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Jiří Hřib, Božena Vooková*, Vilém Neděla

Imaging of native early embryogenic tissue of Scots pine (*Pinus sylvestris* L.) by ESEM

Abstract: Environmental scanning electron microscopy enables the investigation of uncoated pine early embryogenic tissue samples *in situ*. The samples were examined under low vacuum conditions (air pressure 550 Pa) at a temperature of around -18°C by the AQUASEM II non-commercial environmental scanning electron microscope. The native extracellular matrix surface network was imaged by the environmental scanning electron microscope and in dark field mode of the optical microscope too. The backscattered electron detector disclosed brightness loci in the cells of early embryogenic culture. This work shows images of native pine embryogenic tissues. The continuity of extracellular matrix with structural integrity of plant organism is discussed.

Keywords: Somatic embryogenesis, backscattered electron detector, ionization detector, bottle cells, extracellular matrix, structural integrity

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1 Introduction

In several plants cultured *in vitro*, scanning electron microscope (SEM) analysis revealed that induction of morphogenesis is linked to the appearance of a fibrillar network referred to as the extracellular matrix (ECM) or extracellular matrix surface network (ECMSN). A structural subcellular marker such as ECM was found to be a characteristic feature for embryogenic tissues [1-4]. The ECMSN plays an important morphoregulatory role during somatic embryogenesis and organogenesis, implying an active role in plant morphogenesis [5,6].

Nevertheless, complex preparation processes is required to make the sample stable in vacuum conditions, and also electrically conductive. Each step of sample preparation, from chemical fixation, dehydration and drying up to metal coating, can cause various artefact formation and/or destruction of the finer features. High pressure of gas, mostly water vapour in a specimen chamber of the environmental scanning electron microscope (ESEM) together with suitably reduced sample temperature, allows the direct observation of fully hydrated or electrically nonconductive samples in their native state, without the necessity of their surface covering by a conductive layer [7]. It allows to overcome above mentioned SEM disadvantages.

In Scots pine (*Pinus sylvestris* L.) somatic embryogenesis was initiated from immature embryos by Keinonen-Mettälä [8] in 1996. Embryogenic tissue contained first stages of early embryos. Lelu *et al.* [9] obtained induction and plantlet regeneration from immature seeds. Later, the effect of parent genotype on somatic embryogenesis in *P. sylvestris* was described. No mother × father interaction was found, indicating that mothers successful at initiation and after 6 months in tissue culture, pollinated by any of the successful fathers, produced somatic lines and mature somatic embryos [10]. Enhancement of somatic embryogenesis from immature zygotic embryos of *P. sylvestris* and with optimized culture conditions and tight quality control, over 90% of the embryos germinate and develop into vigorously growing emblings [11]. Secondary phenolic compounds (stilbenes) in somatic embryogenesis of *P. sylvestris* were studied [12]. Somatic embryogenesis is a useful tool to propagate conifers vegetatively. The major limitation in many pine species is the low quality of cotyledonary somatic embryos. The presence of supernumerary suspensor cells in early somatic embryos of Scots pine is caused by disturbed polar auxin transport and results in aberrant embryo development [13].

In the present experiments the new method of environmental scanning electron microscopy (ESEM) was used, which is essentially different from low temperature (LT) ESEM or cryo SEM. The study of the ECMSN with

***Corresponding author: Božena Vooková:** Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, SK-95007 Nitra, Slovak Republic, E-mail: nrgrvook@savba.sk
Jiří Hřib, Vilém Neděla: Institute of Scientific Instruments, Academy of Sciences of the Czech Republic, CZ-612 64 Brno, Czech Republic

higher resolution and larger depth of field than is possible by optical light microscopy can be very important for better description of embryogenic tissue cells in situ.

The aim of this study was to present the structure of *Pinus sylvestris* embryogenic tissue detailed using ESEM studies of native ECMSN.

2 Experimental Procedures

2.1 Induction of *Pinus sylvestris* embryogenic tissue

Immature cones of *Pinus sylvestris* L. were collected in 2010, June 28th, from trees in Oravský Biely Potok. The zygotic embryos at the time of collection were at the precotyledonary stage of development. Megagametophytes containing embryos were cultured on DCR medium [14] containing 0.5 mg L^{-1} BAP, 2 mg L^{-1} 2,4- dichlorophenoxyacetic acid (2,4-D), 200 mg L^{-1} myo-inositol, 500 mg L^{-1} casein hydrolysate, 50 mg L^{-1} glutamine, 20 g L^{-1} sucrose, solidified with 3 g L^{-1} Phytagel. The pH was adjusted to 5.8 before autoclaving. The medium was poured into Petri dishes (6 cm in diameter). The cultures were grown in darkness at 25 ± 1°C. Induction of *Pinus sylvestris* embryogenic tissue was observed after five week cultivation of immature zygotic embryos on initiation medium. This tissue contained mucilaginous cell mass proliferated from the micropylar end of megagametophyte. Proliferation occurred on the same initiation medium in darkness. The tissue was transferred to a fresh medium at 2 - 3 week intervals. For the observation in ESEM the 30th subculture of embryogenic tissue was used (Fig. 1).

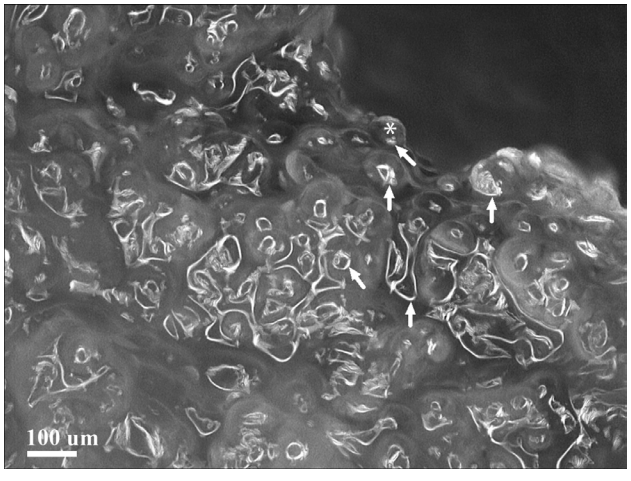

Fig. 1: Early embryogenic tissue of Scots pine (Pinus sylvestris L.) in the dark field (Olympus Lext OLS 3100) (the presence of the mucilaginous matrix is indicated by arrows, the present of head is indicated by *).

2.2 Observation in ESEM

Native samples (free of chemical fixation and drying) of embryogenic tissue in proliferation stage cultured on fresh medium during 10 days, were placed on a cooled specimen holder (Peltier stage) and their temperature was gradually decreased and then maintained between -18 and -22°C. Due to the relatively low heat conductivity of the conifer samples (dimensions of 2–3 mm^2 and thickness of 2 mm), the real temperature of the sample surface can be higher. At the beginning of the pumping the pressure was equal to the atmospheric pressure in all parts of the microscope. The pumping process started approximately one minute after the decrease of the sample holder temperature. Imaging was started after ten minutes when the sample was inserted. Every one of selected parts were observed approximately 8-10 minutes. Whole experiment was finished after 30 minutes including manipulation and imaging. Described method was tested on 36 sites of early embryogenic tissue. Paper shows results of two observed series each contains four samples.

The conifer samples were examined under low vacuum conditions (air pressure 550 Pa) by the AQUASEM II non-commercial ESEM. It was designed in the Institute of Scientific Instruments of the Academy of Sciences of the Czech Republic for research on detection systems and ESEM techniques [15]. The single crystal YAG:Ce^{3+}, used for detection of backscattered electrons (BSEs) in high and low vacuum conditions, has a hole in the center so that it simultaneously acts as a pressure-limiting aperture of the AQUASEM II [14]. Generally, the system of pressure limiting apertures in the ESEM enables maintaining low pressure (from 10^{-3} to 10^{-6} Pa) of a gas in the vicinity of the electron source and simultaneously, the use of relatively high pressure of the gas (from 1 to approx. 2000 Pa) in the specimen chamber of the microscope. In low vacuum and environmental modes the secondary electron (SE) signal can be detected by an ionization detector using an electrode with the inner diameter of 4 mm deposited on the input surface of the YAG single crystal. With a positive bias of 280 V with respect to the sample, it acts in a similar fashion to the environmental secondary electrons detector.

The combination of the BSE-YAG detector of high-energy BSEs emitted from deep layers of the sample and thus yielding information on the material contrast (electron emission is strongly dependent on the atomic number of the sample) with a special ionization detector [16] recording signals composed of predominantly low-

energy SE emitted from the surface layers of the sample and giving topographic contrast enables us to detect information on the surface structure of the studied samples, and moreover on their material composition.

All experiments were carried out under constant operating conditions (beam accelerating voltage 20 kV, probe current 70 pA, sample distance 2.5 mm between the bottom surface of the YAG single crystal and the surface of the sample, positive bias of the detection electrode system 280 V) and in the gas environment with relative humidity equal to 40%.

3 Results and Discussion

Study of *P. sylvestris* embryogenic tissue (embryonal suspensor mass) by the methodology of natural low temperature ESEM showed that this tissue contained long suspensor cell aggregates (Fig. 2A,B), aggregates of bottle shape cells (Fig.3 A-F) and early somatic embryos composed from embryonal heads (Fig. 4A-F) with suspensor cells. It was observed that suspensor cells in early somatic embryos of Scots pine play important roles in embryo development [13]. The surfaces of the bottle shape cells as well as the embryonal heads were covered with mucilaginous matrix – ECM (ECMSN). It seems that consistency of this matrix is different from the network-like or fibrillar structure observed in other species [4,17,18]. Consistent with Lai *et al.* [4] we suppose that the ECM layer forms bridges connecting the neighboring cells together and may be the early structural marker for embryogenic tissues. Bottle shape cells were described by Šamaj *et al.* [18] as well developing somatic embryos. It is interesting that bottle cells in animals contribute significantly to gastrulation. The role of bottle-shaped cells in Scots pine somatic embryogenesis in not known.

The most readily observable result of the interaction of cells with the ECM is cell adhesion. The adhesion of plant cells to one another is fundamental to the formation and maintenance of plant structure [19]. Our experiments indicate significant adhesion of early embryogenic tissue of *Pinus sylvestris*.

Hoenemann *et al.* [20] described that in the non-embryogenic cell line of Cyclamen persicum the much looser cell adhesion might be caused by reduced pectin content, which might be an important factor for the loss of embryogeneity due to reduced cell adhesion. It is probable that in the conifers, cell adhesion is important in the process of *Pinus* somatic embryogenesis.

The BSE detector allowing for images with contrast relating to the atomic weight of the elements in the sample was used in this study. The signal of BSEs has been widely used for the investigation of specimen surface in scanning electron microscopy for many years [21,22]. A new type YAG – BSE detector for high resolution SEM imaging of large biological samples was presented [23]. A variable-pressure scanning electron microscope (VP-SEM) equipped with a high-sensitive BSE detector of the YAG type was applied to studies of fixed biological tissue samples [24]. Our micrographs (Fig. 2B; 3B, D, F; 4B, D, F) are from ESEM on non-fixed samples with a BSE-YAG detector. Other subsequent experiments display more accurate determination of elements in the cells of pine

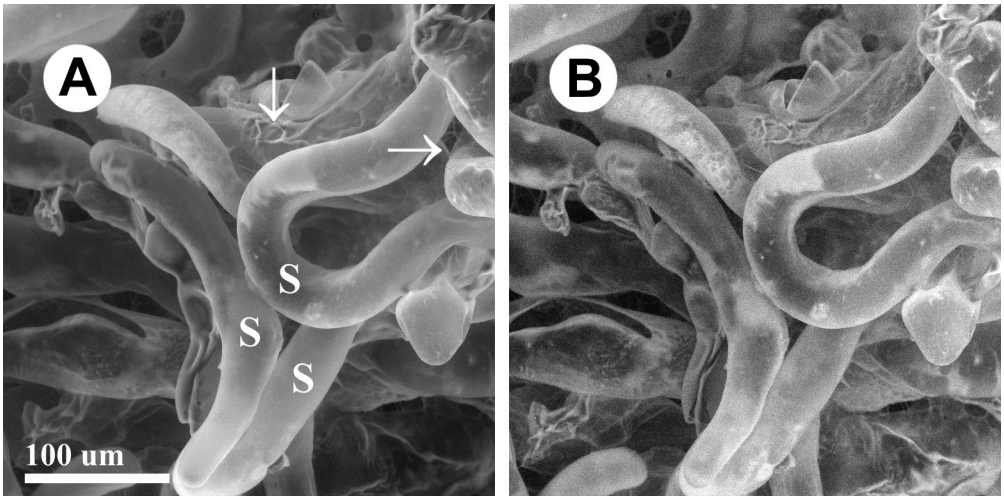

Fig. 2: ESEM observations of early embryogenic tissue (*Pinus sylvestris* L.). (A- using ionization detector, B- using the BSE YAG detector (accelerating voltage 20 kV, probe current 70 pA, ionization detector 270 V, pressure of air in the specimen chamber 550 Pa), the presence of the ECMSN is indicated by full arrows, S-long suspensor cells).

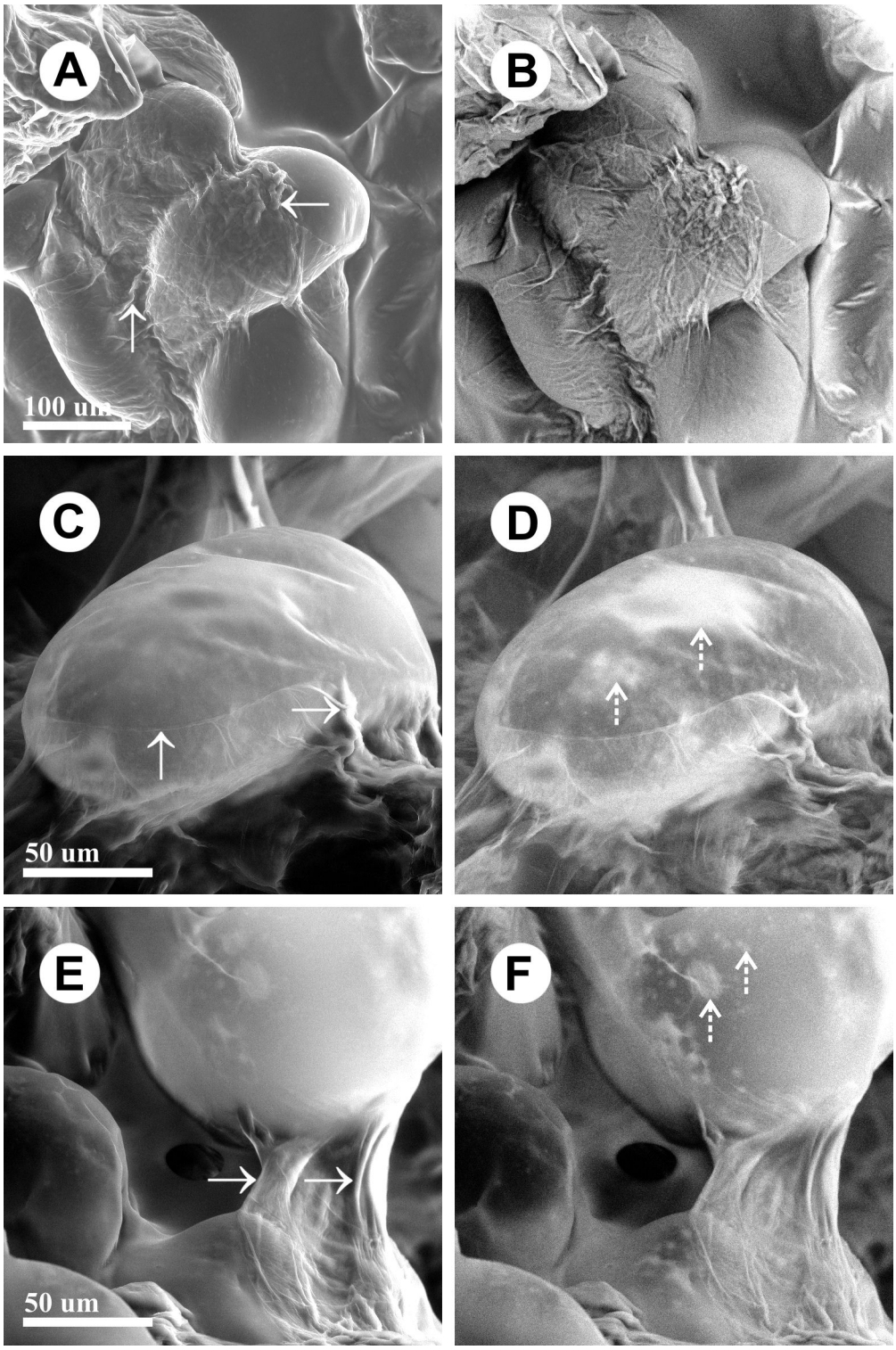

Fig. 3: ESEM observations of bottle shape cells in Scots pine (*Pinus sylvestris* L.) somatic embryogenesis. (A,C,E - using ionization detector, B,D,F - using the BSE YAG detector; the presence of the ECMSN is indicated by full arrows, the presence of brightness loci by dashed arrows).

early embryogenic tissue which appeared as brightness loci (Fig. 3 D, F). Preliminary our experiments suggest the displaying of calcium and potassium distributions in the cells.

The interactions between cells play a fundamental role in the growth and development of multicellular organisms. Structural integrity of plants was described by Šebánek *et al.* [25]. It may also be mentioned that the

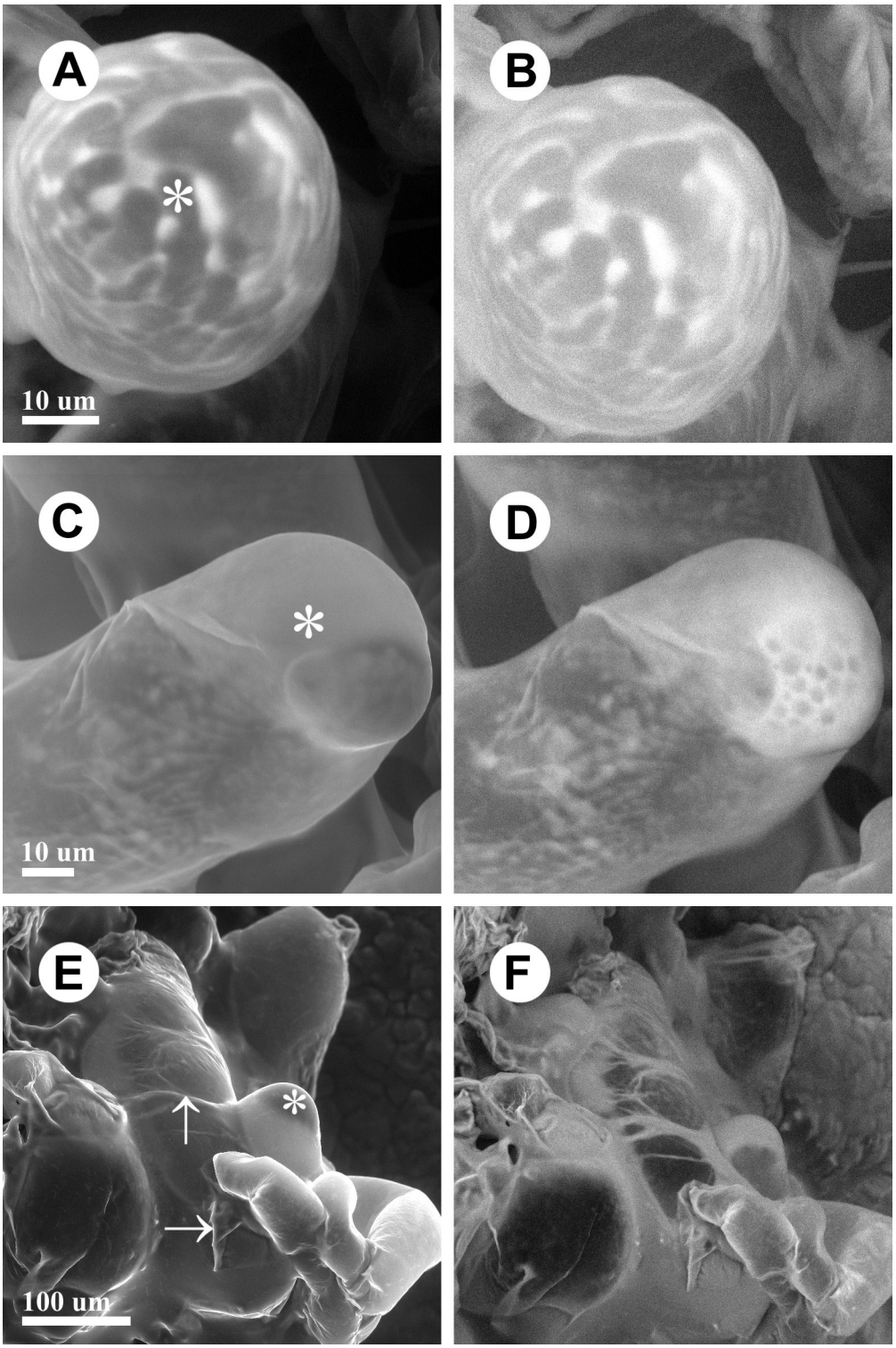

Fig. 4: ESEM images of pine embryogenic head. (A,C,E - using ionization detector, B, D, F - using the BSE YAG detector; the presence of the ECMSN is indicated by full arrows, the presence of own heads by *).

extracellular matrix issue pertains to Dostal´s [26] topic of structural integrity of plant organism.

This paper introduces the native structure of pine embryogenic tissues investigated using electron microscope. For the future, the use of the methodology with low temperature presented in this work is offered for the study of organisms for example algae, lichen and following directly from Arctic or Antarctic nature and with it, to apply this also generally in polar biology.

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Conflict of interest: Authors declare nothing to disclose.

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