BIOLOGIA PLANTARUM 45 (2): 173-182, 2002

Morphology and anatomy of *Pisum sativum* somatic embryos

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Abstract

The morphological and anatomical aspects of direct and indirect somatic embryogenesis in pea were described. Direct embryos were induced from shoot apical meristems of 3 to 5-d-old pea seedlings, embryogenic callus originated from immature pea zygotic embryos or shoot apices. Auxin (picloram, 2,4-dichlorophenoxyacetic acid) was necessary to induce somatic embryos. The developmental stages typical for pea zygotic embryos were detected. Globular and heart-shaped somatic embryos were morphologically similar to their zygotic counterparts; in contrast, torpedo and cotyledonary somatic embryos displayed great morphological variation, which affected mainly cotyledons (size, shape, number). Based on anatomical sections, possible ways of somatic embryo formation and localization of initiation sites within primary explant tissue have been proposed. The multicellular origin of somatic embryos is supposed in both systems of pea somatic embryogenesis under investigation.

Additional key words: auxins, direct and indirect somatic embryogenesis, grain legumes, pea regeneration *in vitro*.

Introduction

The understanding of both the induction, initiation and development of somatic embryos is crucial for a better regulation of these processes for various experimental or practical objectives. The knowledge of regeneration mechanisms including the initiation sites of regeneration and the cells/tissues involved in embryo formation is of high importance for choosing optimum strategy in particular technology. The data obtained with somatic embryos may also improve our knowledge of embryo and seed development *in planta* and thus may enable to exploit these results in plant breeding programmes (regulation of storage substances accumulation and their deposition in legume seeds). Somatic embryogenesis in grain legume crops is recently a relatively well elaborated system (Griga 1999). In pea, both direct (Tétu *et al.* 1990, Bencheikh and Gallais 1996, Griga 1998) and indirect, callus-mediated (Kysely *et al.* 1987, Kysely and Jacobsen 1990, Stejskal and Griga 1992, Van Doorne *et al.* 1995, Loiseau *et al.* 1995, 1996) somatic embryogenesis was reported. However, only limited information exists about morphological and anatomical aspects of the process (Tétu *et al.* 1990, Kysely and Jacobsen 1990, Loiseau *et al.* 1998, Griga 1999), which may affect the possible use of the system for generating of somaclonal variation on one hand (Griga *et al.* 1995, Griga 1999) and for transgenic plant production on the other hand (no transgenics have been produced via somatic embryogenesis in pea up to date - Griga 1999).

In this paper, we present the morphological and anatomical description of *1*) pea somatic embryo initiation from initial explants via direct and indirect way of regeneration, and of *2*) pea somatic embryo development.

Received 28 March 2001, *accepted* 8 August 2001.

Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; MSB-medium - medium with mineral salts according to Murashige and Skoog (1962), vitamins according to Gamborg *et al.* (1968); NAA - α-naphthaleneacetic acid.

Acknowledgements: The author wishes to thank Ms. J. Vysloužilová for excellent technical assistance. This research was supported by the Grant Agency of the Czech Republic (grant No. 206/96/K188) and INCO-COPERNICUS grant No. IC15-CT96-096.

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Materials and methods

Plants: Forty seven pea (*Pisum sativum* L., *P. arvense* L.) cultivars/breeding lines were induced to form somatic embryos (Stejskal and Griga 1992, Griga 1998). Anatomical and morphological observations were mainly done with dry seed pea line HM-6 and canning pea cv. Oskar. Several hundreds of somatic embryos were investigated morphologically and selected (mostly typical) ones were subjected to anatomical analyses.

Indirect somatic embryogenesis: Embryogenic callus was induced from the whole immature zygotic embryos on MSB-medium with 2.26 μM 2,4-D (Stejskal and Griga 1992) or from shoot apices (3 to 5 mm in size) isolated from 3 to 5-d-old etiolated seedlings on MSB-medium with 2.5 μM picloram (Griga 1998). Development of somatic embryos took place completely on auxin induction medium or the embryos were transferred to BKZ-medium (Lazzeri *et al.* 1987) or MSB-medium without growth regulators.

Direct somatic embryogenesis: Somatic embryos were induced directly (without callus intervention) from shoot apical meristems (0.5 - 1.0 mm in size) isolated from 3 to 5-d-old etiolated seedlings and cultured on MSB-medium with 2.5 μM picloram (Griga 1998). Development of somatic embryos took place completely on auxin induction medium or the embryos were transferred to MSB-medium without growth regulators.

Morphology of somatic embryo initiation and development was studied and photographed with stereomicroscope *Technival* (*Carl Zeiss*, Jena, Germany) on colour reversal *Fuji* film *Sensia II 100*, *Provia 100*, and *Velvia 50* (Tokyo, Japan).

Anatomy: For anatomical/histological studies, cultured explants as well as particular developmental stages of somatic embryos were fixed in Navashin's fixation for 24 h and then rinsed in running tap water for 24 h. After ethanol/xylene dehydration objects were embedded into paraffin. Sections of 10 μm in thickness were cut and stained with basic fuchsin and picroindigocarmine. Samples were analysed and photographed with cytological microscope *Jenaval* (*Carl Zeiss*, Jena, Germany) on *Ilford* black and white negative film Pan 100 and Pan F Plus 50 (Mobberley, UK).

Results

Morphology and anatomy of embryo initiation - direct somatic embryogenesis: Somatic embryos initiated directly (without callus intervention) from meristematic domus of cultured shoot apical meristem, preferentially from leaf primordia/axillary bud primordia area. First visible morphological structures (pale green globular embryos) were evident within two weeks of culture on induction medium (Fig. 1*A*). Somatic embryos appeared as single ones and/or in rows or clumps (Fig. 1*A,B*). In the course of further week of culture on induction or hormone-free medium still another embryos initiated, while older ones rapidly grew and developed (asynchronous development); more developed embryos often exhibited dark green color (Fig. 1*B*). Two types of embryo initiation were observed. Frequently, the initial explants were represented for a relatively long culture period (1 to 2 weeks) by more or less homogenous tissue composed predominantly of meristematic cells, with delayed cell expansion (Fig. 1*C*). The globular structures originated from the apical part of these explants (meristematic dome) as extrusions of epidermal and several subepidermal cell layers. At least some of these structures may be identified as leaf/axillary bud primordia, whose original developmental programme was terminated and changed into embryogenic development. Distinct epidermis usually covered directly developed pea somatic embryos (Fig. 1*C*). The embryogenic nature of such structures was proved by tracing their further morphological and anatomical development as well as by the presence of storage proteins typical for pea sexual seeds (Griga *et al.* 2001). Nevertheless, in some cases when auxin induction stimulus was not sufficient enough (early subculture from auxin induction medium to hormone-free medium), this development could be converted back to organogenesis (or organogenesis – leaf and shoot bud differentiation – started to dominate over embryogenesis). Thus, such explant may contain both somatic embryos and shoot buds or developing leaves. In another type of development, the cells of explanted apical meristem gradually entered the expansion phase (Fig. 1*D*) and only some epidermal and subepidermal cells/group of cells still maintained division ability, which resulted in formation of globular proembryos (Fig. 1*E,F*). The location of embryo initiation sites in leaf/axillary bud primordia area was again often observed (Fig. 1*D,E,F*). Protoderm formation preceeded the true epidermis formation in this type of embryo initiation. In both types of direct embryogenesis the globular proembryos/ embryos originated as a result of coordinated growth of a group of embryogenically competent cells of the explant rather than a result of division of a single cell (Williams and Maheswaran 1986). Thus, multicellular origin of direct pea somatic embryos is supposed. Somatic embryos were mostly attached to the maternal

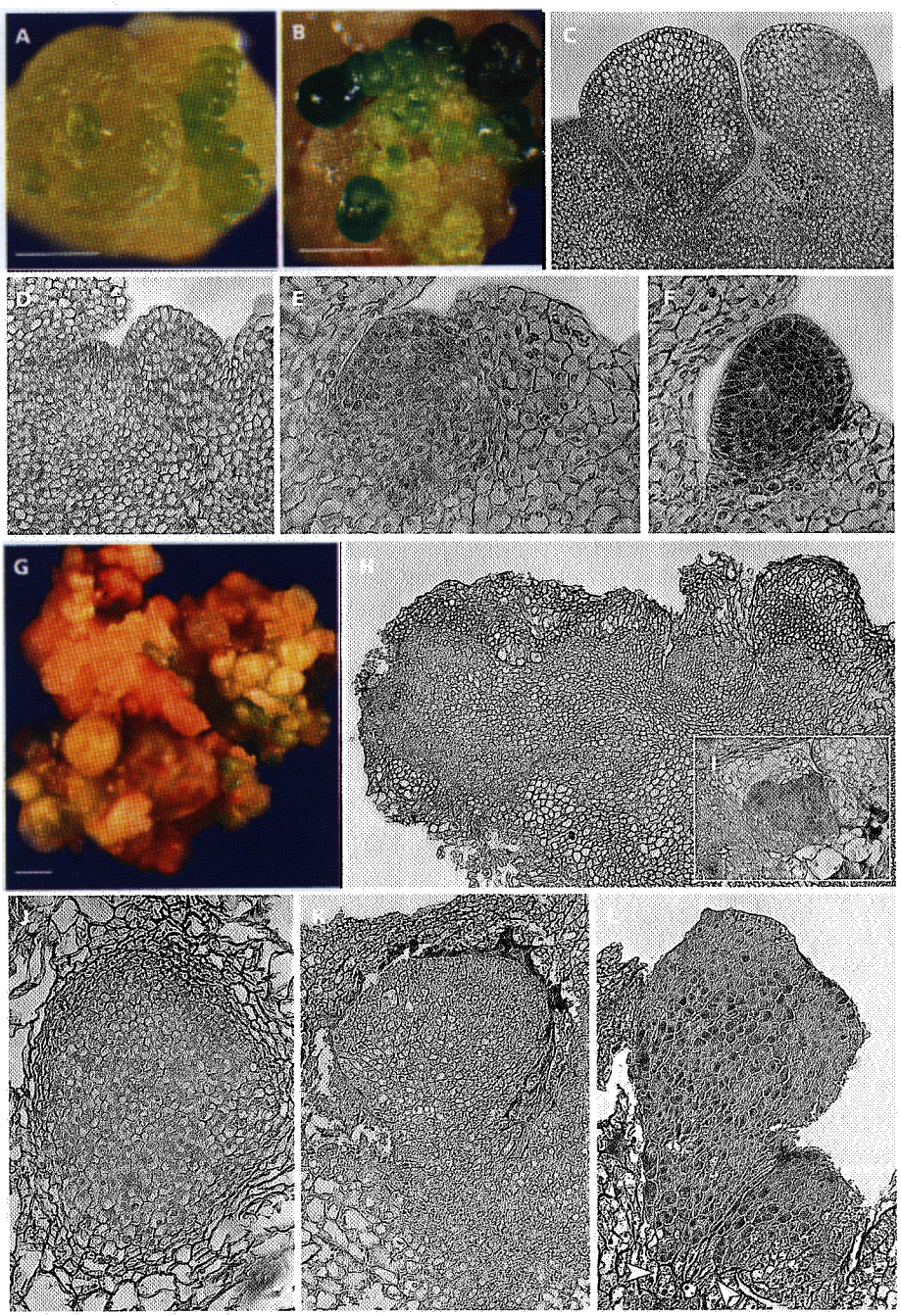

Fig. 1. Morphology and anatomy of pea somatic embryo initiation. *A - F* - direct somatic embryogenesis; *G - L* - indirect (callus-mediated) somatic embryogenesis. *A, B* - initiation of globular somatic embryos from leaf/axillary bud primordia area of meristematic dome in 2-week-old (*A*) and 3-week-old (*B*) explant; *bar* (*A*, *B*) represents 1 mm. *C* - anatomical representation of situation in *A*, *B*; globular embryos originating from homogenous meristematic tissues of meristematic dome; the change of development of preexisting leaf primordia/axillary buds into somatic embryos assumed; × 40. *D* - cell expansion in apical meristem explant; section through leaf primordium/axillary bud primordium area shows a group of still meristematic cells; × 50. *E, F* - distinctly formed globular proembryos surrounded by expanding cells which lost division potential; × 100. *G* - 8-week-old callus derived from shoot apex with somatic embryos in various stages of development; *bar* represents 1 mm. *H* - gradual cell expansion in pea shoot apex explant and its change into callus (4-week-old explant); some cells or groups of cells inside the callus still retain division potential - probable initiation sites of somatic embryos; × 25. *I* - detail of embryogenic callus consisted of various types of cells from small meristematic ones to highly parenchymatous cells; proembryo initiation in the centre; × 50. *J* - globular proembryo surrounded by parenchymatous callus cells; × 50. *K* - globular embryo inside the callus connected by broad basis to maternal explant tissue; × 31.25. *L* - globular proembryo and elongated (heart-shaped) embryo with densely stained meristematic cells; connection with maternal tissue via suspensor-like structures (*arrows*); × 31.25.

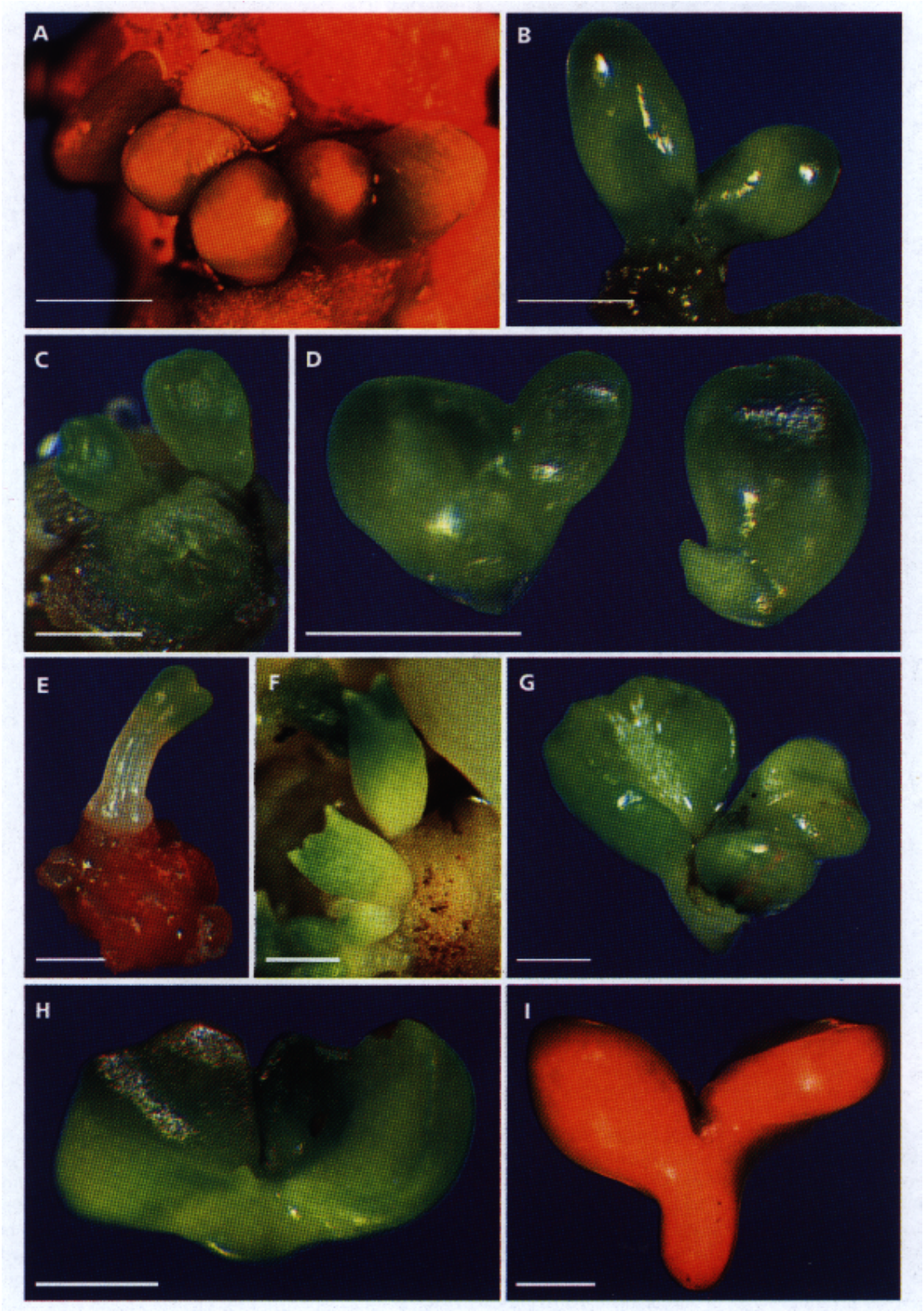

Fig. 2. Morphology of pea somatic embryo development. *A* - globular embryos. *B* - oblong embryos. *C* - transition to heart-shaped embryos. *D* - heart-shaped embryos. *E, F* - torpedo stage embryos. *G, H, I* - cotyledonary embryos. *H* - cotyledonary somatic embryo corresponding with its organ proportions (cotyledons versus embryo axis) to pea zygotic embryo. This morphological type was observed less frequently. *I* - Fully morphologically and physiologically mature cotyledonary somatic embryo. More frequent type with robust hypocotyl and proportionally smaller cotyledons. *Bars* represent 1 mm.

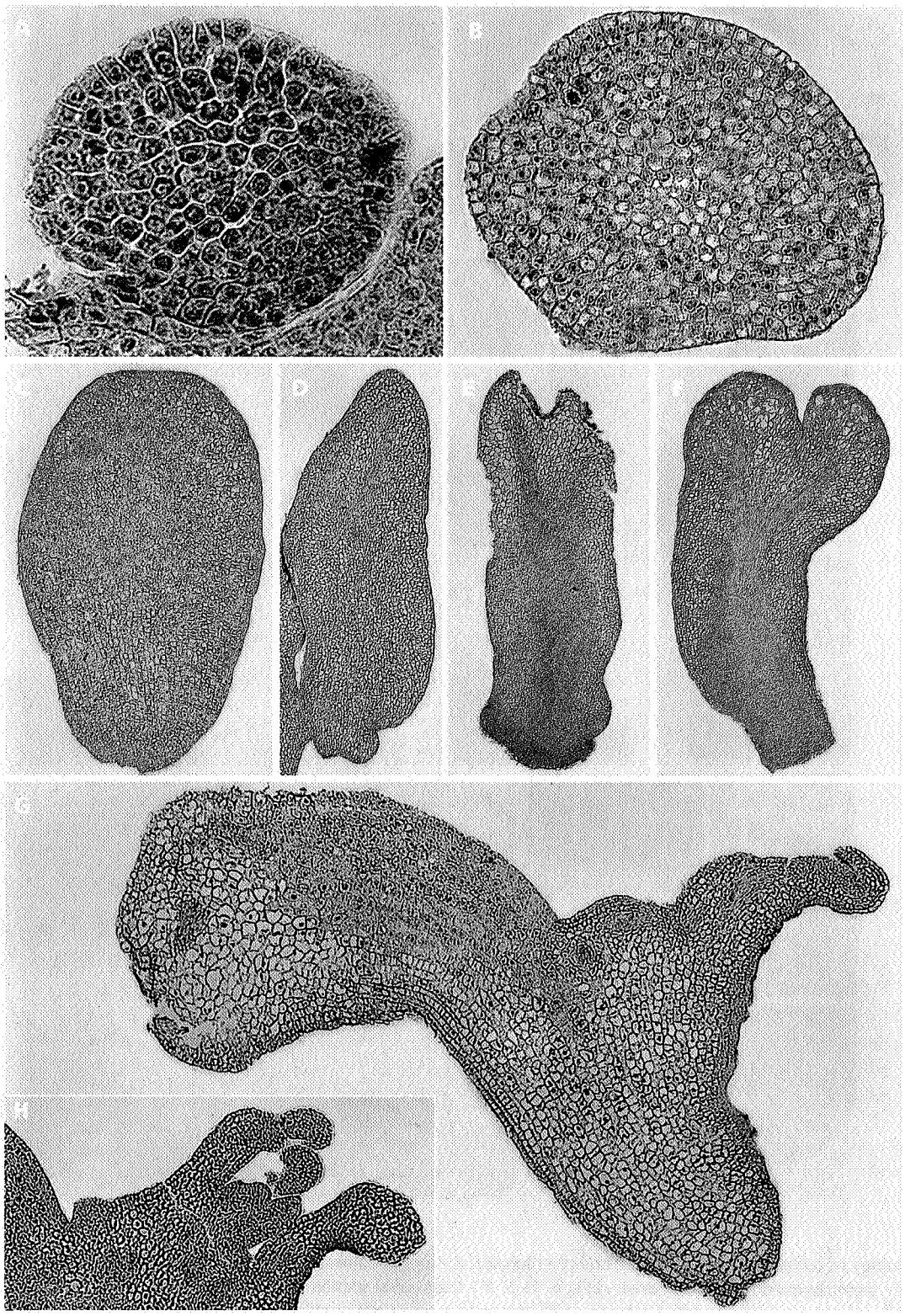

Fig. 3. Anatomy of pea somatic embryo development. *A* - globular proembryo; × 50. *B* - globular embryo with distinct epidermis; × 100. *C* - oblong stage embryo; transition to torpedo stage; × 20. *D*, *E* - torpedo stage; (D - × 12.5, E - × 25). *F* - transition from torpedo to early cotyledonary stage; × 10. *G* - cotyledonary embryo with differentiated root and shoot pole; × 12.5. *H* - detail of shoot apex of the same explant as in *G* in another section plane; × 12.5.

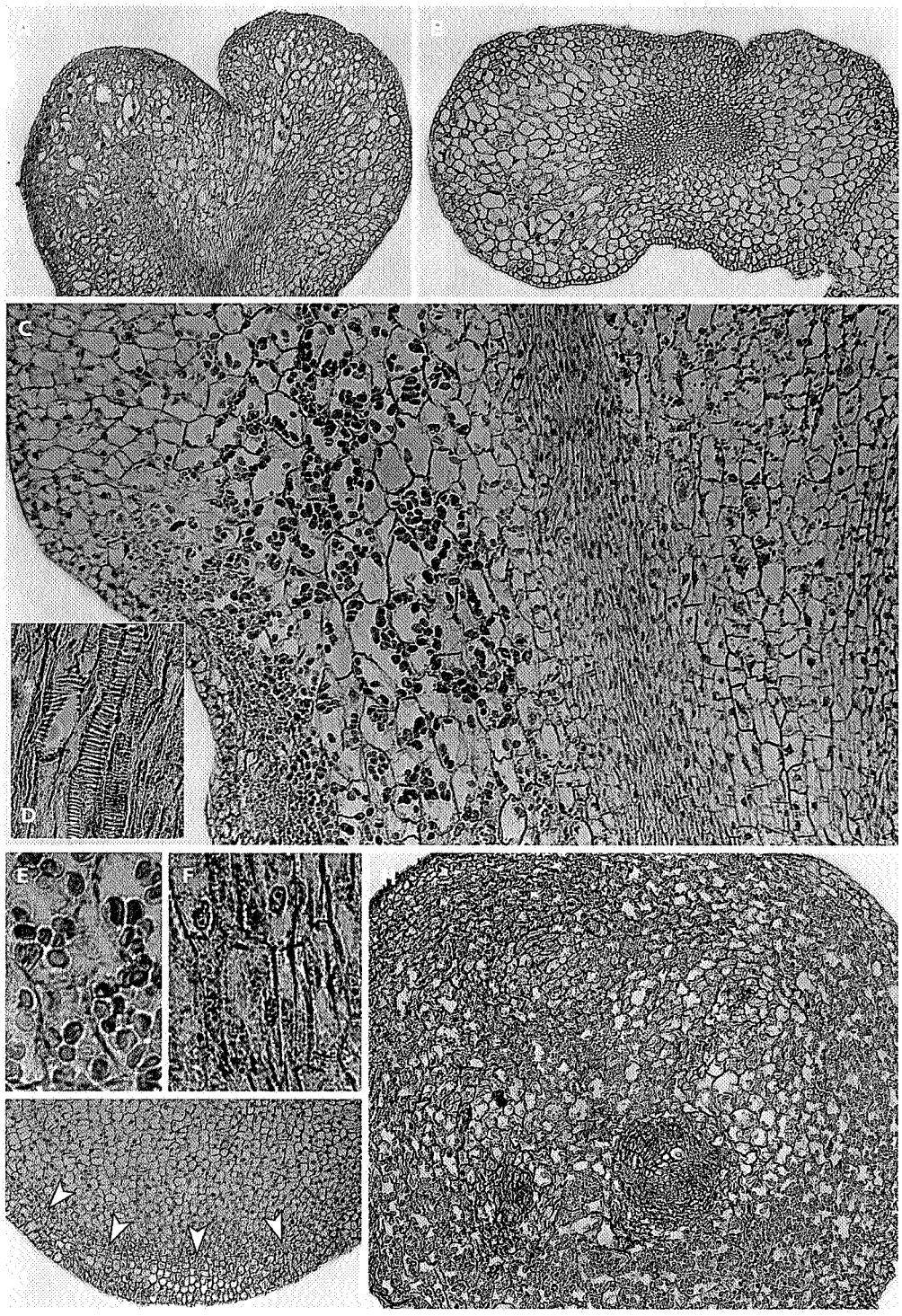

Fig. 4. Histology of pea cotyledonary somatic embryo (details). *A* - longitudinal section of upper part of embryo in early cotyledonary stage; highly parenchymatous primary cortex cells; × 15.6. *B* - transversal section in the basal part of cotyledons; epidermis, highly parenchymatous primary cortex, central cylinder with two meristematic procambial bundles; × 20. *C* - longitudinal section of the mature cotyledonary embryo in the upper part of hypocotyl; advanced differentiation of tissues visible: epidermis, primary cortex with numerous starch grains, central cylinder with two procambial bundles branching into cotyledons; × 31.25. *D* - differentiation of procambial cells into vascular elements; × 125. *E* - detail of parenchymatous primary cortex cells with starch grains; × 125. *F* - detail of elongated procambial cells with elongated nuclei; × 125. *G* - distinctly closed root pole of an embryo with a cell zone (*arrows*) resembling a root cap; × 50. *H* - transversal section in the similar position as in (*C*); × 25.

tissue by broad basis (Fig. 1*C,F*), suspensor-like structures were rarely observed. This fact may be also considered as an argument for multicellular origin of somatic embryos. In general, callus formation was located mainly in the cut area of the explant (basal part) and was delayed as compared to embryo initiation. We did not find any evidence that callus cells played a role in embryo initiation.

Morphology and anatomy of embryo initiation - indirect somatic embryogenesis: The cells of initial explant expanded and the whole explant (immature zygotic embryo or shoot apex) gradually turned into callus. Epidermal and subepidermal tissues were usually completely disrupted and lost their cell division ability. Only in the more inner parts of the explant the regions of meristematic, still dividing cells remained (Fig. 1*H,I*). These cell groups, which were markedly delineated from surrounding parenchymatous callus cells or even necrotic tissues, later gave rise to globular proembryos/embryos, which subsequently emerged through the callus on the explant surface (Fig. 1*G,J,K,L*). Formation of suspensor-like structures was more frequent as compared to direct somatic embryogenesis (Fig. 1*K,L*). We have never observed somatic embryogenesis in pure callus culture, *i.e.*, after separation of calli from initial explants (containing meristematic tissues) and subculturing them for several subcultures. Somatic embryogenesis was always restricted to tissues containing meristematic regions of initial explant.

Morphology of somatic embryo development: Developing pea somatic embryos exhibited clearly distinct globular, heart-shaped, torpedo and cotyledonary stages (Fig. 2*A-I*). The absolutely symmetrical individuals resembling zygotic embryos in shape, organ proportions (heart-shaped somatic embryo) and size were observed in globular and heart-shaped somatic embryos. Nevertheless, typicall heart-shaped somatic embryos were observed in our experiments relatively rarely. In contrast to zygotic embryogenesis in pea where torpedo stage is practically absent (Liu *et al.* 1995, 1996), we may frequently find this stage during somatic embryogenesis. While the globular and heart-shaped somatic embryos exhibited regular shapes, the torpedo and particularly cotyledonary stage exhibited broad spectrum of morphological abnormalities involving mainly cotyledons: reduced or increased number of cotyledons, various size of cotyledons in dicotyledonary somatic embryos, fused cotyledons, trumpet- or horn-shaped somatic embryos, foliar- or cauliflower-shaped somatic embryos, or quite irregular somatic embryos (Fig. 5). The proportion of gross morphological abnormalities may vary from 40 to 80 % as related to particular culture protocol and genotype used. In some cases we may observe absence of heart-shaped stage or torpedo stage, *i.e.*, globular somatic embryos elongated through the oblong-stage (Fig. 2*B*) into torpedo stage or heart-shaped stage developed directly into cotyledonary stage. This influenced final shape of cotyledonary somatic embryos and mainly the proportions of individual organs (cotyledons versus hypocotyl) from types with robust hypocotyl and small cotyledons and *vice versa* (Fig. 6). Thus, some types proportionally more corresponded to mature zygotic embryo of pea (Fig. 2*H*). Globular, oblong and heart-shaped somatic embryos exhibited usually light to dark green colour (Fig. 2*A,B,C,D*), torpedo and cotyledonary ones displayed colour gradient – cotyledons and upper part of hypocotyl were green, lower part of hypocotyl and root pole were creamy or yellowish (Fig. 2*E,F,G*). However, the coloration of somatic embryos may be significantly affected by culture regime (*e.g.* media for development and maturation; data not shown).

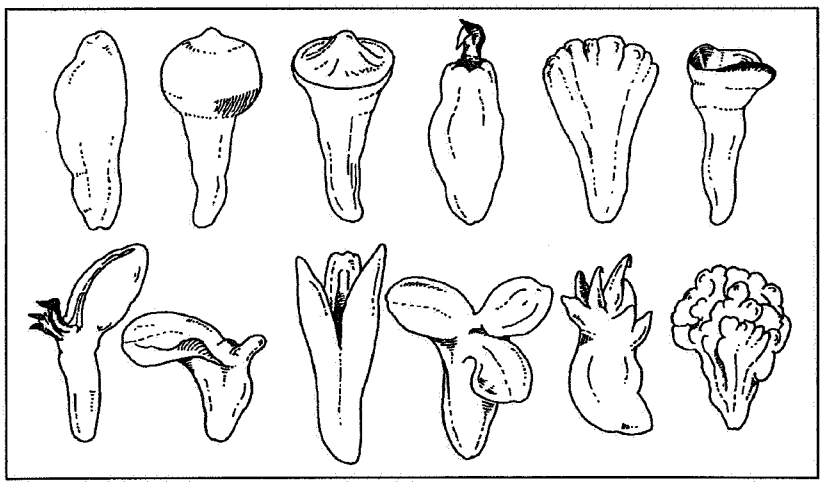

Fig. 5. Morphological abnormalities of pea cotyledonary somatic embryos.

Anatomy of somatic embryo development: Anatomical sections showed distinct radicular ends of somatic embryos without vascular communication to maternal tissue of initial explant (Fig. 3*C,D,E,G*). Early stages of somatic embryos (globular, heart-shaped) exhibited homogenous meristematic character of tissue (Fig. 3 *A,B*), the cells contained dense cytoplasm with large round-shaped nuclei. A protoderm constitution preceeded the true epidermis formation in callus-mediated somatic embryogenesis. In direct somatic embryogenesis, an epidermis of initial explant covered emerging somatic embryos originating from epidermal/subepidermal cells. Successively, the vacuolated parenchymatous embryonic cortex as well as meristematic procambial strands progressively differentiated (Figs. 3*C,D,E,F*, 4*A,B,C*). Morphologically normal cotyledonary somatic embryos had differentiated distinct root and shoot pole (Fig. 3*G,H*). In the root pole of torpedo and cotyledonary embryos, the cell zone resembling root cap was sometimes observed (Fig. 4*G*). Procambial cells contained elongated nuclei (Fig. 4*C,F*) reflecting the shape of the cells; procambium bifurcated into forming cotyledons. In mature somatic embryos procambial cells started to differentiate into elements of vascular system (Fig. 4*D*). Parenchymatous cells of embryonic cortex of hypocotyl and cotyledons contained frequently numerous granules of starch (Fig. 4*C,E,H*) as one of the main storage substance of pea seed.

Discussion

Pea somatic embryogenesis is connected predominantly with embryogenically competent cells of initial explants (shoot apices, shoot apical meristems, immature zygotic embryos and their parts, *i.e.*, embryonic axes and cotyledons, and nodes from cotyledon-free seedlings - Tétu *et al.* 1990, Kysely and Jacobsen 1990, Stejskal and Griga 1992, Van Doorne *et al.* 1995, Loiseau *et al.* 1995, 1996, 1998, Griga 1998, Griga and Klenotičová 2000).

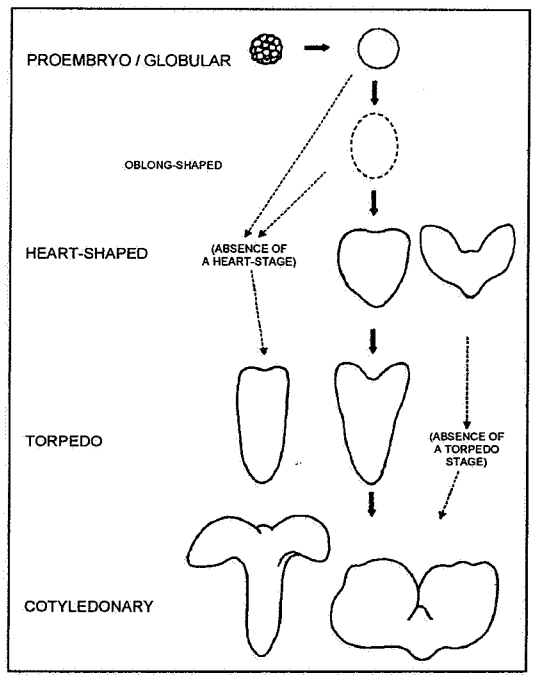

Fig. 6. Observed morphological modifications of pea somatic embryo development.

Despite of some exceptions (cotyledon callus; Ozcan *et al.* 1993, Nadolska-Orczyk *et al.* 1994, Loiseau *et al.* 1998), these explants contain organized meristems whose cells play a key role in embryo intitiation (Kysely and Jacobsen 1990, Van Doorne *et al.* 1995). These competent meristematic cells give rise to somatic embryos either directly or after several mitotic cycles. These observations may be supported by the fact that no complete somatic embryogenesis in pea was reported in pure callus culture (maintained for a longer period of time) or cell suspension, but somatic embryos developed always in connection with initial explant. The only exception is induction of pea somatic embryos from protoplast-derived calli (Lehminger-Mertens and Jacobsen 1989). Unfortunately, this pioneering work was not repeated later by other laboratories. In addition, no fully differentiated plant parts (with specialized non-meristematic cells) were successfully used for pea somatic embryogenesis.

The location of initiation sites within the explant as well as the mode of embryo initiation and the cells/tissues involved may be affected by a number of factors, of which the most important are: explant type, its physiological state and position on agar medium, type and concentration of auxin used, and duration of auxin treatment (Hartweck *et al.* 1988, Hepher *et al.* 1988, Yeung 1995). Thus, certain combinations of these factors led to different observations by various authors describing anatomically pea somatic embryogenesis. Tétu *et al.* (1990) induced direct somatic embryos from immature cotyledons after 4 - 5 weeks of culture on medium with NAA, in darkness. Embryos appeared to arise directly from small cells of meristematic character scattered among the differentiated epidermal and subepidermal cells without intermediate callus formation. In contrast, Kysely and Jacobsen (1990) induced embryogenic callus from embryo axes of immature embryos and from shoot apices on medium with 2,4-D or picloram. Embryogenic callus contained regions of meristematic cells, located near the callus surface. At the periphery of such meristematic regions the earliest globular somatic embryo stages could be observed. Loiseau *et al.* (1998) were able to induce embryogenic

callus from shoot apices and cotyledons of immature zygotic embryos with the use of picloram. The whole shoot apices evolved into embryogenic calli. Proembryos arose after 2 to 3 weeks from peripheral cell layers of the callus. In contrast to shoot apex explants, embryogenic calli from immature cotyledons were formed only from the wounded area caused by embryonic axis excision. First embryos initiated from peripheral cell layers of the callus after 10 - 15 d. The authors suggested a concept of positive effect of starch development in maternal explant tissues (shoot apex versus immature cotyledon) on somatic embryo initiation and development.

Using shoot apical meristems we were able to induce direct somatic embryogenesis with relatively low auxin concentration (2.5 μM picloram). In contrast to data of Tétu *et al.* (1990) and Loiseau *et al.* (1998), the initial explant stayed relatively long in homogenous, nearly meristematic stage; callus formation in the first two weeks was absent or negligible (located on cut surface of slightly swollen apical meristem). Thus, both morphologically and from anatomical sections, it was clearly evident that globular proembryos/embryos originated superficially - from epidermal, subepidermal and sometimes outer cortical tissues. Embryos originated as single ones or in rows or clumps preferentially in the area of leaf primordia/axillary buds of the apical meristem. We assume the direct involvement of leaf/axillary bud primordia in the formation of at least some embryos (Kysely *et al.* 1987, Kysely and Jacobsen 1990, Kara 1995, Bencheikh and Gallais 1996, Griga 1998, Loiseau *et al.* 1998). Similarly as Ozcan *et al.* (1993) we have observed that somatic embryogenesis may convert to adventitious shoot development (organogenesis) if the auxin stimulus is not sufficient (when the initial explant is subcultured very early from auxin induction medium to hormone-free medium). In contrast, the indirect embryos in our experiments initiated exclusively from the groups of meristematic cells inside the explant (shoot apex, immature zygotic embryo, embryo axes) which after auxin treatment (2,4-D, NAA, picloram) completely turned into callus (Stejskal and Griga 1992, Griga 1998). As we reported previously (Griga *et al.* 1995, Griga and Létal 1995, Griga 2000), callus mediated somatic embryogenesis may generate somaclonal variation. There are no available data about genetic status of pea plants produced via direct somatic embryogenesis, but high degree of genetic uniformity may be expected due to the use of shoot apical meristem as initial explant. Based on morphological and anatomical data presented here we presume the pea shoot apical meristems as a suitable target for genetic transformation via particle bombardment.

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