Histology of somatic embryogenesis in rice (*Oryza sativa* cv. 5272)

Rafael Vega[1, 4], Nelly Vásquez[2], Ana M. Espinoza[3], Andrés M. Gatica[1] & Marta Valdez-Melara[1]

1. Escuela de Biología, Universidad de Costa Rica, 11501-2060, San Pedro, Costa Rica.
2. Centro Tropical de Investigación y Enseñanza (CATIE), Turrialba, Costa Rica.
3. Centro de Investigación en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica, 11501-2060, San Pedro, Costa Rica.
4. Dirección actual: Fundación para la Innovación Tecnológica Agropecuaria, El Salvador.

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Abstract: Rice (*Oryza sativa* cv. 5272) embryogenic calli were obtained from mature zygotic embryos culture on Murashige & Skoog (1962) medium supplemented with 2.5 mg/l 2,4- dichlorophenoxyacetic acid. Histological analysis of somatic embryogenesis revealed that after two weeks of culture of explants on the callus induction medium, somatic embryo development began with a cluster of proembryogenic cells in the peripheral region of the calli. The outer cell layer of embryogenic calli consisted of small and isodiametric cells with a dense cytoplasm and a prominent nucleus and nucleolus; whereas the inner cell layer is composed of large cells with small nucleus and large vacuole. These embryogenic cells underwent a series of organized divisions and formed the proembryo with a well-defined protodermis. Rev. Biol. Trop. 57 (Suppl. 1): 141-150. Epub 2009 November 30.

Key words: *Oryza sativa,* rice, *in vitro* culture, histology, somatic embryogenesis, morphogenesis, Costa Rica.

The totipotent character of plant cells allow that any differentiated cells that retains its nucleus has the ability to regenerate an entire new plant by organogenesis or somatic embryogenesis (SE) (Reynolds 1997, Fortes & Pais 2000). SE is the developmental process by which bipolar structures that resemble zygotic embryos are developed from haploid or diploid somatic cell through an orderly embryological stage without gametes fusion (Jiménez 2001, Jiménez 2005, Quiroz-Figueroa *et al*. 2006, Namasivayam 2007). Two types of somatic embryogenesis are recognized: direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE). DSE is characterized by the induction of somatic embryos directly from pro-embryogenic cells from leaves, stem, microspores or protoplasts without the proliferation of calli, whereas in ISE somatic embryos are developed from friable embryogenic calli (Jiménez 2001, Molina *et al*. 2002, Quiroz-Figueroa *et al*. 2002b, Quiroz-Figueroa *et al*. 2006).

Somatic embryogenesis is a unique process in plants and it is of remarkable interest for biotechnological applications such as clonal propagation, artificial seeds and genetic engineering (Quiroz-Figueroa *et al*. 2006, Namasivayam 2007). Precisely, when somatic embryogenesis is integrated with conventional breeding programs and molecular and cell biological techniques, it provides a valuable tool to enhanced genetic improvement of crop species (Quiroz-Figueroa *et al*. 2006).

As rice (*Oryza sativa*) is the most important staple crop for one third of the world population, there is considerable interest in the development of new cultivars tolerant to biotic and abiotic stresses (Valdez *et al*. 1996b). Plant biotechnology represents an alternative to conventional breeding programs; nevertheless, integration of biotechnology into rice

improvement through genetic engineering or mutagenesis requires a reliable and efficient *in vitro* culture system.

In rice, somatic embryogenesis is the most common regeneration pathway and has been obtained from roots, leaf bases of young seedlings, mature embryos, immature embryos, caryopses, microscopes, cell suspension, protoplast and young inflorescences (Kawata & Ishihara 1968, Inoue & Maeda 1980, Wernicke *et al*. 1981, Heyser *et al*. 1983, Abe & Futsuhara 1984, Raghavan Ram & Nabors 1984, Abe & Futsuhara 1985, Chen *et al*. 1985, Abe & Futsuhara 1986, Kavi Kishor & Reddy 1986, Raina *et al*. 1987, Hartke & Lorz, 1989, Koetje *et al*. 1989, Chowdhry *et al*. 1993, Valdez *et al*., 1996a,b, Gairi & Rashid 2004, Hoque & Mansfield 2004, Meneses *et al.* 2005, Ge *et al*. 2006). Some author present organogenesis and embryogenesis, occurring simultaneously, as the regeneration pathway (Boissot *et al*. 1990, Gairi & Rashid 2004).

Nevertheless, successful application of genetic engineering or mutagenesis techniques cannot be achieved if the processes leading to morphogenesis are not well understood (Fortes & Pais 2000). Therefore, the aim of this work was to describe the events leading to the development of plantlets from embryogenic calli obtained from Costa Rican rice "CR-5272" through histological analysis.

MATERIALS AND METHODS

Plant material and embryo isolation: Seeds of the commercial Costa Rican cultivar CR-5272 were surface-sterilized following the procedure described by Valdez *et al*. (1996). Briefly, the seeds were immersed in an aqueous 30% (v/v) Domestos™ (Lever Ltd. Warrington, G.B.) solution for 35 min and then rinsed five times in sterile distilled water. Disinfected seeds were soaked overnight in sterile distilled water to facilitate embryo isolation without damaging the scutellum. Embryos were excised under a binocular microscope using fine scalpels and forceps.

Callus induction: Ten disinfected seeds were cultured in each Petri dish (100x15 mm) containing 40 ml of callus induction medium (CIM) which consisted of MS mineral salts (Murashige & Skoog 1962) supplemented with 100 mg/l myo-inositol, 50 mg/l tryptophan, 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/l sucrose and 3 g/l Phytagel. The pH was adjusted to 5.8 before autoclaving for 21 min at 121°C and 1.07 kg cm^{-2}. Petri dishes were sealed with polyethylene food wrap (Glad, Costa Rica) and the explants were cultured in the dark at 26±2 °C.

In order to investigate the effect of particle gun bombardment on the regeneration capacity, embryogenic calli were bombarded with tungsten particles coated with DNA or tungsten particles only using 80 psi and a Particle Inflow Gun (Finer *et al*. 1992). Additionally, non-bombarded embryogenic calli were included as controls. The number of shoots and *in vitro* plants per embryogenic calli were determined after ten weeks of culture on regeneration medium that consisted of MS mineral salts supplemented 0.5 mg/l 6- benzylaminopurine (BAP), 0.05 mg/l naphthaleneacetic acid (NAA), 30 g/l sucrose and 3 g/l Phytagel. The explants were cultured in the dark at 26±2 °C.

The percentage of embryogenic calli with shoots [(Number of calli with shoots /total of embryogenic calli)x100] and the percentage of regeneration [(Number of *in vitro* plants /total of embryogenic calli)x100] were calculated.

Histological study: Histological analysis of the embryogenic calli and zygotic embryos were performed according to Boissot *et al*. (1990). Embryogenic calli were collected 5, 10, 15 and 20 days after cultured on callus induction medium. Both types of tissues were fixed in FAA (formalin-acetic acid-ethanol) for 24 h. Then, the samples were dehydrated in a graded series of ethanol (70, 95 and 100%) for 1 h each one and embedded in paraffin wax. Samples were cut into 9-12 μm sections and were stained with PAS-Hematoxylin, PAS-aniline blue black or eosine-alcyan blue. Macroscopic

features were photographed using a stereoscope and the photographs of the histological study were taken using an OLYMPUS IX51 inverted microscope.

RESULTS

Mature embryos produced friable yellowish calli (Fig. 1A-C) derived from the scutellum (Fig. 1D) after two weeks of culture on callus induction medium. The epithelial cells of the scutellum are columnar with a dense cytoplasm and a prominent nucleus and nucleolus (Fig. 1D). Three days after zygotic embryos were culture on the CIM, transverse section showed that embryogenic regions were formed from the more mitotically active epithelial cells of the scutellum (Fig. 1E).

Histological observations on embryogenic calli revealed two types of cells (Fig. 2A). The outer cell layer consisted of 9-12 rows of small and isodiametric cells with a dense cytoplasm and a prominent nucleus and nucleolus (Fig. 2B). The initial divisions of these cells were periclinal and anticlinal. These observations denote an active metabolism and indicate that external calli cells resembled meristematic cells. In contrast, in the inner cell layer, large cells with small nucleus and large vacuole were observed (Fig. 2C). Between these two cell layers, there is a layer of 3 rows composed of laterally compressed cells with green stained primary cell walls. The center of the embryogenic calli consisted of large cells with small nucleus and starch granules and abundant intercellular spaces (Fig. 2C).

After 10 days of culture, cluster of proembryogenic cells in the peripheral region of the calli were observed (Fig. 3A). It was observed that immediately after the proembryogenic

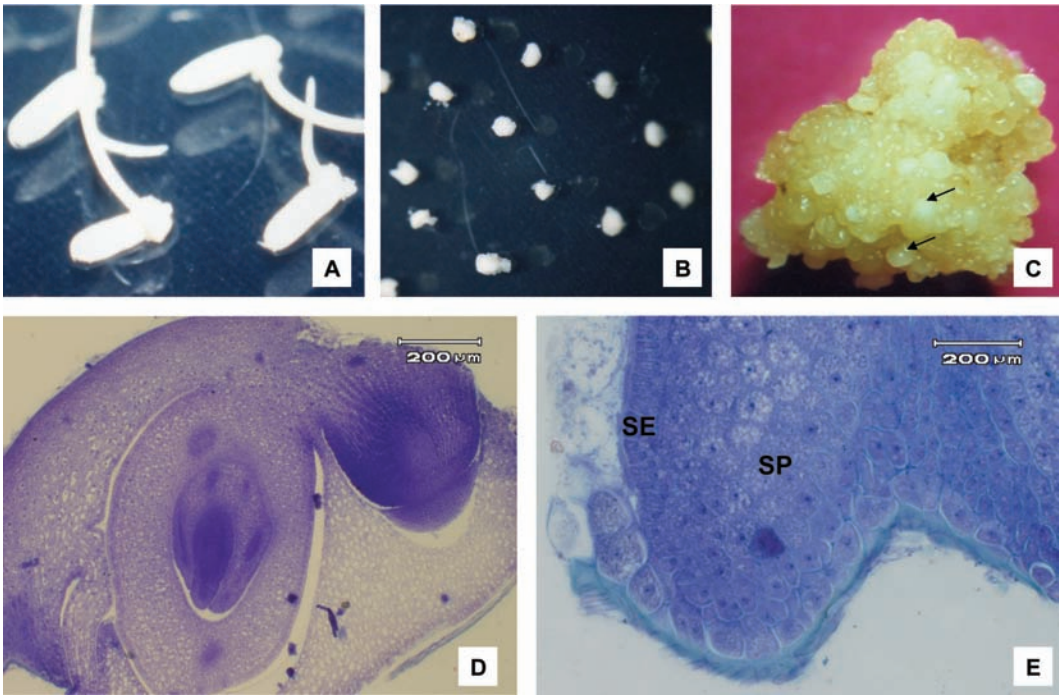

Fig. 1. Somatic embryogenesis in rice (*Oryza sativa* cv. 5272). (A) Two-week-old embryogenic calli derived from mature seeds cultured on callus induction medium (B) Four-week-old embryogenic calli (C) Embryogenic callus with somatic embryos (arrows) (D) Transverse section of the scutellum of mature embryo showing epithelial cells (E) Enlarge view of the epithelial cells of the scutellum (SE) and scutellum parenchyma (SP) of mature embryo. Note prominent nuclei.

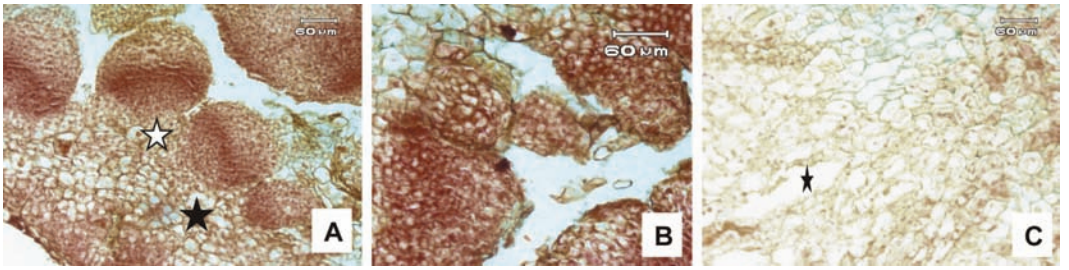

Fig. 2. Histological analysis of somatic embryogenesis in *Oryza sativa* cv. 5272. (A) Embryogenic cells (☆) and non-embryogenic cells (★) (B) Embryogenic cells showing isodiametric cells (C) Non-embryogenic cells with large vacuole, small starch granules and abundant intercelullar spaces (★).

region the cells increased in size with respect to the formers indicating different stages of differentiation (Fig. 3A). The next step in SE is the formation of the proembryo (Fig. 3B) which showed a well-defined protodermis (Fig. 3B). At 20 days after initiation of culture, these proembryos continued a series of organized division and gave rise to globular somatic embryos which are delimited by a well defined layer of epidermal cells with conspicuous nucleus (Fig. 3D). These somatic embryos had no apparent vascular connection with the mother tissue and had a suspensor–like (Fig. 3D). After ten days of culture on regeneration medium, globular somatic embryos developed into heart-shape somatic embryos, which consisted of cells with prominent nucleus and dense cytoplasm (Fig. 3E). After 15 days of culture on regeneration medium torpedo-shaped somatic embryos were observed. These somatic embryos showed signs of polarization with apical and radical meristems in opposite poles (Fig. 3F).

GUS expression was determined on rice embryogenic calli after 24 h of particle bombardment. The presence of abundant and prominent foci in the calli periphery were observed, which indicated that tungsten particles coated with the DNA has been delivery into the cell and cause no damage to the embryogenic cells (Fig. 4). The number of shoots, regenerated *in vitro* plants, percentage of embryogenic calli with shoots and the percentage of regeneration was similar among non-bombarded calli and the calli bombarded with tungsten or tungsten plus DNA (Table 1).

DISCUSSION

In the application of genetic engineering or mutagenesis techniques in rice improvement through *in vitro* culture, knowledge on the morphogenetic pathway and location of the precise origin of competent cells is important (Mendoza *et al*. 1993). This study presents histological aspects of callus initiation and somatic embryo formation on epithelial cells of the scutellum of mature zygotic embryo of rice (*Oryza sativa* cv. 5272). Our results confirmed previous observations in wheat immature embryos (Ozias-Akins & Vasil 1983), maize immature embryos (Kamo *et al.* 1985), immature and mature zygotic embryos of *Pannicum maximum* Jacq. (Lu & Vasil 1985) and rice (Hartke & Lorz 1989, Jones & Rost 1989, Koetje *et al.* 1989, Biswas *et al.* 1994, Rueb *et al.* 1994, Valdez *et al.* 1996).

Maeda & Radi (1991) emphasized that the main function of the epithelial cells of the rice scutellum is the absorption of sugars and plant growth regulators, which could explain their high metabolic activity and the facility to dedifferentiate and gave rise to embryogenic calli in comparison with the rest of cells of the scutellum. Jones & Rost (1989) indicated that the scutellum epithelium is a modified layer of columnar cells at the interfase of the embryo

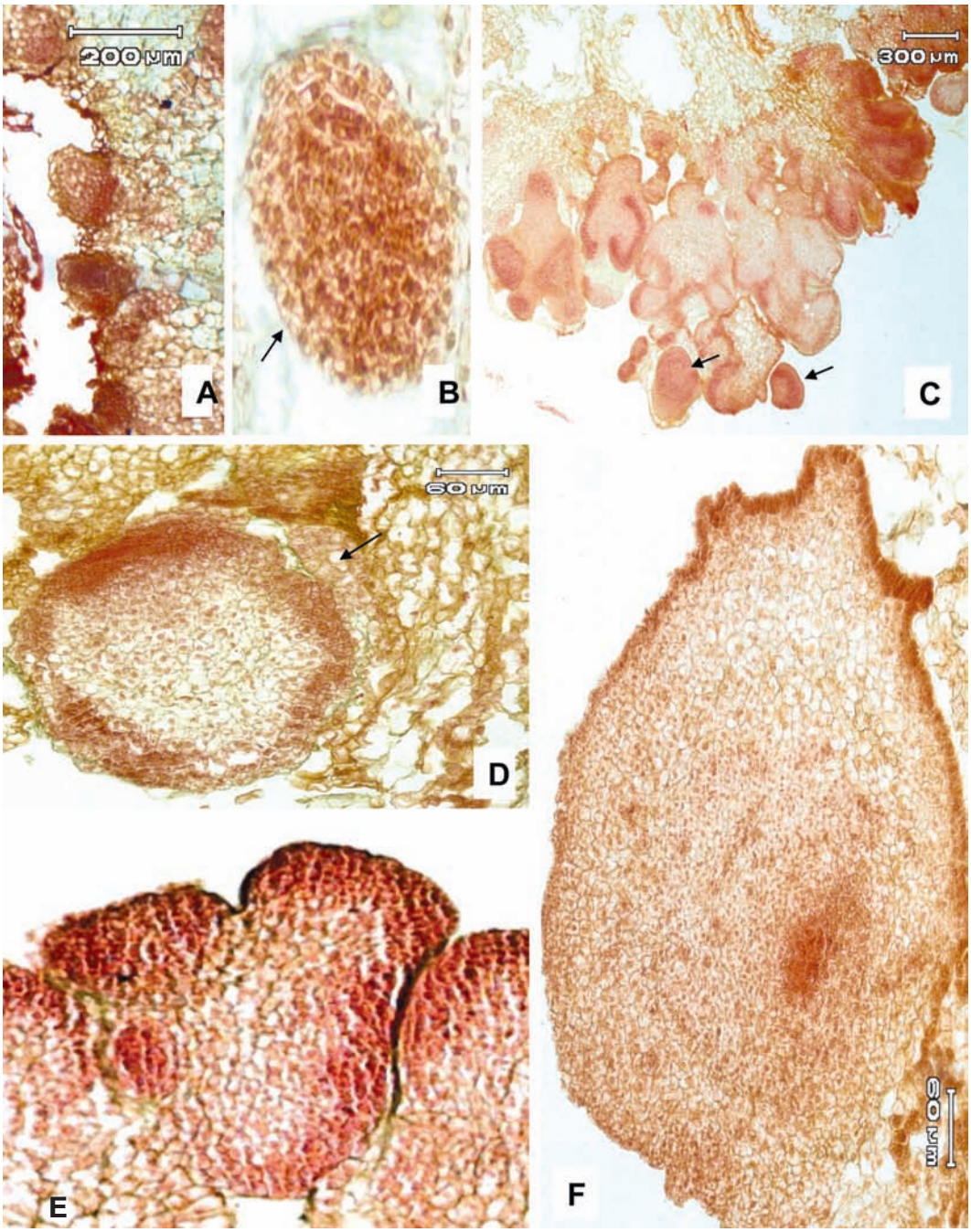

Fig. 3.Histological analysis of somatic embryogenesis in *Oryza sativa* cv. 5272. (A) Cluster of proembryogenic cells in the peripheral region of the calli (arrows). (B) Proembryo with well defined protodermis (arrow) (C) Appearance of the embryogenic calli with multiples globular somatic embryos (arrows) (D) Globular somatic embryo without connection to mother tissue and suspensor–like (arrow) (E) Heart-shaped somatic embryo (F) Torpedo-shaped somatic embryo.

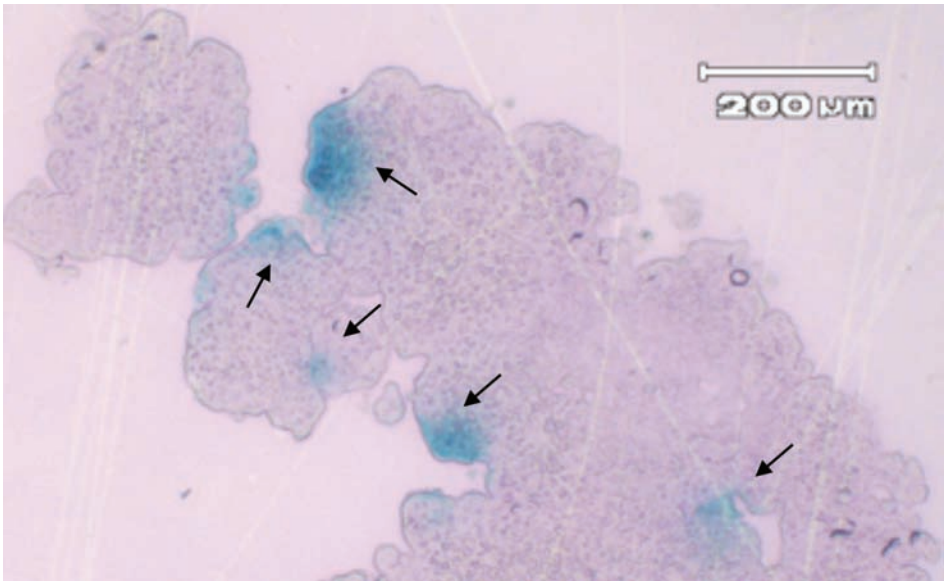

Fig. 4. GUS expression (arrows) in globular structures in the peripherial region of 5-days-old embryogenic calli of rice.

TABLE 1
*Effect of particle bombardment on regeneration capacity of embryogenic calli of rice (*Oryza sativa *cv. 5272) after 10 weeks of culture*

Treatment	Number of initial embryogenic calli	Number of shoots	Number of regenerated *in vitro* plants	Embryogenic calli with shoots (%)[1]	Regeneration (%)[2]
Non-bombarded	36	21	28	58	78
Tungsten	39	22	25	56	64
Tungsten + DNA	45	27	30	60	67

1. [(Number of calli with shoots /total of embryogenic calli) x 100].
2. [(Number of *in vitro* plants /total of embryogenic calli) x 100].

with the endosperm and it is an active tissue during germination and has been reported as the site of amylase synthesis in grasses.

The morphological and anatomical observations indicate that somatic embryos may arise from one cell or a group of cells (Quiroz-Figueroa *et al*. 2002b, Quiroz-Figueroa *et al*. 2006). When somatic embryos have unicellular origin, coordinated cell divisions are observed and the embryos are connected to the maternal tissue by a suspensor-like. In contrast, multicellular origin is characterized by no coordinated cell divisions and somatic embryos are observed as a protuberance and fused to the maternal tissue (Quiroz-Figueroa *et al*. 2006). Our histological observations showed that somatic embryos originated from the more external cells of the embryogenic calli, agreeing with previous observations in sugarcane (Jane-Ho & Vasil 1983), Guinea Grass (Lu &

Vasil 1985) and oil palm (Schwendiman *et al.* 1988).

During somatic embryogenesis induction in the rice cultivar 5272 two distinguishable clusters, embryogenic and non-embryogenic cells, were observed. In the embryogenic systems described until now in maize (Fransz & Schel 1991a,b), sugarcane (Falco *et al*. 1996), pearl millet (Taylor & Vasil 1996), cork oak (Puigderrajols *et al*. 2001), sugar beet (Moghaddam & Taha 2005) and coffee (Quiroz-Figueroa *et al*. 2002a, Gatica *et al*. 2007a) the embryogenic cells show characteristics common to meristematic cells: high division rates, cells are isodiametric and small with a dense cytoplasm with several starch grains, large nucleus and prominent nucleolus, small vacuoles, thin cell walls and a higher metabolic activity (Quiroz-Figueroa *et al*. 2006). Moreover, the main morphological characteristic of somatic embryos is the bipolarity and the absence of connection with the explant vascular tissue (Falco *et al*. 1996, Gatica *et al*. 2007a). The cells containing starch grains observed in rice somatic embryogenesis have been related to the acquisition of embryogenic potencial and indicates the high nutritional requirement of cell populations during the process (Fransz & Schel 1991b, Apezzato-Da-Gloria & Machado 2004).

The histological observations of the rice embryogenic calli at different developmental stages showed an internal organization of the calli. Thus, the external layers consisted of meristematic cells, which gave rise to clusters of embryogenic cells and somatic embryos. Whereas, the interior of the callus consisted of parenchymatic cells with a less visible nucleus and many vacuoles. Some of these parenchymatic cells are broken and gave rise to the internal space of the callus. These observations confirmed the study of Fransz & Schel (1991a) in maize that indicate that the formation of prominent air spaces is due to the differentiation and ultimate death of vacuolated cells in the cell aggregates.

Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Namasivayam 2007). In DSE it has been suggested that embryogenic cells are present and simple require favorable conditions for embryo development while in ISE requires the re-determination of differentiated cells (Quiroz-Figueroa *et al*. 2002b). Jiménez (2001) clearly distinguishes between embryogenic and competent cells. The former ones are those cells that have completed their transition from a somatic state to one in which no further application of exogenous stimuli are necessary to produce somatic embryo. On the other hand, the term competent cells is restricted to that cells that have reached the transitional state and have started to become embryogenic but still require exogenous stimuli application. In this sense, some of the epithelial cells of the scutellum in presence of 2,4-D acquired an competent state and gave rise to the embryogenic calli alter 5 days of culture. It has been showed that the auxin 2,4-D plays an important role in the dedifferentiation and cell division in rice somatic embryogenesis (Meneses *et al*. 2005).

On the other hand, optimization of physical, biological and environmental parameters for particle bombardment is necessary for transient or stable gene expression in any plant tissue (Tee & Maziah 2005). Moreover, size of tissue is an important factor that needs to be considered for multiplication and regeneration of bombarded explants. Since particle bombardment involves the penetration of heavy metal particles into intact cells or tissues, microparticles hit may provoke various levels of tissue wounding and damage that can hind plant regeneration (Tadesse *et al*. 2003). Our histological observations suggest that biolistic cause no damage to the embryogenic calli and therefore these technique could be used for genetic transformation as reported in rice (Li *et al.* 1993), maize (Valdez *et al*. 2004) and coffee (Rosillo *et al*. 2003, Ribas *et al*. 2005, Gatica *et al*. 2007b).

In conclusion, our results provide further information on the morphology and development of friable callus in rice (*Oryza sativa* cv. 5272) tissue culture.

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RESUMEN

Los estudios anatómicos e histológicos de los callos embriogénicos de arroz (*Oryza sativa*) mostraron que estos se originan del epitelio escutelar de los embriones cigóticos maduros. Al crecer en un medio Murashige y Skoog (1962) suplementado con 2.5 mg/l 2,4-D, presentan grupos de células embriogénicas en las zonas periféricas, las cuales a su vez darán lugar a la formación de proembriones y de embriones somáticos. Las células de los callos embriogénicos se caracterizan por tener núcleo y nucleolo conspícuos, forma isodiámetrica, citoplasma denso y están acompañadas de células adyacentes con abundantes gránulos de almidón. Los embriones somáticos completan su desarrollo para dar formación a plántulas completas, al estar en un medio de regeneración. Los estudios histológicos permitieron observar la expresión transitoria del gen *uidA* en grupos de células de las capas más externas de los callos embriogénicos sometidos al método de transformación genética por biobalística.

Palabras clave: *Oryza sativa*, arroz, cultivo *in vitro*, embriogénesis somática, histología, morfogénesis, Costa Rica.

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