Histological studies on somatic embryogenesis in rice (*Oryza sativ* **L.**)

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ABSTRACT

Rice (*Oryza sativa*) embryogenic calli were obtained from mature zygotic embryos culture using two varieties of rice, Sakha 101 and Sakha104 on Murashige and Skoog (1962) medium supplemented with 1mg/l, 2mg/l and 3mg/l 2,4- dichlorophenoxy acetic acid. The results indicated that the best medium was that containing 3mg/l. 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, each with a dense cytoplasm and a prominent nucleus and nucleolus, whereas the inner cell layer is composed of large cells with small nuclei and large vacuoles. These embryogenic cells underwent a series of organized divisions and formed the proembryo with a well-defined protodermis. At 20 days after initiation of culture, these proembryos continued a series of organized divisions and gave rise to globular somatic embryos which are delimited by a well-defined layer of epidermal cells with conspicuous nuclei. These somatic embryos had no apparent vascular connection with the mother tissue and had a suspensor. After ten days of culture on regeneration medium, globular somatic embryos developed into heart-shaped somatic embryos, which consisted of cells with prominent nuclei and dense cytoplasm. After 15 days of culture on regeneration medium torpedo-shaped somatic embryos were observed. Then, after 25 to30 days, it turned into a monocotyledon form**.**

INTRODUCTION

The totipotent character of plant cells allow that any differentiated cell that retains its nucleus has the ability to regenerate an entire new plant by organogenesis or somatic embryogenesis (Reynolds, 1997; Fortes and Pais, 2000). Somatic embryogenesis (SE), is the developmental process by which bipolar structures that resemble zygotic embryos are developed from haploid or diploid somatic cells through an orderly embryological stage without gametes fusion (Jiménez, 2001; Namasivayam, 2007).Two types of SE 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). These are of remarkable interest for biotechnological applications such as clonal propagation, artificial seeds and genetic engineering and provement of crop species (Quiroz-Figueroa *et al*., 2006; Namasivayam, 2007).

Rice (*Oryza sativa*) is one of the most important staple crop for one third of the world population, and 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 (Heyser *et al*., 1983; Ge *et al*., 2006; Sharma *et al*., 2017). Some authors presented that organogenesis and embryogenesis, occurring simultaneously, as the regeneration pathway (Boissot *et al*., 1990; Gairi and 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 and Pais 2000). Therefore, the aim of this work was to describe the events leading to the development of plantlets from embryogenic calli obtained from Sakha 101 and Sakha104 through histological analysis.

MATERIALS AND METHODS
Explants preparation:

Seeds of the commercial Sakha 101 and Sakha104 sterilized following the procedure described by Valdez *et al*. (1996a).Seeds were surface-sterilized by immersion in 70% ethanol for 90 sec., followed by 3 minutes shaking in sterile distilled water and then 60 minutes shaking in 50% Clorox (Sodium hypochlorite 5%) and finally rinsing three times in sterile distilled water.

Callus induction:

100 explant (mature embryos) of each varieties were cultured, (ten 15mm calli, 15cru plate) on callus induction Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 7g/L agar and 3 mg/L 2,4-D.

The pH of the medium was adjusted to 5.7 to 5.8 prior to autoclaving. Cultured seeds were maintained in the dark room at 25 to 27˚C under diffuse cool white fluorescent light and the percentage of callus induction was calculated after 3 to 4 weeks.

Somatic embryogenesis and regeneration:

After 3 weeks somatic embryogenesis of calli were measured for all treatments. To obtain regeneration, embryogenic calli were transferred to the same medium containing 2 mg/L BAP + 0.05 mg/L NAA. For experiment analyzing proportion of plants regeneration were calculated as follows: (The number of plantlets regenerated /The number of embryogenic callus) × 100.

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 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. Microscopic features were photographed using a stereoscope and the photographs of the histological study were taken using an optical microscope (Vega1 *et al*. 2009).

Statistical analysis:

The experiment was carried out in factorial experiment (two factorial, variety × embryogenesis treatments) with a completely randomized design with 3 replications. Differences between means were scored with Duncan's multiple range test. Statistical analysis was performed using SPSS.

Histological studies on somatic embryogenesis in rice (*Oryza sativ* L.)

Results and discussion
Response of mature rice seeds to hormones

Response of mature seeds varied according to the concentration of growth regulators. The present results indicated that growth regulator (2, 4-D) alone played an essential role in callus induction. De-husked rice seeds cultured in the free-hormone MS medium was germinated (Table 1) and the absence of calli revealed that 2, 4-D is a key factor to stimulate its formation. Gatica-Arias *et. al.* (2007) reported that the presence of auxins increased the DNA methylation than usual and this might be required for programming of differentiated cells and made them able to begin division.

Table (1). Effect of different hormone concentrations on callus induction, and callus nature of *Oryza sativa* L. drived from explanted embryos after 21 days.

Treatments	Variety	Callus induction	Callus nature			
			Shape	color	Size	Type
Control	Sakha 101	NR	NR	NR	NR	NR
	Sakha 104	NR	NR	NR	NR	NR
MS-1mg/l.2,4-D,	Sakha 101	100%	Unequal	Light yellow	medium	Semi compact
	Sakha 104	100%	Round	Light yellow	medium	Semi compact
MS-21mg/l.2,4-D,	Sakha 101	100%	Round	Light yellow	massive	Friable
	Sakha 104	100%	Unequal	Light yellow	massive	Friable
MS-31mg/l.2,4-D,	Sakha 101	100%	Unequal	Light yellow	medium	Semi compact
	Sakha 104	100%	Globular	Light yellow	medium	Semi compact

NR= No response.

The mature embryos produced friable yellowish calli (Fig. 1A-C) derived from the scutellum after two weeks of culture on regeneration medium. The epithelial cells of the scutellum are columnar with a dense cytoplasm and a prominent nucleus and nucleolus. 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). The 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*). The results confirmed the previous observations on wheat immature embryos of maize (Kamo *et al*., 1985), immature and mature zygotic embryos of *Pannicum maximum* and rice (Rueb *et al*., 1994). Maeda and 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 and Rost (1989) indicated that the scutellum epithelium is a modified layer of columnar cells at the interfase of the embryo with the endosperm and it is an active tissue during germination and has been reported as the site of amylase synthesis in grasses (Azhar*et al.,* 2019; Ghobeishavi *et al.,* 2015).
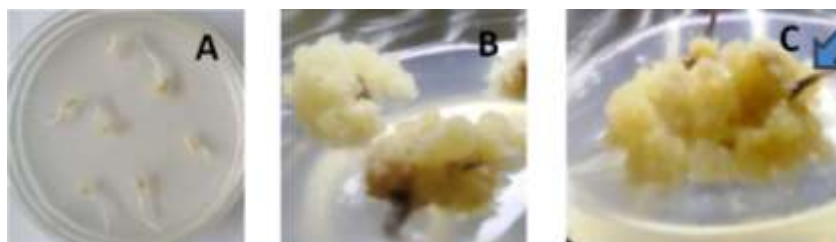
Fig. 1. Somatic embryogenesis in rice (*Oryza sativa,* L (A) week-old embryogenic calli derived from mature seeds cultured on callus induction medium, (B) Four-week-old embryogenic calli, (C) embryogenic callus with somatic embryos (arrows)

Histological observations on embryogenic calli showed two types of cells (Fig. 2A). The inner cell layer consisted 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 outer 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).

Fig. 2.Histological analysis of somatic embryogenesis in *Oryza sativa*. (A) Embryogenic cells (★)and nonembryogenic cells (☆) (B) Embryogenic cells showing isodiametric cells (C) Non-embryogenic cells with large vacuole, small starch granules and abundant intercelullar spaces (↗).(X 200)

The morphological and anatomical observations indicate that somatic embryos may arise from one cell or a group of cells (Sharma *et al.*2017,Quiroz-Figueroa *et al.* 2002, 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). The cuurent histological observations showed that somatic embryos originated from the more external cells of the embryogenic calli. This agrres with previous observations in sugarcane, Guinea Grass (Lu and Vasil, 1985) and oil palm (Schwendiman *et al*. 1988). During somatic embryogenesis induction in the rice cultivars distinguishable clusters, embryogenic and non-embryogenic cells were observed. The embryogenic system was described in maize (Puigderrajols *et al*., 2001), and coffee (Gatica-Arias *et al*. 2007).The embryogenic cells show characteristics common to meristematic cells with high division rates, cells are isodiametric and small with a dense cytoplasm with several starch grains, large nucleus and prominent

Histological studies on somatic embryogenesis in rice (*Oryza sativ* L.)

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 (Gatica *et al*. 2007). The cells containing starch grains observed in rice somatic embryogenesis indicated the high nutritional requirement of cell populations during the process (Apezzato-Da-Gloria and 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 Sharma *et al.* (2017) and Abbasi *et al*. (2011) for rice, Fransz and Schel (1991) for maize which indicated that the formation of prominent air spaces is due to the differentiation and ultimate death of vacuolated cells in the cell aggregates.

After 10 days of culture, cluster of proembryogenic cells in the peripheral region of the calli were observed. It was obvious that immediately after the proembryogenic region the cells increased in size with respect to the formers indicating different stages of differentiation. The next step in SE is the formation of the proembryo, which showed a well-defined protodermis. 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. 3A). These somatic embryos had no apparent vascular connection with the mother tissue and had a suspensor–like (Fig. 3B). 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. 3C-D). 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. 3E-F). Then, after 25 to30 days, it turned into a monocotyledon form (Fig. 3G-H).
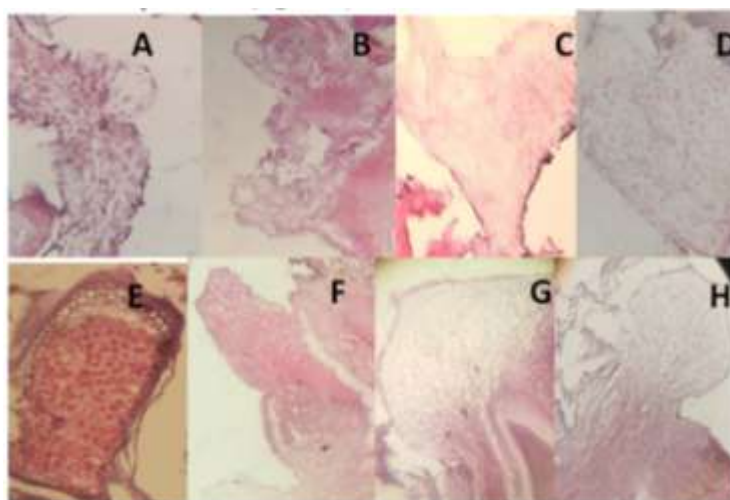

Fig. 3. Histological analysis of somatic embryogenesis in *Oryza sativa,* (A-B) Appearance of the embryogenic calli with multiples globular somatic embryo, (D) Globular somatic embryo without connection to mother tissue and suspensor–like, (C-D) Heart-shaped somatic embryo, (E-F) Torpedo-shaped somatic embryo, (G-H) monocotyledon somatic embryo. (X 60.)

The presence of abundant and prominent foci in the calli periphery were observed, which indicated that tungsten particles coated with the DNA have been delivery into the cell and cause no damage to the embryogenic cells. Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Dina and Reem, 2018) (Miroshnichenko *et al.,* 2017; Mishra and Narashima, 2016; Namasivayam, 2007). In direct somatic embryogenesis (DSE), it has been suggested that embryogenic cells are present and simple, which require favorable conditions for embryo development, while in indirect somatic embryogenesis (ISE) requires the re-determination of differentiated cells (Quiroz-Figueroa *et al*., 2002). 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 a competent state and gave rise to the embryogenic calli after 5 days of culture. It has been showed that the auxin 2,4-D plays an important role in the differentiation 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 and 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). The present histological observations suggest that biolistic causes no damage to the embryogenic calli and therefore these technique could be used for genetic transformation as reported in rice (Li *et al*. 1993), inmaize (Valdez *et al*. 2004) and in coffee (Rosillo *et al*. 2003; Ribas *et al*. 2005, Gatica-Arias *et al*. 2007).

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

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دراسات هستولوجية علي الاجنة الجسمية لنبات الارز

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المستخلص

تم الحصول على تجمعات خلوية من زراعة الأجنة الناضجة باستخدام صنفين من الأرز، سخا 101 وسخا 104 على وسط موراشيجوسكوج (1962) باضافة 1 ، 2 ، 3 مجم / لتر فى وجود 2 ،4 – حمض ثنائي كلوروفينوكسي أسيتيك. تشير النتائج إلى أن أفضل وسط يحتوي على 2 مجم / لتر. أظهر التحليل النسيجي لتكوين الاجنة الجسدية أنه بعد أسبوعين من زراعة الاجنة الناضجة على وسط انتاج الكالسبدأ نمو الجنين الجسدي بمجموعة من الخلايا الجنينية في المنطقة المحيطية من الكالس. طبقة الخلية الخارجية من الخلايا الجنينية المتكونة من خلايا صغيرة ومتساوية مع السيتوبلازم الكثيف ونواة بارزة. بينما تتكون طبقة من خلايا داخلية كبيرة ذات نواة صغيرة وفجوة كبيرة. خضعت هذه الخلايا الجنينية لسلسلة من الانقسامات المنظمة وشكلت الأجنة مع غشاء أولي محدد جيدًا. بعد 20 يومًا من بدء الاستنبات، استمرت هذه الأجنة في سلسلة من الانقسامات المنظمة وأدت إلى ظهور أجنة جسدية كروية محددة بطبقة محددة جيدًا من خلايا البشرة ذات النواة الواضحة. لم يكن لهذه الأجنة الجسدية أي علاقة أوعية ظاهرية بالنسيج الأم وكان لها معلق. بعد عشرة أيام من الاستنبات على وسط التجديد، تطورت الأجنة الجسدية الكروية إلى أجنة جسدية على شكل قلب، والتي تتكون من خلايا ذات نواة بارزة وسيتوبلازم كثيف. بعد 15 يومًا من نقل الكلس على وسط التجديد، لوحظت أجنة جسدية متوسطة الشكل على شكل طوربيد. ثم تحولت بعد 25اا لي 30 يومًا إلى شكل أحادي الفلقة.