BIOLOGIA PLANTARUM 55 (3): 549-553, 2011

BRIEF COMMUNICATION

Extracellular matrix as the early structural marker for *Centella asiatica* embryogenic tissues

K.S. LAI[1], K. YUSOFF[2] and M. MAZIAH[1]*

Department of Biochemistry[1] and Department of Microbiology[2], Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor DE, Malaysia

Abstract

Embryogenic and non-embryogenic calli were induced from the *Centella asiatica* leaf explants on Murashige and Skoog medium supplemented with kinetin and 2,4-dichlorophenophenoxyacetic acid. The extracellular matrix (ECM) layer was seen on the surface of embryogenic cells but not on the non-embryogenic cells. The ECM formed bridges with net-like material between the embryogenic cells. This network like structure was believed to play an important role in plant morphogenesis and can serve as an early structural marker of embryogenic competence in *Centella asiatica* calli culture.

Additional key words: auxin, cytokinin, embryogenic cells, SEM, TEM.

Centella asiatica is an important medicinal plant belonging to the family of *Umbelliferae*. *In vitro C. asiatica* has been routinely regenerated and serves as a basic platform for the production of medicinally important secondary metabolites. To date, regeneration of *C. asiatica* has been achieved through callus culture (Patra *et al*. 1998), shoot tip culture (Sangeeta and Alak 2003), somatic embryogenesis (Martin 2004), and cell suspension culture (Sangeeta and Alak 2005). Somatic embryogenesis is one form of asexual reproduction which starts from isolated somatic or gametic (microspore) cells (Zimmerman 1993) and whereby these cell under favorable experimental condition are induced to form a somatic embryo *in vitro* (Namasivayam 2007). Early identification leading to the acquisition of embryogenic competence in somatic cells is essential for regeneration of complete new plants.

Recently, a considerable attention is paid to extracellular matrix (ECM) layer which belong to an integral part of the ECM-plasma membrane-cytoskeleton continuum (Popielarska-Konieczna *et al*. 2008). It plays a fundamental role in the reception and transduction of signals connected with positional information, recognition, cell fate determination and plant development (Popielarska-Konieczna *et al*. 2008). Previously Namasivayam *et al*. (2006) review the presence of ECM as the potential structural marker during the acquisition of embryogenic competence in *Brassica napus*. ECM may play fundamental role in cell recognition, cell-to-cell interaction, cell division and differentiation and also in generation and maintenance of some traits in plant cell populations (Bobák *et al*. 2003/4). Besides, Šamaj *et al*. (1999) also reports that the ECM plays an important morphoregulatory role during somatic embryogenesis and organogenesis, implying an active role in plant morphogenesis. ECM was also observed in coffee proembryoid cells (Sondahl *et al*. 1979), *Drosera rotundifolia* proembryo (Šamaj *et al*. 1995), *Papaver* proembryo (Ovečka and Bobák 1999), coconut (Verdeil *et al*. 2001) and endosperm derived callus in kiwifruit (Popielarska *et al*. 2006). Thus the aim of the present study was to identify the morphological differences that indicate

Received 4 October 2009*, accepted* 16 March 2011.

Abbreviations: 2,4-D - 2,4-dichlorophenophenoxyacetic acid, ECM - extracellular matrix; Kn - kinetin, SEM - scanning electron microdscopy; TEM - transmission electron microscopy.

Acknowledgements: We thank Mr. Ho Ooi Kuan from the Microscopy Unit of Institute of Bioscience, Universiti Putra Malaysia for his excellent technical assistance.

* Author for correspondence; fax: (+603) 8946 6703, e-mail: maziahm@biotech.upm.edu.my

embryogenic competence in *C. asiatica* embryogenic tissue and to describe detailed ECM ultrastructure.

The embryogenic and non-embryogenic tissues were induced from the leaf explants of *Centella asiatica* (L.) Urban on Murashige and Skoog (1962; MS) medium supplemented with 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4 D) and 1 mg dm^{-3} kinetin (Kn) grown under 16-h photoperiod, irradiance of 25 µmol m^{-2} s^{-1} and temperature of 24 ± 2 ºC.

For scanning electron microscopy, calli were prefixed in 4 % buffered glutaraldehyde [0.1 M phosphate buffer saline (PBS), pH 7.2] for 2 h at 4 ºC. After washing with PBS, the samples were postfixed in 1 % OsO_4 for 2 h at 4 ºC before subjecting to dehydration through a graded ethanol series. Samples were dried by CO_2 critical point dryer (*BAL-TEC*, model *CPD 030*, Balzers, Liechtenstein) and sputtered with gold coater (*BAL-TEC*, model *SC 030*). Finally they were observed with *JEOL JSM-6400* (Tokyo, Japan) scanning electron microscope.

For light and transmission electron microscopy experiments, callus samples were fixed in 4 % buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 5 h at 4 ºC. Then samples were washed with three changes (10 min each) of PBS and postfixed in 1 % OsO_4 for 2 h at 4 ºC. After fixation, they were dehydrated in acetone series and embedded in resin mixture. For light microscopy, semithin sections (1 - 3 µm) were cut with *Leica ultracut UCT* (Wetzlar, Germany) and the slides were stained with 1 % toluidine blue before examining under *Leica CME* light microscope. Meanwhile ultrathin sections (70 - 90 nm) were cut using *Leica ultracut UCT* and stained with uranyl acetate and lead citrate (Reynolds 1963). The sections were analysed with *Phillips HMG 400* (Eindhoven, The Netherlands) transmission electron microscope.

In this study, we distinguished the two types of *C. asiatica* callus: embryogenic or non-embryogenic. Previously, we have identified the embryogenic calli of *C. asiatica* based on its compact and greenish morphotype compared to the non-embryogenic calli which were friable and whitish. Embryogenic calli regenerated into complete plantlets when subjected to regeneration medium. On the other hand, very low or no regeneration was observed in non-embryogenic calli. Through light microscopy observations, the embryogenic tissue was composed of similar isodiametric cells, possessing plastids and dense cytoplasm (Fig. 2*A*,*C*). In contrast, non-embryogenic cells were bigger and highly vacuolated (Fig. 2*B*,*D*). Similar observations were also reported in maize callus by Šamaj *et al*. (1999). They reported that the embryogenic cells were characterized by centrally located round shape nucleus, small protein bodies, and numerous starch grains while non-embryogenic cells were much bigger and highly vacuolated. Moreover histological observations on various plant species such as carrot (Halperin and Jansen 1967), sugarcane (Ho and Vasil 1983), pearl millet (Taylor and Vasil 1996), and cork oak (Puigderrajols *et al*. 2001) also concluded that, the embryogenic cells forming somatic embryos were generally small and have densely staining nucleus and cytoplasm.

Based on the SEM observation, ECM layer was present on the surface of the embryogenic cells (Fig. 1*D*). This ECM layer was seen covering and forming a network-like structure on the surface of the cells. Its forms bridges connecting the neighboring cells together (Fig. 1*E*,*F*). Different thicknesses of fibrils were seen and they were linked to other fibrils in bundle (Fig. 1*G*,*H*). The presence of the ECM layer was only seen covering certain region of the cells surface and were absent or partly damaged in other regions. This was probably due to the shrinkage and hole formation in the ECM layer cause by the critical-point drying during the sample preparation as reported by Šamaj *et al*. (1999). However, the ECM layer was absent from the non-embryogenic cells (Fig. 1*B*,*C*). The observation is also consistent with studies of *Papaver somniferum* (Ovečka *et al*. 1998), *Cichorium intybus* (Dubois *et al*. 1991, Chapman *et al*. 2000), *Brassica napus* (Namasivayam *et al*. 2006) and *Actinidia deliciosa* (Marzena *et al*. 2006).

Detailed TEM studies have shown the presence of a distinct layer outside the embryogenic cells wall (Fig. 2*E*,*G*). This additional layer corresponded to the same ECM layer covering the surface of the embryogenic cells which were observed under the SEM previously. Under the higher magnification, the ECM layer was observed to contain fibrillar structures and dark staining materials (Fig. 2*H*,*I*,*J*). On the other hand, no additional layer outside the cell wall was observed on the non-embryogenic cells (Fig. 2*F*). Similar observations were also reported in *Brassica napus* embryogenic tissue by Namasivayam *et al*. (2006). The authors reported that the layer was an amorphous layer filled with many small osmiophilic granules associated with fibres extending from the outer cell wall. Besides, parts of this ECM layer contained small granules or vesicle-like structures. Some cross sections of the embryogenic cells showed the presence of vesicles adjacent to the plastids, Golgi body, and endoplasmatic reticulum. The presence of the polysomes and endoplasmatic reticulum in the cytoplasm of the epidermal cells suggest active protein synthesis either for cell division or contributing to the formation of ECM (Namasivayam 2004). ECM has been identified as an important structural feature that plays an essential role during the process of somatic embryogenensis. This is because the occurrence of the ECM in developmentally restricted stage indicates its essential role in fixing cell position and morphogenesis before the formation of protodermis (Bobák *et al*. 2004). In addition, it can serve as an early morphological structural marker on the surface of regeneration competent cells during direct embryogenesis (Bobák *et al*. 2003/04).

Although not much has been known about its chemical nature, there have been reports on the presence

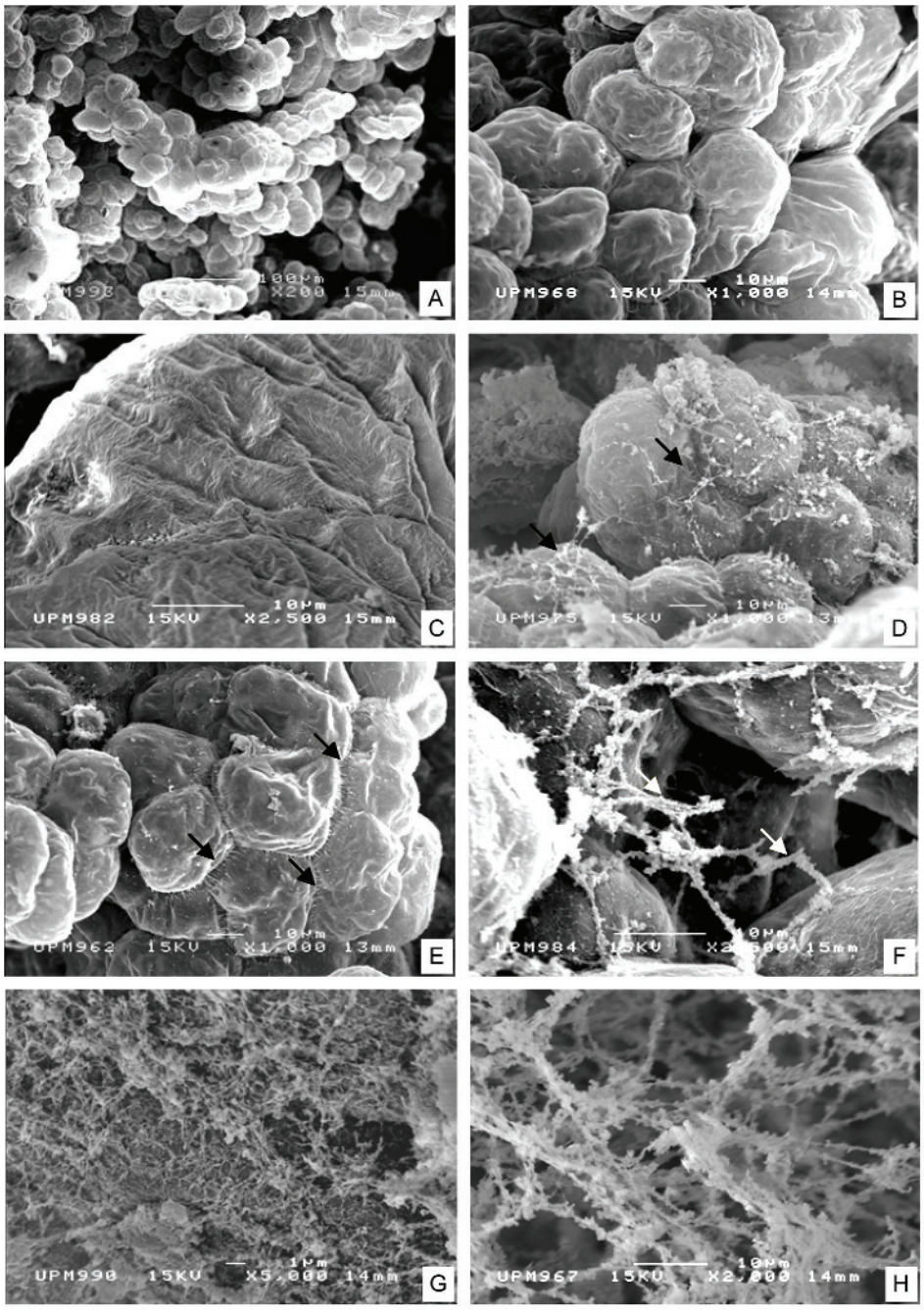

Fig. 1. SEM observations of embryogenic and non-embryogenic calli. *A* - embryogenic calli, *B,C* - non-embryogenic calli showing no fibrillar network on the surface of the cells, *D,E,F* - embryogenic calli with fibrillar network forming fine bridges between the cells and covering the surface of the cells (*arrows*), *G,H* - higher magnification of the fibrillar network of ECM.

of the arabinogalactan protein (AGP) in maize (Šamaj *et al*. 1999) and lipid, protein and weakly esterified pectic polysaccharides in *Cichorium* (Dubois *et al*. 1991, Verdus *et al*. 1993, Chapman *et al*. 2000). Nonetheless, this early structural marker is essential in the acquisition of the embryogenic competence during the somatic embryogenesis. To our knowledge, this is the first report on the location of ECM layer in the embryogenic cells of the *C. asiatica*. It can be used as an early structural marker for screening embryogenic competent cells and improved current tissue culture techniques for this medicinal important herb.

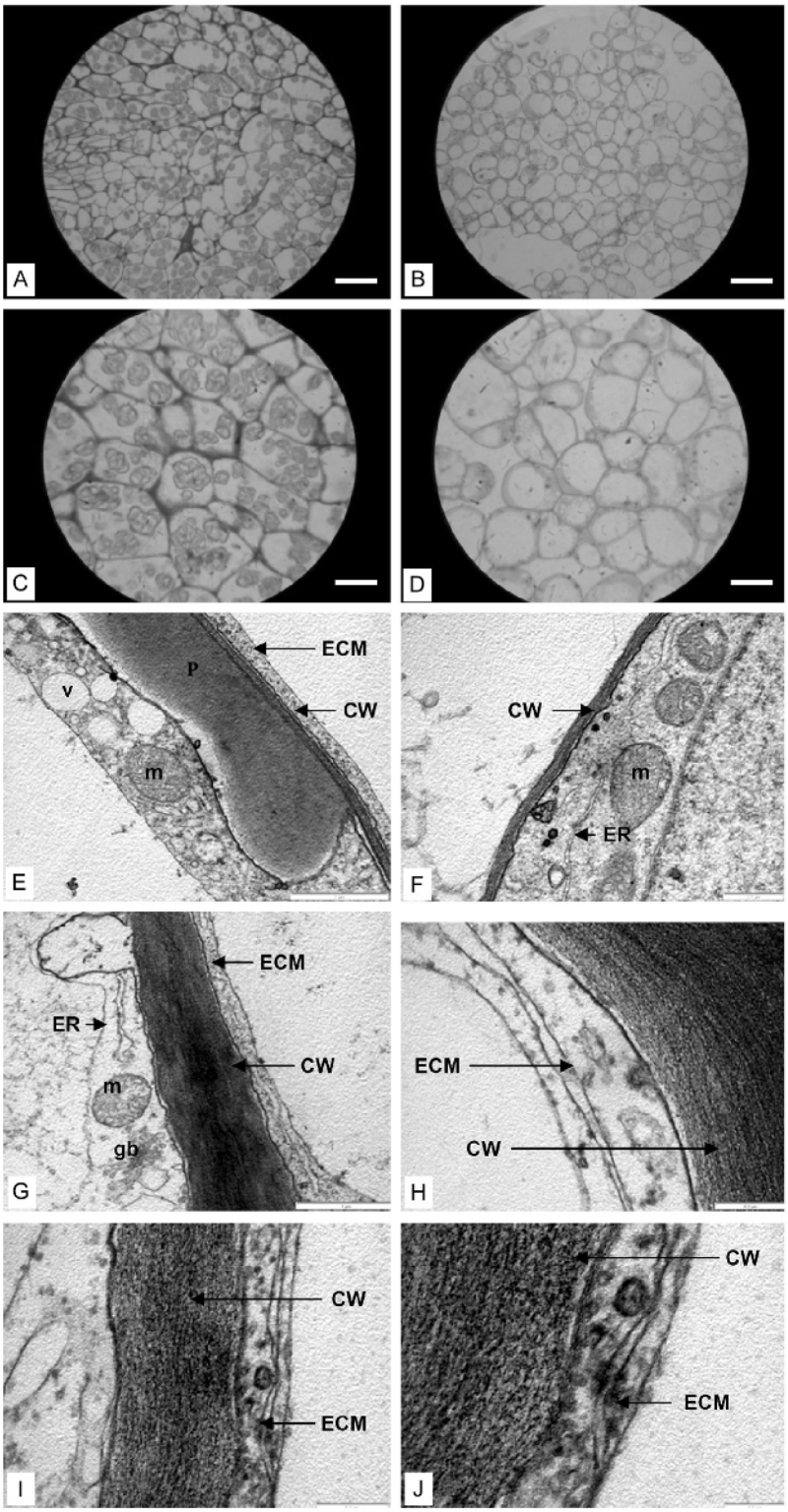

Fig. 2. Light micrographs and TEM observations of embryogenic and non-embryogenic calli: *A*,*C* - embryogenic calli showing presence of isodiametric cells and plastids, *B,D* - non-embryogenic cells showing the presence of highly vacuolated cells, *E,G* - embryogenic cell showing presence of an amorphous ECM layer outside the cell wall, *F* - non-embryogenic cell showing no presence of ECM layer, *H,I,J* - ECM layer containing dark-staining material, vesicle and fibrillar structures. ECM - etracellular matrix, v - vesicles, CW - cell wall, p - plastid, ER - endoplasmic reticulum, gb - Golgi bodies, m - mitochondria. *Bars*: *A*,*B* = 100 µm, *C*,*D* = 50 µm, *E*,*G* = 1 µm, *F* = 0.5 µm, *H*,*I* = 0.2 µm, *J* = 0.1 µm.

References

Bobák, M., Šamaj, J., Hlinková, E., Hlavačka, A., Ovečka, M.: Extracellular matrix in early stages of direct somatic embryogenesis in leaves of *Drosera spathulata*. - Biol. Plant. **47**: 161-166, 2003/04.

Bobák, M., Šamaj, J., Pretová, A., Blehová, A., Hlinková, E., Ovečka, M., Hlavačka, A., Kutamová, Z.: The histological analysis of indirect somatic embryogenesis on *Drosera spathulata* Labill. - Acta Physiol. Plant. **26**: 353-361, 2004.

Chapman, A., Blervacq, A.S., Hendriks, T., Slomianny, C., Vasseur, J., Hilbert, J.L.: Cell wall differentiation during early somatic embryogenesis in plants. II. Ultrastuctural study and pectin immunolocalisation on chicory embryos. - Can. J. Bot. **78**: 824-831, 2000.

Dubois, T., Guedira, M., Dubois, J., Vasseur, J.: Direct somatic embryogenesis in leaves of *Cichorium*: a histological and SEM study of early stages. - Protoplasma **162**: 120-127, 1991.

Halperin, W., Jensen, W.A.: Ultrastructural changes during growth and embryogenesis in carrot cell cultures. - J. Ultrastructure Res. **18**: 428-443, 1967.

Ho, W., Vasil, I.K.: Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryo. - Protoplasma **118**: 169-180, 1983.

Martin, K.P.: Plant regeneration through somatic embryogenesis in medicinally important *Centella asiatica* L. - *In Vitro* cell. dev. Biol. Plant **40**: 586-591, 2004.

Marzena, P., Halina, Ś., Grzegorz, G.: Histological and SEM studies on organogenesis in endosperm-derived callus of kiwifruit (*Actinidia deliciosa* cv. Hayward). - Acta biol. cracov. Ser. Bot. **48**: 97-104, 2006.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Namasivayam, P.: Molecular and cell biology study of winter oil seed rape (*Brassica napus* L. spp. *oleifera*) embryogenic cultures. - Ph. D. Dissertation. University of Cambridge, Cambridge 2004.

Namasivayam, P.: Acquisition of embryogenic competence during somatic embryogenesis. - Plant Cell Tissue Organ Cult. **90**: 1-8, 2007.

Namasivayam, P., Skepper, J., Hanke, D.: Identification of a potential structural marker for embryogenic competency in the *Brassica napus* spp. *oleifera* embryogenic tissue. - Plant Cell Rep. **25**: 887-895, 2006.

Ovečka, M., Bobák, M.: Structural diversity of *Papaver somniferum* L. cell surfaces *in vitro* depending on particular steps of plant regeneration and morphogenic program. - Acta Physiol. Plant. **21**: 117-126, 1999.

Ovečka, M., Bobák, M., Blehová, A., Krištin, J.: *Papaver somniferum* regeneration by somatic embryogenesis and shoot organogenesis. - Biol. Plant. **40**: 321-328, 1998.

Patra, A., Rai, B., Rout, G.R., Das, P.: Successful plant regeneration from callus cultures of *Centella asiatica* (Linn.) Urban. - Plant Growth Regul. **24**: 13-16, 1998.

Popielarska, M., Ślesak, H., Góralski, G.: Histological and SEM studies on organogenesis in endosperm-derived callus of kiwifruit (*Actinidia deliciosa* cv. Hayward). - Acta. biol. cracov. Ser. Bot. **48**: 97-104, 2006.

Popielarska-Konieczna, M., Kozieradzka-Kriszkurna, J., Świerczyńska, J., Góralski, G., Ślesak, H., Bohdanowicz., J.: Ultrastructure and histochemical analysis of extracellular matrix surface network in kiwifruit endosperm-derived callus culture. - Plant Cell Rep. **27**: 1137-1145, 2008.

Puigderrajols, P., Mir, G., Molinas, M.: Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in cork oak (*Quercus suber* L.). - Ann. Bot. **87**: 179-189, 2001.

Reynolds E.S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. - J. cell. Biol. **17**: 208-212, 1963.

Šamaj, J., Baluška, F., Bobák, M., Volkmann, D.: Extracellular matrix surface network of embryogenic units of friable maize callus contains arabinogalactan-proteins recognized by monoclonal antibody JIM4. - Plant Cell Rep. **18**: 369-374, 1999.

Šamaj, J., Bobák, M., Blehová, Á., Krištin, J., Auxtová-Šamajová, O.: Developmental SEM observations on an extracellular matrix in embryogenic calli of *Drosera rotundufolia* and *Zea mays*. - Protoplasma **186**: 45-49, 1995.

Šamaj, J., Ensikat, H.J., Baluška, F, Knox, J.P., Barthlott, W., Volkmann, D.: Immunogold localization of plant surface arabinogalactan-proteins using glycerol liquid substituition and scanning electron microscopy. - J. Microscopy **193**: 150-157, 1999.

Sangeeta, N., Alak, K.B.: *In vitro* method for propagation of *Centella asiatica* (L) Urban by shoot tip culture. - J. Plant Biochem. Biotechnol. **12**: 167-169, 2003.

Sangeeta, N., Alak, K.B.: Establishment of callus and cell suspension cultures of *Centella asiatica*. - Biol. Plant. **49**: 411-413, 2005.

Sondahl, M.R., Salisbury, J.L., Sharp, W.R.: SEM characterization of embryogenic tissue and globular embryos during high frequency somatic embryogenesis in coffee callus cells. - Z. Pflanzephysiol. **194**: 185-188, 1979.

Taylor, M.G., Vasil, I.K.: Histology of, and physical factors affecting transient GUS expression in pearl millet (*Pennisetum glaucum* (L.) R. Br.) embryos following microprojectile bombardment. - Plant Cell Rep. **10**: 273-278, 1996.

Verdeil, J.L., Hocher, V., Huet, C., Grosdemange, F., Escoute, J., Ferriere, N., Nicole, M.: Ultrastructural changes in coconut calli associated with the acquisition of embryogenic competence. - Ann. Bot. **88**: 9-18, 2001.

Verdus, M.C., Dubois, T., Dubois, J., Vasseur, J.: Ultrastructural changes in leaves of *Cichorium* during somatic embryogenesis. - Ann. Bot. **72**: 375-383, 1993.

Zimmerman, J.L.: Somatic embryogenesis: a model for early development in higher plants. - Plant Cell **5**: 1411-1423, 1993.