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Extracellular matrix in early stages of direct somatic embryogenesis in leaves of *Drosera spathulata*

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Abstract

Leaves from mature *in vitro* grown plants of *Drosera spathulata* Labill. regenerated new plantlets on solid induction medium in light. Especially vascular sheath parenchyma cells located close to basal part of tentacule showed high embryogenic potential. Proembryoids arrising from the tentacule base part were visible by scanning electron microscopy. Their surface cells were linked and covered with thin external, fibrilar network representing an extracellular matrix (ECM). Proembryogenic surface cells were later connected by coarse strands of fibrils. Young protoderm was formed arround globular embryoids and its cells were characterized by "brain-like" surface structure. However, the surface of fully developed protodermal cells was practicaly smooth and cells were stick to each other very tightly in torpedo and cotyledonary shaped embryoids. The presence of ECM was typical only for somatic proembryos and globular embryos. The ECM network was never observed on the surface of heart and torpedo shaped embryos.

Additional key words: benzylaminopurine, cell surface, 2,4-dichlorophenophenoxyacetic acid.

Introduction

Routine regeneration of medical herbs *in vitro* is basic assumption for high production of important secondary metabolites. Plants can regenerate *in vitro* through organogenic or embryogenic route. Various species of genus *Drosera* can regenerate *in vitro* by different pathways. We observed organogenesis in leaves of *Drosera spathulata* (Bobák *et al.* 1989, 1993) and direct plant regeneration in *Drosera rotundifolia* (Bobák *et al.* 1995).

The early stages leading a somatic cell to regenerate a complete new plant seems to be very important. Cell reactivation during induction of somatic embryogenesis is connected with the reprogramming of gene expression and reorganisation of internal cellular architecture, affecting cell morphology and pattern of cell division (Cyr *et al.* 1987, Emons 1994). *Drosera* can serve as a good model for study of early phases of plant regeneration *in vitro*. Recently a considerable attention is paid to extracellular matrix surface network (ECMSN), of which the chemical composition and structural arrangement on the cell surface may play fundamental role in cell recognition, cell-to-cell interaction, cell division and differentiation and also in generation and maintenance of some traits in plant cell populations. ECM was seen first on the surface of *Coffea* proembryoid cells (Sondahl *et al.* 1979). Dubois *et al.* (1992) observed ECM surrounding somatic proembryos in *Cichorium* roots and leaves and discussed its function (in cell recognition before forming of protodermis) and chemical character. The occurrence of ECM was also reported during proembryo formation in *Drosera*, *Zea* and *Papaver* (Šamaj *et al.* 1995, Bobák *et al.* 1999, Ovečka

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenophenoxyacetic acid; MS-medium - medium with mineral salts according to Murashige and Skoog (1962); DMSO - dimethylsulfoxide; ECM - extracellular matrix; ECNSN - extracellular matrix surface network.
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and Bobák 1999) and *Pinus* (Jásik *et al.* 1995). Their structural components of ECM could be evaluated as an integral part of the ECM – plasma membrane – cytoskeleton continuum (Wyatt and Carpita 1993, Reuzeau and Pont-Lezica 1995, Flower and Quatrano 1997).

The cell surface structure of somatic proembryos and embryos in various stages of their development on solid medium was studied by SEM in this paper. It was intended as preliminaries to a cytochemical study determining chemical nature of ECM and ultrastructural study of proembryoids which are in progress.

Materials and methods

Plants and tissue culture: The plants of *Drosera spathulata* Labill. were grown on (Murashige and Skoog 1962) medium without hormones, a photon flux density (PFD) of 32 μmol m^{-2} s^{-1} (400 - 700 nm), 16-h photoperiod and temperature of 24 ± 2 °C. The excised leaves of mature *Drosera* plants were used as explants. After isolation they were placed with the adaxial side on solid induction medium MS with various concentrations of hormones (0.05 mg dm^{-3} or 0.5 mg dm^{-3} 2,4-D; 0.5 mg dm^{-3} BAP) separately or together in light (PFD of 40 - 45 μmol m^{-2} s^{-1}, 24 ± 2 °C. Orientation of explants was very important. When abaxial side of leaf was facing the medium no regeneration was observed on upper adaxial side. Best plant regeneration was achieved in 90 mm sterile plastic dishes containing induction MS medium with low concentration of 2,4-D (0.05 mg dm^{-3}). Many abnormalities were observed using both hormones 2,4-D and BAP in higher concentrations (1 mg dm^{-3}).

Scanning electron microscopy: Material for SEM was prefixed in 5 % glutaraldehyde dissolved in 0.1 M phosphate buffer for 3 h (room temperature, pH 7.2) and postfixed in 2 % aqueous OsO_4 for 2 h at room temperature. Samples were dehydrated in ethanol and dried by CO_2 critical point drying system. Then they were sputtered with gold-paladium and observed with *JEOL JXA 840A* (Tokyo, Japan) scanning electron microscope by 10 kV.

Transmission electron microscopy: Samples for transmission electron microscopy (TEM) were fixed in 5 % glutaraldehyde, buffered with phosphate buffer for 4 h, and postfixed in 2 % osmium tetroxide for 2 h, buffered with the same buffer. After dehydration in acetone, the samples were embedded in *Durcupan ACM* (*Fluca*, Buchs, Switzerland). Ultrathin sections stained with uranyl-acetate and lead citrate were examined using *TEM 2000 FX* (*JEOL*).

Results and discussion

Histological and cytological studies using a light microscope (the step by step section method) show formation of differently large clusters of dividing reactivated parenchymatic cells of leaf vascular bundles (Fig. 1). Globular somatic embryos are formed from these clusters in culture after 10 d (Fig. 2).

The first symptom of direct somatic embryogenesis is visible by SEM, when somatic proembryos are emerging through leaf epidermis in basal part of tentacule on 10^{th} day after placing explants on induction medium (Figs. 3, 4).

More detailed TEM studies have shown that embryogenic cluster cells are characterized by a conspicuous ECMSN composed of fibrillar material (Fig. 5). Such networks have been identified by SEM (Figs. 6, 7). Especially in early stages of proembryoid formation the network is fully developed on the solid induction medium (Fig. 8). Function of ECM in cell recognition and communication during proembryoid differentiation was also reported in *Cichorium* leaves (Dubois *et al.* 1991) and roots (Dubois *et al.* 1992). Authors also proved its protein character by protease digesting and connection with cell cytoskeleton by colchicine and cold treatments, which led to microtubule destabilization and removing off the network. We also obtained useful information about reversibility and irreversibility in the ECM formation using SEM method and cytoskeleton toxin treatments (Bobák *et al.* 1999). We have found that 4 h effect was partly reversed by DMSO, while that of 6 h was irreversible. It seems that toxins (trifluralin, colchicine) probably caused proembryo cell collapse and death after 6 h toxins treatments. Colchicine effect was partly reversed by DMSO, while cold effect was fully reversed by high temperatures. DMSO has been used to improve reparation ability of plasmalema and cell surface (cell wall) during genetic transformation of plant cells. Global reorganization of cell architecture (especially microtubule organization) and polarization during induction of somatic embryogenesis caused by asymmetric cell division are well known (Cyr *et al.* 1987, Dijak and Simmonds 1988). The active function of actin microfilaments in cell shape determination and karyokinesis (especially nuclear positioning) during embryogenesis has also been discussed (Staiger and Schliwa 1987, Katsuta and Shibaoka 1988).

During further differentiation of somatic proembryos (up to 15 d on inducing medium) the network is gradually reduced to coarse strands linking neighbouring surface cells (Fig. 9).

The development of embryoids is not synchronized. Various stages – globular, heart and torpedo shaped embryoids are observed after 21 d on induction medium. While protodermis is formed by periclinal cell divisions of surface cells in globular shaped embryoids the ECM was practically disappeared (Fig. 10). Young protodermal cells have typical "brain-like" surface structure with deeper "furrows" between the cells (Fig. 11). This structural feature is observed under optimal culture conditions, when the influence of stress factors is relatively minimalized. However, "brain-like" aspect has changed gradually during protodermis maturation when the stomata and mother tentacule cells are differentiated. Protodermal cells have become more tightly connected without deep "furrows" and their surface has been remaining more and more plate. The surface of protodermal cells is practically smooth (except slight precipitate caused by sample preparation) and cells are sticking to each other very tightly (similarly like in epidermal leaf cells) in cotyledonary shaped embryoids (Fig. 12). After 21 d in culture, plantlets regenerated via somatic embryogenesis into young complete plants and

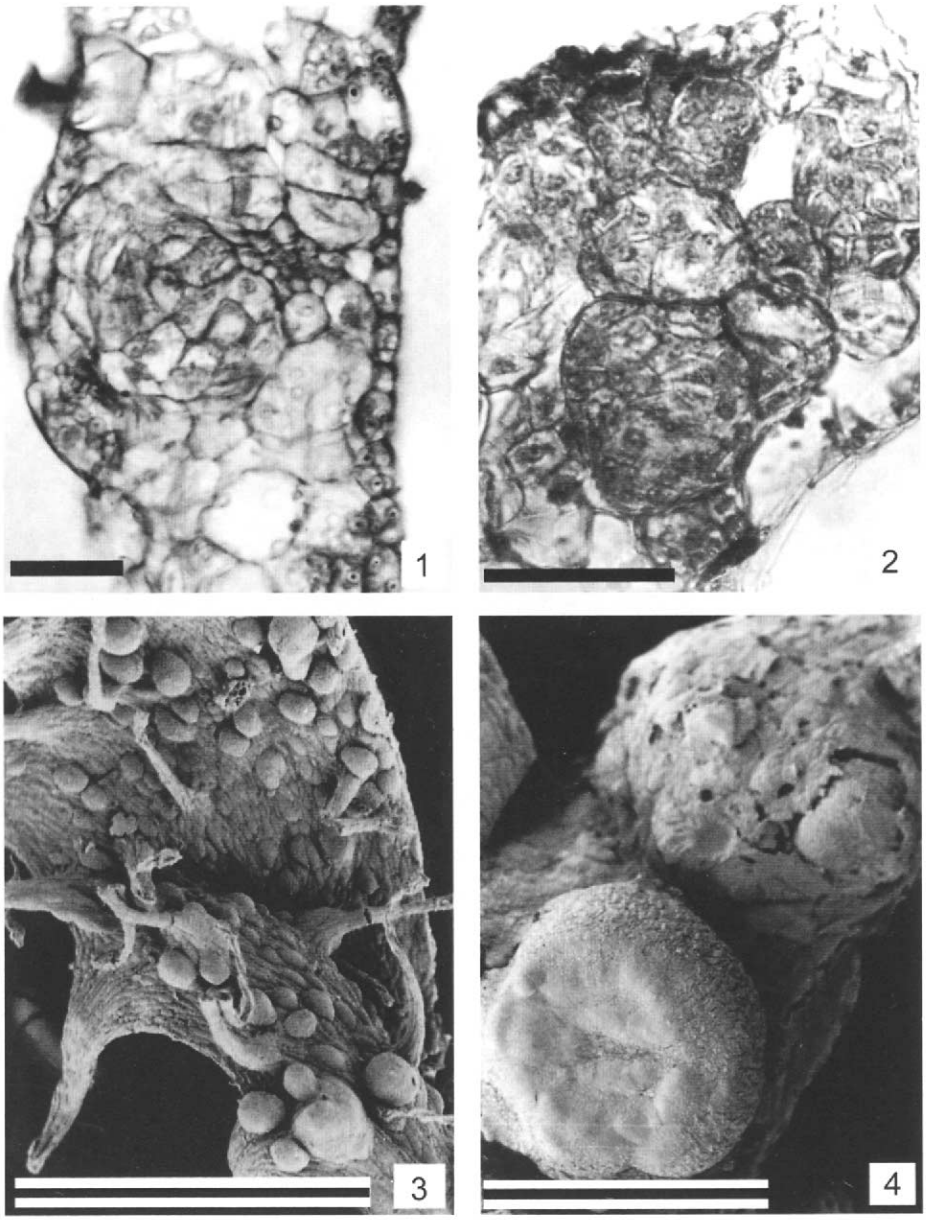

Fig. 1. Leaf cross section showing formation of a large cluster of dividing reactivated cells. *Bar* = 50 μm.
Fig. 2. Multicellular proembryos in central leaf part. *Bar* = 50 μm.
Fig. 3. SEM view of the leaf surface with various stages of somatic embryos after 10 d in culture. *Bar* = 1 mm.
Fig. 4. Two embryos in different stages of differentiation. Note globular structure emerged from explant. *Bar* = 1 mm.

are easily separated from mother explant tissue. They grow to maturity after their transfer to the medium without hormones (Fig. 13).

Several molecular markers have been detected in inductive phase and the progression of somatic embryogenesis (review see Dudits *et al.* 1991). The role of callose (Dubois *et al.* 1990) and extracellular proteins and glycoproteins for development of multicellular structures including somatic embryos, has already been discussed (De Vries *et al.* 1988, Dubois *et al.* 1992, Knox 1995, Kreuger and Van Holst 1995, Nothnagel 1997, Šamaj *et al.* 1999). It seems that ECM network can serve as an early morphological structural marker on the surface of regeneration competent cells during direct embryogenesis.

Changes in cell morphology during somatic embryogenesis reflecting the reorganization of structural components of somatic cells *in vitro* can be a part of their adaptation to stress. We observed the ECM in defined culture conditions (light, solid induction medium) and sample preparation only surrounding developing proembryos. We were able to see very well developed network of ECM and its gradual degradation during further embryoid differentiation in our model. Dubois *et al.* (1991, 1992) claimed that liquid induction medium is much better than solid one for ECM network

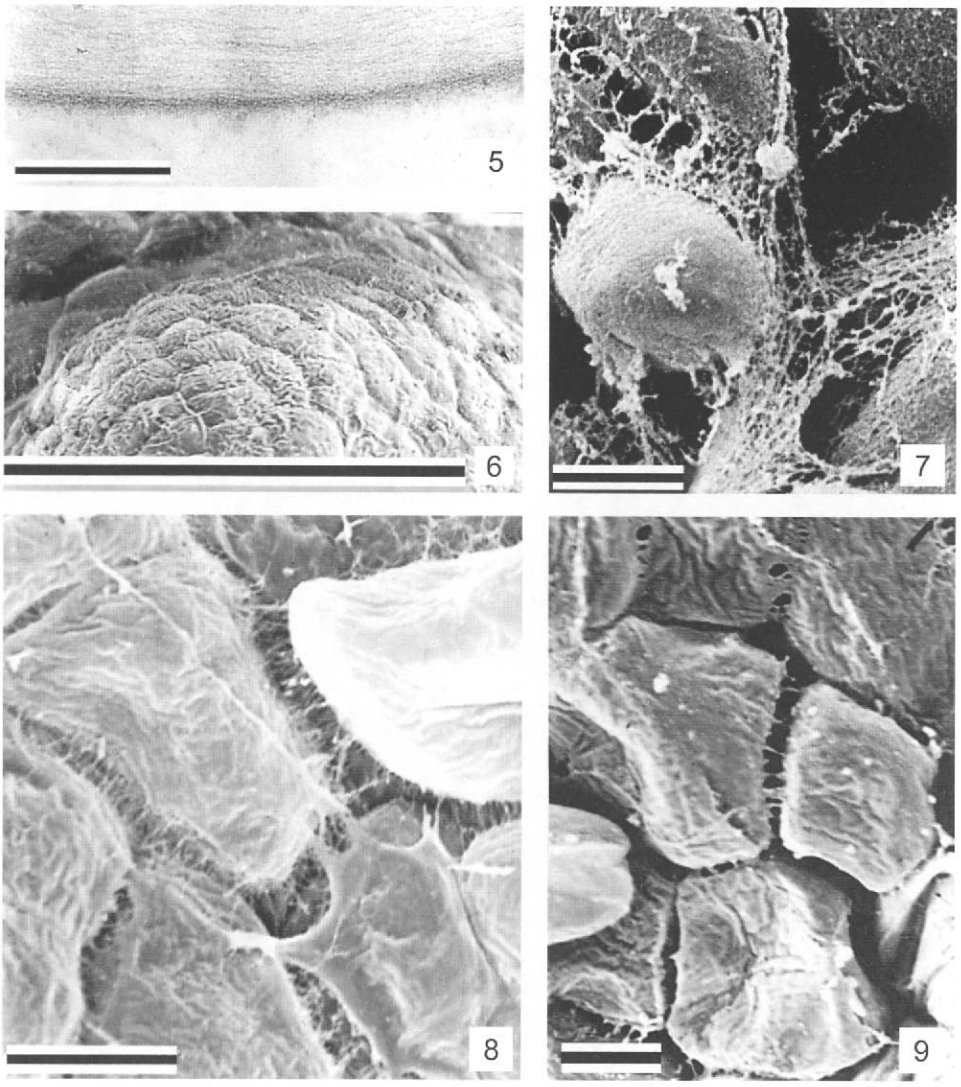

Fig. 5. Transmission electron microscopy of peripheral cells of somatic embryos with conspicuous fibrillar structures. *Bar* = 600 mm.
Figs. 6, 7. Extracellular matrix organized in the network on the surface cells of somatic embryos after 10 d. *Bar* = 100 μm, 20 μm.
Fig. 8. Detail of fully developed ECM linking surface cells together. *Bar* = 10 μm.
Fig. 9. Fine bridges of net-like material between the cells gradually reduced strands linking neighbouring surface cells. *Bar* = 10 μm.

development in *Cichorium*. The possible effect of liquid induction medium (agitated and nonagitated) in our system was also studied. However, preliminary data did not prove it, which can be caused by genotype specificity and different culture requirements leading to somatic embryogenesis in *Cichorium* and *Drosera* representing evolutionary very distant plant species. The chemical nature of the ECM network is still not well known. Now it became more obvious that some cell wall molecules might be involved in determination of cell position within plant organs and embryogenic clumps as well as in cell-cell communication (Knox 1995, McCabe *et al.* 1997, Nothnagel 1997).

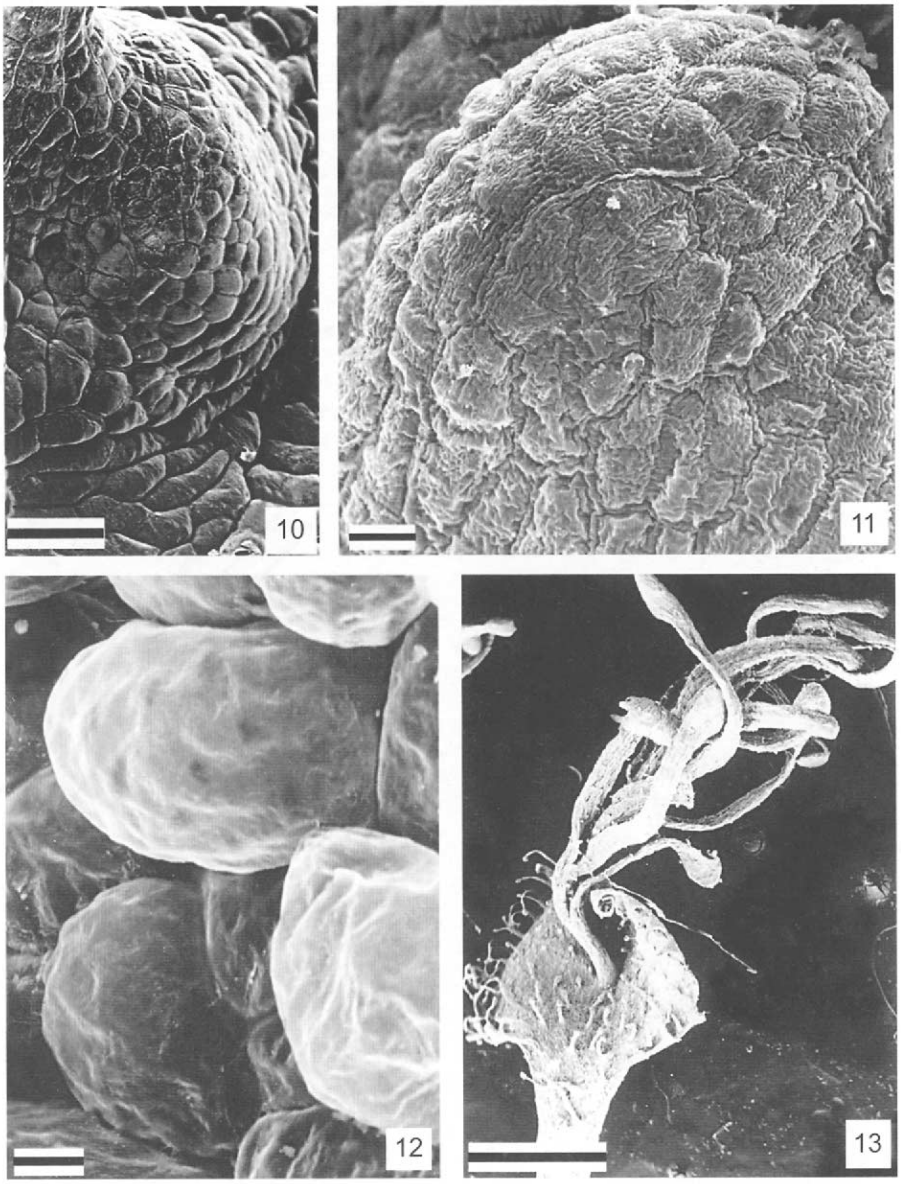

Fig. 10. Globular somatic embryo with young protodermal cells without extracellular matrix after 15 days of culture. *Bar* = 10 μm.
Fig. 11. Globular somatic embryo with "wrinkled" protodermal cells. *Bar* = 10 μm.
Fig. 12. Protodermal cells having smooth surface structure. *Bar* = 10 μm.
Fig. 13. Regenerated young plant on the surface of a mother leaf explant after 40 d of culture. *Bar* = 1 mm.

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