Microscopic Analysis of Polyembryony in *Opuntia ficus-indica*

Cecilia Vélez-Gutiérrez and Benjamín Rodríguez-Garay[1]
Centro de Investigación y Asistencia en Tecnología
y Diseño del Estado de Jalisco, A.C.
Departamento de Cultivo de Tejidos Vegetales
División de Biotecnología
Avenida Normalistas 800
Guadalajara, Jalisco, 44270, México

ABSTRACT

The genus *Opuntia* is widely distributed in the arid and semiarid regions of Mexico and it is an important element for the conservation of arid ecosystems as well as a high-value crop for human and animal consumption. The most common means of propagation in *Opuntia* is through the use of cladodes; this vegetative propagation system ensures genetic integrity of propagated plants. However, seeds are also important units to be considered for genetic improvement and germplasm conservation. Microscopic analysis showed that high numbers of somatic embryos per seed are present and originate from the nucellar tissue at the micropylar end of the ovule. All somatic embryos showed well-developed embryo stages with a close similarity to zygotic embryogenesis.

Key words: *In vivo* somatic embryogenesis, scanning electron microscopy, nucellar embryogenesis.

INTRODUCTION

The genus *Opuntia* is widely distributed in the arid and semiarid regions of Mexico. This genus is a highly valuable plant resource, due to the great diversity of uses for human and animal consumption (Pimienta-Barrios, 1990). Furthermore, it is an important element for the conservation of arid ecosystems and the control of desertification.

Genetic improvement and germplasm conservation of *Opuntia* have been important concerns for the international scientific community (FAO, 1995). The status of the most important species of the genus *Opuntia*, with regard to their use and conservation has been analyzed in international meetings. In June 1996, an International Workshop on the Use and Status of Nopal and Mesquite was held at CINVESTAV (Irapuato, Guanajuato, Mexico) under the auspices of the Food and Agriculture Organization. In this conference, the importance of the strategic use of plant biology and biotechnology for the genetic improvement and germplasm conservation of the genus *Opuntia* was widely discussed.

The most common means of propagation in *Opuntia* is through the use of cladodes, which bear good numbers of meristematic tissue called "areoles". In cactus pear, cladodes, flowers, and even developing fruits are capable of further differentiation; however, cladodes are the typical propagation unit (Pimienta, 1990). Efficient *in vitro* tissue-culture techniques emulating

[1]Corresponding author

conventional/vegetative cloning, have been developed for the rapid, large-scale multiplication of *Opuntia* (Escobar *et al.*, 1986). Efficiency in this context implies a high multiplication rate, genetic uniformity, and reduced volume and weight, compared to the conventional method (Villalobos, 1995). The *in vivo* and *in vitro* vegetative propagation systems ensure the genetic integrity of the propagated plants, in contrast to seed propagation.

Although sexual propagation has been attempted, genetic segregation and slow growth and development represent serious practical problems. However, seeds are important elements to be considered for plant improvement and germplasm conservation, because they are so easily stored for long-term use.

The diploid or sporophytic generation of a plant begins after the fertilization of the egg cell by the male gametophyte, giving rise to the single-celled zygote. In angiosperms, the zygote is typically a polarized cell that divides transversely to form two cells with different features and developmental fates: a basal cell and a terminal cell. Cell polarization has been explained by several authors by using *in vivo* and *in vitro* models. Attention has been paid to the influence of auxins, calcium, and transcellular electrical patterns (Quatrano, 1978; Goldsworthy and Rathore, 1985; Dijak *et al.*, 1986; Hush *et al.*, 1991; Liu *et al.*, 1993) The terminal cell (of the transversely divided zygote) will develop into the embryo proper, whereas the highly vacuolated basal cell will give rise to the suspensor (Yeung and Meinke, 1993; Rodriguez-Garay *et al.*, 1996). Throughout many years, the function assigned to the suspensor has been that of holding the embryo in a fixed position in the seed. However, recently, there is increasing evidence that suggests that the suspensor may play an active role during early embryo development (Yeung and Sussex, 1979; Yeung and Meinke, 1993).

The embryo differentiates into two organ systems (the axis and the cotyledon) that have different developmental fates. The cotyledon is a terminally differentiated organ system that senesces after germination. By contrast, the axis contains the root and shoot meristems that will give rise to sporophytic organ systems through the life cycle (Goldberg, 1988).

Besides the normal origin and formation of the zygotic embryo, many plant species produce asexual embryos from somatic tissue. Among these species there are several that belong to the genus *Opuntia (O. aurantiaca* Lindl., O. *dillenii* Haw., O. *glaucophylla* Wendl., *O. leucantha* Link., O. *rafinesquii* Engelm., O. *tortispina* Engelm., and O. *ficus-indica* (L.) Mill.). As a rule, this kind of embryogenesis *in vivo* has been confined to intraovular structures, mainly to the nucellar tissue (Tisserat *et al.*, 1979). Also, there is a limited number of genera that produce somatic embryos *in vivo* on leaves, e.g., *Asplenium, Cardamine, Kalanchoe, Ranunculus,* and *Tolmiea* (George, 1993). More recently, E. N. Vivas de la Torre and B. Rodríguez-Garay (unpublished) have detected early stages of somatic embryogenesis *in vivo* on leaves of *Begonia* sp. which have continued their development *in vitro.* Somatic embryogenesis *in vivo* is powerful evidence of the totipotency of plant cells.

The objective of this work was to microscopically analyze the origin and development of polyembryony in *O. ficus-indica.*

MATERIALS AND METHODS

Plant Material

Intact seeds were removed from mature and immature fruits of *O. ficus-indica* collected in the summer of 1995 and 1996 from plants cultivated in orchards in Acatic, Jalisco, Mexico. Seeds were dissected with a scalpel under a stereo-microscope to obtain intact embryo sacs containing zygotic and somatic embryos. In some cases, the embryo sacs were ruptured to expose embryos for microscopic examination.

Embryo Germination Tests

Germination tests *in vitro* were conducted by aseptic culture of isolated embryos in a basal MS medium (Murashige and Skoog, 1962), and incubated at 27°C under darkness to test their viability.

Light Microscopy

In some cases, isolated embryos were double stained with acetocarmine/Evans blue following Gupta and Durzan (1987) to differentiate between suspensor and embryo proper. Also, intact embryo sacs were transferred to a microscope slide and cleared in Hoyer's solution (7.5 g arabic gum, 5 ml glycerin, 100 g chloral hydrate, and 30 ml H_2O) for examination of the position and origin of embryos (Vernon and Meinke, 1994).

Exposed embryos were examined with an Olympus CK2 inverted microscope and an Olympus BH2 compound microscope, and photographed with an Olympus AD Exposure Control Unit.

Scanning Electron Microscopy

After the embryo sac was carefully ruptured under a stereo-microscope, exposed embryos were frozen at –40°C for 12 hours. The frozen embryos were dehydrated in an Usifroid SMH15 lyophilizer operated at 30°C and a pressure of 30 Pa for 10 hours.

The dehydrated specimens were mounted on stubs and coated with gold in an Ernest Fuller sputter coater. Observations were made with a JEOL JSM-5400 LV scanning electron microscope operated at 20 kV.

RESULTS AND DISCUSSION

In the germination tests it was found that only well-developed mature zygotic and somatic embryos could germinate *in vitro* after seven days of culture. Two to three seedlings per seed were obtained (Figure 1). Earlier embryo stages (globular, heart, and torpedo) failed to germinate. These results are similar to those observed *in vivo* (Mondragon-Jacobo and Pimienta-Barrios, 1995).

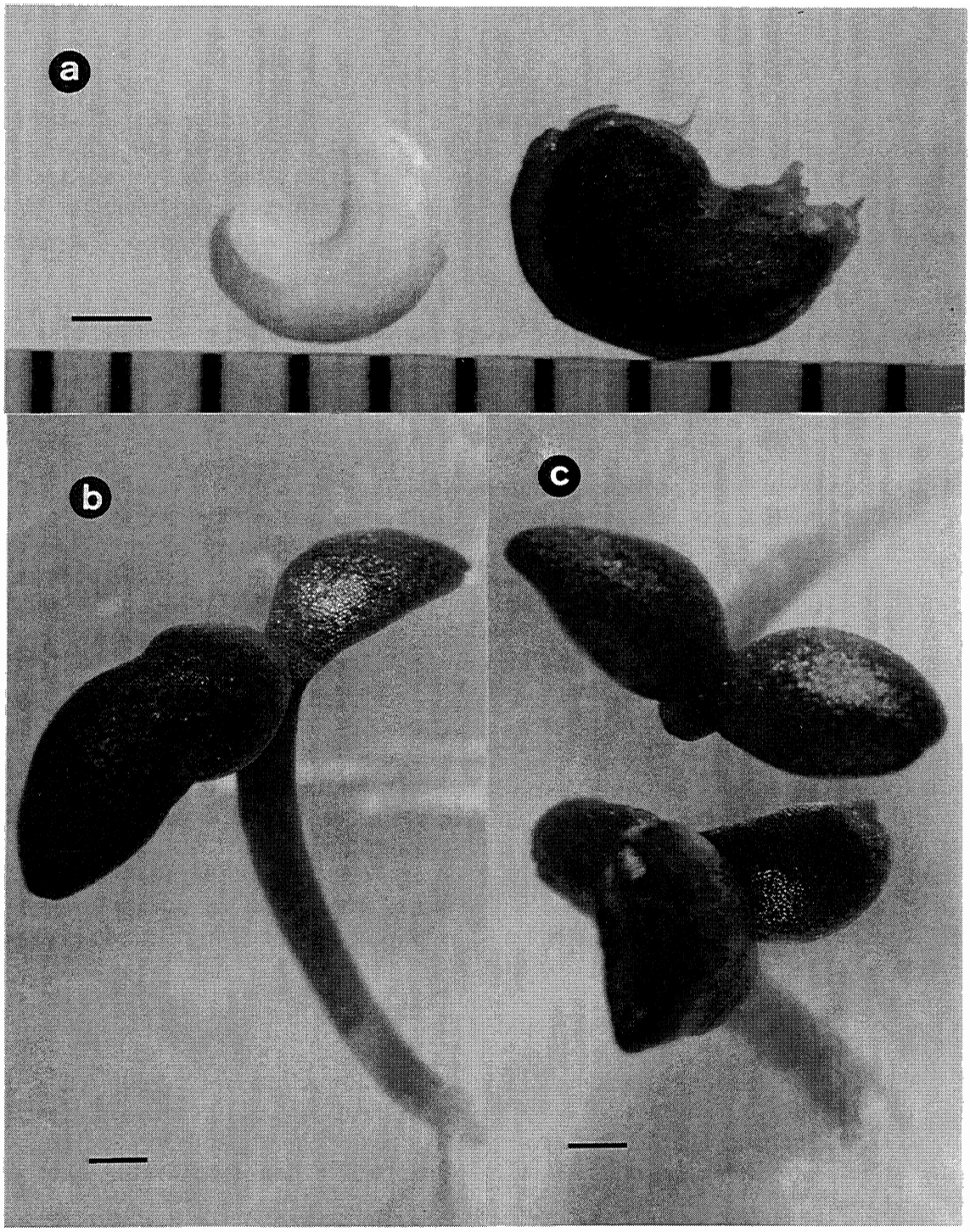

Figure 1. *In vitro* germination of embryos taken from a mature seed of *O. ficus-indica.*
(a) Isolated embryos from a mature seed. (b) and (c) Germinated embryos from a single seed. Bar = 1.0 mm

Figure 2a shows a diagramatic representation of polyembryony in an ovule. The formation of the somatic embryos was found to be confined at the micropylar end of the ovule, and no embryos were observed at the chalazal region. The tissue-clarifying Hoyer's solution allowed the observation of the position of somatic embryos originating in the nucellar tissue inside the intact embryo sac (Figure 2b). This technique allows the study of the embryos without disruption of the whole ovular unit and avoids other tedious histological techniques.

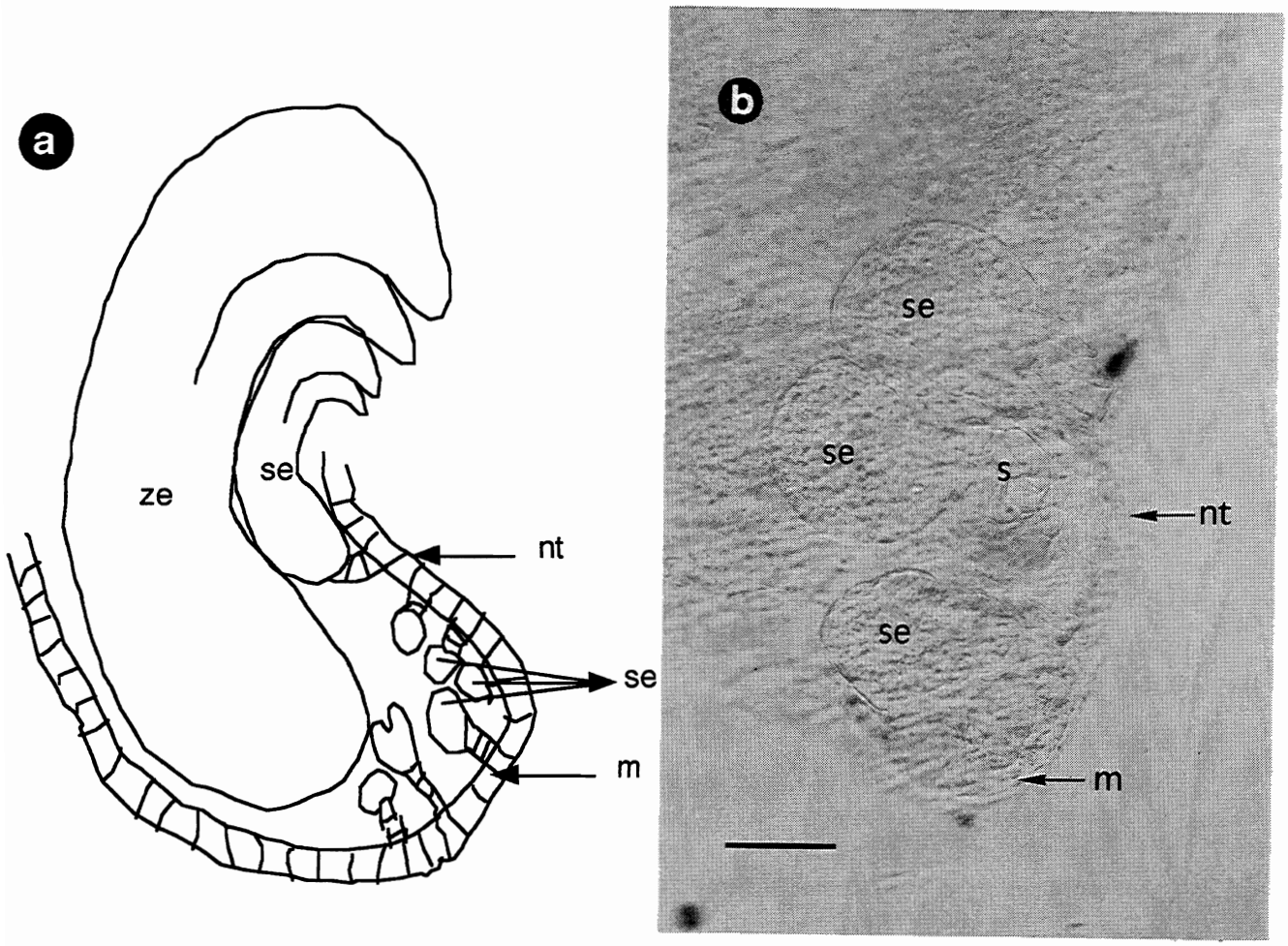

Figure 2. Diagramatic representation of origin and position of zygotic and somatic embryos in *O. ficus-indica* (a). Light micrograph of somatic embryos positioned inside an embryo sac cleared with Hoyer's solution (b).

Bar = 0.1 mm. nt = nucellar tissue; ze = zygotic embryo; se = somatic embryo; m = micropylar end; s = suspensor.

Previous reports on polyembryony in the genus *Opuntia* (Mondragon-Jacobo and Pimienta-Barrios, 1995) have mentioned the production of only two to four embryos per seed, just based on germination. However, the present work shows the evidence of the formation of up to 15 embryos per seed by light-microscope analysis (Figure 3).

Figure 3b shows a heart-shaped somatic embryo originated from the nucellus and still connected to it by its suspensor. The rest of the embryos in this figure are at different globular stages. It was found that all analyzed embryos showed suspensors, and the evidence of their highly vacuolated cells was confirmed by their deep staining with Evans blue (blue color not visible in

the photograph of Figure 4). All embryo developmental stages shown in Figure 4 are representative of several embryos found in one seed, being Figure 4a the putative zygotic embryo. A scanning electron micrograph of a typical heart-shaped embryo is shown in Figure 5. The presence of well-developed suspensors may allow the maturation and germination of the largest and oldest nucellar embryos, since suspensors play an active role in early embryo development (Yeung and Sussex, 1979; Yeung and Meinke, 1993).

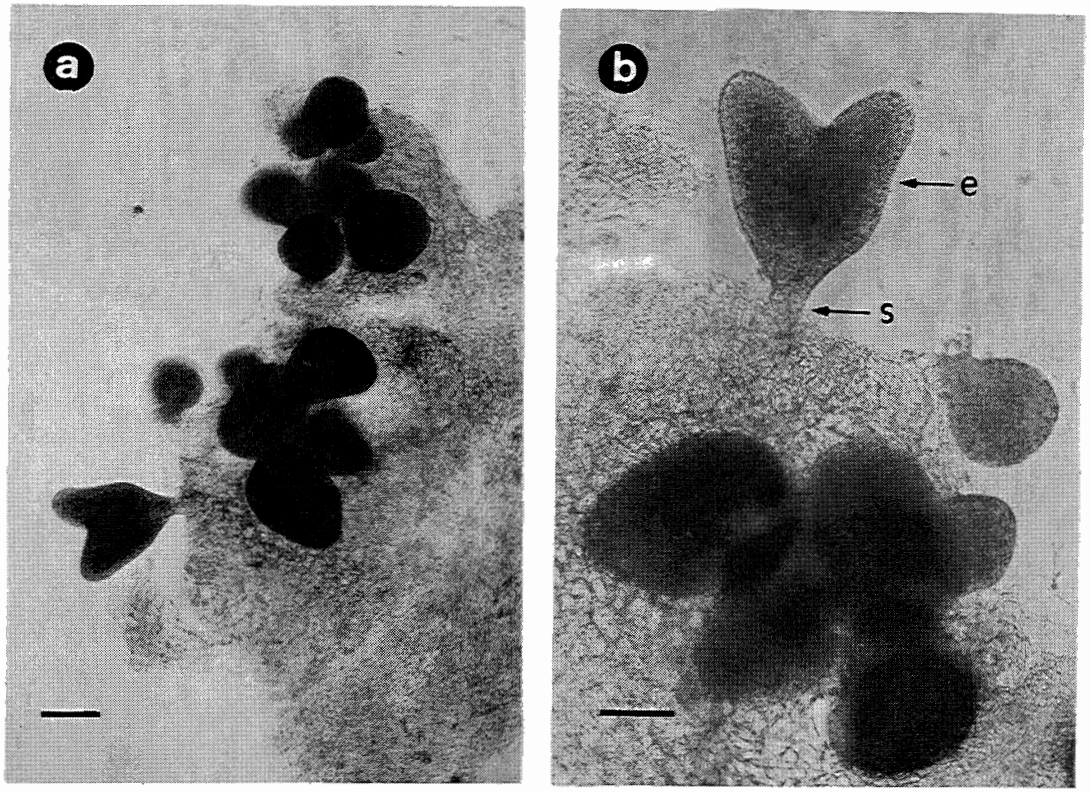

Figure 3. (a) Micrograph of a ruptured embryo sac showing up to 15 somatic embryos in different developmental stages originated on the nucellar tissue. Bar = 0.2 mm. (b) Close-up micrograph of the previous Figure 3a showing the suspensor (s) of a heart-shape somatic embryo (e). Bar = 0.1 mm.

The embryogenic capacity of the nucellar tissue of *O. ficus-indica* may be explained by the ease for polarization of genetically predetermined embryonic cells and the putative influence of calcium ions, endogenous auxins, and transcellular electrical patterns (Quatrano, 1978; Goldsworthy and Rathore, 1985; Dijak *et al.*, 1986; Hush *et al.*, 1991; Liu *et al.*, 1993), as it has been found in other plant species.

The presence of well-developed suspensors and all the normal developmental embryo stages in the nucellar embryos show a close similarity to zygotic embryogenesis. The nucellar cells give rise to somatic embryos which are genetically identical to the mother plant. This feature may be used for diverse purposes, such as genetic improvement (genetic transformation of nucellar cells) and germplasm banks, among many others, with the help of biotechnological tools.

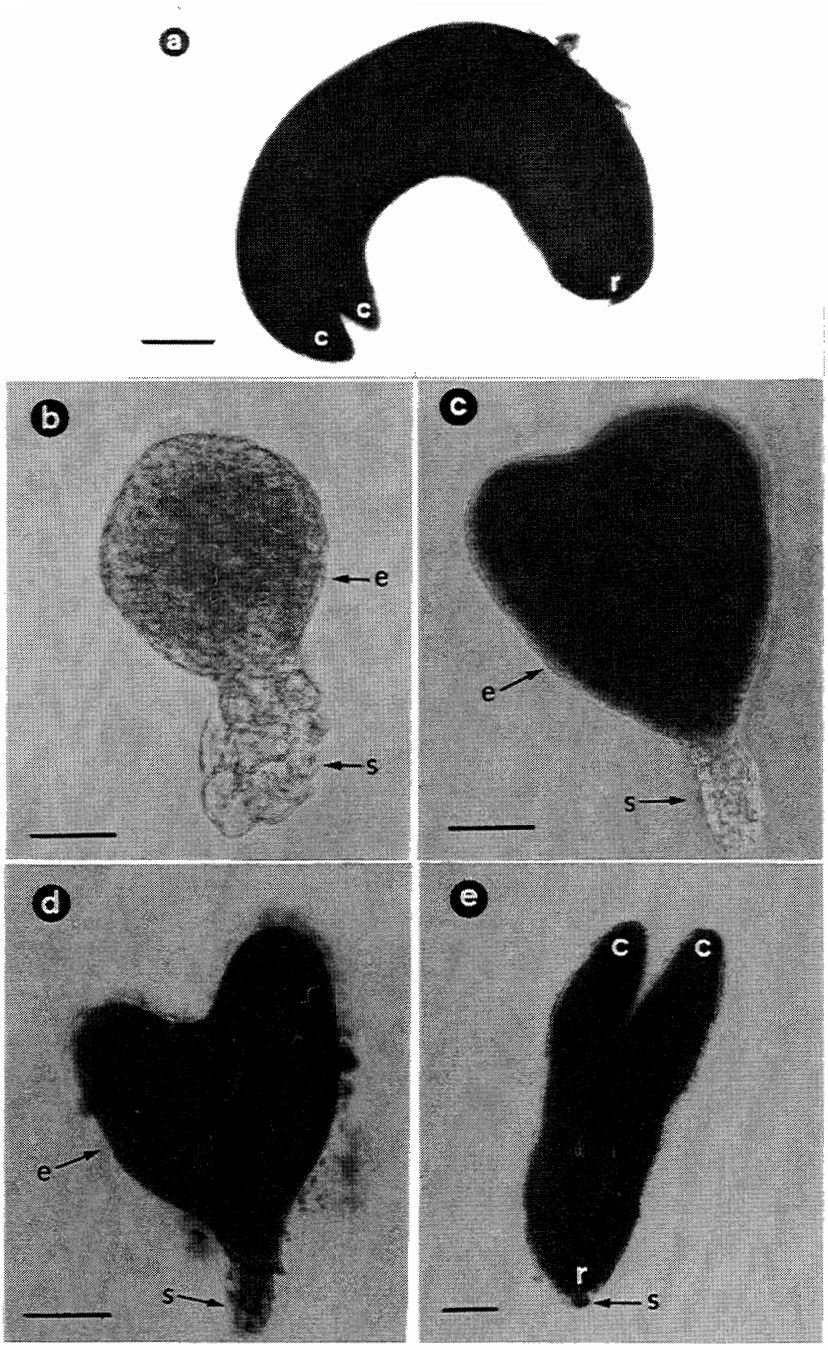

Figure 4. Different embryo developmental stages in *O. ficus-indica*.
(a) Putative mature zygotic embryo. Bar = 0.5 mm; (b) Globular stage. Bar = 0.05 mm;
(c) Early heart-shaped embryo. Bar = 0.1 mm; (d) Late heart-shaped embryo. Bar = 0.05 mm;
(e) Torpedo-shaped embryo. Bar = 0.15 mm.

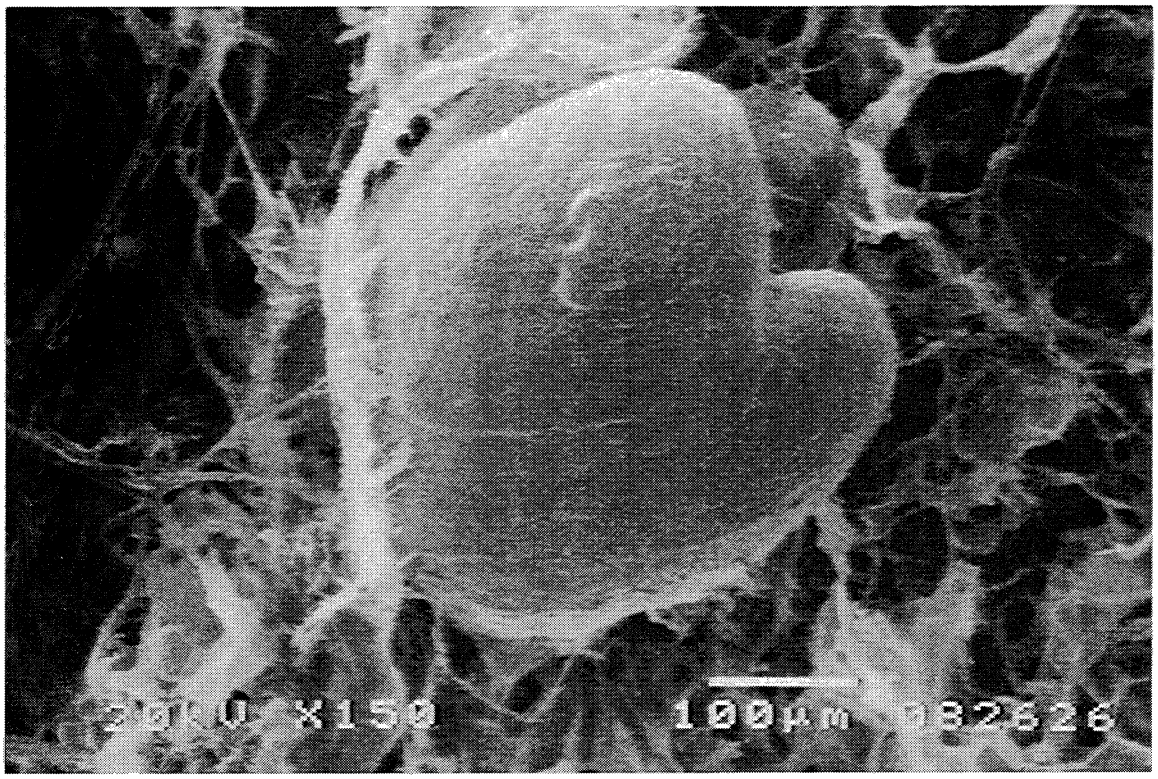

Figure 5. Scanning electron micrograph of a heart-shaped embryo of *O. ficus-indica*

ACKNOWLEDGMENTS

The authors thank Hermes Ulises-Ramírez at the Universidad de Guadalajara for the Scanning Electron Microscope (SEM) work and Jorge García-Fajardo for useful suggestions in preparing the plant specimens for SEM observations, and Oscar Olmedo-Arcega and Fernando Santacruz-Ruvalcaba in preparing the manuscript.

C. Veléz-Gutiérrez is an M.S. graduate student financially supported by CONACyT-México.

BIBLIOGRAPHY

Dijak, M., D. L. Smith, T. J. Wilson, and D. C. W. Brown. 1986. Stimulation of direct embryogenesis from mesophyll protoplasts of *Medicago saliva.* Plant Cell Rep. 5:468–470.

Escobar, A., V. M. Villalobos, and M. Villegas. 1986. *Opuntia* micropropagation by axillary proliferation. Plant Cell Tiss. Org. Cult. 7:269–277.

FAO. 1995. Report of the meetings of the working groups on cactus pear productivity and cactus pear genetic resources collection, conservation and evaluation. FAO International Cooperation Network on Cactus Pear. Guadalajara, Jalisco, Mexico. Nov. 1995.

Gupta, P. K., and D. J. Durzan. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Technology 5:147–151.

George, E. F. 1993. Plant Propagation by Tissue Culture. Part 1. The Technology. Exegetics Limited. England.

Goldberg, R. B. 1988. Plants: novel developmental processes. Science. 240:1460–1467.

Goldsworthy, A., and K. S. Rathore. 1985. The electrical control of growth in plant tissue cultures: The polar transport of auxins. J. Exp. Bot. 36:1134–1141.

Hush, J. M., R. L. Overall, and I. A. Newman. 1991. A calcium influx precedes organogenesis in *Graptopetalum.* Plant Cell Env. 14:657–665.

Liu, C.-M., Z.-H. Xu, and N.-H. Chua. 1993. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell 5:621–630.

Mondragón-Jacobo, C. and E. Pimienta-Barrios. 1995. Propagation. *In:* Barbara, G., P. Inglese, and E. Pimienta-Barrios (eds.). Agro-ecology, cultivation and uses of cactus pear. FAO Plant Production and Protection Paper No.132. Rome, Italy.

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

Pimienta-Barrios, E. 1990. El nopal tunero. Universidad de Guadalajara. Guadalajara, Jalisco. Mexico.

Quatrano, R. S. 1978. Development of cell polarity. Ann. Rev. Plant Physiol. 29:487–510.

Rodríguez-Garay, B., A. Gutiérrez-Mora y F. Santacruz-Ruvalcaba. 1996. Métodos de propagación biotecnológicos y convencionales en agaváceas pare zones áridas. *In:* Izquierdo, J. y G. Palomino (eds.). Técnicas convencionales y biotecnológicas pare la propagación de plantas de zones áridas. Oficina Regional de la FAO pare America Latina y el Caribe. Santiago, Chile. (In press).

Tisserat, B., E. B. Esan, and T. Murashige. 1979. Somatic embryogenesis in angiosperms. Hort. Rev. 1:1–78

Vernon, D. M. and D. W. Meinke. 1994. Embryogenic transformation of the suspensor in *Twin,* a polyembryonic mutant of *Arabidopsis.* Dev. Biol. 165:566–573.

Villalobos, V. M. 1995. Tissue culture application for *Opuntia* sp. pl. Micropropagation. *In:* Barbara, G., P. Inglese and E. Pimienta-Barrios (eds.). Agroecology, cultivation and uses of cactus pear. FAO Plant Production and Protection Paper No. 132. Rome, Italy.

Yeung, E. C. and 1. M. Sussex. 1979. Embryogeny of *Phaseolus coccineus:* The suspensor and the growth of the embryo-proper *in vitro.* Z. Pflanzenphysiol. 91:423–433.

Yeung, E. C. and D. W. Meinke. 1993. Embryogenesis in angiosperms: Development of the suspensor. Plant Cell 5:1371–1381.