised structures was observed (Figure 1E). Embryogenic regions, with defined protoderm and numerous meristematic cells were present. The meristematic calluses were formed by small, isodiametric cells with a dense cytoplasm. The cells had a high nucleus/cytoplasm ratio (Figure 1F). At 30 days after inoculation on induction medium, it was possible to observe the initiation of the proliferation of the globular structure (Figure 1G). The histological sections showed intense cell multiplication and divisions towards the periclinal globular structures. The cells have prominent nuclei and nucleoli and reduced vacuoles, and intense callus proliferation throughout the explant regions (Figure 1H). The calli were maintained in the induction medium for 30 days and the samples were collected at the end of this period for histological analysis.

The embryogenic callus was compact, whitish in colour and had a well-defined protoderm tissue, dense cytoplasm and a large nucleus. In contrast, non embryogenic cells had elongated and vacuolated cells. At 60 days after induction, only the globular embryogenic masses were transferred to medium containing low concentrations of 2.4-D (0.5 mg L^{-1}). The somatic embryos did not have a connection with the vascular tissues of the initial explant (Figure 2B). After 15 days in medium with low concentrations of 2.4-D, a histochemical test was carried out using Evans blue (0.1%) and carmine acetic acid (2%). The embryogenic cells reacted with carmine acetic acid and turned red while non-embryogenic cells were blue in colour (Figure 2A).

After 20 days in maturation medium, somatic embryos were observed (Figure 2C). The histological analysis showed somatic embryos with caulinar and root apex, protodermal tissue, and the vascular system, which apparently has no connection with the vascular tissue explant that gave rise to it (Figure 2D). After approximately 10 days in a maturation medium; embryos

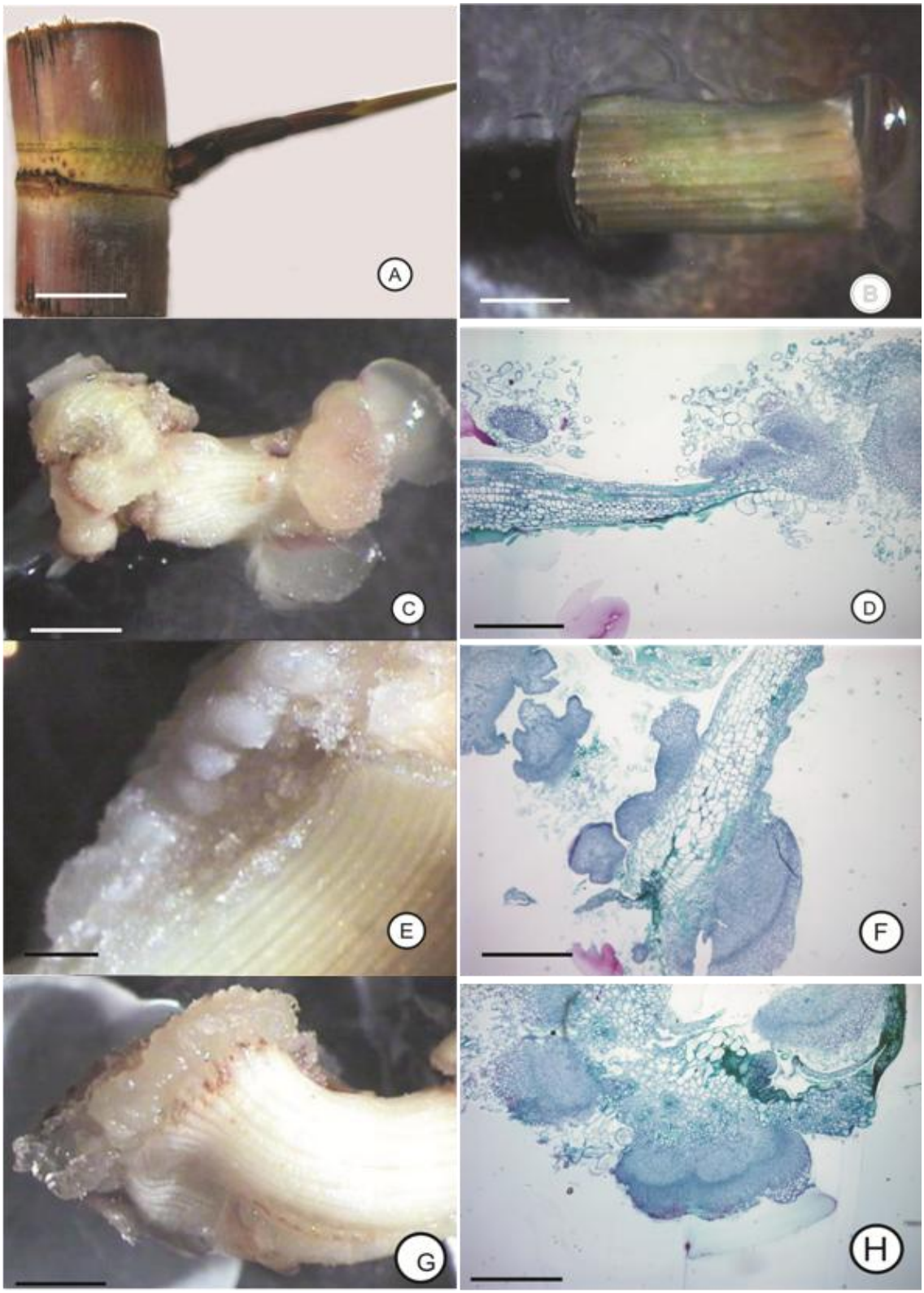

Figure 1. Initiation of embryogenic callus formation in sugarcane leaves. **A,** Sugarcane nodal segment with the developed buds. **B,** explants inoculated in induction medium. **C,** formation of callus 10 days after induction. **D,** longitudinal section of the explant at 10 days after the beginning of induction. **E,** formation of calluses 15 days after induction. **F,** longitudinal section of the explant at 15 days after induction, G, formation of callus 30 days after induction. H, transversal section of the explant, 30 days after induction. Bars: A = B = C = E = G = 1 mm, B = D = F = H = 400 µm.

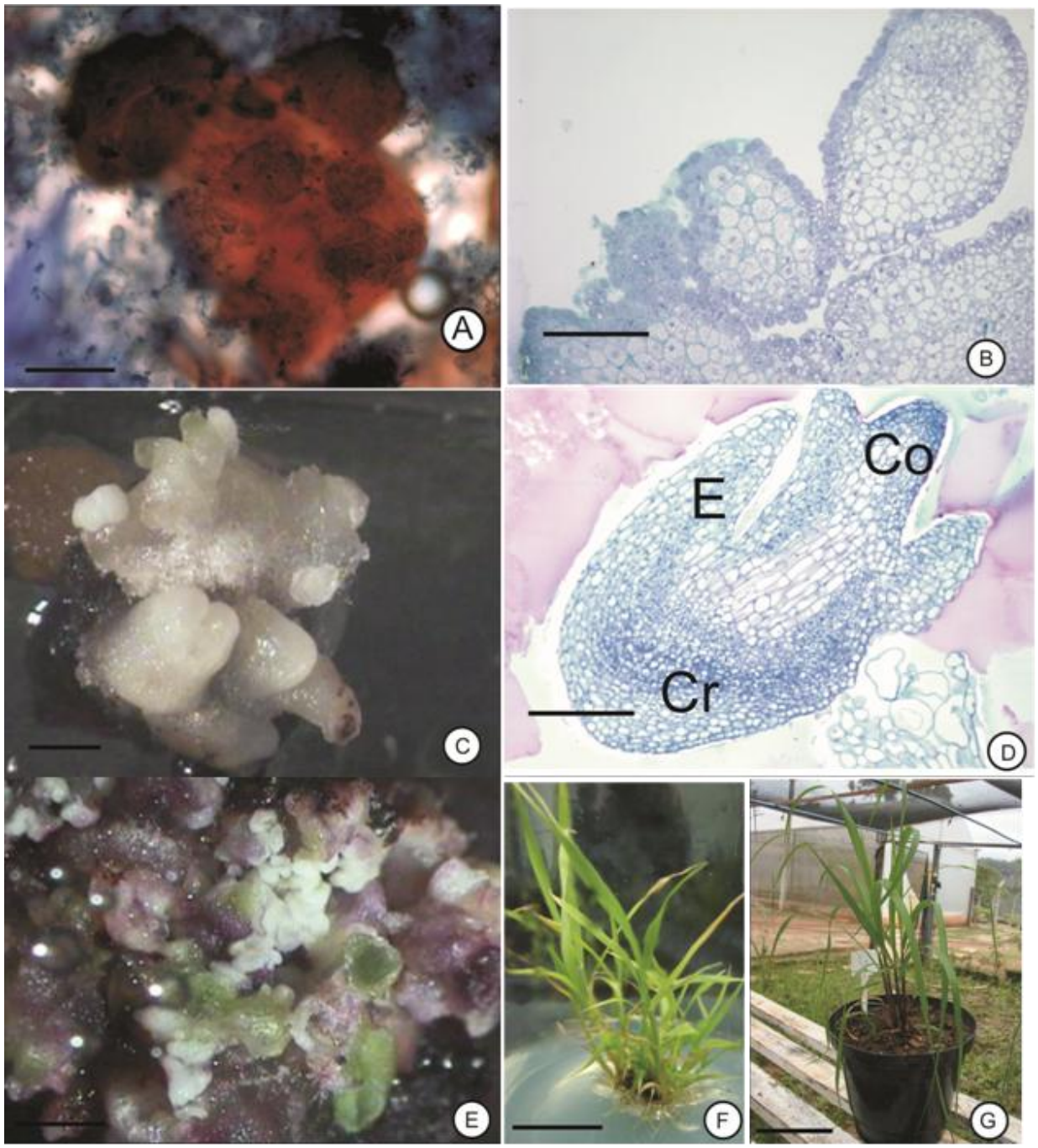

Figure 2. Development of callus and the somatic embryogenesis of sugarcane, RB867515 cultivar. **A,** Carmine acetic acid and blue Evans histochemical test 30 days after subculture. **B,** histological sections of globular embryogenic masses at 30 days. **C,** somatic embryos in maturation medium. **D,** cross section of a somatic embryo of sugarcane, showing the caulinar and root apex. **E,** somatic embryo in early germination in regeneration medium. **F,** plants in MS medium for development of the roots. G, plant in the soil and acclimatized in the greenhouse. co, coleoptile. cr, coleorhizae. e, scutellar. Bars: A = C = E = 1 mm, B = 400 µm, D = 200 µm, F = 1 cm, G = 10 cm.

were transferred to regeneration medium containing 1 mg L^{-1} GA_3 and BAP, and after 1 to 2 weeks of culture, green points were observed, indicating the beginning of the formation of shoots (Figure 2E). The plantlets were transferred to MS medium containing the same concentration of BAP and GA_3 and supplemented with 0.1 mg L^{-1} NAA to induce rooting (Figure 2F). After 30 days, the plants were transferred to soil and acclimatised in a greenhouse. The plants grow normally and uniformly (Figure 2G).

DISCUSSION

This study shows the histological features of the induction and somatic embryo formation from vascular tissues of sugarcane plants. The explants were cultured in the induction medium where callus formed following the expansion of the explants. Five days after induction, it was possible to observe the expansion of the explants. The rapid swelling of explants could have been caused by 2.4-D, which has the ability to promote the rapid elongation of cells (Gill et al., 2004). These callus presented different aspects: Embryogenic callus was nodular, compact and yellowish, while the non-embryogenic callus appeared watery and soft. These observations in the present work are consistent with those of other studies (Gill et al., 2004; Garcia et al., 2007). Histochemical analysis of the callus in this study was performed using the blue Evans and carmine acetic acid tests. Embryogenic cells stained red while non embryogenic cells remained blue (Astarita and Guerra, 1998). This shows that callus was heterogenous; hence, embryogenic callus was manually separated in the subsequent subculture stages. The differences between embryogenic and non-embryogenic callus could elucidate the mechanisms involved in the determination of the types of cells (Jimenez and Bangert, 2001). In order to achieve success in the establishment of embryogenic competent strains cell types, it is important to have internal reorganisation of callus (Quiroz-Figueroa et al., 2006) and the knowledge of the morphogenetic pathway; the location of the precise origin of competent cells can give this result.

Our results confirm previous work (Ho and Vasil, 1983; Garcia et al., 2007), which observed the formation of callus from parenchymal cells of the bundle sheath near the phloem tissue. Histological analysis also revealed that the globular embryos had no connection with the adjacent tissues and it was possible to observe the presence of a well-defined layer of epidermal cells, confirming the embryogenic pathway of the material under study. However, in the previous work, Falco et al. (1996a) observed the presence of shoot tips, but these had connections with the initial explant. These authors also described the bipolar structures, with no connection to the vascular tissues, suggesting that they are somatic embryos, concluding the presence of the two morphogenetic pathways (somatic embryogenesis and organogenesis) in the same explant. Franklin et al. (2006) observed the presence of globular structures with no connection to the tissues after 10 days on regeneration medium. The shoot apical meristem differentiated first, followed by the differentiation of the root. Apparently, the shoot apex was connected with the root by the vascular system; thus, bipolarity is an important characteristic of somatic embryogenesis pathway. Ho and Vasil (1983) reported in sugarcane somatic embryo, that the shoot apex was the first region to be organised followed by the root apex. According to these authors, the scutellum consists of large cells with starch grains. The embryo has the coleoptile that protects the leaf primordia and coleorhiza which protects the root apex. Most of the somatic cells are not naturally embryogenic and the induction phase is required for the acquisition of embryogenic competence (Namasivayam, 2007).

According to Garcia et al. (2007), auxins such as 2.4-D and picloram induce the formation of embryogenic calluses from the bundle sheath cells, and cell growth is attributed to the rapid metabolism of 2.4-D, which leads to the irreversible differentiation in somatic embryos (Vasil, 1988). Consequently, a potent auxin, 2,4-D was used in this study to induce somatic embryogenesis in sugarcane bud explants. This paper also provides important information on the morphology and the development of somatic embryos and on the regeneration of sugarcane somatic embryos, RB867515 cultivar, which may contribute toward more studies in other sugarcane cultivars.

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