Plant Cell, Tissue and Organ Culture **43:** 43–49, 1995.

Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*

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Received 4 May 1994; accepted in revised form 15 June 1995

Key words: Histology, morphogenesis, scanning electron microscopy

Abstract

Shoot regeneration was obtained from isolated leaves of *Drosera rotundifolia* L. cultured on MS media with various concentrations of 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA). The best direct shoot organogenesis was obtained on growth regulator-free medium or medium supplemented with 10^{-8} M NAA. Liquid culture medium significantly increased regeneration capacity of leaf tissue. Histological and scanning electron microscopy investigations verify direct plant regeneration without intermediate callus formation. Leaf epidermal cells showed the highest regeneration potential leading to the regeneration of buds. Young shoots with three to seven leaflets rooted spontaneously on the growth regulator-free medium within 38 days of culture and isolated mature plants produced fertile seeds.

Abbreviations: BA – 6-benzyladenine; FAA – 40% formalin (5%) + 90% acetic acid (5%) + 70% ethanol (90%); MS – Murashige and Skoog's (1962) medium; NAA – α-naphthaleneacetic acid; plumbagin – 5-hydroxy-2-methyl-1,4-naphthoquinone; 7-methyljuglone – 7-methyl-5-hydroxy-1,4-naphthoquinone; SEM – scanning electron microscopy; TEM – transmission electron microscopy; PPF – photosynthetic photon flux

Introduction

The carnivorous plant *Drosera rotundifolia* L. is a medicinal herb containing valuable secondary metabolities. The most important are 1,4-naphthoquinones, especially plumbagin and 7-methyljuglone (Schölly & Kapetanidis 1989; Crouch *et al.* 1990). Extracts from the plants containing these naphthoquinones have antimicrobial, antifungal and antitumoural effects (Fujii *et al.* 1992). Moreover, plumbagin can also inhibit development of insects and parasitic nematodes (Fetterer & Fleming 1991).

Most of the research on the tissue culture of sundew until now has stressed micropropagation. The main goals were have been the optimization of culture conditions (Simola 1978; Anthony 1992), leading to the higher production of important secondary metabolites *in vitro* (Blehová *et al.* 1992). Bonnet *et al.* (1984) have reported that the content of active secondary substances is up to six-fold higher in cultures of *Drosera rotundifolia* than in plants.

However, morphological studies describing the direct regeneration of *Drosera rotundifolia in vitro* are absent in the literature. There are only two reports on the regeneration of *Drosera spathulata* from callus and leaf cultures through organogenesis (Bobák *et al.* 1989, 1993).

In this study 49 different media were screened to evaluate their effect on regeneration from leaf explants of *Drosera rotundifolia* L. This is the first report on the precise shoot origin and development of shoots from leaf epidermal cells using both histological and scanning electron microscopy methods.

Table 1. Influence of various concentrations of BA and NAA on shoot regeneration on *Drosera rotundifolia* leaves after 60 days of culture.

NAA (M) \ BA (M)	0	10^{-8}	10^{-7}	10^{-6}	5×10^{-6}	10^{-5}	2×10^{-5}
0	R[1]	R	R	C	C	C	C
10^{-8}	R	R	R	C	C	C	C
10^{-7}	R	R	R	C	C	C	C
10^{-6}	ΔN	ΔN	ΔN	ΔC	ΔC	ΔC	ΔC
5×10^{-6}	ΔN	ΔN	ΔN	ΔC	ΔC	ΔC	ΔC
10^{-5}	ΔN	ΔN	ΔN	ΔC	ΔC	ΔC	ΔC
2×10^{-5}	ΔN	ΔN	ΔN	ΔC	ΔC	ΔC	ΔC

[1]R – shoot regeneration, C – callus formation, N – necrosis;
Δ – red pigmentation of explants and calli induced by medium composition

Materials and methods

Plant material and tissue culture

Leaves from aseptically grown plants of *Drosera rotundifolia* (MS medium, 16-h photoperiod, PPF of 32 μmol $m^{-2}s^{-1}$, cool white fluorescent bulbs, 24 ± 2 °C) were used as explants. After isolation they were placed with the abaxial part in contact with 49 variants of MS medium supplemented with various concentrations of BA and NAA, 3% sucrose, and 100 mg l^{-1} myoinositol (Table 1). The pH of the media was adjusted to 5.8 before the addition of 0.7% agar (Difco Bacto), and the mixture was then autoclaved at 105 kPa for 15 min. Growth regulators and agar were omitted from liquid MS medium. Liquid cultures were placed on a gyratory shaker at 120 rpm and all cultures were maintained at 24 ± 2 °C under a 16-h photoperiod using cool white fluorescent lights with a PPF of 32 μmol m^{-2} s^{-1} (400–700 nm). The numbers of regenerated shoots per leaf explant were counted on both solidified and liquid shoot induction media (in three independent experiments, with a total of 90 leaf explants on each medium) after 28 days of culture. Statistical analyses were performed with the two-way ANOVA test.

Light microscopy

Explants for light microscopy were sampled at 0, 8, 16, 24, 48, 72 h and 10, 14 and 18 days of culture. Samples for light microscopy were fixed in a mixture of ethanol-acetic acid (3:1) or FAA for 24 to 48 h, dehydrated in a graded butanol series with 30-min intervals and embedded in paraffin wax. Sections of 15 ± 1 μm were stained with basic fuchsin and picroindigocarmine. Samples for semithin sections were prepared for examination by transmission electron microscopy (TEM). They were fixed in 3% glutaraldehyde for 5 h, postfixed in 1% OsO_4 for 2 h, washed in 0.1 M phosphate buffer, dehydrated in a graded acetone series with 30-min intervals and embedded in Durcupan ACM (Fluka). Sections of 10 μm were cut on a LKB Nova ultramicrotome and stained with toluidine blue and basic fuchsin.

Scanning electron microscopy

Explants for SEM were sampled at 0, 24, 72, 120 h and 7, 10, 14, 20, 25, 30 and 38 days of culture. Samples were fixed in 2% buffered glutaraldehyde (0.1 M phosphate buffer) for 3 to 5 h and postfixed in 1% buffered OsO_4 (0.1 M phosphate buffer) for 1 h and dehydrated in a graded series of acetone or ethanol. Ethanol was then gradually removed with amyl acetate before drying. The samples were critical point dried with liquid CO_2 using a Balzers Critical Point Dryer CPD 020, mounted on metal blocks with silver print (Balzers), and covered with gold using a Jeol sputter coater. The samples were examined in a Jeol JXA840B scanning electron microscope.

Results

Change in leaf pigmentation

Two days after culture initiation a change in leaf pigmentation from green to red or dark red in some spots or on the whole surface of the isolated leaf was observed.

Table 2. Quantitative evaluation of direct shoot regeneration after 30 days of culture. Mean number of shoots per leaf explant ± SD on solidified MS media with various concentrations of NAA and BA.

NAA (M) \ BA (M)	0	10^{-8}	10^{-7}
0	6.8 ± 1.5	4.1 ± 1.4	3.4 ± 1.2
10^{-8}	6.5 ± 1.4	1.4 ± 0.8	1.3 ± 0.9
10^{-7}	1.3 ± 1.0	1.6 ± 1.4	1.5 ± 1.0

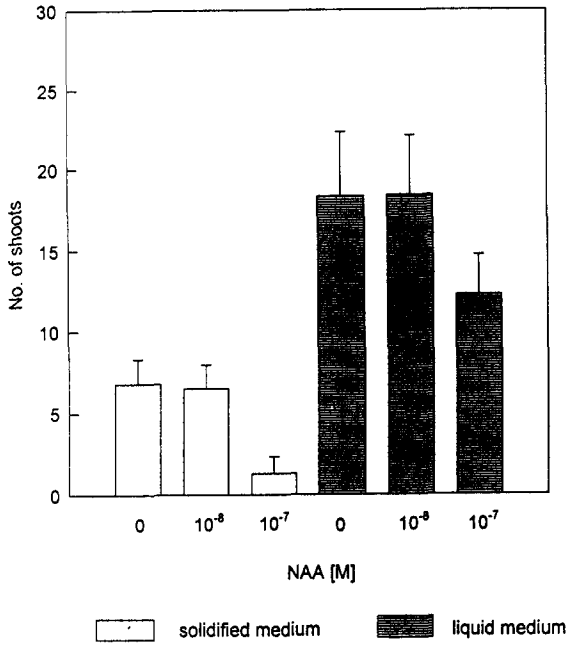

Fig. 1. Differences between direct shoot regeneration on solidified and liquid MS media with various concentrations of NAA. Vertical lines on each bar are equal to 1 standard deviation.

This appears to be due to the conversion of chloroplasts to chromoplasts induced by the medium resulting in red pigment accumulation. Media supplemented with 0 to 2×10^{-5} M BA and 10^{-6} to 2×10^{-5} M NAA induced intensive red pigment accumulation in leaves and produced some necrosis after 28 days of culture (Table 1).

Callus induction

Callus was induced in the light at BA concentrations higher than 10^{-6} M, or in combination with most concentrations of NAA tested (Table 1). However, optimal callus formation and subsequent proliferation in light took place only on media containing 10^{-6} or 5×10^{-6} M BA. The callus was light green and very compact, forming nodule-like structures. Media containing BA and NAA at concentrations from 0.1 to 2×10^{-5} M induced red pigmentation of the calli, usually localized in several spots (Table 1).

Regeneration from leaves

Direct shoot formation from leaf tissue occurred on media containing BA and NAA at low concentrations. The highest amount of shoot regeneration was achieved using media without growth regulators or supplemented with the lowest concentration of NAA (10^{-8} M; Tables 1, 2). Buds formed on the whole leaf surface, but especially near the tentacules.

Using liquid culture media statistically significantly increased regeneration potential (Fig. 1). The average number of buds increased to 18.3 per explant on the liquid medium without growth regulators and to 18.4 on the liquid medium with 10^{-8} M NAA. Within 38 days of culture initiation, the young shoots with three to seven leaflets had rooted spontaneously on the mother leaf explant and could be separated easily from the leaf explant. After isolation and transfer to fresh solidified MS medium without growth regulators, they grew to maturity, flowered and produced fertile seeds.

Histological observations on shoot regeneration

Leaf epidermal cells began to divide within 48 h of placement on the shoot-inducing media. These cells showed the highest regeneration potential and were the cells from which the new shoots were regenerated. The first divisions of the epidermal cells were usually anticlinal or transversal followed by periclinal ones (Fig. 2A). Mesophyll cells contained visible plastids with high starch deposits (Fig. 2). Young cells of protracheal elements and parenchymatous cells around the vascular bundles were also involved in division, but usually only after 72 h of culture (Fig. 2B). The division of epidermal cells led to the formation of cell clusters on the explant surface (Fig. 2C). Globular structures containing 30 to 40 cells were formed from these clusters after 10 to 12 days of culture. These structures that become young buds are evidently of epidermal origin (Fig. 2D). Asymmetrical cell divisions led to the formation of a new epidermis on the surface with meristematic cells inside the developing shoot buds after 14 to 16 days in culture. These cells were highly cytoplasmic with small provacuoles or vacuoles and a large

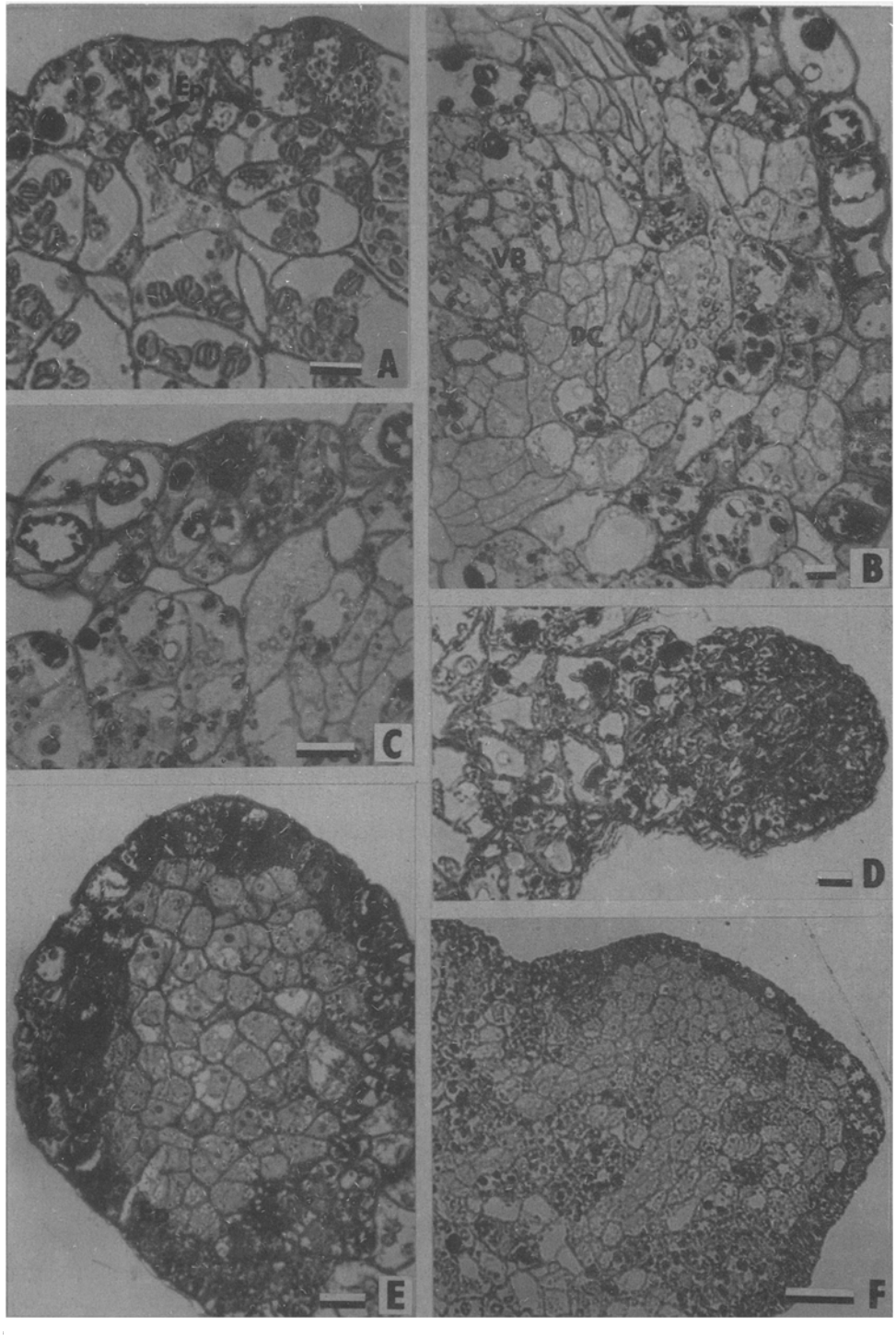

Fig. 2. Histological-anatomical study of shoot regeneration on transversal sections. (*A*) First anticlinal and transversal divisions of epidermal (Ep) cells followed by periclinal ones at 48 h after culture. A cell in late metaphase is marked by arrow. (*B*) Dividing parenchymatic cells formed a layer resembling procambium (PC) arround vascular bundle (VB) in the inner part of the leaf explant after 72 h of culture. (*C*) Clusters of gradually dedifferentiated epidermal cells. (*D*) Young bud after 10 days of culture. (*E*) Bud with young epidermis (Ep) and meristematic cells inside after 14 days of culture. Cells of epidermal origin separate the bud from the mother explant tissue. (*F*) Bud with wide basal part after 18 days of culture. Scale bar = 10 μm for A, B, C, D, E and 100 μm for F.

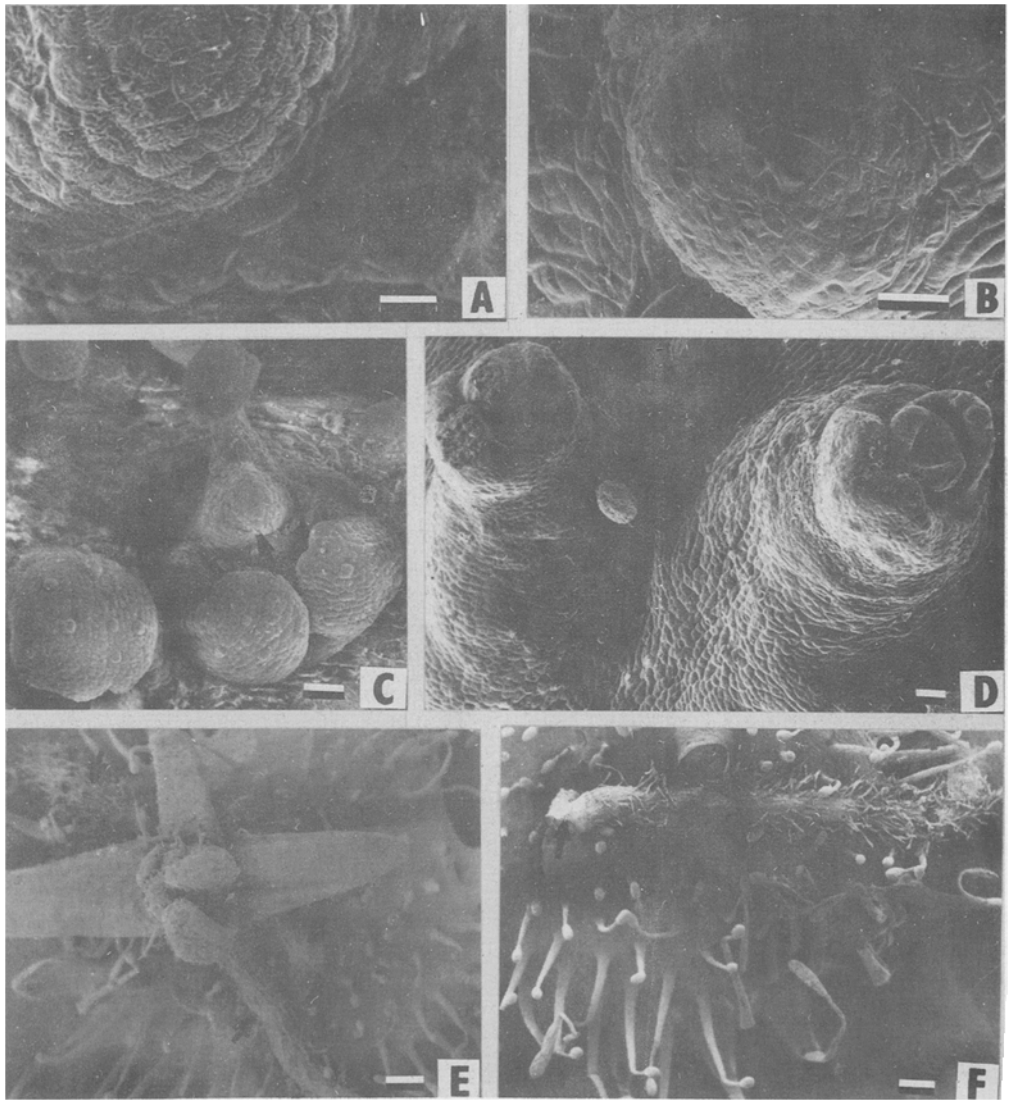

Fig. 3. SEM study of shoot regeneration. (*A*) Net-like extracellular matrix (ECM) was present on the meristematic nodal surface composed of promeristemoid cells after 10 days of culture. (*B*) Globular bud (meristemoid) with young epidermis on its surface without extracellular matrix after 14 days of culture. (*C*) Bud localized on the basal part of tentacule (arrow) with three leaf primordia on solidified medium after 20 days of culture. (*D*) Elongated and thickened buds on liquid medium after 25 days of culture. (*E*) Regenerated young plant with three primary roots (arrows) on the surface of a mother leaf explant on solidified medium after 38 days of culture. (*F*) Region from which shoots and roots originated (arrow). Scale bar: 50 μm for A, B, C, D and 1 mm for E, F.

prominent nucleus with a visible nucleolus. Mostly, the central vacuole was not differentiated. However, several smaller vacuoles often contained dense deposit. Young epidermal cells surrounded the buds. Moreover, cells of epidermal origin often separated the buds from the mother mesophyll tissue, especially in early stages of bud development (Fig. 2E). After 18 to 20 days of culture, these cell layers, which were coherent and composed from original epidermal cells, remained only as several clusters of cells with dense deposits in their vacuoles. Meristematic cells on the basal part of shoot bud were localized (Fig. 2F).

SEM observations of shoot regeneration

Globular shoot buds formed on the leaf surface were first visible by SEM after 10 to 12 days of culture (Fig. 3A). They were composed of small cells surrounded by a well-developed extracellular matrix. These cells were quite different in size and shape from the leaf epidermal cells. They were probably dedifferentiated epidermal cells from which the new epidermis and shoot meristem were formed. The extracellular matrix was organized in a network linking neighbouring surface cells (Fig. 3A). It was present only in the early stages of bud development and disappeared when the new epidermis had formed on the bud surface after 14 to 16 days in culture (Fig. 3B). After 20 days in culture the leaf primordia were visible. Usually three leaf primordia were formed per bud on the solidified medium (Fig. 3C). The bud bases were short and the root primordia were formed later in this region . In contrast, the bud bases were very elongated and thickened on the liquid medium (Fig. 3D). The rooting potential of such elongated buds was very poor. Young shoots differentiated on the solid medium spontaneously and rooted directly on the explant surface within 32 days of the culture. They had three to seven leaflets and primary roots with numerous root hairs (Fig. 3E). After removing the young leaflets, we obtained a detailed picture of the region where shoot and roots were connected (Fig. 3F). Young complete plants were easily separable from the mother explant tissue. They grew to maturity after transfer to solidified medium without growth regulators.

Discussion

The screening of 49 medium variants showed that the growth regulator-free medium and the medium supplemented with 10^{-8} M NAA was the most favourable for direct plant regeneration. Similarly, Simola (1978) and Anthony (1992) reported that growth regulator-free medium induced organogenesis in leaf cultures of *Drosera rotundifolia* and complete plants could be regenerated. Simola (1978) found that, in contrast to other carnivorous plants, *Drosera rotundifolia* is able to grow and flower on medium with inorganic nitrogen as the sole source of nitrogen. We induced direct bud formation on both solidified and liquid medium. Liquid medium was best for bud formation (the average number of regenerated buds per explant increased significantly) but not for rooting. In contrast to our results, Bonnet *et al.* (1984) reported very high bud production (up to 20 buds per explant) on solidified medium supplemented with 10^{-7} M BA. We obtained the best bud induction and plant regeneration on the media where cytokinin was omitted. Moreover, the highest concentrations of BA (over 10^{-7} M) led to an intensive callus induction.

Direct regeneration from somatic tissue is a rare phenomenon and has been described in a few species such as *Ranunculus sceleratus* L. (Konar & Nataraja 1965), *Daucus carota* L. (McWilliam *et al.* 1974), *Dactylis glomerata* L. (Conger *et al.* 1983), *Lycopersicum* (Young *et al.* 1987), *Cichorium* (Dubois *et al.* 1991), *Nicotiana tabacum* L. (Stolarz *et al.* 1991). In our work we have shown direct regeneration from leaves of *Drosera rotundifolia.*

In this study, buds of *Drosera rotundifolia* originated from epidermal cells on the leaf explants as shown by detailed histological and SEM observations. Epidermal cells firstly dedifferentiated and then underwent *de novo* shoot differentiation.

The first divisions of the epidermal cells were usually anticlinal or transversal followed by periclinal ones. This way of shoot regeneration is strictly different from the direct and indirect regeneration of *Drosera spathulata* in isolated leaves and callus cultures reported previously (Bobák *et al.* 1989; 1993). In contrast to *Drosera spathulata*, the protracheal and parenchymatous cells around the vascular bundles were not involved in the shoot regeneration of *Drosera rotundifolia.* They began to divide later than epidermal cells, which indicates that regeneration competency of various cells in an explant can be genotypically dependent in *Droseraceae.*

Cellular changes in thin cell layer (TCL) explants of stem *Brassica napus* L. cv. Vega support the concept that the location of mitotic activity in already differentiated parenchymatous cells of an explant is determined not only by optimal growth regulator and nutritional concentrations in the culture medium but also by intertissue relations (epidermis-parenchyma, parenchyma-vascular tissues and intercellular relations among parenchymatous cells) (Pihakaski-Maunsbach *et al.* 1993). More detailed scanning electron microscopic (SEM) studies of the bud surface cells showed a fibrillar network representing an extracellular matrix (ECM) covering them. The structural arrangement of ECM depended on the developmental stage of embryogenic cells. When protodermis was formed, cells were practically smooth (Šamaj *et al.* 1995). The extracellular network could be a point of comparision with ani-

mal cells, where surface glycoproteins of ECM play a role in adhesion and recognition, and are linked to the cytoskeleton (Ruoslahti & Pierschbacher 1987).

Acknowledgements

We thank J. Šramková and J. Polonyi (Faculty of Medicine, Bratislava) for excellent technical assistance.

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