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Repetitive Observation of Coniferous Samples in ESEM and SEM.

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Environmental scanning electron microscopy (ESEM) is well known for its ability to observe nonconductive moist or wet samples in optional conditions including the possibility for in-situ study of their dynamical changes. ESEM allows the observation of a wide range of biological samples from different kinds of fully hydrated fixed cells to live animals, as well as plant samples in their native state, free of any treatment [1]. Nevertheless, observation of wet susceptible biological samples is burdened with the low possibility for repetitive imaging of samples due to sample collapse and relatively low resolution in comparison with scanning electron microscopy (SEM).

The observations of wet samples in ESEM are usually performed in high pressure water vapor conditions with cooling of the sample to a temperature slightly higher than 0 °C. Our newly published methodological study of early somatic embryos (ESEs) of conifer [2] pointed out high sample stability and increased resistance to beam damage in nonstandard conditions (low temperature around -20 °C and 400 Pa air environment). The low-temperature (LT) method allows the observation of samples in conditions of reduced gas pressure and relative humidity, hence with higher resolution.

The aim of this paper is to introduce and discuss the utilization of the LT method for ESEM [2] as an alternative method for the preparation and repetitive observation of suitable plant samples. The method allows the study of the same plant surface microstructure in a fully hydrated, freeze-dried or sputter coated state and making a compromise between adequate advantages and disadvantages (naturally wet structure and in-situ study of dynamical changes vs. low resolution, the highest beam sensitivity and impact of free radicals in ESEM; chemical free freeze-dried surface structure with higher resolution, lower beam sensitivity and possibility of repetitive observation in SEM/ESEM vs. skills in LT method and necessity of precise control of the environment in the ESEM specimen chamber; expensive and time-consuming sample preparation, shape modification and susceptible microstructure damage vs. the highest resolution, the lowest beam sensitivity and repetitive observation in SEM).

The embryogenic cultures of *Picea abies* were obtained from the collection of Mendel University (Brno). Firstly, the ESEs were observed in their native state according to the LT method protocol using a non-commercial ESEM AQUASEM II (Figure 1A) and dried by controlled pressure decreasing to 10 Pa (Figure 1B). Due to the process of freeze substitution, water from the sample surface as well as from the inner structure was dried; while natural surface structure including specific morphological features of ESEs [3] (Figure 1C-light microscopy) was preserved. When the dried samples were stabilized and the absorption of environmental air moisture was prevented, the sample was prepared for transportation, storage or sputter coating and repetitive observation, see Figure 2. For the possibility of comparison, the dry sample was firstly observed in a low vacuum (10 Pa of air) in ESEM (Figure 2A), and then gold coated and observed in SEM (Figures 2 B and C). Image comparison shows minimum morphological changes but also the possibility to observe the susceptible extracellular matrix in SEM (detail from the white box is visible in Figure 2C). Shrinkage visible in Figure 2B (white arrow) was caused during sputter coating.

In our experiment we observed that the possibility for the accurate setting of the working environment in the specimen chamber of the ESEM can be utilized for fast and cheap sample preparation and for repeatable morphological studies of the ESEs of conifer in SEM. The quality of results is strongly dependent on the process conditions; however high variability of ESEM parameters, such as appropriate temperature, freezing velocity and humidity, can be found and set. Despite the fact that this alternative method has many limitations, the surface microstructure is well preserved with minimum artifacts and without expensive and time-consuming chemical treatments.

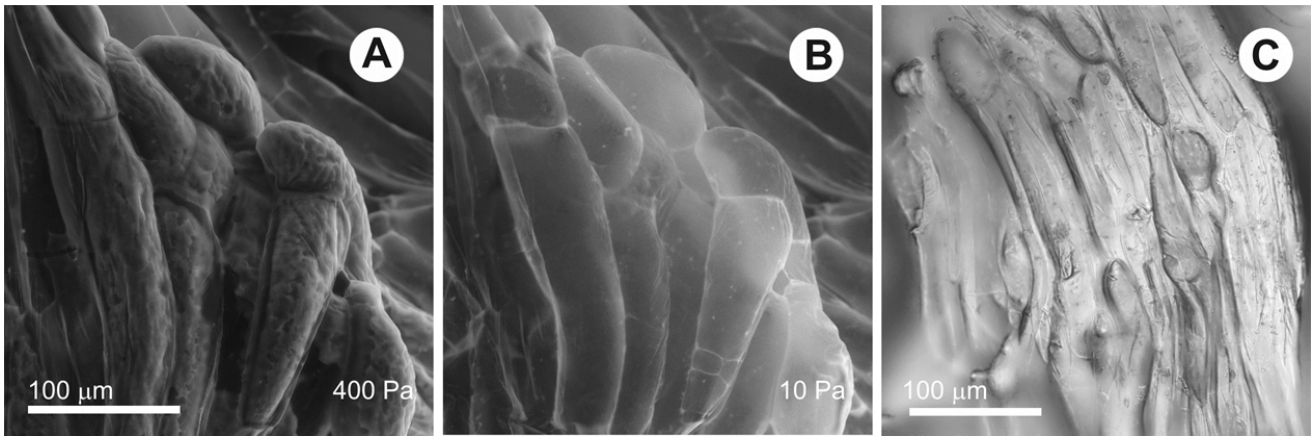

Figure 1: A,B Process of freeze substitution of the ESEs in the ESEM AQUASEM II. The wet mucous layer on the sample surface (A) is slowly removed from the sample until the surface is exposed (B). The light microscopy of the ESEs shows specific structures in their native state (C).

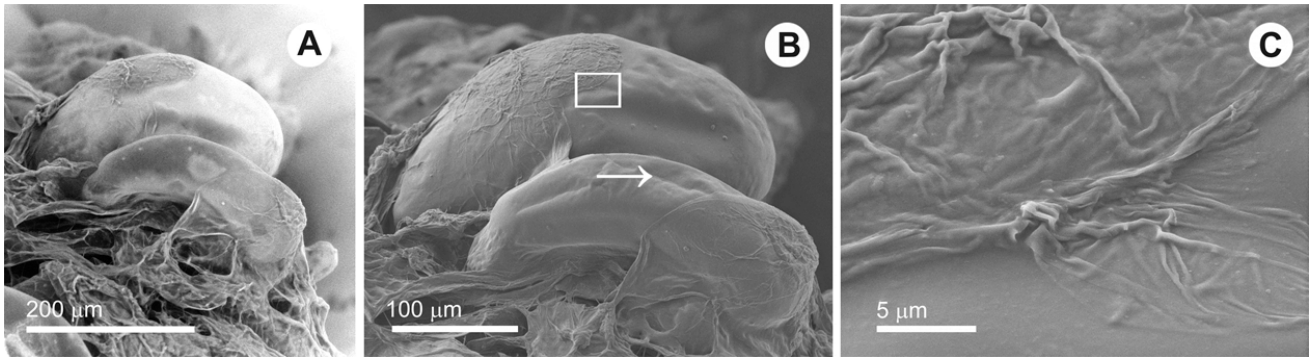

Figure 2: A) The dry sample of ESEs prepared by the LT method observed in low vacuum mode of the ESEM AQUASEM II (10 Pa). (B, C) After gold coating ESEs were repetitively observed in SEM JEOL 6700F in high vacuum 1.5 e^{-5} Pa. High stability of the sample during observation in different vacuum conditions and microscopes is evident. The white arrow points out shrinkage caused by sputter coating.

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