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BRIEF COMMUNICATION

Imaging of early conifer embryogenic tissues with the environmental scanning electron microscope

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Abstract

This article describes the usage of non-commercial environmental scanning electron microscope (ESEM) for the visualization of plant extracellular matrix in *Abies alba* and *Abies numidica*. Non sputter-coated samples free of using the common fixation technique observed at the relatively low humidity of the air environment with the pressure 550 Pa and the low temperature of the sample from -18 to -22 °C give surprisingly very good results that show the natural structure of the tissues. This seems to be generally applicable. Moreover, a specially designed ionization detector of secondary electrons and a YAG:Ce^{3+} detector of backscattered electrons were used for better comparison.

Additional key words: *Abies alba*, *A. numidica*, extracellular matrix.

In the environmental scanning electron microscope (ESEM) the specimens can be observed across a wide range of pressure from the vacuum (comparable with the SEM) to the high pressure of various gases (over a thousand Pa) in the specimen chamber. In high pressure conditions very wet non-conductive samples can be observed free of charging artefacts without a conductive coating covering their surface (Danilatos 1988). If the pressure of the gas is sufficiently increased or the sample temperature reduced, their natural and fully hydrated surface structure is preserved (Stokes 2003, Neděla 2007, 2010). Thus, i*n situ* observation could be performed on non-fixed zygotic and somatic embryos. Using ESEM, the surface changes in white spruce somatic and zygotic embryos under various regimes were studied by (Reid *et al*. 1999). The equipment *AQUASEM* (*Tescan*, Brno, Czech Republic) was used by Hřib (2005) for observation of zygotic and somatic embryos of Algerian fir.

The early stages leading to somatic cell regeneration into a completely new plant seem to be very important. In several *in vitro* cultured plants, the 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) (Sondahl *et al*. 1979, Dubois *et al*. 1992, Šamaj *et al*. 1995). Bobák *et al*. (2003/4) suggested that ECM formation can be a stress response of explants, elicited by specific *in vitro* conditions. Previous authors used the SEM to describe the extracellular matrix on fixed biological material in buffered glutaraldehyde, dehydrated, dried by the CO_2 critical point drying system and sputtered with gold-palladium, *etc*. Popielarska-Konieczna *et al*. (2010) showed the ultrastructure of the ECM in *Actinidia deliciosa* endosperm-derived callus culture using SEM and ESEM.

Here we presented detailed ESEM studies of native ECM structure of early conifer embryogenic tissues in specific conditions of observation (gas type, gas humidity and temperature of sample) and using the *BSE YAG* detector of high energy electrons and a specially designed ionization detector of low energy secondary electrons.

The embryogenic tissue of silver fir (*Abies alba* Mill.) and Algerian fir (*Abies numidica* De Lann.) was initiated from immature zygotic embryos of cones from open-

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Abbreviations: SH medium - Schenk and Hildebrandt medium; AGPs - arabinogalactan proteins; ECM - extracellular matrix; ECMSN - extracellular matrix surface network; SEM - scanning electron microscope; ESEM - environmental SEM; SE - secondary electrons, BSE - back secondary electrons; YAG : Ce^{3+} - yttrium aluminum garnet activated with trivalent cerium
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pollinated trees. Immature seeds were surface-sterilized for 10 min in 10 % (v/v) H_2O_2 and then rinsed several times with sterile distilled water. The immature cones of *A. alba* were collected on 26 July 2006 in the Dobroč primeval forest. The embryogenic tissue was initiated and proliferated on the same medium used for *A. numidica* seeds (Vooková and Kormuťák 2009). The embryogenic tissue of *A. numidica* was obtained from immature zygotic embryos of cones collected in the Arboretum Mlyňany on 8 July 1998. The megagametophytes containing embryos were cultured on Schenk and Hildebrandt (1972; SH) medium supplemented with 1 g dm^{-3} myo-inositol, 1 g dm^{-3} casein hydrolysate, 0.5 g dm^{-3} L-glutamine, 5 μM 6-benzylaminopurine, 20 g dm^{-3} sucrose and 3 g dm^{-3} *Phytagel*. The pH was adjusted to 5.8 before autoclaving. The medium was poured into Petri dishes or Erlenmeyer flasks. The cultures were grown in darkness at 25 ± 1 °C and transferred to a fresh medium in 2 - 3 week intervals.

Native samples, in the mid stage of the subcultivation, 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. Approximately 1 min after the decrease of the sample holder temperature the pumping process started.

The conifer samples were examined under low vacuum conditions (air pressure 550 Pa) by the non-commercial environmental scanning electron microscope *AQUASEM-II*. 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 (Neděla *et al*. 2007). The single crystal *YAG:*Ce^{3+}, used for detection of backscattered electrons (BSEs) in high and low vacuum conditions, has a hole in the centre so that it simultaneously acts as a pressure-limiting aperture of the *AQUASEM II* (Neděla 2007). Generally, the system of pressure limiting apertures in the ESEM enables to keep low pressure (from 10^{-3} to 10^{-6} Pa) of a gas in the vicinity of electrons source and simultaneously to use 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 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 270 V) and in the gas environment with relative humidity equal to 40 %.

Early embryogenic tissues (calli) of *A. alba* and *A. numidica* were white and mucilaginous 10 -12 d after the last passage and displayed a distinctive structure. Embryogenic tissues in spruce have been described *e.g.* by Hakman and Fowke (1987). Generally, the embryogenic tissue of conifers is characterized by the presence of bipolar structures composed of an embryonic part with embryogenic cells and a suspensor formed by long cells. Potentially embryogenic structures, despite some similar morphological features, show a different structural organization when observed in more detail. Šamaj *et al*. (2008) show that one of these structural features is the presence of an extracellular matrix network (ECMSN) on the surface of somatic embryos/ embryogenic cells with a high regeneration potential.

To obtain a detailed image of the extracellular matrix *in situ* we applied the techniques and conditions described above and compared micrographs obtained using the *BSE-YAG* and the ionization detectors. The individual micrographs from the embryogenic tissue of *A. alba* show high-resolution images of embryogenic and suspensor cells and the native surface of extracellular matrix obtained by the ionization detector (Fig. 1*b*,*d*). On the contrary, with the *BSE-YAG* detector, a high distinction of chemical characteristics can be achieved (Fig. 1*a*,*c*). Thus, by combining the two detectors the scope of received information can be expanded. Similar images of *A. numidica* native embryogenic tissue obtained using the BSE-YAG detector and the ionization detector can be seen in Fig. 2.

Structures (or particles) of embryonic parts with high level of brightness, particularly apparent in Figs. 2*a*,*c* recorded using the BSE YAG detector, are very similar to the particles with activated Ca^{2+}-binding protein, calmodulin, which were visualized by fluphenazine fluorescence during somatic embryogenesis of carrot (Timmers *et al*. 1989, Overvoorde and Grimes 1994). The hypothesis will be verified by subsequent experiments.

In the Norway spruce, Jalonen and Von Arnold (1991) divided the embryogenic cell lines into two groups differing in secretion of extracellular proteins and regeneration capacity. In the same species, a close correlation between the presence of specific extracellular proteins and somatic embryo morphology was found (Mo *et al*. 1996).

The chemical composition of the extracellular matrix is only partially known, however, Šamaj *et al*. (2008) characterized arabinogalactan proteins (AGPs) as mole-

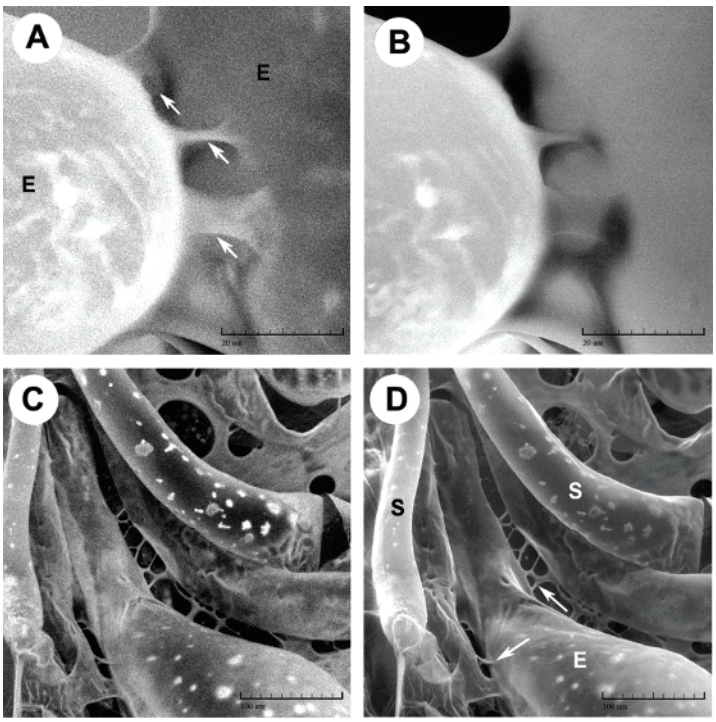

Fig. 1 Comparison of early embryogenic tissue of *Abies alba* using *AQUASEM II* ESEM with two detectors. *A* and *C* were recorded using the *BSE YAG* detector, *B* and *D* using the ionization detector (accelerating voltage 20 kV, probe current 70 pA, ionization detector 280 V, pressure of air in the specimen chamber 550 Pa), the presence of the ECM is indicated by *arrows*; E - embryonic part, S - suspensor cell.

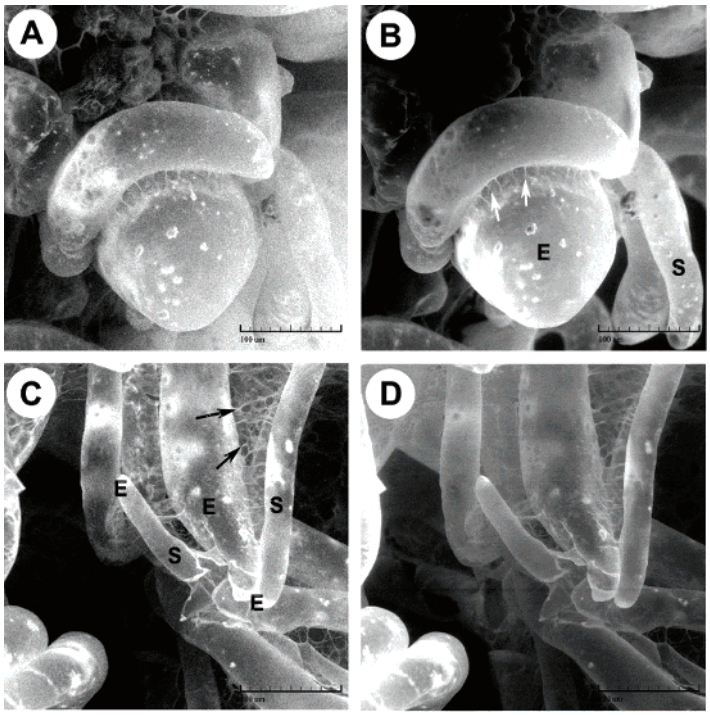

Fig. 2. Comparison of early embryogenic tissue of *Abies numidica* using two detectors. Others details as in Fig. 1.

cular components of the ECMSN and localized them in hybrid fir (*A. alba* × *A. cephalonica*) embryogenic cell lines with different embryogenic and regenerative potential. The plant ECM could be a point of comparison with animal cells, where surface glycoproteins of the extracellular matrix play an important role in adhesion

and recognition, and are linked to the cytoskeleton (Ruoslahti and Pierschbacher 1987).

Somatic embryogenesis is frequently the model for the early development in plants. We suppose that the early somatic embryogenic tissues ECM might present exceptionally suitable structures for studying the structural integrity of plants.

It is beneficial to present our methodology of the research on another species of the *Pinaceae* family. Finally, our methodology, which combines both types of detectors, can largely contribute to the study of native cell surface structures in somatic embryogenic tissues of plants.

In summary, our methodology combining both types of detectors, can largely contribute to the study of the cell surface structures of somatic and zygotic embryos.

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