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Early State of Spruce Somatic Embryos in Native State Observed Using the ESEM and Cryo-SEM

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The aim of this paper was the study of morphology of cultured ESE. This is the first report of ESEM and SEM with cryo-attachment observation of ECM in early somatic embryogenic tissue of Norway spruce.

Our samples, ESE, clone 2/2 from the collection of the Department of Plant Biology of Mendel University in Brno, were originally derived from a mature zygotic embryo of tree no. 12345 of Norway spruce (*Picea abies* (L.) Karst.) mountain climate type of an experimental area in the Beskyd Mountains. The ESE cultures were maintained on medium designated LP/2 in 100 mm diameter Petri dishes [1] with 9 μM 2.4-D and 4.4 μM BAP. The ESM from the upper parts of their aggregates (2.5 – 5 mg) were subcultured in 10-14 day periods. The cultures were maintained in the dark at 23±1°C.

Samples of collected ESE were placed on a cooled specimen holder (Peltier stage) and their temperature was gradually decreased and then maintained between -18°C and -22°C [2]. 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 on the sample surface can be slightly higher. At the beginning of the pumping, the pressure was equal to the atmospheric pressure in all parts of the microscope. Approximately 1 minute after the decrease of the sample holder temperature, the pumping process started. The conifer samples were examined under low vacuum conditions (air pressure from 550 Pa to 690 Pa) by the non-commercial environmental scanning electron microscope AQUASEM-II among others also equipped with a special ionization detector of secondary electrons and a halved YAG:Ce^{3+} detector of backscattered electrons (BSE-YAG). 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 secondary electrons 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 in ESEM 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 a gas environment with relative humidity equal to 40%.

For comparison, our samples were observed by using the field emission scanning electron microscope with cryo-attachment. The sample was placed on the aluminium target and the excess liquid drained. Then the sample was frozen by plunging it into liquid nitrogen and immediately transferred under vacuum into the chamber of the cryo-attachment CryoALTO 2500 (Gatan). At a

temperature of -95ºC vacuum sublimation was performed for 2 minutes to decontaminate the surface of the sample. The frozen sample was observed free of sputtering by the conductive layer in the FESEM JEOL 7401F operated at acceleration voltage 1 kV for the detector of secondary electrons situated in the specimen chamber of the microscope and 4 kV for the BSE-YAG detector. The working distance was around 18.3 mm for low magnification mode and 8.5 mm for classic SEM mode and the stage temperature, -130ºC [3].

The smooth and compact surface layer was observed using the ionization detector of low energy secondary electrons, see Fig. 1A, while the detector of backscattered electrons, giving the information about the material contrast from the bulk of sample, shows a structure looking like surface-porosity, see Fig. 1B. But in the fact the figure shows an undersurface matrix distribution of fibres containing high atomic number elements. The presence of a smooth and compact surface of extracellular matrix was proven by our results obtained using the Cryo-SEM, see Figs. 1C, D [4].

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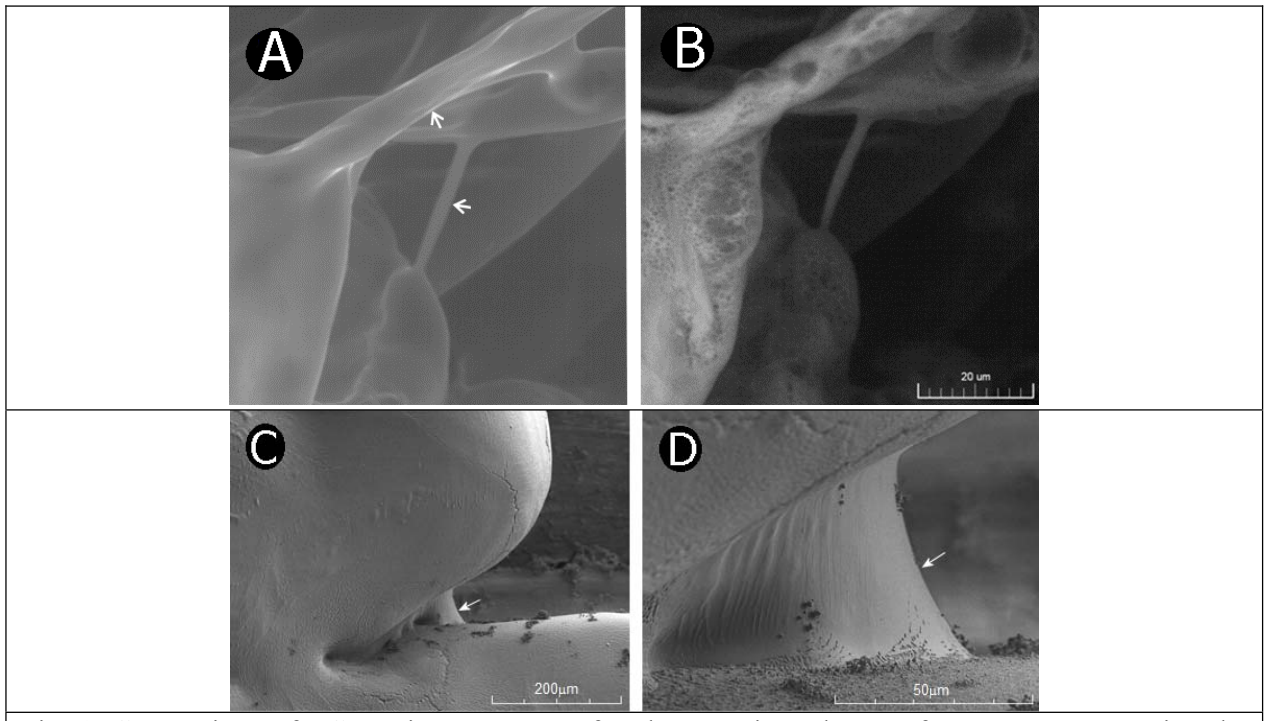

Fig. 1. Comparison of ECM microstructure of early somatic embryos of Norway spruce using the ESEM and Cryo-SEM (A – ionization detector in ESEM; B – BSE YAG detector in ESEM; C, D – detector of secondary electrons in Cryo-SEM; the presence of ECM indicated by arrows).

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