BIOLOGIA PLANTARUM 40 (3): 321-328, 1997/98

Papaver somniferum regeneration by somatic embryogenesis and shoot organogenesis

M. OVEČKA, M. BOBÁK*, A. BLEHOVÁ* and J. KRIŠTÍN*

Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 14, 842 23 Bratislava, Slovakia
Department of Plant Physiology, Comenius University,
*Mlynská dolina B-2, 842 15 Bratislava, Slovakia**

Abstract

Secondary somatic embryogenesis and shoot organogenesis from primary somatic embryos of *Papaver somniferum* L. are described. The embryos became malformed, the root meristem expressed dividing activity without position-dependent cell differentiation, causing abnormal development or arrested growth of primary somatic embryos. The adventitious shoots regenerated from embryo hypocotyl, but secondary somatic embryos had an epidermal origin close to the root meristem. The regeneration occurred without hormonal treatment, indicating endogenous nature of triggering signals. These signals are probably related to the integrity loss of morphogenetic steps during development of primary somatic embryos, which appeared to induce an activation of cells competent to regeneration.

Additional key words: embryo hypocotyl, poppy, root meristem, shoot organogenesis.

Introduction

Somatic embryogenesis, as an expression of plant cell totipotency, is widely used way of *in vitro* plant regeneration and propagation. Many experimental systems for the establishment of somatic embryo cultures, with particular characterization of embryo origin and the most effective hormonal treatment were developed. The external hormonal stimuli, such as amount and duration of synthetic auxin application, were shown to be the crucial aspects of somatic embryogenesis (Nomura and Komamine 1985, Komamine *et al.* 1990, Dudits *et al.* 1991, Masuda *et al.* 1995, Masuda and Tokuji 1996). Somatic embryos originate from somatic cells which are more or less differentiated. Embryonic or highly juvenile tissue types are the most appropriate materials for somatic embryogenesis (Maheswaran and Williams 1985, Litz and Gray 1995). Cultures with secondary-repetitive somatic embryogenesis from primary somatic embryo tissue were successfully used to improve the embryogenic

Received 21 March 1997, *accepted* 11 August 1997.
Fax: (+421) 7 371948, e-mail: botuove@savba.savba.sk

potential (Williams and Maheswaran 1986, Maheswaran and Williams 1985, 1986, Dubois *et al.* 1990, Marousky and West 1990, Nadel *et al.* 1990, Gyulai *et al.* 1993).

In *P. somniferum* L., hormones were necessary for induction of indirect somatic embryogenesis from the seedling hypocotyl tissue. Regeneration included formation of the embryogenic tissue - meristemoids (white globular bodies), and somatic embryos proliferating on hormone-free media from the meristemoids (Nessler 1982, Schuchmann and Welmann 1983, Galewsky and Nessler 1986, Wakhlu and Bajwa 1986, 1987, Hsu and Pack 1989, Griffing *et al.* 1989). Embryogenic culture from septa and unriped seeds of poppy capsules was established (Erdelský *et al.* 1990). In this culture, development and morphology of somatic embryos were characterized (Ovečka *et al.* 1996). Somatic embryos with abnormal morphology were impaired in further development (Ovečka *et al.* 1996), but expressed an ability to undergo secondary regeneration without additional hormonal treatment. We reported here a detailed histological view on the process of secondary regeneration with particular characterization of the origin of activated cells and tissues of primary somatic embryos during organogenesis and somatic embryogenesis.

Materials and methods

Primary somatic embryogenesis of *Papaver somniferum* L. cv. Amarín was induced from septa and unriped seeds of poppy capsules with different combinations of naphtaleneacetic acid (NAA) and kinetin, followed by transfering the culture on the hormone-free medium (Erdelský *et al.* 1990, Ovečka *et al.* 1996). Primary somatic embryos arrested in torpedo stage with swollen hypocotyl and without root proliferation were isolated and placed on the solidified MS medium (Murashige and Skoog 1962) without hormones in Erlenmeyer flasks (photosynthetic photon flux of 10 μmol m^{-2} s^{-1}, 16-h photoperiod, temperature of 25 ± 2°C) and subcultured every three weeks.

Embryos for histological studies were fixed in FAA (formalin 40 %, acetic acid 5 %, ethyl alcohol 50 %), dehydrated in ethyl alcohol and embedded in *Histoplast S* (*Serva*, Heidelberg, Germany). Sections cut to a thickness of 7 - 8 μm were stained with hematoxylin-eosin, or periodic acid-Shiff (PAS) reaction. For scanning electron microscopy, embryos were fixed in 3 % glutaraldehyde diluted in 0.1 M phosphate buffer for 48 h at room temperature, pH 7.2. Samples were postfixed in 2 % osmium tetroxide for 1 h, and dehydrated in ethyl alcohol series. After critical point drying in liquid CO_2 the samples were coated with gold and examined in scanning electron microscope *JXA 840A* (*JEOL*, Japan) at 15 kV.

Results and discussion

Some of the primary embryos non-competent for production of plantlets expressed an abnormal proliferation. The embryos were suppressed in torpedo stage of growth, they underwent hypocotyl swelling and shoot development did not occur between the

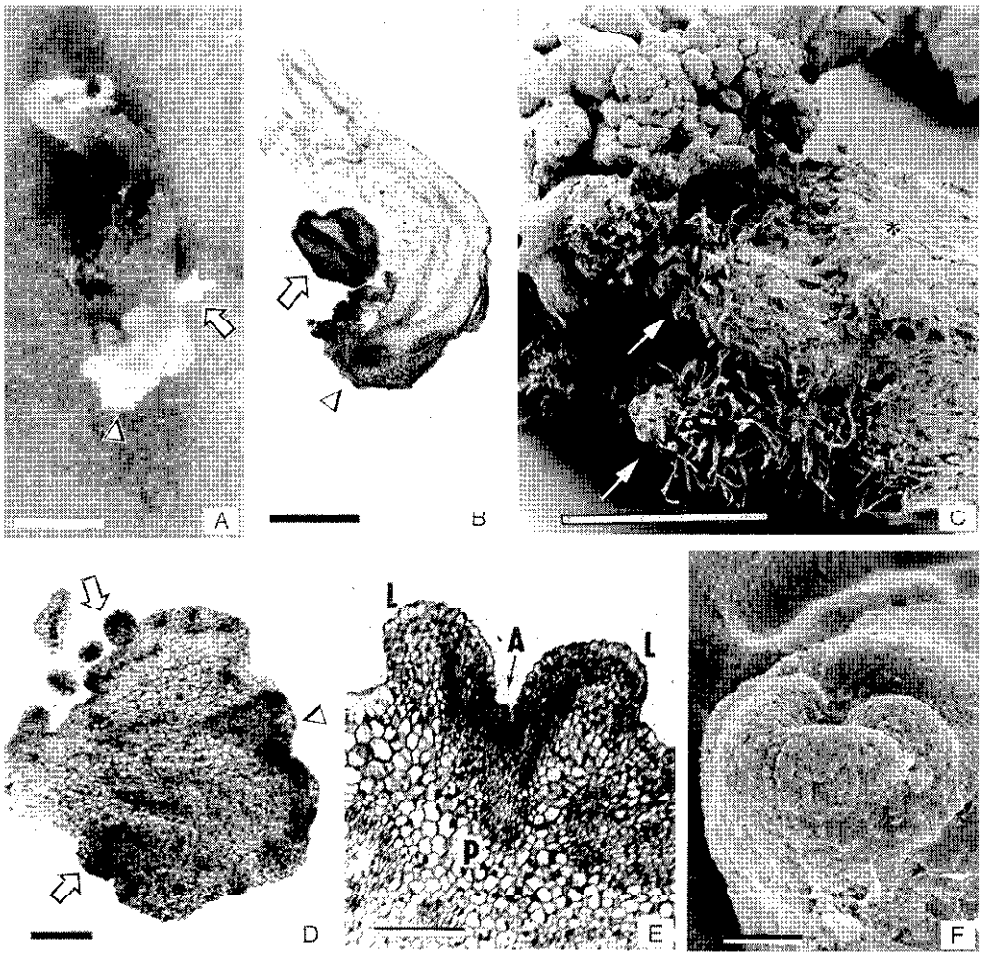


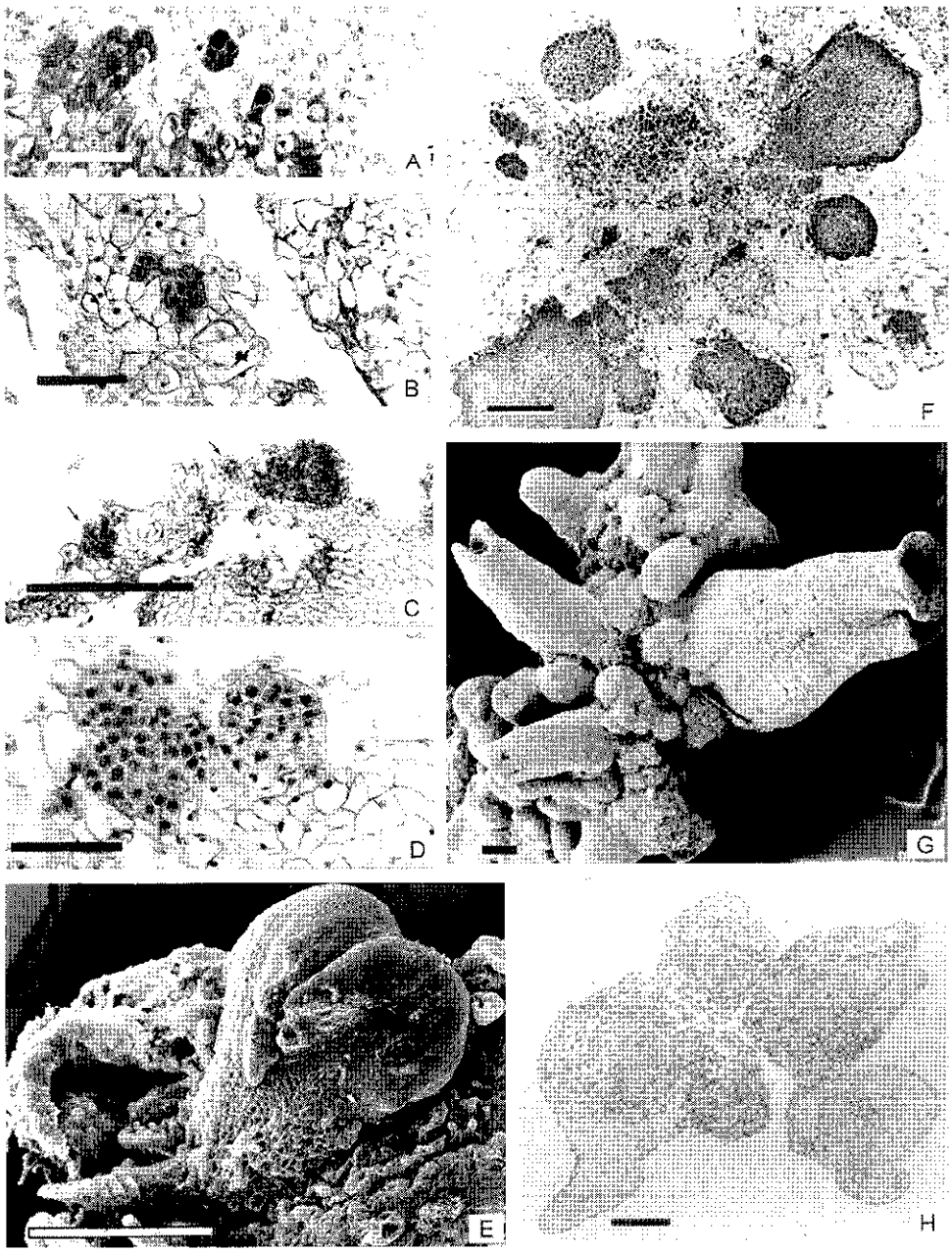
Fig. 1. *A* - arrested primary somatic embryo with secondary somatic embryogenesis on the root pole (*arrowhead*) and shoot bud (*arrow*) emerging from the hypocotyl (bar = 1 mm). *B* - original root meristem of the primary somatic embryo surrounded by dividing tissue (*arrowhead*) with cluster of secondary somatic embryos (*arrow*) (bar = 1 mm). *C* - cluster of secondary somatic embryos on the root pole of primary somatic embryo; adventitious roots (*arrows*) proliferating from thick hypocotyl (*asterix*); primary root is not developed (bar = 1 mm). *D* - meristematic proliferation (*arrows*) on the hypocotyl periphery, leading to the formation of the shoots (*arowhead* - root pole of the embryo; bar = 200 μm). *E* - histology of the regenerating shoot (A - apical meristem, L - leaf primordia, P - procambium; bar = 100 μm). *F* - regenerated shoot apex with leaf primordia (bar = 50 μm).

two cotyledons (Fig. 1*A*). Regardless of embryo proliferation, some embryo cells and tissues expressed the potential for secondary somatic embryogenesis and/or shoot organogenesis in the medium without growth regulators. Growth of the embryos was suppressed due to non proliferating primary root, whereas overdeveloped root meristem was present (Fig. 1*B*). In some cases adventitious roots developed from basal swollen root pole (Fig. 1*C*), but extended root meristem was coupled with dividing tissue in the basal region of the embryo, and the secondary somatic embryogenesis was mostly localised in this region (Figs. 1*A,B,C*). Shoot organogenesis undergone from hypocotyl close to the base (Fig. 1*A*), and other parts of the primary embryos, were not involved in regeneration. Embryonal procambial strands were disorganized and also hypocotyl epidermis formed woven structures (Fig. 1*B*). Similar growth supression of the primary embryonic axis (Williams and Maheswaran 1986, Maheswaran and Williams 1986), or abnormal development with fusing cotyledons (Gyulai *et al.* 1993) were frequently reported during secondary somatic embryogenesis using both zygotic and somatic embrya as an initiating tissue. Abnormal development or inhibited growth could be responsible for inducing promotion conditions for expression of embryogenic potential and proliferation of competent embryo-forming cells.

Shoot organogenesis started with meristematic activity in the hypocotyl tissue (Fig. 1*D*). Peripheral cells were activated, and the origin of the shoots in peripheral hypocotyl cell layers was clearly visible (Fig. 1*E*). Shoot apical meristem produced procambial strands (Fig. 1*E*), and leaf primordia (Figs. 1*E,F*), autonomously apart from the ground embryo tissue and other activated organogenic loci.

Contrary to organogenesis, only basal part of the swollen hypocotyl regenerated secondary somatic embryos. Root pole of primary somatic embryos possessed disorganized root meristem and produced dividing tissue (Fig. 1*B*). Single competent cells within this cell population closely to the hypocotyl epidermis were detected by both high cytoplasmic density and content of many starch grains (Fig. 2*A*). From these cells small proembryos regenerated with decreased cell size and high cytoplasmic density (Fig. 2B). Small proembryos and globular embryos were formed on the surface of the hypocotyl tissue (Figs. 2*C,D*). This observation could be in agreement with other studies of somatic embryogenesis, which documented that the embryonal hypocotyl, particularly the hypocotyl epidermis appears to be an appropriate material for expression of somatic embryogenesis (Konar *et al.* 1972, Williams and Maheswaran 1986, Maheswaran and Williams 1985, 1986, Masuda *et al.* 1995, Masuda and Tokuji 1996). Swollen primary embryo epidermis at the root pole had a teratogenic appearance (Figs. 2*B,C*), but the epidermal and subepidermal

Fig. 2 (*on the following page*). *A* - single competent embryo-forming cells with high cytoplasmic density and starch grains (bar = 50 μm). *B* - two secondary somatic proembryos within the epidermal cell layer - longitudinal section (bar = 100 μm). *C* - proembryo and globular somatic embryo (*arrows*) in cross section (bar = 500 μm). *D* - globular somatic embryos with developed protoderm (bar = 100 μm). *E* - heart-shaped somatic embryo on the surface of the hypocotyl (bar = 500 μm). *F* - many somatic embryos in different stages of development in the disorganized hypocotyl tissue (bar = 200 μm). *G* - cluster of globular, heart-shaped and torpedo secondary somatic embryos (bar = 100 μm). *H* - cluster of individual somatic embryos (bar = 200 μm).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origin of secondary somatic embryos can be recognized. They were present on the hypocotyl surface, not breaking it (Fig. 2*E*).

During development and growth of the secondary somatic embryos the original hypocotyl tissue became disorganized (Fig. 2*F*). Meristematic proliferation on the root pole destroyed cell and tissue organization of the hypocotyl and basal region of the embryo, which resulted in formation of clusters of competent cells and proembryos (Figs. 1*C*, 2*G*). The embryos in the clusters were arranged very closely to each other (Fig. 2*G*), and the histological view has shown a group of individual embryos with no presence of callus tissue, illustrating direct somatic embryogenesis from individual competent cells clustered before (Fig. 2*H*). Both individual embryos and clustered embryos were formed directly with single-cell origin. In *Trifolium repens,* regeneration of secondary somatic embryos through the multicellular proliferation of superficial embryo cells without dedifferentiated callus formation was reported as a direct one (Maheswaran and Williams 1985, 1986), and the multicellular origin of somatic embryos was shown (Maheswaran and Williams 1986). In the somatic embryogenesis of *Medicago sativa* the embryos had multicellular origin in an organized callus and unicellular origin in a friable callus and in the epidermis of cotyledons, hypocotyls and roots of primary somatic embryos on hormone-free medium (Santos *et al.* 1983). Barciela and Vieitez (1993) reported embryo formation of unicellular origin from the "few-celled" nodules on the abaxial surface of the *Camellia japonica* cotyledons together with multicellular origin of embryos in embryogenic areas, and a short, localised callus phase (nodule formation) was necessary for the redetermination (Barciela and Vieitez 1993). Depending on the environmental stimuli, experimental systems with direct and/or indirect somatic embryogenesis were described. Indirect induction was enhanced after a long time of synthetic auxin application and subculturing of calli on the fresh media or pro-embryogenic masses (PEMs) in liquid media, contrary to direct regeneration, which can be induced without subculturing and after a short auxin treatment (Dudits *et al.* 1991, Emons 1994). Establishment of the embryo-forming cells described here was not achieved by external hormonal stimuli [with the respect of the hormonal pretreatment during the establishment of primary embryo culture (Erdelský *et al.* 1990, Ovečka *et al.* 1996)]. This embryogenic cell population (Figs. 1*B*, 2*A*) seems to be similar to the formation of PEMs, established during indirect somatic embryogenesis of carrot in the presence of 2,4-D (Komamine *et al.* 1990, Emons 1994). However, formation of embryo-competent cells on the root pole of primary poppy embryos required no direct hormonal induction and occurred without any general cell dedifferentiation (Figs. 2*G,H*). We suppose that dividing activity of the cells only capable to embryogenesis was released. They expressed embryogenic potential like in the permissive induction, allowing the embryogenic potential of induced cells to be expressed (Christianson 1985, 1987). Cells in root meristem of malformed embryos failed to differentiate after cessation of cell division, which tended to elicit a local stress conditions and their totipotency expression. Production of malformed embryos and high somaclonal variability in the primary culture limited the potential for regeneration of complete plants (Ovečka *et al.* 1996). Secondary somatic embryogenesis reported here did not improve dramatically the conversion

ability (not tested statistically). Organogenesis produced shoots competent to rhizogenesis, however, secondary somatic embryogenesis was a dominant way of regeneration from primary somatic embryos. The process of regeneration was triggered by some stimuli linked to disconnected processes of morphogenesis and cell differentiation within embryo tissues, which induced production of dedifferentiated, competent cells. The stimulus did not represent an external hormonal treatment, indicating endogenous and position dependent manner of the signals, which induced shoot organogenesis and secondary somatic embryogenesis.

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