Somatic Embryogenesis in Some Cactus and Agave Species

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ABSTRACT

Somatic embryogenesis is an asexual form of plant propagation in nature that mimics many of the events of sexual reproduction. Also, this process may be reproduced artificially by the manipulation of tissues and cells *in vitro*. Some of the most important factors for a successful plant regeneration are the culture medium and environmental incubation conditions. *In vitro* somatic embryogenesis is an important prerequisite for the use of many biotechnological tools for genetic improvement, as well as for mass propagation. In this paper the somatic embryogenesis of several agaves and cacti is reviewed.

Key words: cactaceae, zygotic embryos, tissue culture, *Aztekium ritteri, Agave tequilana, Agave victoria-reginae, Mamillaria san-angelensis, Opuntia ficus-indica, Turbinicarpus pseudomacrochele.*

INTRODUCTION

The sporophytic generation of a plant is initiated with the zygote, which is the initial cell (product of gamete fusion) that bears all the genetic information to construct the adult individual.

In angiosperms, the zygote divides transversally, resulting in two cells. One of them, the apical cell, is small and dense with an intense activity of DNA synthesis. Further ordinal divisions of this cell give rise to the embryo head that will be the new plant. The other resultant cell (basal cell) is a large and highly vacuolated one that will conform the suspensor complex, which plays an important role during the early stages of the young embryo (Yeung and Sussex, 1979). *In vivo*-produced somatic embryos generally follow the same pattern; however, they are not initiated from a zygote, but from a somatic cell as occurs in the polyembryony of *O. ficus-indica*.

In vitro-produced somatic embryos are formed from single cells cultivated in liquid or solid medium. Cell suspensions can be plated onto solid media where cells grow into a callus from which plants can often be regenerated.

Embryos can be distinguished from adventitious shoots, because they are bipolar, having both a shoot and root pole, and they do not have any vascular connections with the underlying parental tissue (Haccius, 1978). Somatic embryo production steadily is being increased as essential factors become better understood (Williams and Maheswaran, 1986).

In the sexual cycle, new plants arise after fusion of the parental gametes, and, generally, each seed represents a new combination of genes. When the asexual (vegetative) cycle is used, genes within the plant selected for propagation usually are perpetuated.

For *in vitro*-culture (including somatic embryogenesis), only a small amount of space is required, propagation is carried out under aseptic conditions, and the rate of propagation is much greater than in macropropagation.

Somatic embryogenesis can be initiated by two mechanisms: directly on explanted tissues, where plants are genetically identical (clonation), and indirectly from unorganized tissues (callus). Propagation by indirect embryogenesis carries the risk of producing plants that may differ genetically from each other and from the parental plant. It is thought that the ocurrence of genetic variability within tissue cultures may partly originate from cellular changes that are induced during culture (George, 1993).

The genetic variability associated with tissue and cell culture is named "somaclonal variation" and represents an opportunity where selection pressure can be imposed to isolate unique genetic forms of a clone. The ability to recover plants from single cells has made possible the genetic improvement *in vitro*. Plant tolerance to abnormal temperatures, herbicides, fungal toxins, high levels of salt, etc., can be obtained by exposing cell cultures to a selective agent (Wolf and Earle, 1990; Wrather and Freytag, 1991; Trolinder and Shang, 1991; Olmos et al., 1994).

The most important advantages of the cell- and tissue-culture tools used in plant breeding are:

- Freedom from the effects of the natural environment.

- The ability to handle large numbers of individuals (cells) in a very small spaces.

- Creation of variation within cultures. Variability usually occurs spontaneously; however, it can be induced by mutagenic agents.

- Genetic variability can be created deliberately in cultured cells by using genetic-engineering techniques.

EARLY ONTOGENY OF SOMATIC EMBRYOS IN *Aztekium ritteri* (Cactaceae)

The early ontogeny (from a single cell to the globular stage) of somatic embryos in *Aztekium ritteri* is described, and the histochemical evidence for the presence of the two main elements of the early embryogenesis, the embryo proper and the suspensor, is presented.

Callus that is normally produced in the micropropagation of *A. ritteri* (Rodriguez-Garay and Rubluo, 1992) was used (approximately 5 g/flask) to initiate cell suspension cultures in 500-ml Erlenmeyer flasks containing 250 ml of MS medium (Murashige and Skoog, 1962), supplemented with L2 vitamins (Phillips and Collins, 1979) and with the addition of 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)+2 mg/l 1-naphthaleneacetic acid (NAA)+2 mg/l kinetin (KIN). Cell suspension cultures were transferred to fresh medium every two weeks. The former cell suspension stock was used to inoculate (10% inoculum) five 25-cm^2 canted-neck Corning tissue-culture flasks of each of the following growth-regulator treatments: 2,4-D at 0, 1, 2 and 3 mg/l, NAA at 1 and 2 mg/l and KIN at 1 and 2 mg/l. All cell suspension cultures were incubated at 27 ±2°C on an orbital shaker at 120 rpm under a 16-h photoperiod with fluorescent light (25 $\mu m \cdot s^{-1} \cdot m^{-2}$). Cell-suspension cultures were monitored daily under an inverted microscope for the presence of somatic embryos.

Double staining of the embryo-suspensor complex was carried out following the histochemical procedure described by Gupta and Durzan (1987). Cell samples from suspension cultures were washed with distilled water in a conical test tube by centrifugation to remove excess culture medium. Once the culture medium was removed, enough 2% acetocarmine (approximately 0.3 to 0.5 ml) was added to cover the packed-cell volume. Cells in the test tube were heated for about 10

s, avoiding boiling, then washed twice with distilled water. Finally, 3 to 4 drops of 0.5% Evans blue were added to the packed-cell volume. After 30 sec, the cells were washed twice with distilled water and resuspended in 50% glycerol for microscopic analysis.

Stock cell-suspension cultures initiated from green callus cultures turned red after 48 hours. It was found that most of the cells in the red cultures were destroyed (not shown). It is probable that the red color was the expression of some kind of phytoalexin produced by a stress due to a drastic change in the osmotic potential of the culture medium. Eventually (about 1 in 10), some pale-yellow to green cell-suspension cultures were produced, then used to inoculate the growth regulator treatments and to maintain the stock cell cultures. None of the cell cultures under growth regulator treatments resulted in good cell suspensions and turned red. Nevertheless, when creamy-colored cell suspensions were left with no subculture from 4 to 8 weeks, a mixture of different stages of embryonic structures began to be observed under the inverted microscope. It seems that the medium depleted of growth regulators (by the consumption of the cells) favored the initiation and further development of the somatic embryos as if they had been transferred to a growth-regulator-free medium, as is generally required for embryo expression in many species (Rodriguez-Garay et al., 1996).

It was found that the proembryos were initiated by the unequal transverse division of an avocado-shaped somatic cell, giving rise to a small embryonal cell (one-celled embryo proper) and a vacuolated suspensor-like cell (Figure 1a), similar to those described for angiosperms (West and Harada, 1993; Yeung and Meinke, 1993), which are a common feature in zygotic embryogenesis. As the embryo developed, the boundary between the embryo proper (dense cells apparently unorganized) and the suspensor (highly vacuolated cell) was clearly defined and the polarity of the embryoid was evident, which represents the developmental axis of the putative somatic embryos (Figures 1b through 1d). After a given number of cell divisions of the embryo head, the original suspensor cell started to proliferate forming a multicellular suspensor complex (Figures 1e and 1f). As the embryo proper developed, its dense cells became organized and the suspensor cells were more abundant around the original suspensor cell (Figures 1g through 1k). Finally, the globular stage was observed showing some suspensor remains (Figure 1l). Double-stained proembryos by the histochemical test gave the evidence for the presence of the embryo proper and the suspensor in the observed structures, as it has been done for other species (Gupta and Durzan, 1987). The embryo proper was stained intensively with acetocarmine, which is suggestive of intense RNA synthesis and the highly vacuolated suspensor cells were stained with Evans blue (Figure 1m).

It was observed that some cell clusters (not shown) lacking suspensor cell(s), but resembling the stages shown in Figures (1b) through (1i), did not produce any proembryo-like structure. This blockage of morphogenesis may be due to the organized proliferation of dense cells from an embryo proper, which could not function completely in the absence of gene products from the suspensor cells, which are important sources of growth substances (Yeung and Meinke, 1993).

SOMATIC EMBRYOGENESIS IN *Opuntia ficus-indica*

Opuntia has become an important crop for exotic fruit, vegetable, and forage production in Mexico, USA, Chile, Argentina, Israel, Italy, and South Africa (Pimienta-Barrios and Muñoz-Urias, 1994; Flores-Valdez, 1994). Furthermore, it is an important element for the conservation of arid ecosystems and the control of desertification.

The most common means of propagation in *Opuntia* is through the use of cladodes, which bear large numbers of meristematic tissue called areolas. In cactus pear, cladodes, flowers, and even developing fruits are capable of further differentiation; however, cladodes are the typical propagation unit (Pimienta, 1990).

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 attemped, genetic segregation and slow growth and development represent serious practical problems. However, seeds are important elements to be considered for plant improvement and germ plasm conservation, because they are easily stored for long-term use.

In forming the zygotic embryo, many plant species produce asexual embryos from somatic tissue. Among these species, several belong to the genus *Opuntia* (*O. aurantiaca* Lindl., *O. dilleni* Haw. *O. rafinesquii* Engelm*., O. torstipina* 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., *Kalanchoe*, *Asplenium*, *Ranunculus*, and *Tolmiea* (George, 1993).

Vélez-Gutiérrez and Rodríguez-Garay (1996) analyzed the origin and development of polyembryony in *O. ficus-indica* by microscopic means, where the structures of somatic embryos were identical to the normal stages of zygotic embryos, mainly due to the presence of a typical suspensor (Figure 2a).

More recently, efforts to produce somatic embryos of *O. ficus-indica* have been carried out. Young cladodes have been cultured in a medium where somatic embryogenesis in *Turbinicarpus pseudomacrochele* was obtained (Torres-Muñoz and Rodríguez-Garay, 1996). Embryogenic callus and globular embryos have been obtained and resembled to those of the same stage produced by natural zygotic embryogenesis (Figures 2b and 2c); however, they did not proceed to later stages.

SOMATIC EMBRYOGENESIS IN *Turbinicarpus pseudomacrochele*

Turbinicarpus pseudomacrochele is a Mexican threatened member of the *Cactaceae* family, and it is included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora. (CITES) Appendix I (Hunt, 1992).

The line "B" *Strombocacti* comprises 10 genera and 27 species, which are appreciated by collectors around the world; however, only three of them have been propagated *in vitro*. Axillary shoot proliferation in *Aztekium ritteri* and *Leuchtembergia principis* were reported by Rodríguez-Garay and Rubluo (1992) and Starling (1995), respectively. Also, somatic embryogenesis was reported for *Ariocarpus retusus* (Stuppy and Nagl, 1992).

Recently, Torres-Muñoz and Rodríguez-Garay (1996) reported plant regeneration in *T. pseudomacrochele* through indirect somatic embryogenesis on solid MS medium suplemented with L2 vitamins, 3 mg/l 2,4-D, 2 mg/l NAA, 2 mg/l KIN, 500 mg/l L-glutamine, 250 mg/l casein hidrolizate and solidified with 8 g/l agar.

After four weeks, somatic embryos were evident in suspension cultures and creamy-yellowish embryogenic callus was produced containing primarily proembryos, as well as globular and well-defined embryo structures. Complete plants were produced after somatic embryo germination. Cultures were transferred to the same basal medium without growth regulator, and solidified with 5 g/l of the agar substitute PhytagelÔ. After 16 weeks, somatic embryos started to germinate and developed typical plantlet morphology.

SOMATIC EMBRYOGENESIS IN *Mammillaria san-angelensis*

Recently, Marín-Hernández et al., (1998) reported the *in vitro* somatic embryogenesis in the severely endangered cactus *Mammillaria san-angelensis*. Somatic embryo structures of both unicellular and multicellular origin were originated from ovule integuments cultured in a modified B5 medium supplemented with 2,4-D (4 mg/l) plus KIN (2 mg/l).

After 90 days in culture, the somatic embryo structures appeared in a yellowish callus showing a certain degree of polarity; however, plant regeneration was not obtained.

SOMATIC EMBRYOGENESIS IN *Agave victoria-reginae*

Agave victoria-reginae Moore is a Mexican species named and described in honor of Queen Victoria of England by Thomas Moore. It has been sold internationally since the 1800s as an exceptional ornamental plant (Hertrich, 1942).

Overcollection of plants and seeds as well as habitat destruction have significantly reduced populations of this agave in its native habitat, an arid area of the industrial city of Monterrey, Nuevo Léon in México. Furthermore, this species, like many other agaves, have a long life cycle and sets seed after aproximately 20 to 30 years of vegetative growth. Also, it seldom produces more than 2 or 3 offshoots in the life span of a plant. For these reasons, this species is listed as threatened in CITES Appendix II (Roberts, 1994).

Rodríguez-Garay et al., (1996) described a method for direct somatic embryogenesis that results in large numbers of plants. Somatic embryogenesis from leaf-blade explants from *in vitro*-propagated plants was evident in a 2- to 6-week period on agar-solidified MS medium supplemented with L2 vitamins and 0.3 mg/l of 2,4-D (Figure 3a) and germination of somatic embryos was achieved after 8 weeks on half-strength MS medium and 4 weeks on half-strength SH medium (Schenk and Hildebrandt, 1972), both lacking growth regulators (Figure 3b).

The adaptation of regenerated plants to their natural habitat produced by this method has been nearly 90% (Figure 3c).

SOMATIC EMBRYOGENESIS IN *Agave tequilana* Weber var. Azul

Agave tequilana Weber var. Azul is the most cultivated agave species worldwide (approximately 60,000 ha) and nearly 90% of the cultivated area is located in the state of Jalisco, México. The other 10% is distributed in about four other states in México (Consejo Regulador del Tequila, 1997).

A. tequilana is cultivated for the production of the famous distilled beverage named tequila. This species is conventionally propagated asexually by offshoots that are produced in rhizomes or axillary meristems. This procedure allows the clonal increase of a given genetic stock, although it can faciltate the propagation of diseases (Arizaga and Ezcurra, 1995).

In agaves genetic improvement by conventional plant breeding is complicated mainly by the long life span.

An efficient protocol has been developed for the *in vitro* regeneration of *A. tequilana* through indirect somatic embryogenesis. The best explants for callus formation and the succeding somatic embryo production were leaf sections derived from plantlets propagated and mantained *in vitro* (to be published elsewhere) (Figures 3d and 3e).

Its morphogenetic process represents a useful tool for the plant genetic improvement *in vitro*, and can be used in procedures for cell selection, genetic transformation, and domestication (Van Schaik et al., 1996).

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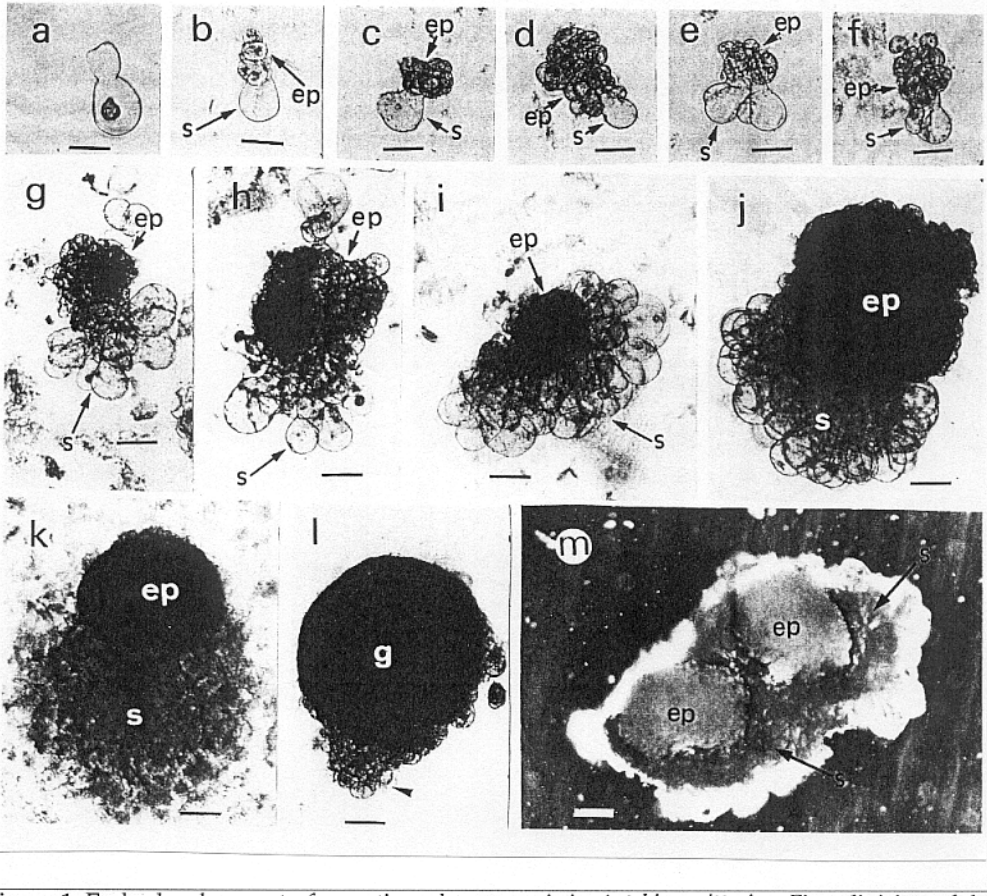

Figure 1. Early development of somatic embryogenesis in *Aztekium ritteri*. a, First division of the initial embryogenic cell; b–d, First cell divisions of the embryo proper. The avocado-shaped suspensor cell remains single; e, The suspensor cell starts to proliferate; f–k, Different stages of development of the embryo proper and the suspensor; l, Globular somatic embryo; g, with suspensor remains (arrowhead); m, Proembryos showing embryo proper and suspensor. Bars in a through m=0.1 mm; ep=embryo proper, s=suspensor.

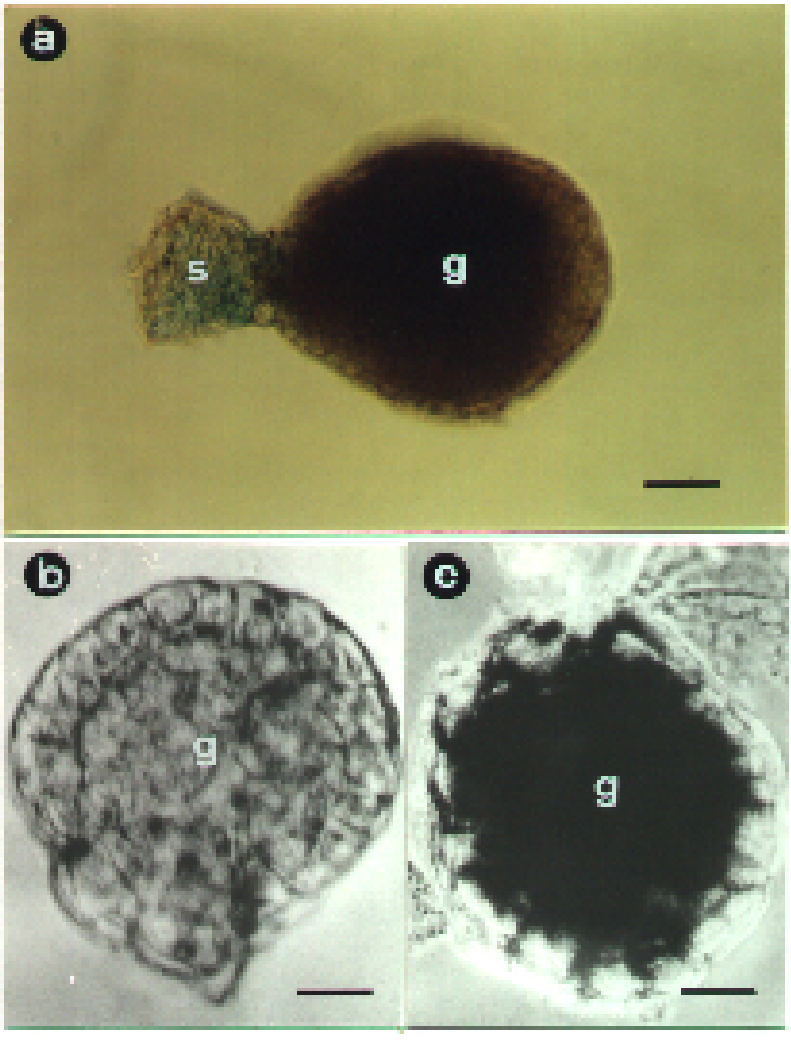

Figure 2. Comparative development of somatic embryos in *O. ficus-indica*. a–b, Globular stage of somatic embryo produced in a seed. Bar in a=0.4 mm. Bar in b=0.35 mm. c, Globular stage of an *in vitro*-produced somatic embryo. Bar in c=0.35 mm; g=globular embryo; s=suspensor.

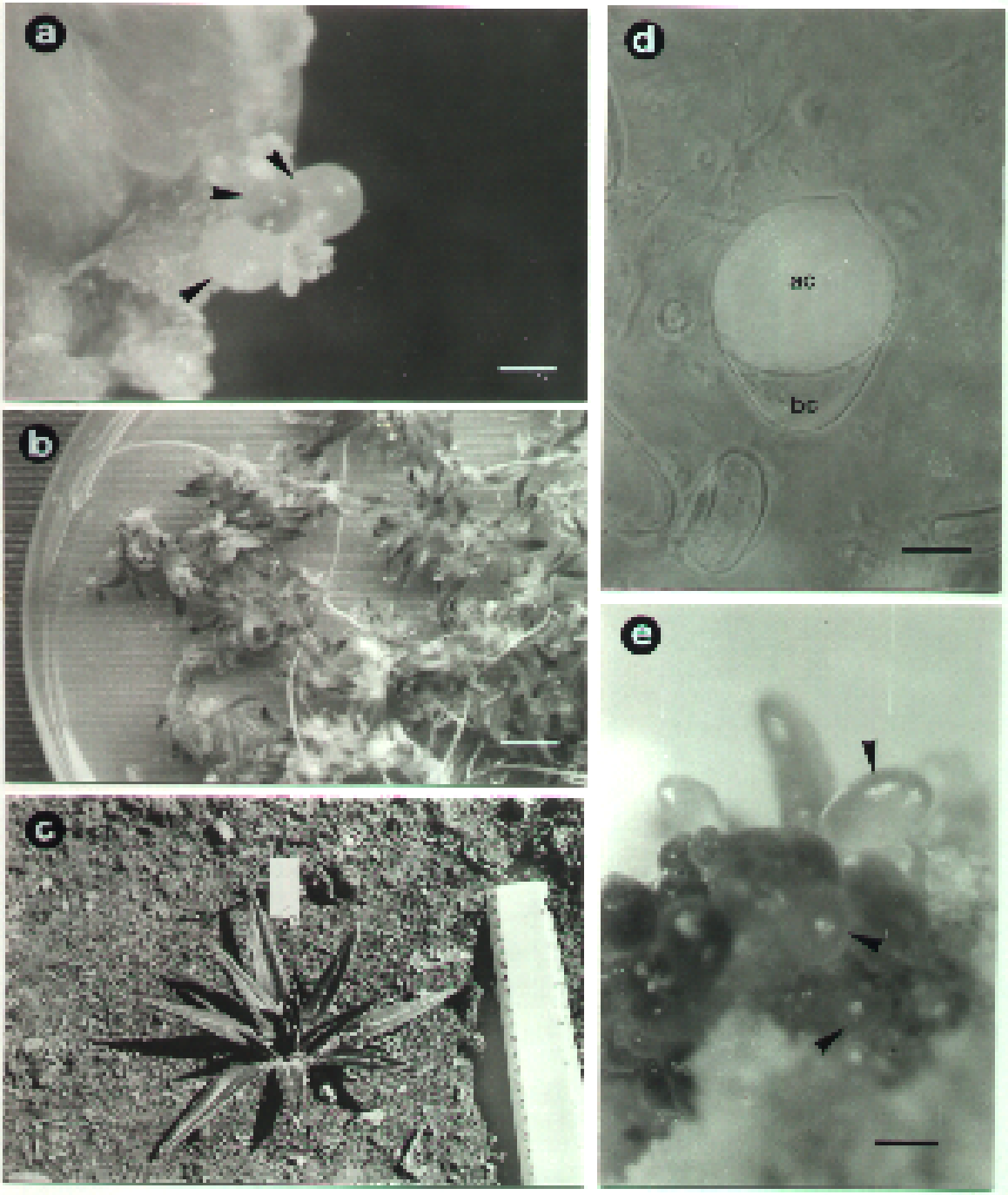

Figure 3. Process of somatic embryogenesis in agave. a, Globular stage of embryos in *Agave victoria-reginae* (arrowheads). Bar=0.5 mm. b, Germinated embryos of *A. victoria-reginae*. Bar=4.0 mm. c, Adaptated plant of *A. victoria reginae* produced by somatic embryogenesis. d, Somatic embryogenic cell of *Agave tequilana*. Bar=0.1 mm. e, *A. tequilana* embryos at globular stage (arrowheads). Bar=0.5 mm; ac=apical cell; bc=basal cell.