Turk J Bot
24 (2000) 91–95

Research Article

Histology of Somatic Embryogenesis in Cultured Leaf Explants of Pistachio (*Pistacia vera* L)

Ahmet ONAY
Dicle Üniversitesi, Fen-Edebiyat Fakültesi, Biyoloji Bölümü, Diyarbakır-TURKEY

Received: 08.12.1998
Accepted: 17.09.1999

Abstract: The histology of somatic embryo initiation and development in pistachio (*Pistacia were* L.) embryogenic masses (EMS) derived from leaf explants was examined using light microscopy. Explants with somatic embryos at different developmental stages were fixed for histological examination, cut into 10 µm thick sections, stained with hematoxylin-eosin and observed microscopically. The histological examination showed that the two types of cell clusters induced originated from explants and were morphologically different. These results clearly demonstrate that the induction and development of somatic embryos from leaf explants of pistachio were from single epidermal or subepidermal cells.

Key Words: *Pistacia vera* L., leaf explants, histology.

Kültürdeki Antepfıstığı (*Pistacia vera* L.) Yaprak Eksplantlarında Somatik Embriyogenesisin Histolojisi

Özet: Antepfıstığı (*Pistacia vera* L.) yapraklarından elde edilen embriyogenik dokularda somatik embriyogenezisin histolojisi ışık mikroskobu kullanılarak araştırılmıştır. Histolojik incelemeler için, farklı gelişme safhalarını içeren eksplantlar, 10 µm kalınlıklarda kesildi ve hematoxylin-eosin boyası ile boyanarak mikroskopta incelendi. Eksplantlardaki histolojik incelemeler sonucunda morfolojik olarak farklı iki tip hücre grubunun bulunduğu görüldü. Yaprak eksplantlarından elde edilen embriyogenik dokudan somatik embriyoların gelişmelerinin, epiderma veya epiderma altındaki tek bir embriyogenik hücreden orijinlendiği görülmüştür.

Anahtar Sözcükler: *Pistacia vera* L., yaprak eksplantı, histoloji.

Introduction

Pictachio (*Pistacia vera* L., *Anacardiaceae*) is a dioecious plant with male and female organs in separate trees. It is an increasingly important nut crop widely cultivated especially in dry and hot areas of Iran, the Middle East, and Mediterranean countries, including Turkey, and also in North Africa and California. The Turkish cultivar grown commercially is Antep (both pistillate and staminate), and it is vegetatively propagated by budding graftwood onto rootstocks of *P. vera,* but with a low multiplication rate. Since Antep is vegetatively propagated clone and the rootstocks are seed propagated, problems such as the production of seedless fruits (blanks) and degree of shell dehiscence affecting overall nut quality and yield have been attributed to the natural variation of seed-derived rootstocks. A potential solution to these problems would be the development of methods for vegetatively propagating the rootstocks. Unfortunately, attempts to propagate rootstocks vegetatively by conventional methods of soft and hard wood cuttings have given inconsistent results (1). In addition, since the species is an outbreeder, propagation from seed results not only in genetically-variable stock but also in undesirable male trees, and grafting can lead to loss of production due to incompatibility between the grafted bud and rootstocks.

The use of tissue culture techniques to propagate pistachio species has become increasingly significant in the past 20 years (2, 3, and 4). To accelerate propagation, in vitro culture techniques based on somatic embryogenesis have been developed (5, 6, 7), but no information is available on the histology of somatic embryo induction and development in pistachio. Information regarding direct or indirect plant regeneration via somatic embryogenesis may be useful in the production of clonal variants, and in clonal multiplication and genetic transformation studies. In this paper, we describe the histological analysis of somatic embryos at different stages of development from embryogenic masses derived from leaf explants of pistachio.

Materials and Methods

Culture Procedure

The Turkish pistachio cultivar Antep was used. Embryogenic cultures were initiated on liquid MS medium supplemented with thidiazuron (TDZ) and maintained on solidified MS medium containing BAP (8). The cultures were incubated at 25°C in light (24-hour photoperiod) and were subcultured at four-week intervals to fresh media.

Light Microscopy

Embryogenic masses for conventional light microscopy were prepared according to the method of Jeffree (9). In brief, a time-course experiment was performed in which samples of embryogenic and non-embryogenic explant material were fixed for histological examination at 0, 3, 9, 15 and 27 days following the establishment of cultures for the induction of somatic embryogenesis. For histological confirmation on the initiation and development of somatic embryos, the explants with somatic embryos at different stages were fixed in 1% OsO_4 in water for three hours. The fixed samples were washed for 30 min three times with distilled water. After washing, the specimens were dehydrated by being passed through ethanol series and infiltrated with resin. The specimens were then transferred into Beem capsules in resin, and placed in an embedding oven (70°C overnight) to cure. The specimens were cut into blocks and sectioned at 10 µm using a rotary microtome. The sections were mounted onto slides and allowed to dry for at least ten minutes before staining. The specimens were stained with hematoxylin-eosin and counterstained with fast green for general histological examinations. Cover slips were mounted with Histoclad mounting medium and dried on a 40°C hot plate. Permanent slides were examined on a Zeiss Universal microscope equipped with a camera.

Results and Discussion

No detailed information is known about the origin and growth of pistachio somatic embryos in culture. However, there seem to be at least two mechanism which could account for their origin. Somatic embryos arise from single cells or small cell aggregates by an initial asymmetric division at the epidermic layer of EMS (8). Somatic embryos also seem to develop from a few small meristematic cells within the EMS.

Histological sections prepared from the embryogenic cultures of pistachio contain somatic embryos at different developmental stages (Figs. 1-10). In an earlier study (8), we demonstrated that juvenile leaf explants of pistachio were the most responsive for induction and development of embryogenic masses (EMS) and somatic embryos. The initiation and further development to from embryogenic masses require transfer of callus to a different culture regime (7).

No meristematic structures have ever been found at the epidermic layer of EMS at day 0. Histological sections of the intact embryogenic masses showed that cells of embryogenic masses were similar in size, but cells appeared to be different in shape. Visible differences between cells, which were taken from different positions in the EMS, were observed. Two distinct regions can be observed in all proliferating EMS (Fig. 1). There were two types of cells: (1) relatively dense cytoplasma, thin cell walls and generally small invisible vacuoles (meristematic cells) and (2) unclear cytoplasma and large vacuoles. A section passing through embryogenic EMS after 3 days of culture revealed mitotic and meristematic activity (Fig. 2). The high frequency of mitotic figures in the meristematic region of sectioned EMS indicates that individual embryos within the EMS may grow rapidly. Some of these cells showed signs of proliferation at the epidermal layer (Fig. 3). The epidermal cells of the EMS elongated vertically, and at certain points the developing somatic embryo emerged. This was also seen in histological studies of EMS examined using scanning electron microscopy (8). The embryogenic cells at the cut end of EMS were also small and compact with densely stained cytoplasma, features associated with early embryogenic EMS development, which gave rise to globular-stage somatic embryos (Fig. 4). They contained densely stained nuclei. It is our interpretation that those embryos originate from the embryogenic cells through asymmetrical cell divisions. Theoretically, all cells in these embryogenic masses are committed to the process of embryo induction, but actually only a limited number may in fact fulfil this commitment. Several observations support the hypothesis that plant growth regulators employed to from embryongenic cells do this by alteration of cell polarity and promotion of subsequent asymmetric divisions. When immature zygotic embryos of *Trifolium* were cultured in the presence of cytokinin, somatic embryos were produced directly from the hypocotyl epidermis (10). It is plausible but unproven that exogenously applied plant growth regulators directly modify cell polarity by interference with pH gradients or the electrical field around cells.

Embryogenic cells under the epidermic layer of the EMS appeared to be smaller in size and more compact, with more densely stained cytoplasm than in the

surrounding cells (Fig. 5). Small cells in the embryogenic region are characterised by a high nucleus: cell volume ratio. Cell divisions in the embryogenic region seemed to give rise to rows of cells which eventually elongated to from suspensor cells (Fig. 6). Although mitotic figures were not observed in the suspensors, tiny cells were

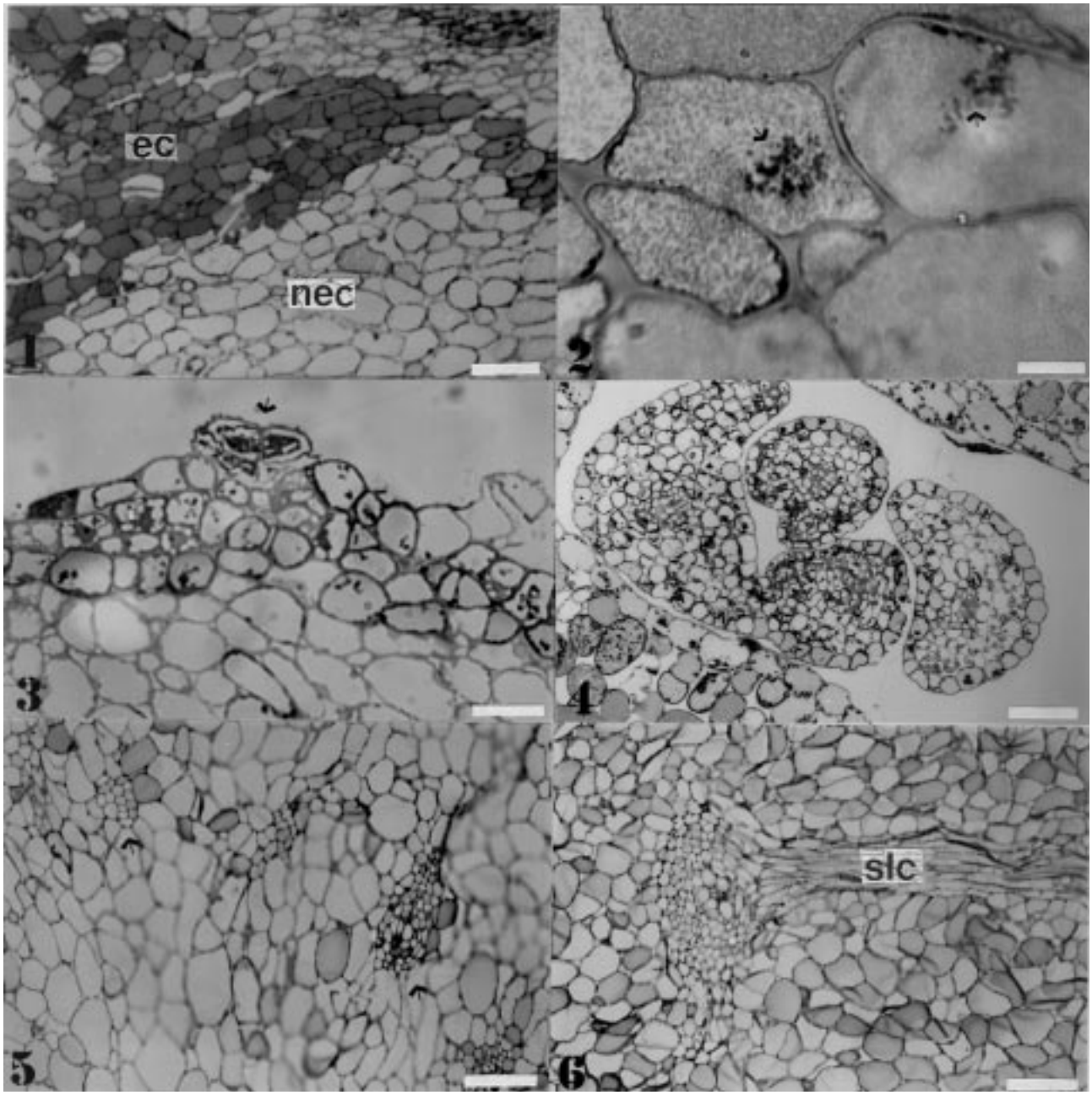


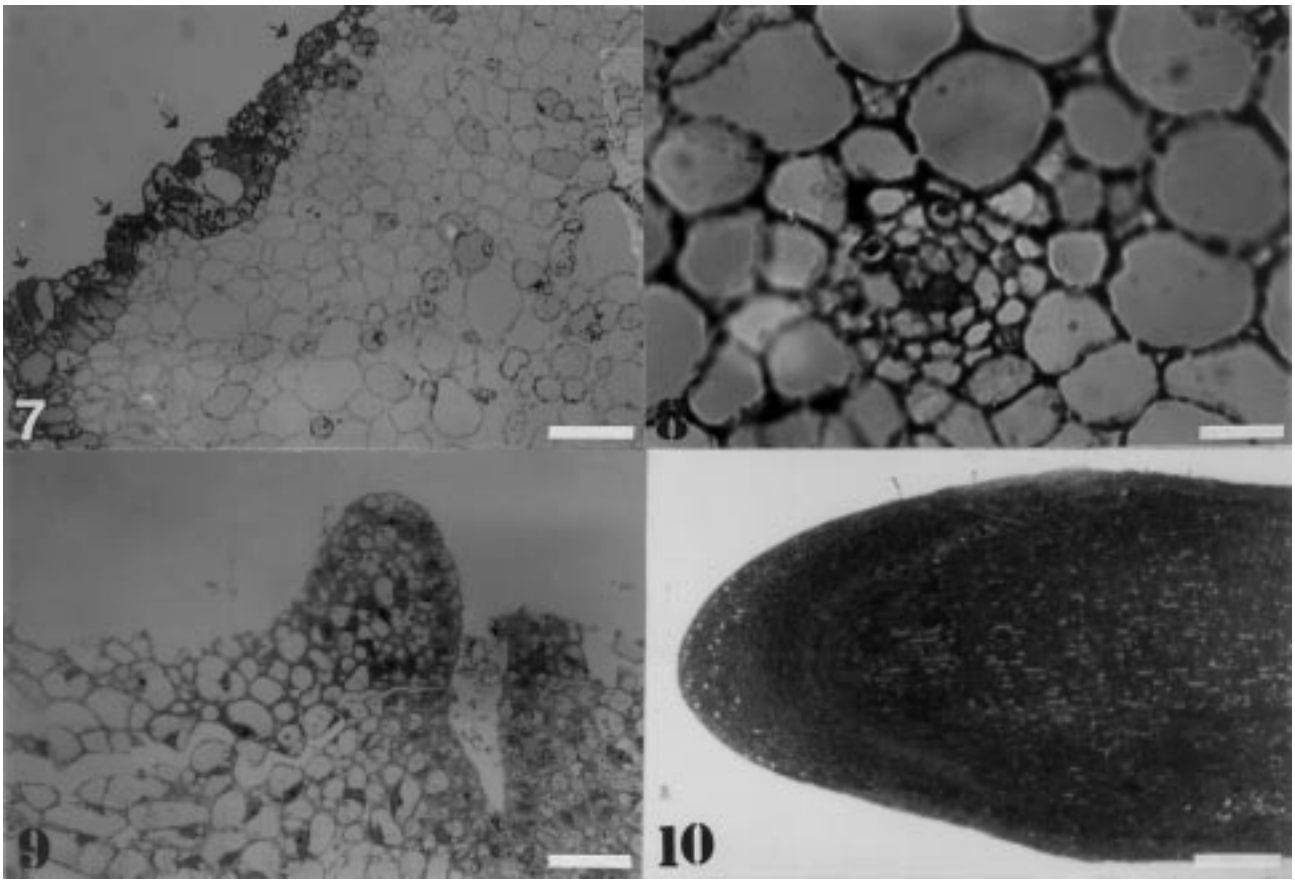
Fig. 1-10. Developmental sequence of the regeneration of somatic embryos on EMS cultures of pistachio (*Pistacia vera* L.) cultured on solid MS medium +1 mg/l BAP) for 27 days; (1) The original status of EMS at day 0, showing vacuolated and parenchymatous cells filled with food reserves. Bar= 100 µm; (2) A magnified view of embryogenic cells arising at cut end of EMS after 3 days of incubation on inducing medium. Note the mitotic figure (arrow) in the embryogenic cells. Bar= 50 µm; (3) Two-cell stage somatic embryo. The arrow indicates epidermal cell divisions. Bar= 150 µm; (4) After 9 days of culture on inducing medium. Arrows indicate vertical section of EMS, showing newly developing embryogenic regions. Bar= 100 µm; (5) After 9 days of culture on inducing medium. Arrow head indicates more newly formed meristemoids within EMS. Bar= 100 µm; (6) Light micrograph of somatic embryo consisting of small embryogenic region and elongate suspensor. Bar= 100 µm;

occasionally found on top of the typical elongate suspensor cells. These small cells may give rise to new somatic embryos by continued cell divisions. In contrast to the cells of the embryogenic region or upper suspensor region, the basipetal suspensor cells seemed to be senescing.

After 9 days of incubation, possibly by a dedifferentiation process, embryogenic cells first began arising along the edge of the EMS, and then after several divisions the cells formed a meristem-like structure (meristemoid), which appeared at the epidermal layer (Fig. 7) and within the EMS (Fig. 8), suggesting that some of cells of the EMS may be preembryonically determined cells (PEDCs).

After 15 days on embryo-induction medium, numerous newly developing meristemoids developed into a meristematic structure within the EMS, and these clusters were developed from the rest of the callus by a specific layer of cells. Their subsequent development into globular embryos (Fig. 9) was also observed in culture. After 27 days on induction medium, the longitudinal section of the developing somatic embryos exhibited root primordia (Fig. 10). From the histological study of initiation and development of somatic embryos in pistachio (*Pistacia vera* L.) reported here, we concluded that the somatic embryos arose indirectly with an intervening callus phase or EMS derived from leaf explants.

The above results clearly demonstrate that the induction and development of somatic embryos from leaf explants of pistachio were only from embryogenic cells at the epidermal and subepidermal layers, whereas, in earlier studies of scanning electron microscopy of



(7) After 11 days of culture on inducing medium. Arrows indicate newly developing somatic embryos. Bar= 100 µm; (8) Globular-stage somatic embryo arising from proliferating meristematic cells within EMS. Bar= 50 µm; (9). Globular-stage somatic embryo arising from proliferating meristematic cells within the embryogenic mass. Bar= 100 µm; (10) Longitudinal section of mature somatic embryo axis passing through root primordia at the time of culture after 27 days of culture on inducing medium. Bar= 100 µm. Embryogenic cells (ec); non-embryogenic cells (nec); epidermis (e); embryogenic mass (ems); suspensor-like cell (slc).

pistachio, they originated from the epidermal layer of cells (8). Cells in the embryogenic tissues are characterised by a dense cytoplasm, diffusely stained chromatin and prominent nuceoli. At present, expression of the embryogenic potential is limited to juvenile tissues.

In the present report, we have documented some morphological characteristics of possible routes of somatic embryo development in pistachio. Embryogenic cells in the callus of leaf explants may be PEDC or induced embryogenic determined cells (IEDC). These parenchymous cells were in a relatively undifferentiated state and may require only the proper hormonal conditions to stimulate divisions. However, it could not be determined in the present study whether embryogenic masses proliferating from leaf explants were of PEDC or IEDC origin. Dedifferentiation during cell divisions and callus formation followed by induction may have occurred. Several molecular markers have been reported that are able to distinguish between embryogenic and non-embryogenic cell clusters (11, 12). To extend the expression of embryogenic potential to explants from mature trees, it is necessary to identify cell or tissue types which are physiologically identical to these juvenile tissues.

Acknowledgements

The research was funded by a grant from the University of Dicle, Faculty of Science and Art, Diyarbakır, Turkey, and carried out under a Scottish Office Agricultural and Fisheries Department licence. Thanks are extended to Mr John Findlay of the Science Faculty Light Microscope Facility for the photography of the tissue cultures.

References

1. Al Barazi, Z., Schwabe, W.W., Rooting softwood cuttings of adult *Pistacia vera*. J. Hort. Sci. 57 (2): 247-252, (1982).
2. Barghchi, M., Alderson, P., Y.P.S., Bajaj (ed.) Biotechnology in agriculture and forestry. vol. 5. Trees II, p. 68-98. Springer-Verlag, Berlin, Heidelberg, (1989).
3. Gonzales, A., Frutos, D., In vitro culture of *Pistacia vera* L. embryos and aged tree explants. NATO ASI series A. 186, 335-338, (1990).
4. Martinelli, A., Loretti, F., Use of in vitro techniques for selection and cloning of different *Pictacia* species. Acta Hort. 227, 436-437, (1988).
5. Onay, A., Jeffree, C.E., Yeoman, M.M., Somatic embryogenesis in cultured immature kernels of Pistachio (*Pistacia vera* L.) Plant Cell Rep., 15: 192-195, (1995).
6. Onay, A., Jeffree, C.E., Yeoman M.M., Plant regeneration from encapsulated embryoids and an embryogenic mass of Pistachio. Plant Cell Rep., 15: 723-726, (1996).
7. Onay, A., Fırat, M.Z., Namlı, O., An improved method for embling production in Pistachio (*Pistacia vera* L.) using liquid medium matured somatic embryos. Turkish Journal of Biology, vol. 21, No 2, 159-174, (1997).
8. Onay, A., Namlı, S., Antepfıstığı'nın (*Pistacia vera* L.) yapraklarında somatik embriyogenesis. XIV. Ulusal Biyoloji Kongresi, Samsun, 7-10 Eylül 1998, Cilt II, Bitki Fizyolojisi-Bitki Anatomisi ve Hidrobiyoloji Seksiyonları, 44-53, Samsun (1998).
9. Jeffree, C.E. Read, N.D., Ambient- and low-temperature scanning electron microscopy. In: Hall JL, Hawes C (Eds.) Electron Microscopy of Plant Cells. Academic Press, London, pp: 313-413, (1991).
10. Maheswaran, G., Williams, E.G., Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens* in vitro. Ann. Bot 56: 619-630, (1985).
11. Pennell, R.I., Janniche, L., Scofield, G.N., Booij H, De Vries S.C., Roberts K., Identification of a transitional cell state in the development pathway to carrot somatic embryogenesis. J. Cell Biol. 119: 1371-1380, (1992).
12. Sterk, P., De Vries, S.C., Molecular markers for plant embryos. In Redenbaugh K (ed.). Synseeds; Applications of Synthetic Seeds to Crop Improvement. pp, 115-132, CRC Press, London, (1992).