Studies on the Induction of Embryogenic Globular Structures in *Opuntia ficus-indica*

Samantha Pinheiro da Costa[1], Arlete A. Soares[2], Birgit Arnholdt-Schmitt[1]

[1]Departamento de Bioquímica e Biologia Molecular e [2]Departamento de Biologia,
Universidade Federal do Ceará (UFC)
Fortaleza, CE, Brazil
e-mail: arnholdt-schmitt@gmx.de
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Dedicated to Prof. Karl-Hermann Neumann, a Pioneer in Tissue Culture Research

ABSTRACT

In studies on the induction of somatic embryogenesis in *Opuntia ficus-indica*, globular structures were obtained from isolated, *in vitro*-germinating zygotic embryos in the presence of 0.45×10^{-6} mol·l^{-1} 2,4-D and 0.91×10^{-6} or 9.1×10^{-6} mol·l^{-1} kinetin. The globuli displayed typical morphological characteristics of embryogenic globuli, i.e., a well-defined globular shape without vascular connection to the original tissue, demonstrating yellowish-white colour. Serial histological analysis during the first 4 weeks of culture revealed that these structures were directly induced after about 2 weeks of primary culture at the periphery, beneath the first cell layers, preferably emerging at subepidermal regions of young cotyledons. Also, a suspensor-like tissue embedding the globuli in the originating material was observed. However, in comparison to young embryonic structures from other species, the globuli from *O. ficus-indica* displayed atypical cytological characteristics that are related to water management in plants.

Keywords: *Opuntia ficus-indica*, somatic embryogenesis, globular structures, histological analysis

INTRODUCTION

Growing interest in research on *Opuntia ficus-indica* is due to its increasing importance as fodder crop or vegetable and exotic-fruit-producing plant in arid and semiarid regions. In northeastern Brazil, about 400,000 ha are covered with this plant species (Santos et al., 1997), which is used there mainly as fodder in small farms. Additionally, planting of *O. ficus-indica* is appropriate to prevent environmental destruction in dry areas caused by erosion. Although breeding efforts are only beginning (Bunch 1997; Mondragon and Bordelon, 1996; Wang et al., 1996), basic biotechnological techniques, such as micropropagation by axillary-bud proliferation, regeneration of isolated-shoot apical meristems, induction of callus and cell suspensions, as well as protoplast isolation and culture and genetic transformation by particle bombardment, are already available for prickly-pear (Arnholdt-Schmitt et al., 2001). Establishment of transgenic plants can be useful in basic research (Arnholdt-Schmitt 1996) or in studies on applied purposes to improve the nutritional value or characteristics of the plants to confront biotic or abiotic stress. In any case, crucial for the application of any transformation technology is the development of regeneration systems. However, the key technology for regeneration that starts at the level of individual cells from tissues or suspension or protoplast cultures, i.e., the induction of somatic embryogenesis, is still missing. Torres-Muñoz and Rodríguez-Garay (1996) reported on successful induction of indirect somatic embryogenesis from the medullar-tissue disk of adult plants of the cactus *Turbinicarpus pseudomacrochele*. The same medium that was used for this species was applied for young cladodes of *O. ficus-indica*. Although embryogenic callus and globular structures had been obtained,

which were analogous to zygotic embryogenic structures, a further development to advanced structures in embryogenesis was not achieved (Santacruz-Ruvalcaba et al., 1998). Vélez-Gutiérrez and Rodríguez-Garay (1996) performed analyses on polyembryonal embryos of *O. ficus-indica*, and demonstrated high comparability to zygotic embryos, including also the occurrence of a typical suspensor (see also Santacruz-Ruvalcaba et al., 1998).

From experience with various species, it is well-known that *in vitro* induction and initiation of somatic embryogenesis (for theoretical considerations on the induction of redifferentiation in tissue culture see DeKlerk et al., 1997) depends on the endogenous concentration of auxins, their distribution in the cultured tissue and interaction with exogenously supplied auxins or antiauxins that are involved in the metabolism or transport of endogenous auxins, or interaction with other growth regulators. In our aim to induce somatic embryogenesis in *O. ficus-indica*, we performed a series of *in vitro*-culture experiments with isolated zygotic embryos of mature seeds of cv. Gigante and exogenously supplied 2,4-dichlorophenoxyacetic acid, kinetin, and abscisic acid in B5 medium.

MATERIAL AND METHODS

Plant Material

For the experiments, seeds of mature fruits of *O. ficus-indica*, cv. Gigante collected at Madalena, Ceará, in the northeastern Brazil were used.

Isolation of Zygotic Embryos and Tissue Culture

Seeds of *O. ficus-indica* were incubated in a approximately 5% hypochlorite solution for about 24h. Mechanical isolation of zygotic embryos from the seeds was performed under sterile conditions using a scalpel and tweezers and viewed through a stereomicroscope. After 2h of incubation in 2% KNO_3, to facilitate germination, the embryos were cultured for 4 weeks in liquid modified B5 medium (Gamborg et al., 1968; 6 embryos per 30 ml nutrient medium in a 125 ml Erlenmeyer flask) on a shaker at 100 rpm, containing various concentrations of dichlorophenoxy acetic acid (2,4-D; 0.45 to 27.0×10^{-6} mol·l^{-1}) as the only source of growth regulator or at 0.45×10^{-6} mol·l^{-1} 2,4-D in combination with kinetin (0.91×10^{-6} or 9.1×10^{-6} mol·l^{-1}) or 10^{-4} mol·l^{-1} abscisic acid (ABA). After 4 weeks of primary culture in the presence of growth regulators, the germinated embryos were subcultured at 4-week intervals by applying the same basal medium without any growth regulator.

Histology

For light microscopy, isolated and *in vitro*-cultured zygotic embryos were fixed in Karnovsky's solution (Karnovsky, 1965), dehydrated in graded alcohol series and embedded in historesin glycol methacrylate. Sections of 7 μm or 10 μm were performed and stained with toluidine blue 0.12% in a water solution containing 5% borax and 0.1% basic fuchsin (Junqueira, 1990). Coomassie brilliant blue R250 was used for protein localization (Cawood et al. 1978). Samples were taken after 1, 2, 3, and 4 weeks of primary culture in the presence of 0.45×10^{-6} mol·l^{-1} 2,4-D and 9.1×10^{-6} mol·l^{-1} kinetin. Further, induced material was investigated after an additional 7 and 9 weeks of subculture in basal B5 medium in the absence of growth regulators.

RESULTS

Induction of Globular Structures on Germinating Zygotic Embryos

Experiments were performed to induce somatic embryogenesis on young tissues of germinating seeds of *O. ficus-indica*. Because germination under *in vitro* conditions has proven to be difficult, a method was established to isolate zygotic embryos from seeds and to induce them to germinate after treatment with 2% KNO_3 in liquid B5 medium. Incubation of the seeds in 5% hypochlorite and mechanical disruption of the seed coat with help of a stereomicroscope enabled the isolation of viable embryos (see Figure 1). Germination rate and development of the embryos in the nutrient medium were influenced by applied growth regulators and light. The presence of 10^{-4} mol·l^{-1} ABA alone or in combination with 0.45 mol·l^{-1} 2,4-D and darkness reduced the rate of germination and postponed development of the zygotic embryo significantly. The highest rate of germination and a strong formation of the root as well as of the cotyledons were observed in the absence of any growth regulator. In the presence of lower concentrations of 2,4-D in the medium, development of the root was reduced or inhibited, malformation of root and shoot was frequently observed and preferably at the regions of the cotyledons, globular structured callus growth was obtained (Figure 1B). However, microscopic analysis revealed that these globular structures did not show the typical morphological characteristics of embryogenic globular structures that we also find, for example, in studies on carrots (Grieb et al., 1994, 1997) or cassava (Pinheiro Bastos et al., 2001). The putative globular structures found in these callus cultures of *O. ficus-indica* could not be separated easily from the originating tissue and the structures were fragile and did not display a distinct outer layer to give a well-defined and stable globular shape.

Nevertheless, by adding kinetin (0.91×10^{-6} or 9.1×10^{-6} mol·l^{-1}) to the medium with 0.45×10^{-6} mol·l^{-1} 2,4-D, induction of globular structures was achieved, which demonstrated the typical morphological characteristics of embryogenic material (Figure 1 C-F). They could be observed about 3 to 4 weeks after inoculation of the isolated zygotic embryos emerging preferably at the cotyledonary tissue. This result shows good repeatability in independent experiments. The higher concentration of kinetin gave more reliable results, i.e., during the primary culture of 4 weeks, a higher number of zygotic embryos with induced somatic embryos was obtained.

Histological Analysis of the Globular Structures and Their Origin in Zygotic Embryos

Figure 2 shows the histological analysis during redifferentiation of somatic cells in cotyledonary tissues of zygotic embryos of *O. ficus-indica* that were cultured in the presence of 0.45×10^{-6} mol·l^{-1} 2,4-D and 9.1×10^{-6} mol·l^{-1} kinetin. Three weeks after inoculation, i.e., about 16 days after germination has taken place, globular structures of various sizes could be observed. Figure 2 A-D indicates the formation of globular structures. The distinct, organized shape of the globuli can be distinguished clearly from areas, which display unorganized callus growth showing a high number of small cells beneath the outer layer of the zygotic embryo (Figure 2 B). During early stages of the globuli, small cells can be found within globular structures, that resulted from recent cell divisions (Figure 2 C, arrow). However, meristematic unvacuolated cells with a dense cytoplasm are difficult to discover. Figure 2 D demonstrates beginning of the formation of an embryogenic globulus at the stage of first cell divisions with meristematic cells (arrow), indicating direct somatic embryogenesis. From Figure 2 A-C it can also be seen, that the globular structures were obtained in peripheral, mostly subepidermal regions of the young original tissue. Induction of globular structures may occur at any peripheral region of the cross section, and, also, was found to be independent of a position near protein-rich cells, which obviously originated from the zygotic embryo. Figure 2 E and F indicate the development of globular structures in either distant or near regions in relation to these storage cells (white arrows). Staining with Coomassie blue confirmed that these cells contained storage protein (data not shown).

In Figure 2 G, H, and J it can be seen that globuli, which separate from the original tissue, are differentiating tracheid cells in the inner region of their structure. Every somatic globulus that was found outside the original tissue during this study, showed this cytodifferentiation without any sign of an advanced morphological stage in embryogenesis (see also Figure 2 J). Additionally, intense violet staining of the inner region indicates a high number of cells with a different cell-wall structure (see Figure 2 G, I, and J). After a subculture of 7 to 9 weeks in B5 medium without any growth regulator, all the globuli found, contained tracheid-cell structures. However, formation of organized vascular structures were not discovered. Initiation of advanced embryonic structures like heart-shapes or torpedo-shapes were not achieved during this subculture.

Figure 2 I and J indicate that induced globular structures of somatic embryos of *O. ficus-indica* developed a suspensor-like tissue, which embedded the embryogenic material into the originating tissue.

DISCUSSION

In the present study, successful induction of embryogenic globular structures at the cotyledons of isolated zygotic embyos of seeds of mature fruits of *O. ficus-indica* (cv. Gigante) is reported. Induction occurred during *in vitro* germination in B5 medium in the presence of 0.45×10^{-6} mol·l^{-1} 2,4-D combined with kinetin (0.91×10^{-6} or 9.1×10^{-6} mol·l^{-1}). Although the obtained globular structures displayed typical morphological characteristics of embryogenic globuli and, even a suspensor-like structure was proven, as it was shown also for zygotic and polyembryonal embryos of *O. ficus-indica* by Vélez-Gutiérrez and Rodríguez-Garay (1996) and Santacruz-Ruvalcaba et al. (1998), cytological analysis of the globuli revealed differences from other plant species. Embryogenic globular structures of other species consist mainly of meristematic, unvacuolated cells, with a marked nucleus and no evidence of tracheid cells. In contrast, in the induced globular structures of *O. ficus-indica*, meristematic cells were only discovered at a very early stage of their development. Highly vacuolated cells were predominant and at the stage of separation from the originating tissue, tracheid cells differentiated already in the inner region. Up to now, tracheid cells or structures have never been observed in globular somatic embryos of Cactaceae, but only in advanced stages during embryogenesis (personal communication by Benjamin Rodríguez-Garay).

A transfer of embryogenic material to the same basal medium but free of any growth regulator, did not result in the initiation of further development of the globular somatic embryos during 9 weeks of subculture so far. But it should be admitted that efforts to perform longterm studies and variations in nutrient components of the medium, such as carbohydrates for example, are yet missing in our studies. Nevertheless, also Santacruz-Ruvalcaba et al. (1998) reported that a medium appropriate to initiate maturation of early somatic embryos of *Turbinicarpus pseudomacrochele* failed to initiate this development in *in vitro* induced globular embryos from young cladodes of *O. ficus-indica*. Observations by Vélez-Gutiérrez and Rodríguez-Garay (1996) may also be of interest, demonstrating that globular, heart and torpedo stages of polyembryonal embryos from the nucellus of seeds of *O. ficus-indica* failed to develop advanced structures in a basal MS medium (Murashige and Skoog, 1962), that was sucessfully used for germination of mature zygotic and somatic embryos. Whether in our system early differentiation of strongly vacuolated cells and the formation of tracheid cells without recognizable organisation in the induced globular structures of *O. ficus-indica* are responsible for difficulties to initiate maturation of the embryos is open for discussion. In our experience, rapid differentiation of meristematic cells is also typical for other meristems in *O. ficus-indica* and may be characteristic for this species.

In future experiments, it would be of interest to analyse when tracheid structures begin to differentiate at early stages in zygotic and apomictic embryos of *O. ficus-indica* and whether organisation of these cells is crucial for further development. Cytodifferentiation to tracheid cells may occur also without preceding cell division (Kohlenbach and Schmidt, 1975; Kohlenbach, 1977). Because auxin is known to induce linear vascular strands upon external application and auxin-transport inhibition interacts with vascular

strand formation (Scheres, 2000), the observation of unorganized tracheid structures in developing embryonic structures of *O. ficus-indica* may indicate disturbances in endogenous auxin metabolism and in the distribution of auxin within the cells. This may also seriously affect nonlinear signaling for the maturation of embryonic structures (see Palme and Gälweiler, 1999; Scheres, 2000).

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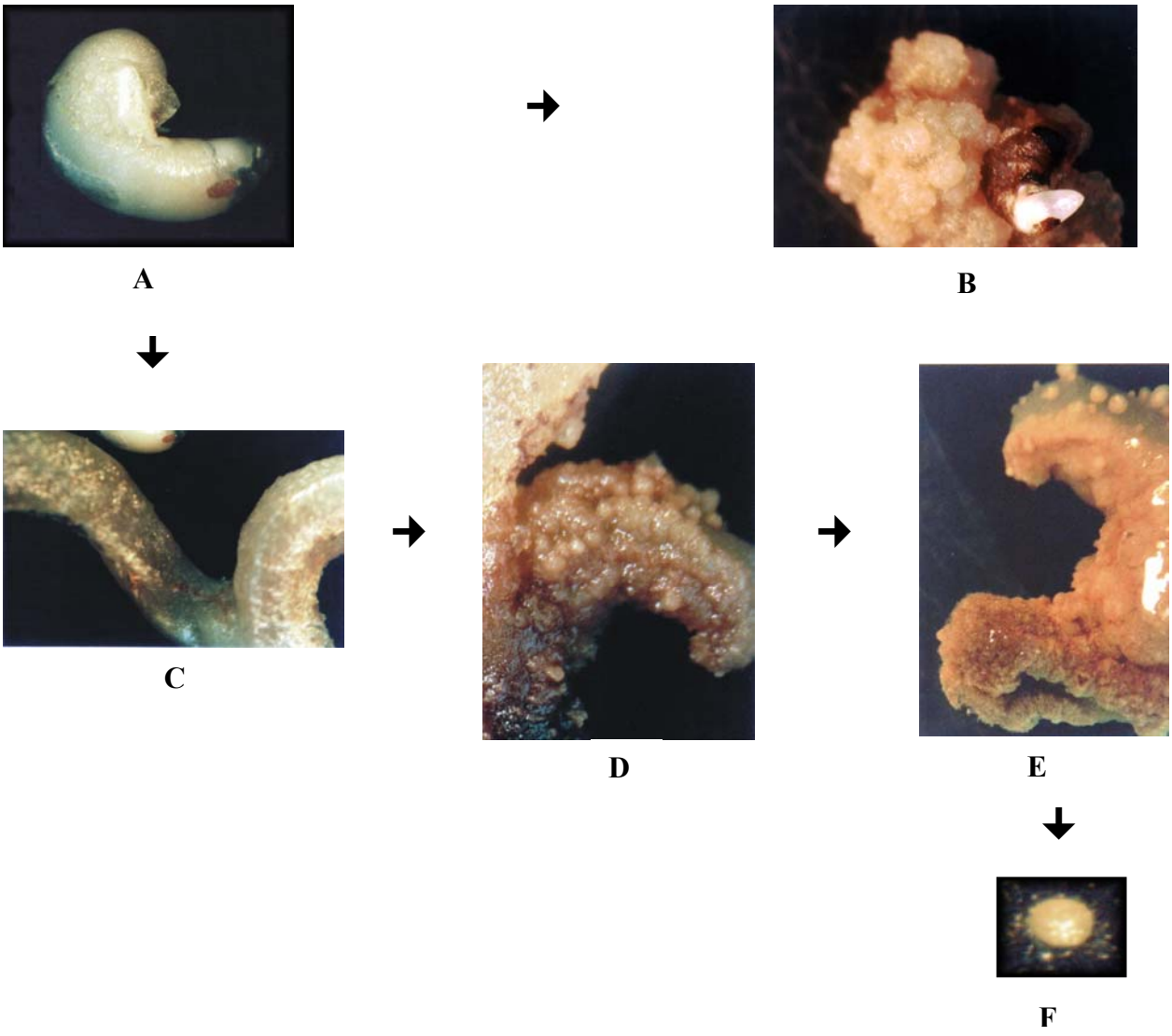

Figure 1. Induction of Globular Structures in Isolated Zygotic Embryos of *Opuntia ficus-indica*. **A**: Isolated zygotic embryo **B**: Globular structured callus, that was induced at the young cotyledonary tissue of an in vitro-germinated zygotic embryo **C**: Cotyledons of an in vitro-germinated zygotic embryo **D**: Induced embryogenic globular structures at the young cotyledonary tissue of an in vitro-germinated zygotic embryo **E**: See D, separation of globuli as autonomous structures **F**: isolated globular structure

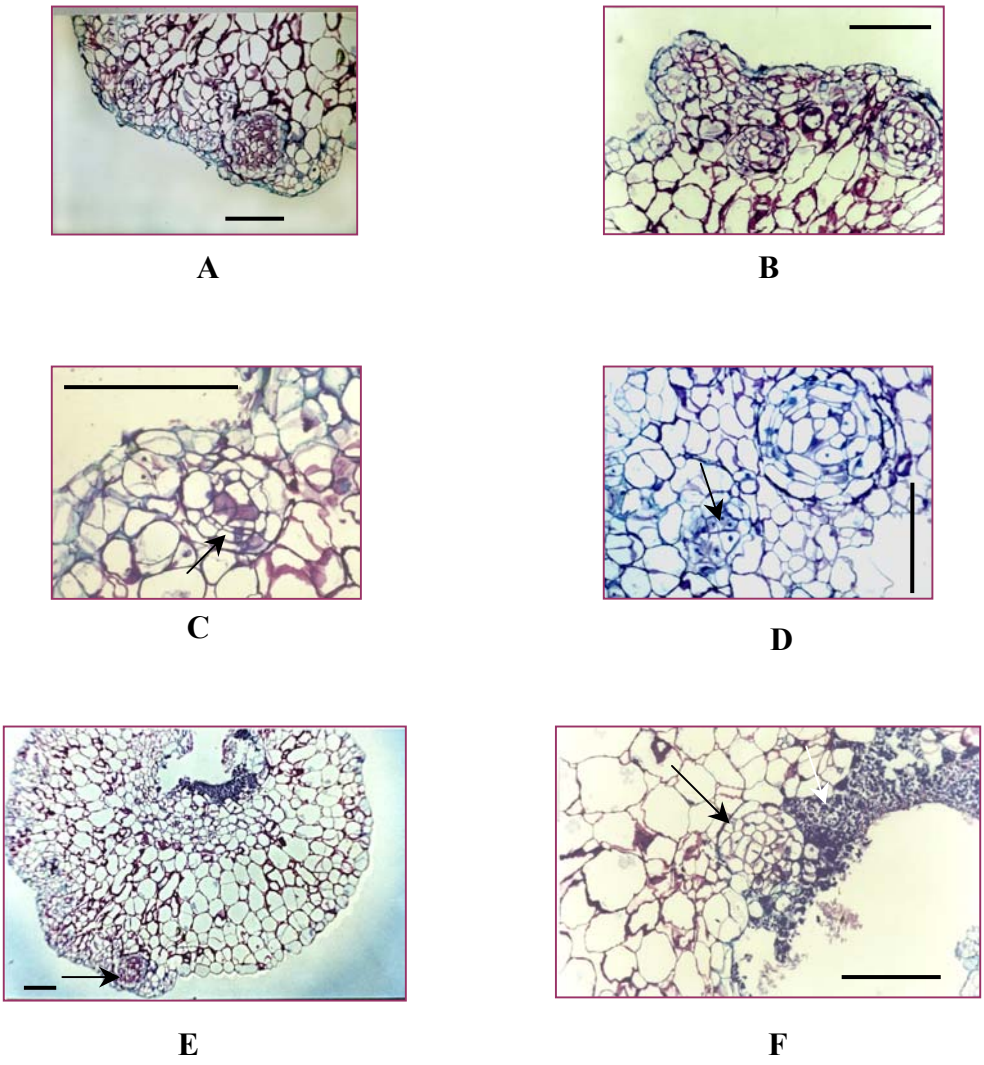

Figure 2. Histological Analysis of the Induction of Embryonic Globular Structures in *Opuntia ficus-indica* (bars: 0.25 mm)
A/B: Globular structures at the periphery of cross sections of in vitro-germinating zygotic embryos **C**: Globulus in the subepidermal region indicating recent cell divisions **D**: Early structure of a globulus showing meristematic cells with marked nuclei **E**: Formation of a globular structure in a distant region related to protein-storing cells **F**: Formation of a globular structure near protein-storing cells

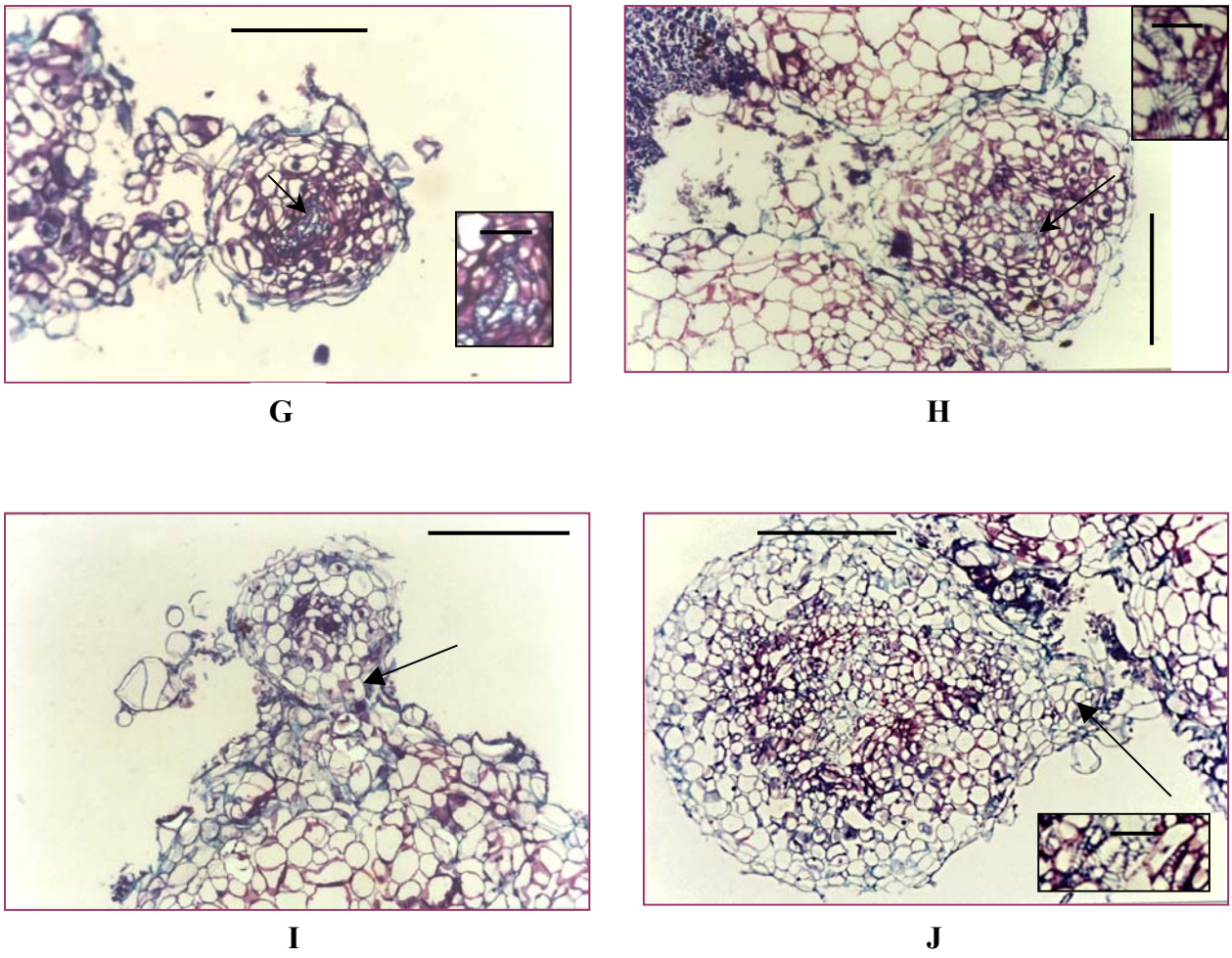

Figure 2. Histological Analysis of the Induction of Embryonic Globular Structures in *Opuntia ficus-indica* (bars: 0.25 mm) (continued)
G: separated globulus with the beginning of tracheid differentiation in the inner region (see also sector, bar 0.05 mm) **H**: Separating globulus with tracheid cells in the center (see also sector, bar 0.05 mm) **I**: Globulus embedded with a suspensor-like structure into the originating tissue **J**: Separating globulus with differentiation of tracheid cells in the inner region (see also sector, bar 0.05 mm) and a distinct suspensor-like structure