Teze disertace
k získání vědeckého titulu "doktor věd"
ve skupině technických věd.

Advanced Environmental Scaning Electron Microscopy

Komise pro obhajoby doktorských disertací v oboru ELEKTROTECHNIKA, ELEKTRONIKA A FOTONIKA

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Environmentální rastrovací elektronová mikroskopie, Pokročilá environmentální rastrovací elektronová mikroskopie, detekční systémy, matematicko-fyzikální simulace, dynamické *in-situ* experimenty, citlivé vzorky.

KEYWORDS

Environmental scanning electron microscopy, Advanced Environmental scanning electron microscopy, detection systems, mathematical and physical simulations, dynamical *in-situ* experiments, susceptible samples.

Obsah

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SUMARY

The first part of the doctoral thesis contains an introduction to the issue of environmental scanning electron microscopy and a description of its current state in the world, including a commented set of citations of author's articles defining his contribution to the field.

In the next part of the thesis, a set of twenty-eight most important author's articles published in impacted scientific journals is systematically arranged. These articles document the gradual development of his scientific work starting with redesigning and rebuilding of the SEM VEGA from Tescan company leading to the creation of the ESEM AQUASEM II prototype followed by the first results of biological samples research.

Subsequently, next articles deals with gas flow simulations to optimize the design of a differentially pumped chamber of ESEM AQUASEM II and the development of the Scintillation SE detector for variable pressure SEM.

Articles dealing with the Monte Carlo simulations of the signal electron-gas and electron-sample interactions to understand and optimize the operation of an ionization detector for ESEM are presented as well. The article High-efficiency detector of secondary and backscattered electrons for low-dose imaging in the ESEM, published in the prestigious scientific journal Ultramicroscopy, brings significant results that push the boundaries of ESEM's possibilities in the field of signal electron detection. This paper presents for the first time a highly sensitive backscattered electron scintillation detector for ESEM and especially a combined secondary electron detector containing the new ISEDS, which is currently one of the most sensitive detectors for ESEM in the world. The ISEDS is also the first in the world to enable energy filtration of detected electrons in ESEM.

The following series of articles contains unique results of in-situ study of biological samples in dynamically changing conditions of the ESEM specimen chamber. From the point of view of interdisciplinary research in the field of plant biology, the articles presenting the new Low-Temperature method for ESEM and its extended version Extended-Temperature method in the article In-situ preparation of plant samples in ESEM for energy dispersive x-ray microanalysis and repetitive observation in SEM and ESEM are crucial. The above mentioned methods, in combination with ISEDS, enabled for the first time in the world to display highly sensitive rotifers in their native state in ESEM and thus contributed to the discovery of new species of these aquatic organisms. Combination of these methods with a new prototype of Wide Field Aperture Detector helped to discover of new candidate sex-linked genes and their putative role in *Silene latifolia* female flower development.

A series of articles focused on the development of a method for imaging highly sensitive PEC capsules and beads culminated in the article named Simulation-based optimization of thermodynamic conditions in the ESEM for a dynamic in-situ study of spherical polyelectrolyte complex particles in their native state, published in the journal Ultramicroscopy. This article pushes the boundaries of ESEM's current possibilities with its breakthrough results in the field of studying samples of biopolymers and hydrogels.

Further articles present the results of dynamic in-situ experiments focused on changes and behaviour of bentonite as a suitable material for use in nuclear waste repositories.

The doctoral thesis continues with several high impact factor articles in the field of physical and environmental chemistry. The articles deal with the research of morphological changes of

ice containing salt impurities in dynamically changing conditions of the ESEM specimen chamber, e.g. during melting, sublimation and temperature cycling. The published results make it possible to understand and explain the important processes associated with the formation of sea ice, its importance as a reaction medium and the release of salts back into the atmosphere in the form of aerosols and reactive halogens depleting the ozone layer. The findings are summarised in the article introducing a new methodology for research of ice samples at environmentally relevant sub-zero temperatures. The methodology opens new possibilities to observe intact ice samples at close to natural conditions.

The doctoral thesis ends with an article focussed on development of a novel Correlative Light Electron Microscopy method (CLEM) using Total Internal Reflection Fluorescence Microscopy (TIRFM) and Advanced Environmental Scanning Electron Microscopy (A-ESEM). In this paper the A-ESEM as the next generation of the ESEM was firstly introduced.

The doctoral thesis summarizes author's results from many years of research and development of unique methods and instrumentation in the field of environmental scanning electron microscopy. These results broaden the boundaries of possibilities and applicability of ESEM for interdisciplinary and internationally excellent research. The author focused on several areas: 1) development and implementation of prototypes of unique ultrasensitive detectors of signal electrons which, unlike commercially available detectors, are able to image sensitive samples under low energy and very low current of electron beam, hence with minimal radiation damage; 2) development of own Monte Carlo simulation programs to understand the physical processes accompanying signal generation and its amplification in gas for image formation in ESEM, which is crucial for the optimization of the efficiency of our detectors; 3) thermodynamics simulations for accurate setting of optimal conditions (values of gas pressure, temperature and relative humidity) in the proximity to the observed sample to prevent its drying, or for the implementation of advanced in-situ studies of samples in dynamically changing conditions in the ESEM specimen chamber; 4) advanced and dynamical in-situ experiments in defined environmental conditions, which are beyond the capabilities of commercially available electron microscopes. Thanks to the achieved results, new methods, unique instrumentation and sophisticated modifications of electron microscopes, significant progress has been made not only by transformation of environmental scanning electron microscopy to new A-ESEM, but also in many other disciplines.

ÚVOD

Environmentální rastrovací elektronová mikroskopie je jedním z novějších vývojových stádií rastrovací elektronové mikroskopie, a proto jedna z elektronově mikroskopických metod. Z instrumentálního hlediska představuje environmentální rastrovací elektronový mikroskop (EREM) nadstavbu/rozšíření klasického rastrovacího elektronového mikroskopu (REM) a to zejména o systém diferenciálně čerpaných komor, speciální detektory, systémy pro regulaci tlaku plynu v komoře vzorku, systémy pro chlazení vzorku a hydratační systémy. EREM ve svých volitelných pracovních režimech/modech umožňuje práci v podmínkách relativně vysokého tlaku různých plynů v komoře vzorku (obvykle od 10 Pa do 2700 Pa), ovšem také v podmínkách tlaku blížícímu se vakuu tak, jako klasický REM, viz vakuové schéma EREM na obrázek 1. EREM proto nabízí veškeré výhody klasického REM a současně další výhody i nevýhody spojené s přítomností vyššího tlaku plynů v komoře vzorku. Výhodou EREM pracujícího v podmínkách vyššího tlaku plynů je možnost pozorovat přirozeně vlhké vzorky bez jejich poškození dehydratací v podmínkách termodynamické rovnováhy, elektricky izolační vzorky nebo polovodiče bez nutnosti zvodivění jejich povrchu, ale také v podmínkách vyšších energií a proudů svazku analyzovat prvkové složení nenabíjejících se elektricky izolačních vzorků pomocí energiově disperzního X-Ray analyzátoru. Další výhodou je možnost detekovat, v optimálních podmínkách tlaku vodních par a intenzity elektrického pole detektoru až 1000x zesílený signál převážně sekundárních elektronů. Hlavní výhodou EREM je však možnost přímého pozorování vzorků v dynamicky se měnících podmínkách a studium jejich změn při působení různých fyzikálních či chemických vlivů v komoře vzorku EREM. Hlavní nevýhodou EREM je nižší rozlišení než v REM, komplikovanější a těžko automatizovatelné ovládání mikroskopu a při pozorování vlhkých vzorků v nativním stavu nebo realizaci dynamických in-situ experimentů možná vyšší kontaminace mikroskopu. EREM má také v důsledku přítomnosti tlak omezující clony omezené zorné pole, což může komplikovat orientaci na vzorku.

INTRODUCTION

The environmental scanning electron microscopy is one of the newer developmental stages of scanning electron microscopy and therefore one of the electron microscopic methods. From an instrumental point of view, the Environmental Scanning Electron Microscope (ESEM) is an extended version of the conventional scanning electron microscope (SEM) additionally equipped with a system of differential pumping chambers, special detectors, gas chamber pressure control systems, sample cooling and hydration systems. In its selectable operating modes, ESEM allows operation under relatively high pressure conditions of various gases in the specimen chamber (usually from 10 Pa to 2700 Pa of water vapors), but also under near-vacuum conditions like conventional SEM, see vacuum scheme of ESEM in Fig. 1. ESEM therefore offers all the advantages of conventional SEM and at the same time other advantages and disadvantages associated with the presence of higher gas pressure in the specimen chamber. The advantage of ESEM operating under higher gas pressure conditions is the possibility to observe naturally wet samples under the conditions of thermodynamic equilibrium without damaging them by dehydration (Eliášová et al. 2018; Vlašínová et al. 2017), electrically insulating samples or semiconductors without the need to conduct their surface and also the possibility to analyse samples under conditions of higher energies and beam currents using an energy-dispersive X-Ray analyser. Another advantage is the possibility to detect (under optimal conditions of water vapor pressure and electric field intensity of the ionisation detector) up to 1000x amplified signal of mostly secondary electrons. However, the main advantage of ESEM is the possibility of direct observation of samples under dynamically changing conditions and study of their changes under various physical or chemical influences in the specimen chamber of ESEM. The main disadvantage of ESEM is lower resolution than SEM, difficult-to-automate microscope control and possibly higher contamination of the microscope when observing wet samples in native state or performing dynamic in-situ experiments. ESEM also has a limited field of view due to the presence of a pressure limiting aperture, which may complicate the orientation on the sample.

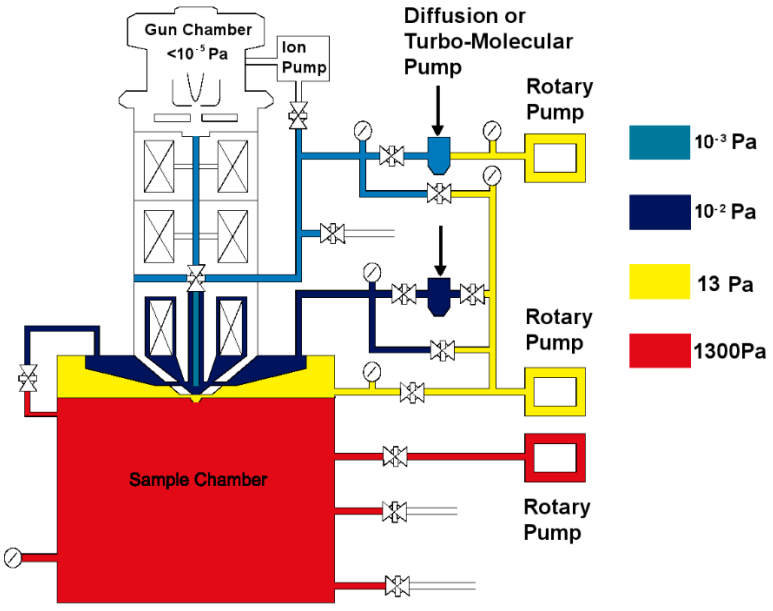

Fig.1: Vacuum scheme of ESEM.

AUTHOR'S SCIENTIFIC CONTRIBUTION IN FIELD OF ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY

One of the author's most important results in the field of research and development of detection systems for ESEM is a new combined system for highly efficient detection of secondary and backscattered electrons (CSSBE). (Neděla et al. 2018). The CSSBE consists of three combined detectors allowing very high efficiency detection of secondary and backscattered electrons (surface topography and material contrasts) at the same time under very low-dose conditions in ESEM, see Fig.2.

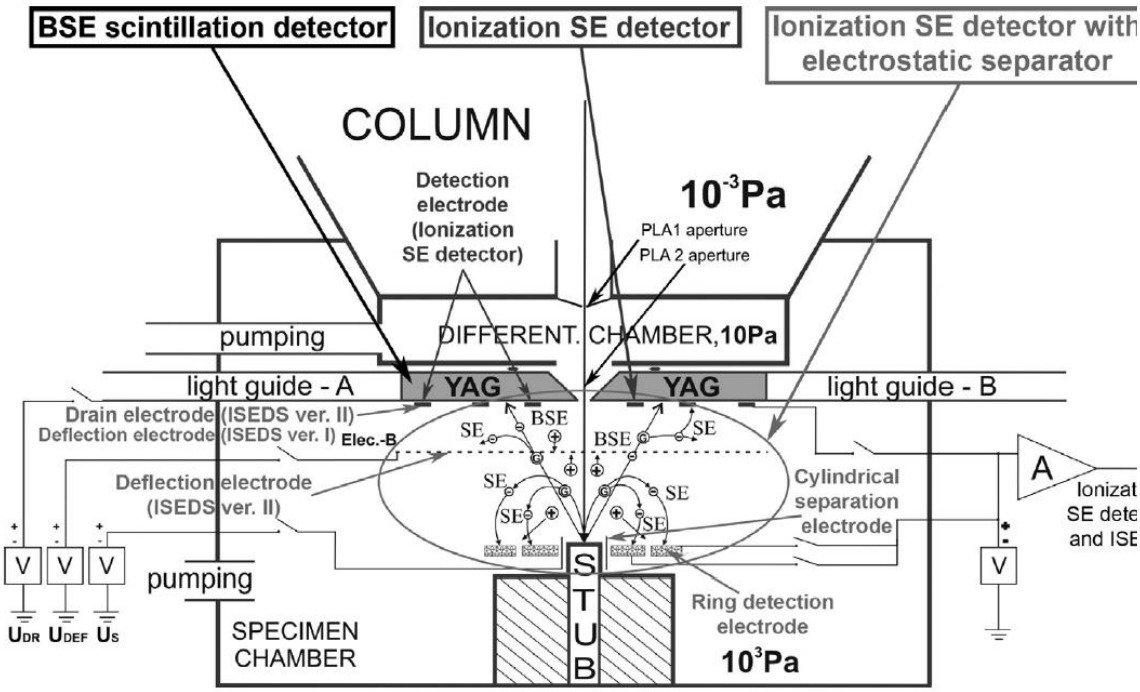

Fig. 2. Schematic of the position of the individual parts of the CSSBE in the ESEM specimen chamber.

One of these detectors is the Ionisation Secondary Electron Detector with the electrostatic Separator (ISEDS) (Neděla et al. 2006). This detector allows recording a strongly amplified signal of SEs with a minimal influence of the BSEs and, as the world's first detector, allows an energy separated detection of signal electrons for the gas pressure from 50 Pa to 300 Pa (Neděla et al. 2010b). The outstanding efficiency of the ISEDS allows imaging of susceptible samples with low emissivity of signal electrons (biological samples, latex particles) under low beam energy 5 keV, up-to-date lowest beam current of 0.2 pA, dwell time of 1.5 ms and pressure of hundreds of Pa (Neděla et al. 2018), see Fig.3.

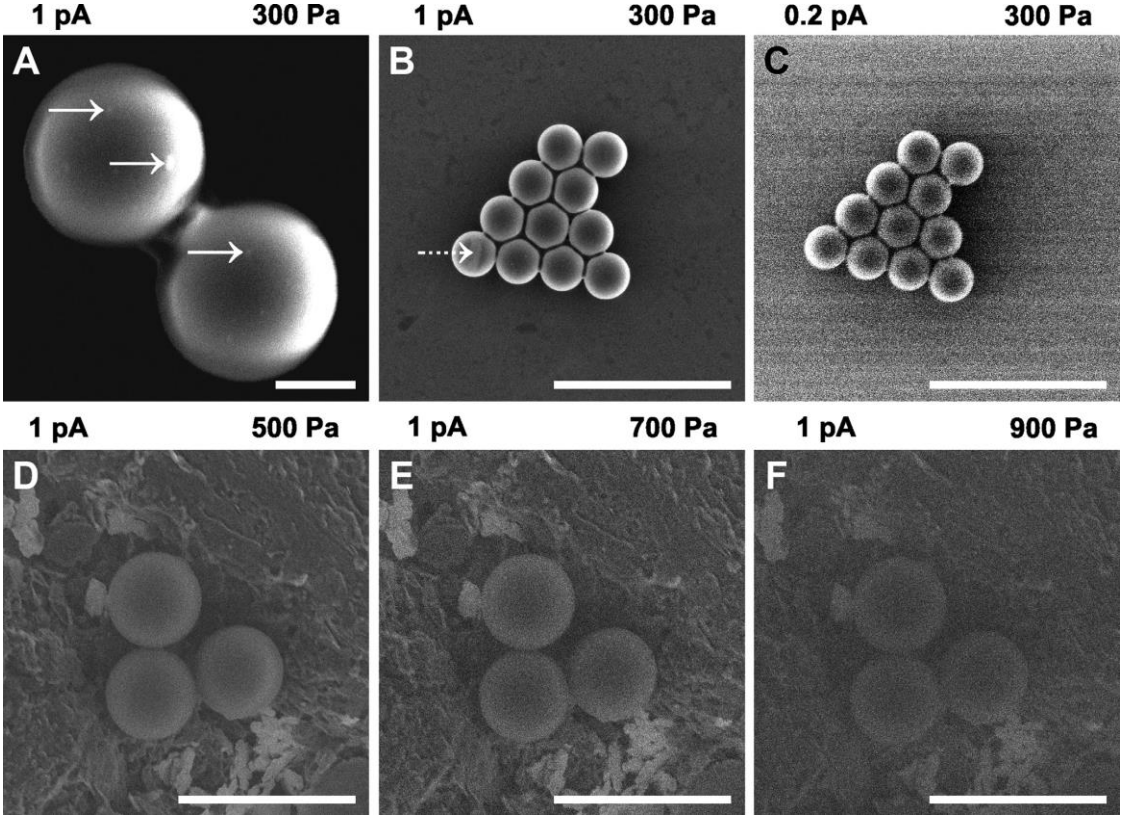

Fig. 3. Polystyrene spheres observed using ISEDS in ESEM AQUASEM II, beam energy 5 keV; A) dwell time 0.63 ms, B) dwell time 1 ms, C) dwell time 1.5 ms; D,E,F) dwell time 0.38 ms; Databar A) 2 μm; B,C,D,E,F) 20 μm.

Another detector we developed is scintillation SE detector for SEM and ESEM (Jirák et al. 2010). The main advantage of this detector is very high detection efficiency in the both SEM and ESEM modes (gas pressure from 0.001 Pa up to 1,000 Pa) without the need to exchange the detectors (Jirák et al. 2010), see Fig. 4.

Fig. 4. Scintillation SE detector for VPSEM (total length of detector prototype is 360 mm).

For macroscopic imaging of wet plants in their native state under large field of view and depth of field and water vapor pressure up to 600 of Pa a new prototype of wide field aperture detector (WFAD) was developed by our group (not published up to date). In combination with the LTM (described later) and software MAPS (Thermofisher Scientific), the WFAD was used for phenotype characterisation of *Silene latifolia* wild-type and epi-mutants, see Fig. 5, where new candidate sex-linked genes (located on the X and Y chromosomes) and their putative role in the female flower development were discovered and described (Bačovský et al. 2021).

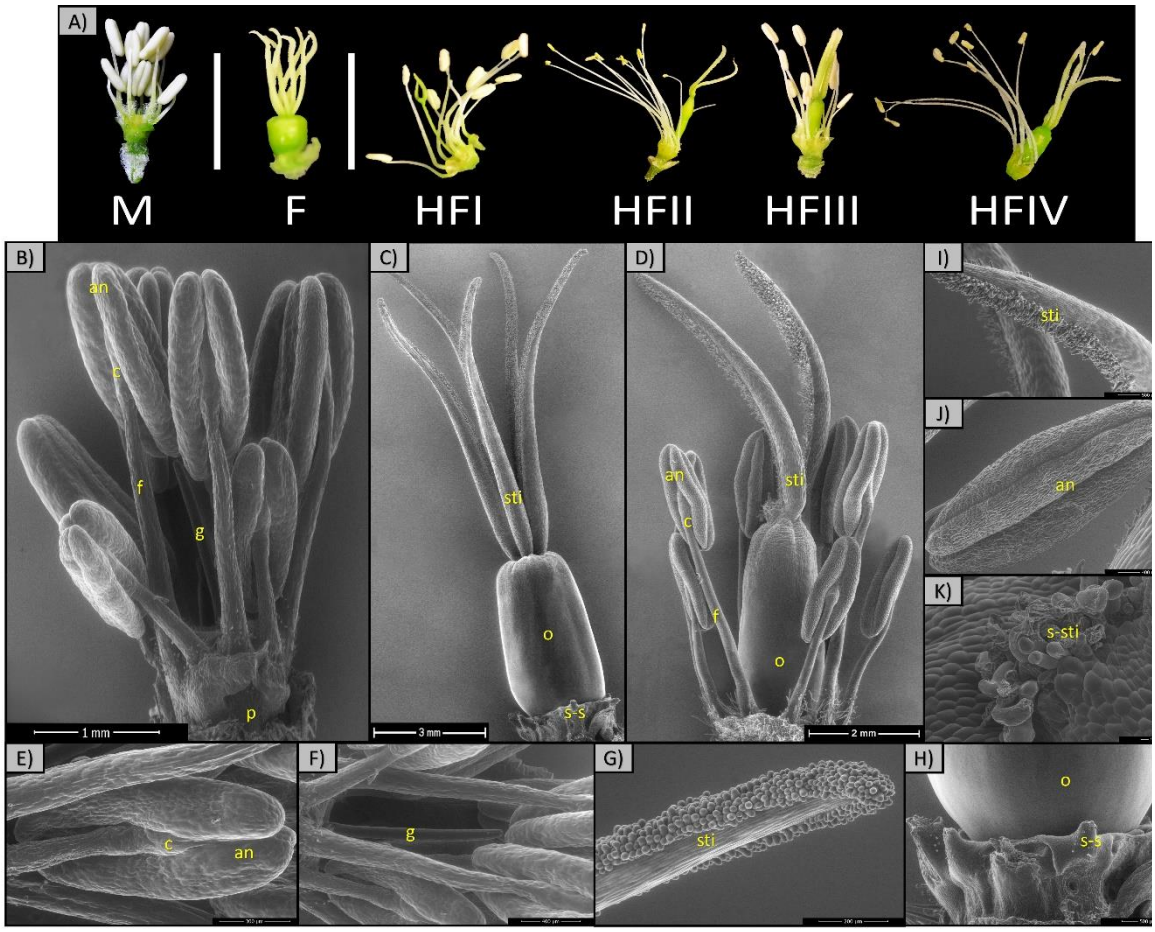

Figure 5. Phenotyping of flower organs in S. latifolia male, female and androhermaphrodite, and flower comparison using A-ESEM. A, Comparison of NT male, female, and four types of androhermaphrodite flowers. B to D, A-ESEM of stage 11 NT male flower, NT female flower, and androhermaphrodite. E to F, Detail of fully developed anther and rudimentary gynoecium in NT male flower. G to H, Detail of fully developed stigma and suppressed stamen in NT female. I to K, Detail of androhermaphrodite stigma, androhermaphrodite anther and suppressed stigma. Note the differences in the carpel size and in the number of stigmas between NT females and androhermaphrodites (C, D). Anther (an), anther connective (c), filament (f), pedicel (p), gynoecium (g), stigma (sti), ovary (o), suppressed stigma (s-sti).

In order to understand the signal generation in a gaseous environment, the program EOD (Lencová et al. 2007), used for the design of charged particle optics devices and detectors, has been extended with a Monte Carlo (MC) module to include the collision phenomena of electrons with the gases in the specimen chamber of the ESEM (Neděla et al. 2011a). Neděla et al. published computed dependencies of signal amplification of detected electrons with selected energies, see Fig. 6 and total signal amplification by including a realistic simulation of the secondary emission from gold sample on the water vapor pressure in ESEM, see Fig. 7 (Neděla et al. 2011b, 2015c). Results of the simulations computed using the EOD software equipped with a Monte Carlo plug-in were compared with experimental measurements and the dependencies of published analytical models.

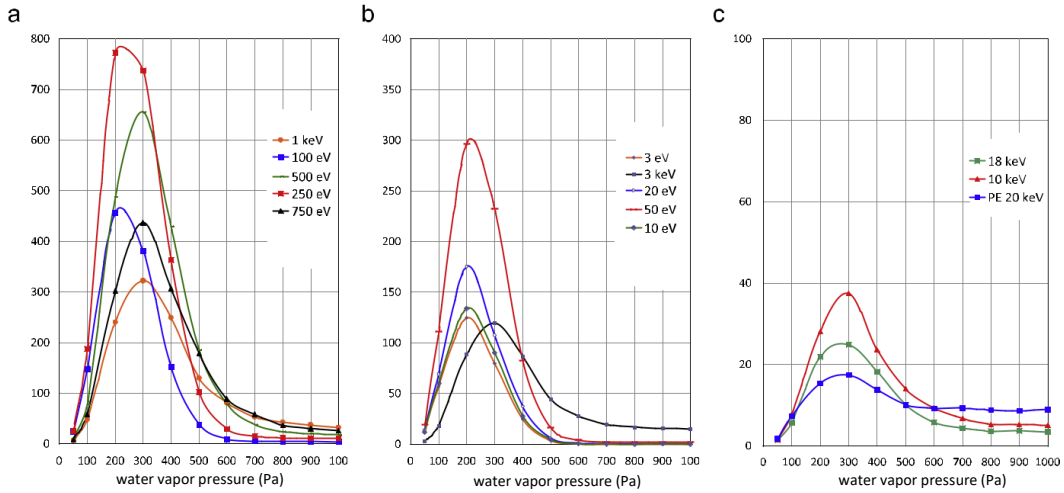

Fig. 6. (a–c) Dependencies of signal amplification in gaseous environment on the water vapor pressure and on the energy of electrons. Two thousand electrons were simulated for each value of pressure. Sample to detection electrode distance is 6 mm and potential on the detection electrode is 300 V.

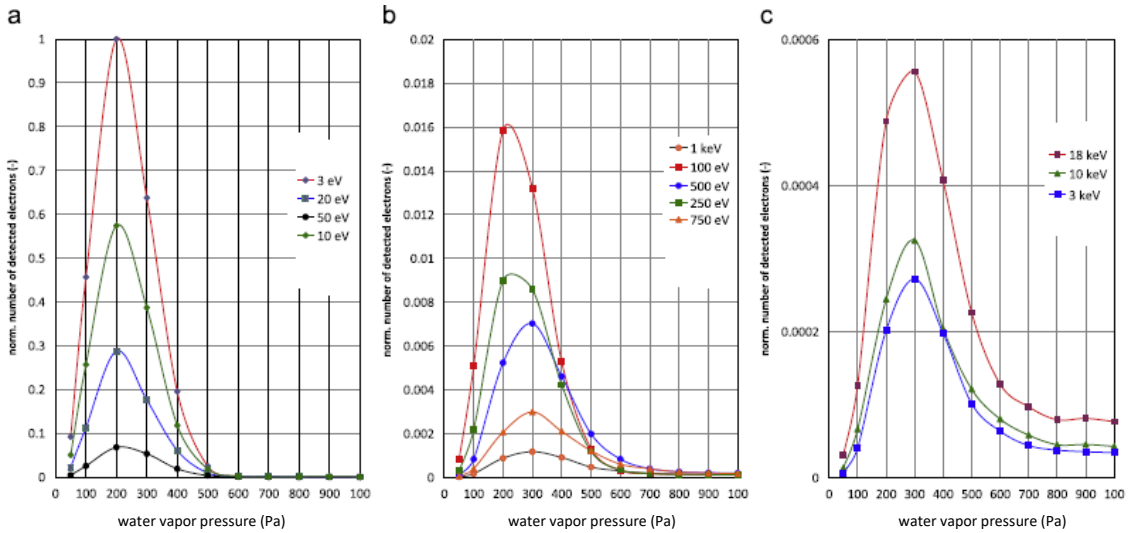

Fig. 7. (a–c) Dependence of the normalized number of detected electrons on the water vapor pressure and the energy of electrons emitted from the gold. It is calculated with respect to simulated energy distribution of signal electrons from gold, energy of primary electrons 20 keV, sample to detection electrode distance 6 mm, potential on the detection electrode 300 V.

Electron-gas interaction in ESEM results not only in the ionization of gas molecules and the emission of new accelerated and cascade-amplified signal electrons (Fletcher et al. 1999, Jirák et al. 2010) by the detectors' electrostatic field (Neděla et al. 2011a,b), but also in the production of positive and negative ions (Toth et al. 2002), see Fig. 8. Positive ions return to the sample surface where its negative charge is neutralized and allows the observation of electrically non-conductive samples free of a conductive layer, see Fig. 9. Ion bombardment also reduces surface contamination of samples.

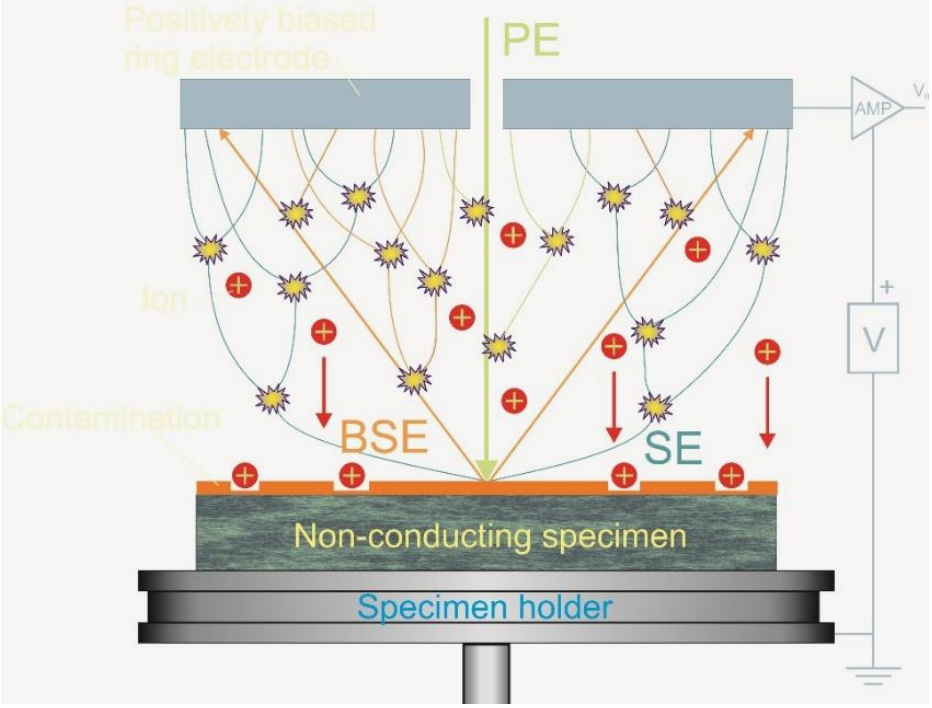

Fig. 8. Model of interaction of beam and signal electrons with gas shows generation and cascade-amplification of signal electrons and ions in high pressure conditions of ESEM equipped with ionization detector.

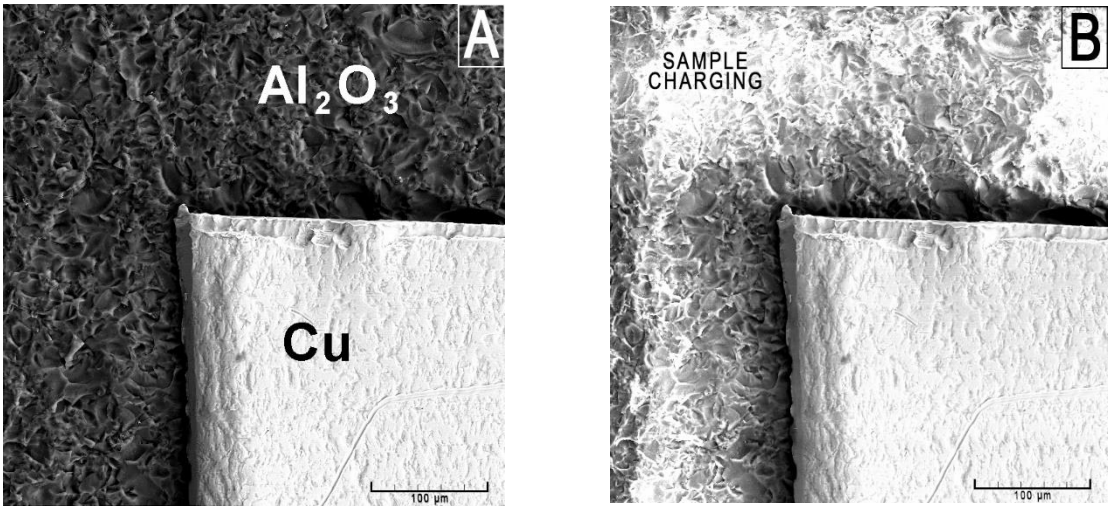

Fig. 9. Electrically non-conductive sample observed in ESEM and SEM, beam energy 30 keV. A) sample without charging in ESEM, water vapor pressure 60 Pa and B) charging sample surface observed in SEM. Bar 100 µm.

If the samples can be simultaneously cooled (usually down to 10°C) and observed under the environment of water vapor (values of temperature and water vapor pressures are set according to the values of the thermodynamic equilibrium, which means under relative humidity 100%, e.g. 2°C and 706 Pa, 5°C and 866 Pa 10°C and 1226 Pa, see Fig. 10), then the samples can be observed in their fully wet state, without the need for any chemical fixation, i.e. in their native state in ESEM.

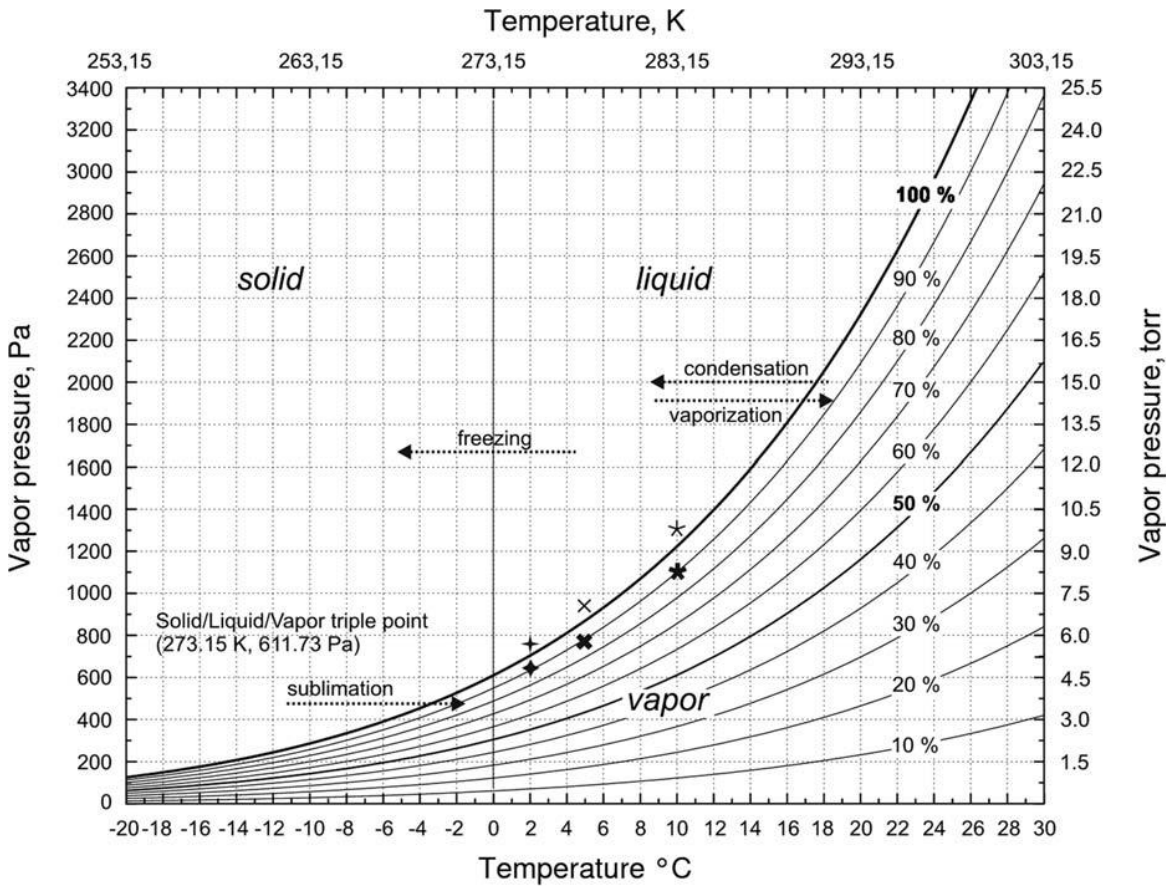

Fig. 10. The saturated water vapor pressure curve with marked relative humidity and water phase changes. Light symbols indicate high-relative humidity conditions at the start of observation in ESEM and bold symbols denote decreasing of water vapor pressure in order to expose sample surface micro-structure.

The ability to precisely control the thermodynamic conditions in the vicinity of the sample opens possibilities for studying samples in dynamically changing conditions, alternatively also under the influence of various physical and chemical processes (Neděla et al. 2020). Tihlaříková et al. (2013) introduced new methods for the gentle in-situ study of live and surviving mites observed under low beam currents in ESEM, see Fig. 11.

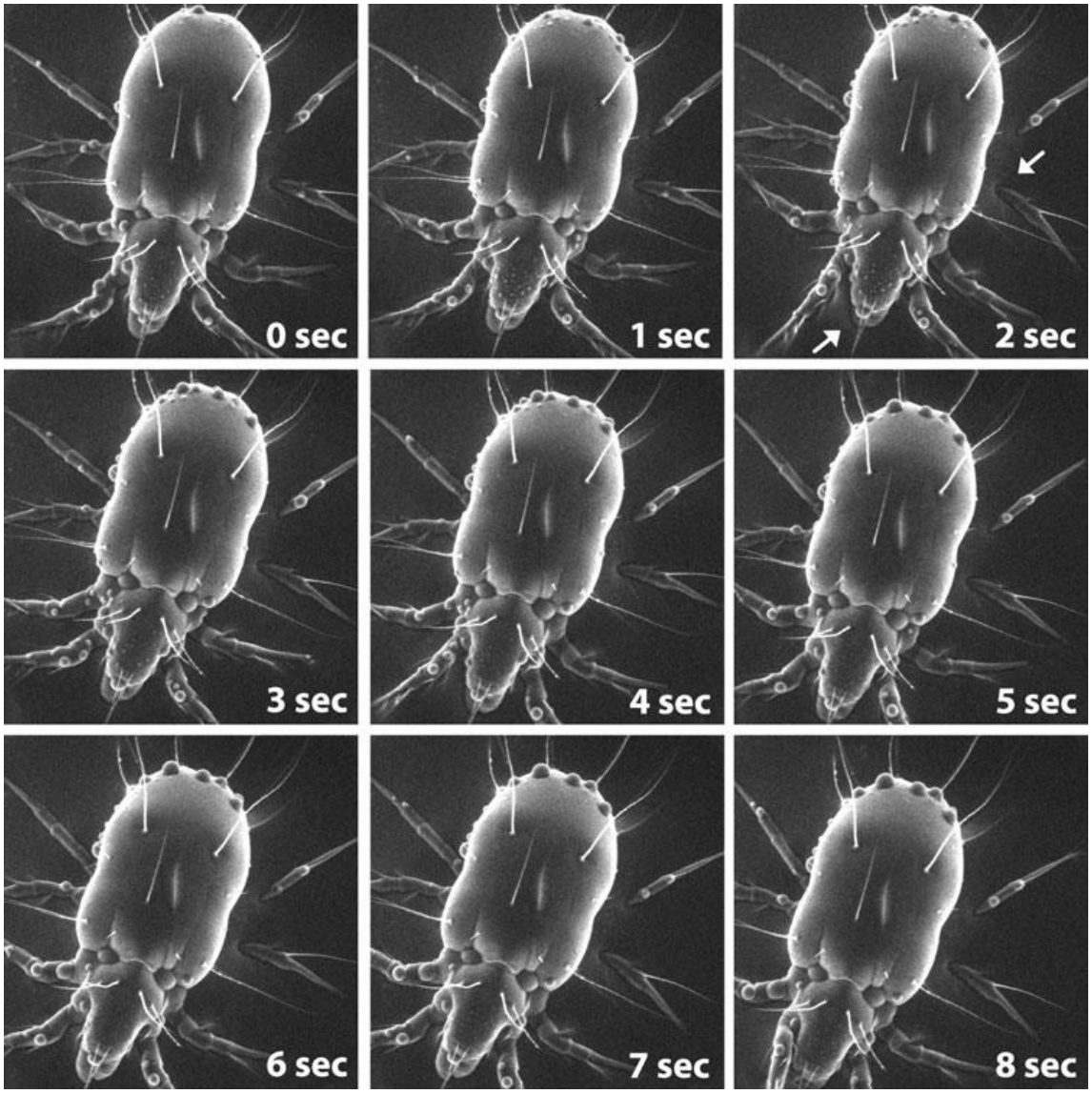

Figure 11. Observation of a live mite in the ESEM AQUASEM II. Frames recorded at marked times and selected from the moving image documenting movement of the mite. All frames show the liquid water underlay in which the mite is immersed, marked by white arrows in a frame of 2 s. Activity of the mite is obvious in the change in its leg.

The possibility to study the morphology of the extracellular matrix covering early somatic embryos of conifers (hundred nm thin layer of biopolymer) in their native state by ESEM was shown by Neděla et al. (2015a). We also introduced new methods for additive in-situ hydration of wet samples in ESEM. This method is based on the usage of small needle connected to hydration system situated outside the ESEM specimen chamber. Alternatively, we can use a wet agar sample holder, which extends observation time of wet biological samples by additional hydration of the sample caused by water evaporating from the agar (Neděla 2007).

A great contribution to the morphological characterization of surfaces of fresh plant sample was the introduction of the Low Temperature method for ESEM (LTM) (Neděla et al. 2015a), see fig. 12. This method allows the increase of resistance of plant sample to radiation damage as well as its shape fixation and stabilization without necessity to use chemicals for long-term observation in ESEM (Neděla et al. 2012).

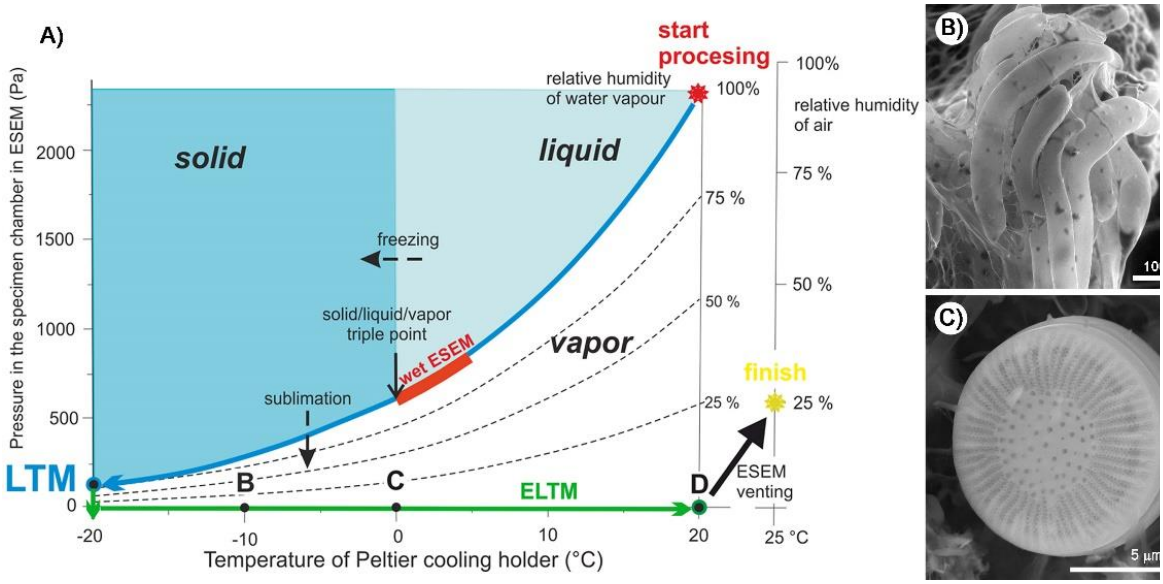

Fig. 12. The phase diagram (A) shows the dependence of the saturated water vapor pressure in the ESEM on the sample temperature. Here are the coloured lines corresponding to the conditions used for the three observation strategies in ESEM. The red line shows conditions for observation of fully wet samples. The blue line are conditions for process of freeze stabilization during the low temperature method (LTM) for ESEM. The green line illustrate process of the extended low temperature method (ELTM) which is used for sample drying and its preparation for later observation or other analysis. The LTM allow in-situ preparation of plant samples in ESEM, for example highly susceptible early somatic embryos (B) or fresh epiphytic diatoms (C).

The results of LTM were validated by other methods such as cryo-SEM and light microscopy (Neděla et al. 2016). The LTM for ESEM was also used for the study of morphological abnormalities caused by copper and arsenic stress on the development of Norway spruce somatic embryos (Đorđević et al. 2019). Using LTM in combination with a new high-efficiency Ionisation Secondary Electron Detector with an electrostatic Separator (ISEDS) for ESEM (Neděla et al. 2018), we recorded the world´s first images of unaffected fresh water rotifers in their native state, and two new species of rotifers were subsequently discovered (Michaloudi et al. 2018), see Fig. 13.

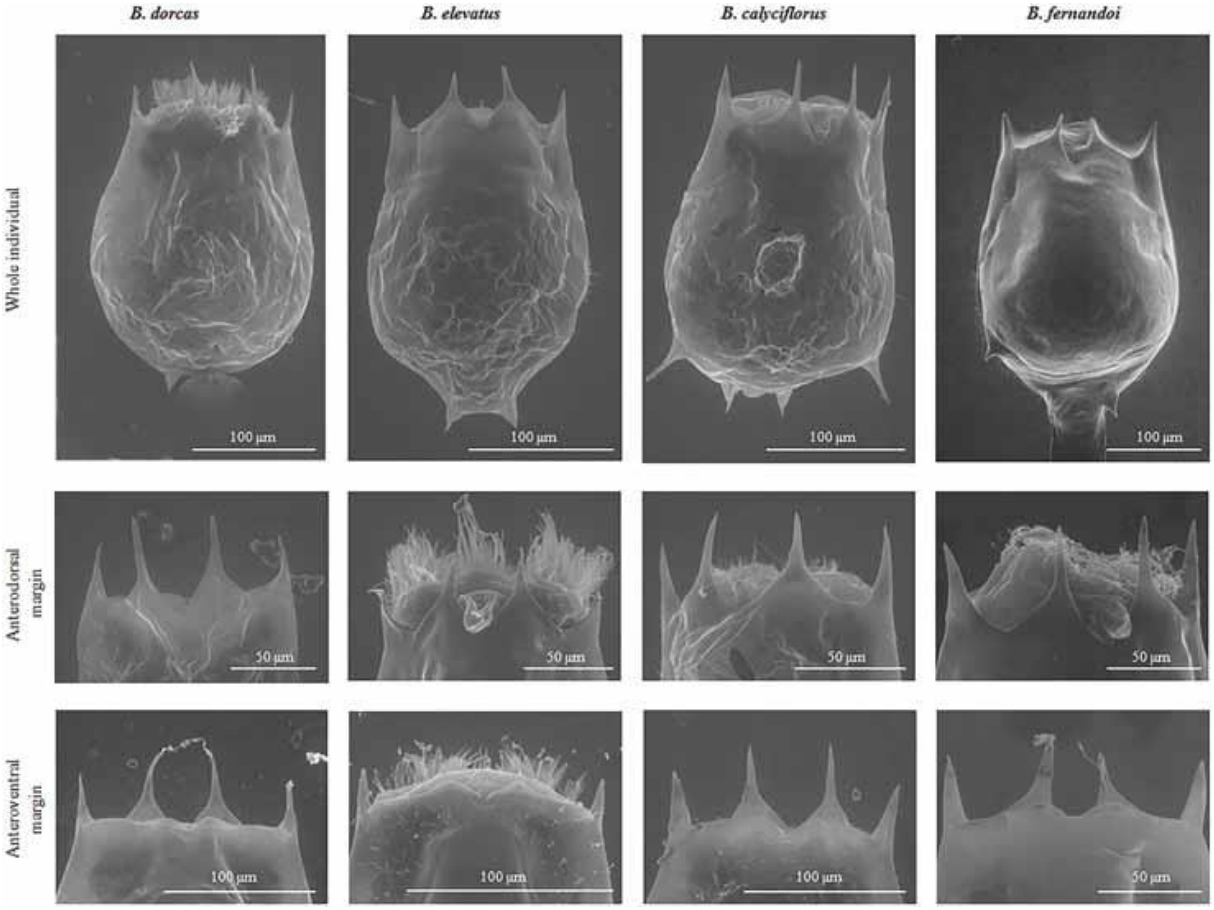

Fig. 13. World unique images of B. *calyciflorus s.s.*, B. *dorcas*, B. *elevatus sp. nov*., B. *fernandoi sp. nov* in their fresh state. Whole individual, Anterodorsal margin, Anteroventral margin. Modified ESEM Qaunta 650 FEG equipped with ISEDS. Observed using LTM for ESEM.

An extended version of the LTM (ELTM) allows in-situ preparation of close-to-native state plant samples and repetitive topographical and material analysis at a higher resolution in the vacuum conditions of SEM or in the low gas pressure conditions of ESEM (Tihlaříková et al. 2019). The ELTM was also utilized for in-situ preparation of plant samples analyzed using an EDX analyzer in an ESEM with no coating or chemical fixation. We were able to quantify traces of silicon, which are not measurable in a conventional manner. We also clarified the relationship between colossal synthesis and transport into the cell wall of the trichomes and the ability to store silicon there, see Fig. 14.

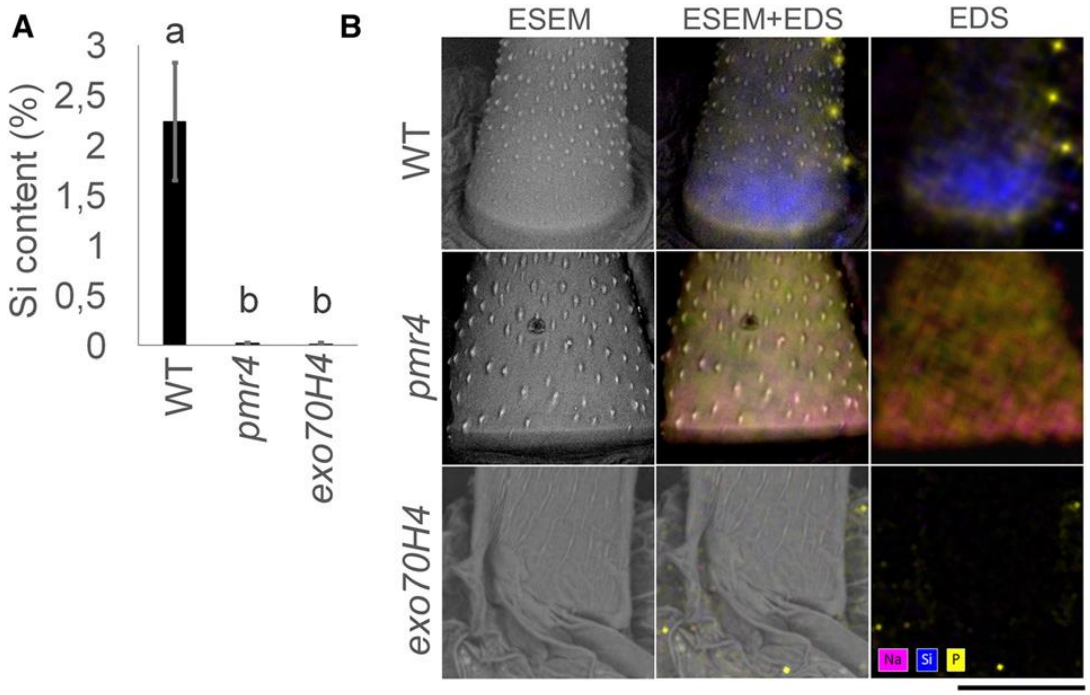

Figure 14. The presence of silica in Arabidopsis trichomes is dependent on callose. A) Quantification of the silica content in multiple genotypes grown on a soil containing 2 mM sodium silicate. Error bars represent SEs. Letters above the bars: mean significant differences (HSD Tukey post hoc test, P, 0.01). B) Examples of trichomes accumulating silicon (blue signal). The accumulation is prominent in the apical domain above the OR. Note that the exo70H4-1 cell wall collapsed due to its mechanical properties. Elements such as sodium (magenta) and phosphate (yellow) also are indicated. Scale bar = 30 mm. WT, Wild type.

The original use of micromanipulators and EDX analysis in ESEM has a crucial contribution to the application of X-Ray EDS micro-analysis for plant biology (Kulich et al. 2018).

In the field of dynamical in-situ experiments, we published several articles with outstanding results. We showed morphological changes of rat tongue at changing thermodynamic conditions in the specimen chamber of ESEM equipped with a new prototype of hydration system (Neděla 2010a). We were the first ones to observe the dynamic in-situ process of sublimation of uncoated frost flowers using our environmental SEM AQUASEM II, see Fig. 15. Based on this observation we experimentally disproved a suspected major role of frost flowers in ozone depletion events as a source of sea-salt aerosol (Yang et al. 2017). Ice contamination processes at grain boundaries in environmentally compatible conditions of a high gas pressure and a relatively high temperature in ESEM in a combination with fluorescence microscopy were studied (Krausko et al. 2014, Vetráková et al. 2019). We also reported partial vitrification of the freeze concentrated solution and the pH change during freezing of NaCl solutions. The ESEM study provided us a unique insight into the morphology of the frozen samples revealing lamellar arrangement of ice and salt (Imrichová et al. 2019). Based on the knowledge and experience gained so far, new methodology for research of ice samples at environmentally relevant sub-zero temperatures, thus under conditions in which it is extremely challenging to maintain the thermodynamic equilibrium of the specimen was published (Imrichová et al. 2021). The methodology opens new possibilities to observe intact ice samples at close to natural conditions. The successful static observation of the intact sample in Figure 16 demonstrates the benefit and novelty of our new method, since such preserved sample shape was never reached in the electron microscope specimen chamber at environmentally relevant temperature before.

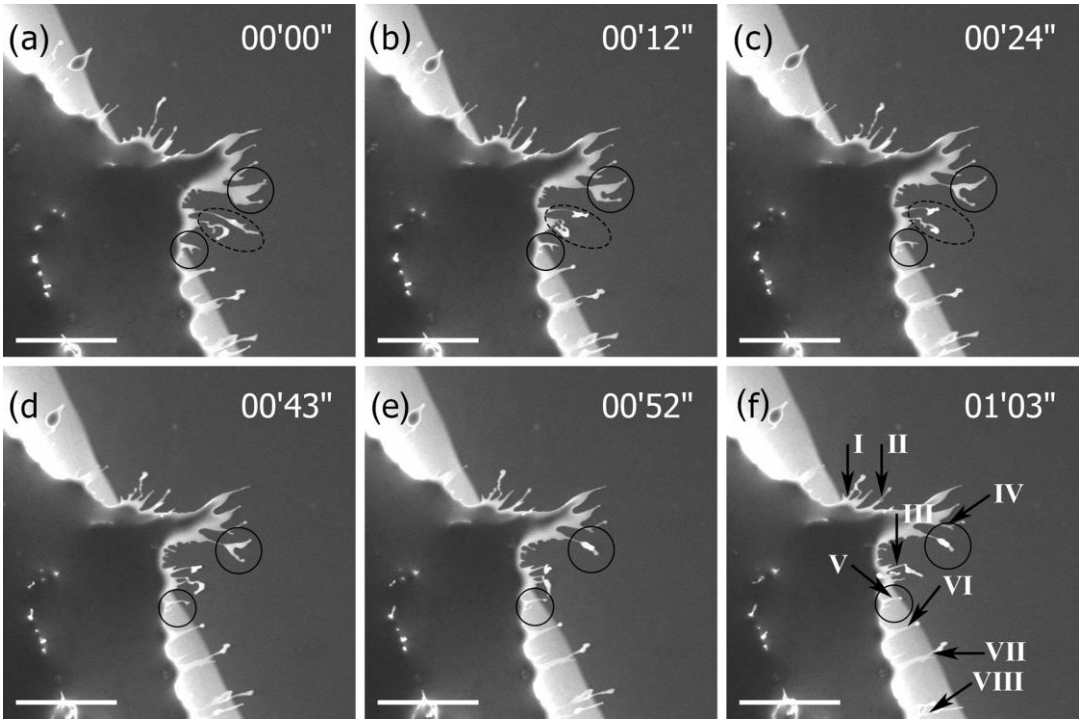

Figure 15. The dynamical in situ images of the formation of brine fingers during slow evaporation of water from the frost flower. The individual fingers bending and flapping around are highlighted in circles. The width of the seven indicated necks in Fig. 3f is measured as dI 2.23±0.43 μm, mean ± standard error of the mean. Imaged with the applied ESEM AQUASEM II; beam energy at 20 keV, ionization detector, water vapor pressure of 348 Pa, sample holder temperature of -5.2 °C, and sample-to-aperture distance of 2 mm. Scale bar: 100 μm.

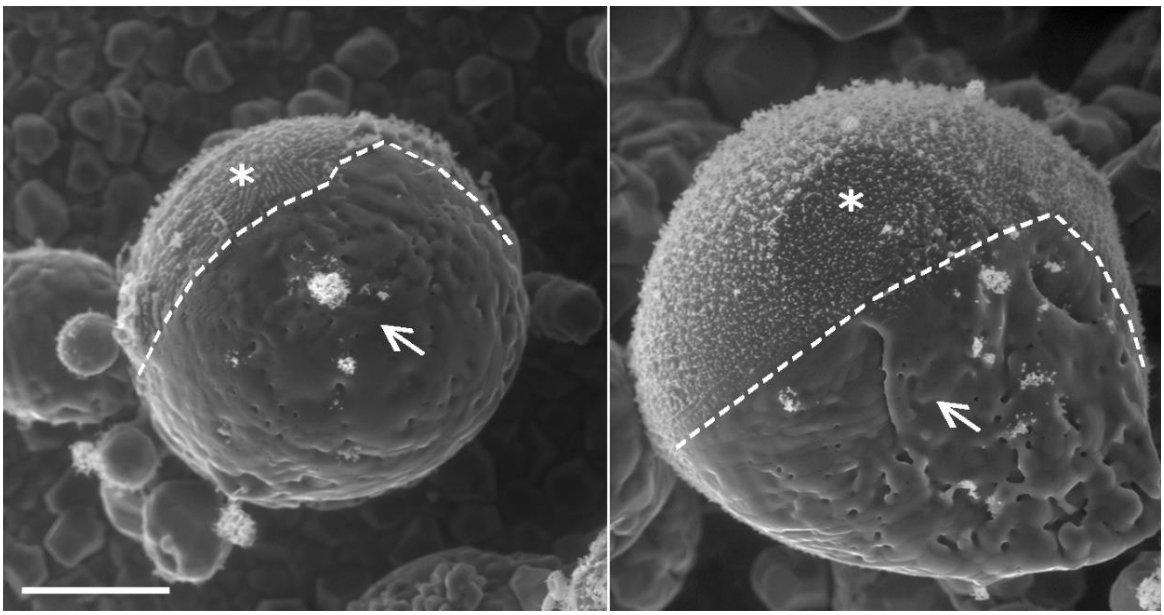

Figure 16: A-ESEM static observation of ice spheres made from 0.06 M NaCl solution using an ISEDS detector. Partial sublimation of a small portion of ice was visible on the upper left part of the spheres (marked with an asterisk). On the opposite pole of the sphere a thin layer of condensed ice can be seen (marked with a solid arrow). Observation parameters: tp -26°C, pN2 420 Pa, pw ~ 60 Pa, beam current 125 pA, dwell time 61 μs. Scale bar = 100 μm for both micrographs.

Morphological and size/shape changes of various types of bentonites and micro-pores inside the bentonite observed under various relative humidity in ESEM were published in collaboration with our partners (Sun et al. 2019a, b). The study of various preparation procedures of lime putties and their surface microstructures monitored by our ESEM was published by Navrátilová et al. (2017). The new way of lime putty preparation was introduced. The preparation consists of mechanical crash of the lime particles immediately after hydration.

One of the most important problems of the study of beam susceptible samples like polymers by electron beam is radiation damage (Grubb 1974), predominately resulting from inelastic collisions between beam electrons and the sample (Egerton et al. 2004). This causes temporary or permanent chemical and physical changes resulting in the formation of volatile products, mass loss and formation of unsaturated structures, chain scissions and cross-linking. Resent results of our group focused on low-dose morphological characterization of extremely beam and environmentally susceptible polyelectrolyte complex particles containing live *E. coli* cells under lowered radiation damage were published by Neděla et al. (2020). We showed that application of lower beam energy enhances electron energy loss and beam scattering in the ESEM, hence the final interaction volume in the sample is smaller. Consequently, the number of free radicals decreases, but their concentration increases, and so the probability of recombination increases. The decrease of beam energy also causes an increase of the emission coefficient of secondary electrons from the sample; however, in the case of a 500 nm water layer, all of the secondary electrons are absorbed by the layer. Inevitable consequence of electron beam-sample interactions is local heating of the inspected area. Due to the relatively low heat conductivity of biological samples and polymers, the real temperature of the sample surface, see Fig. 17 A-C and electron beam affected area, see Fig. 17 D-F, are higher than expected as well as optimal ESEM observation conditions (beam current, gas type and pressure, working distance, etc.), can be different for each sample.

The local increase in the surface temperature, especially when thin polymer layers or hollow beats are observed, can cause irreversible sample dehydration due to the violation of the thermodynamic equilibrium between gas pressure and sample temperature. (Neděla 2010a). Moreover, the samples in ESEM are covered with aqueous layer, which may contain other substances.

According to Raoult's Law, conditions for the thermodynamic equilibrium of clean water did not correspond with the liquid on the sample (Tihlaříková et al. 2013). Furthermore, the presence of the liquid layer can also influence the sample temperature. Calculation of the total amount of heat delivered by the beam to the sample per exposed area is also complicated, because the electron beam is in high pressure conditions of ESEM scattered out from the initially focused spot to the diffused skirt. Also, the chemical composition of the liquid which the beam goes through is difficult to define and its composition can be changed during the experiment.

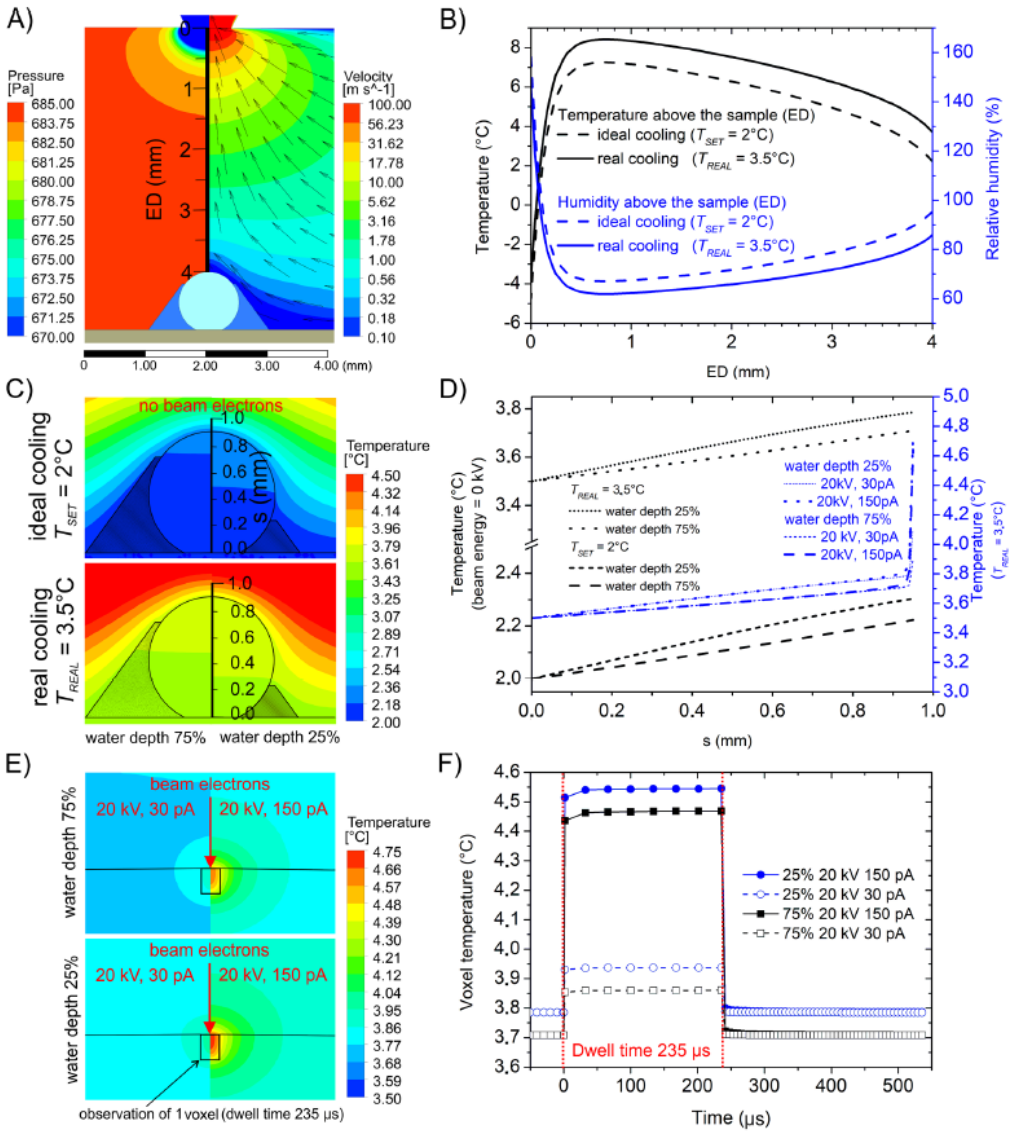

Fig. 17. Simulations of thermodynamic conditions in the vicinity of the sample under experimental conditions in the ESEM AQUASEM II. A, B) the dependence of water vapor pressure, velocity, temperature and relative humidity on the ED; C, D) the temperature gradient inside and closely above the sample under the conditions of ideal and real cooling at 25% and 75% of the surrounding water depth; E) The thermal impact of different beam currents and water depth in the sample's voxel; F) the dependence of voxel temperature on dwell time at various conditions of levels of surrounding water depth and beam current.

Given the above-mentioned findings the current level of commercially available environmental scanning electron microscopes prohibits the study of very susceptible samples like polyelectrolyte complex capsules/beads (PEC) in their native, fully wet, hence unfrozen, state free of damage. The Scientific Group of Environmental Electron Microscopy of the ASCR in Brno currently represents the world's leading workplace in the field of environmental scanning electron microscopy. In an effort to push the boundaries of application capabilities of ESEM, a breakthrough results have recently been published by our group (Neděla et at 2020). We introduced the most complex analysis and optimization of thermodynamic conditions in the specimen chamber of ESEM to allow in-situ observation of extremely delicate wet PEC particles in their native state, see Fig. 18. Based on all experimental and simulation results we also introduce a Delicate Sample Observation Strategy for the ESEM (DSOS).

We show how this strategy can be applied to the characterization of polyelectrolyte complex spherical particles containing immobilized recombinant cells *E. coli* overexpressing cyclohexanone monooxygenase, used as a model biocatalyst (Bertóková et al. 2015). We present the first native-state electron microscopy images of the viscous core of a halved polyelectrolyte complex capsule containing living cells, see Fig. 18I.

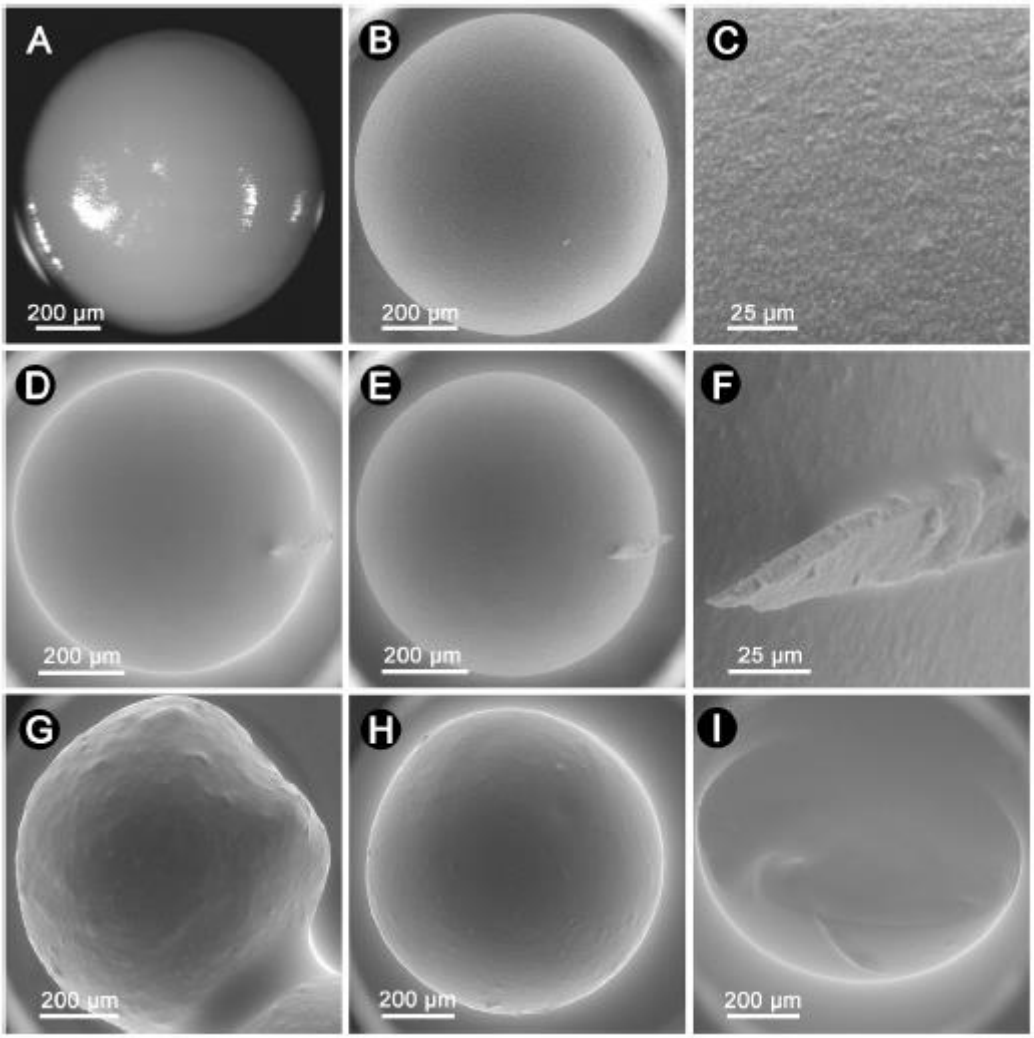

Fig. 18. The DSOS for ESEM applied on different types of spherical PEC particles. Light microscopy image (A) validates that minimal shape changes occur using ESEM, shown in image (B). (C) shows the detail of the surface micromorphology of the same sample (B). Images D-H show impurities and defects of the PEC particle. (F) shows the detail of the impurity visible in figures D and E. The irregular shape of sphericity can be seen in G (10% of E.coli cells causes the surface corrugation) and H. The most sensitive viscous core of the halved spherical PEC particle completely without damage or shape deformation is shown in (I). Observation conditions: TSET 0.2 °C ≈TREAL 2.0 °C, 684 Pa, RHREAL 97%, ED 4 mm; beam energy 10 keV, beam current 30pA (15 pA for I).

The exceptionality of our methods (Neděla et al. 2015b, Tihlaříková et al. 2013) and the ESEM AQUASEM II were also demonstrated by the possibility to image and measure dimensions of unaffected PEC beds prepared by a novel two-step reaction of oppositely-charged polymers including highly defined cellulose sulphate (Krajčovič et al. 2017). Polyvinyl alcohol lens-shaped particles were also imaged for the first time using our microscope under reduced temperature and at the air pressure 540 Pa (Schenkmayerova et al. 2014).

We have also developed a novel correlative light electron microscopy method (CLEM) using total Internal Reflection Fluorescence Microscopy (TIRFM) and Advanced Environmental Scanning Electron Microscopy (A-ESEM), see Fig. 19. This technique enables to determine the number of auxin efflux carriers from the PINFORMED (PIN) family (*Nt*PIN3b-GFP) within plasma membrane (PM) nanodomains of tobacco cell PM ghosts (Stelate et al.2021). In this paper we also firstly introduce A-ESEM as the next generation of the ESEM. A-ESEM is based on number of technological and methodological improvements that have been developed by our group at ISI AS CR.

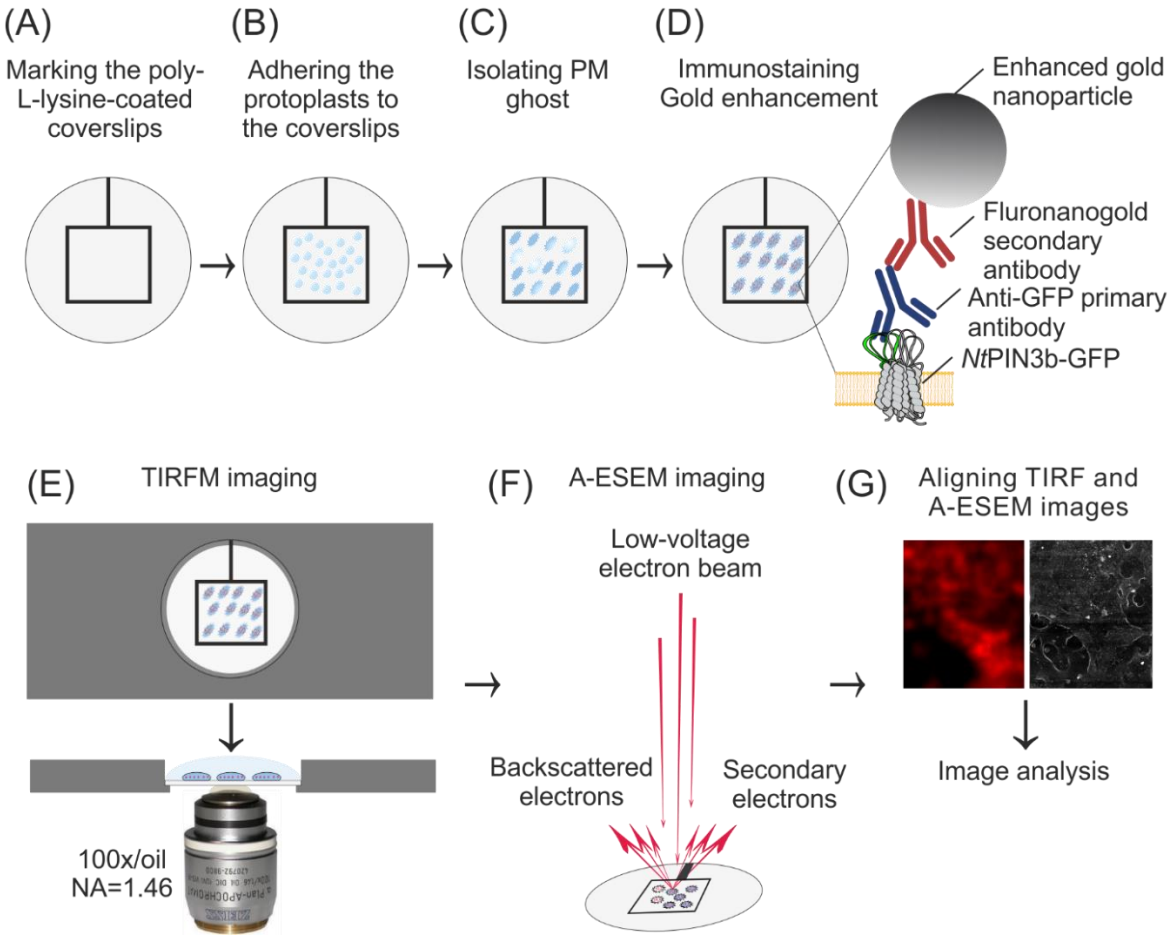

Figure 19. Workflow of TIRFM/A-ESEM CLEM of NtPIN3b auxin efflux carriers in the PM of tobacco cell protoplast ghosts. (A-C) Poly-L-lysine coated coverslips are labelled with adhesive tape, protoplasts are adhered on coverslips and PM ghosts are isolated by several quick flicks. (D) Indirect immunofluorescence staining of NtPIN3b-GFP. Protein, antibodies and gold after enhancement are shown in real size ratios. (E) TIRF microscopy on wet samples using a custom aluminium stage insert and a high numerical aperture objective. (F) A-ESEM performed on dried samples. (G) Software-assisted alignment of TIRFM and A-ESEM images using fiducials defined by superimposing bright field and A-ESEM overview images.

Some modern electron microscopes allow not only the high-resolution observation or usage of the focused ion beam to etch or machine surfaces of samples under vacuum conditions, but also enabling easy switching to the environmental mode and take full advantage of the ESEM described above. These advantages today lie mainly in the possibility of in-situ characterization of samples in conditions of dynamically changing environment, under various physical or chemical influences, all in combination with the possibility to directly measure electrical and non-electrical quantities. Local injection of liquids and gases on the sample, integrated into

micromanipulators, is also possible. Transmission scanning electron microscopy of nanoparticles in liquids with sub nanometer resolution, highly sensitive elemental micro analysis of native and uncoated samples, and in combination with other microscopic methods also correlative microscopy represent a group of specialized equipment.

New technologies are being developed for the world´s unique low-energy ESEM system to observe samples using 1 keV electron beam energy, which will be presented by the ISI ASCR Environmental Electron Microscopy group in the next few years. This will enable to study sensitive samples in an even more gentle way and bring several advantages when studying semiconductors and other electrically non-conductive samples. These technologies will be implemented into our modified ESEM QUANTA 650 FEG equipped with Schottky electron source allowing high resolution imaging of samples.

CONCLUSION

The first part of the doctoral thesis contains an introduction to the issue of environmental scanning electron microscopy and a description of its current state in the world, including a commented set of citations of author's articles defining his contribution to the field. In the next part of the thesis, a set of twenty-five most important author's articles published in impacted scientific journals is systematically arranged. These articles document the gradual development of his scientific work starting with redesigning and rebuilding of the SEM VEGA from Tescan company leading to the creation of the ESEM AQUASEM II prototype followed by the first results of biological samples research. Subsequently, next articles deal with gas flow simulations to optimize the design of a differentially pumped chamber of ESEM AQUASEM II and the development of the Scintillation SE detector for variable pressure SEM. Articles dealing with the Monte Carlo simulations of the signal electron-gas and electron-sample interactions to understand and optimize the operation of an ionization detector for ESEM are presented as well. The article High-efficiency detector of secondary and backscattered electrons for low-dose imaging in the ESEM, published in the prestigious scientific journal Ultramicroscopy, brings significant results that push the boundaries of ESEM's possibilities in the field of signal electron detection. This paper presents for the first time a highly sensitive backscattered electron scintillation detector for ESEM and especially a combined secondary electron detector containing the new ISEDS, which is currently one of the most sensitive detectors for ESEM in the world. The ISEDS is also the first in the world to enable energy filtration of detected electrons in ESEM. The following series of articles contains unique results of in-situ study of biological samples in dynamically changing conditions of the ESEM specimen chamber. From the point of view of interdisciplinary research in the field of plant biology, the articles presenting the new Low-Temperature method for ESEM and its extended version Extended-Temperature method in the article In-situ preparation of plant samples in ESEM for energy dispersive x-ray microanalysis and repetitive observation in SEM and ESEM are crucial. The above mentioned methods, in combination with ISEDS, enabled for the first time in the world to display highly sensitive rotifers in their native state in ESEM and thus contributed to the discovery of new species of these aquatic organisms. A series of articles focused on the development of a method for imaging highly sensitive PEC capsules and beads culminated in the article named Simulation-based optimization of thermodynamic conditions in the ESEM for a dynamic in-situ study of spherical polyelectrolyte complex particles in their native state, published in the journal Ultramicroscopy. This article pushes the boundaries of ESEM's current possibilities with its breakthrough results in the field of studying samples of biopolymers and hydrogels. Further articles present the results of dynamic in-situ experiments focused on changes and behaviour of bentonite as a suitable material for use in nuclear waste repositories. The doctoral thesis ends with several articles with a high impact factor in the field of physical and environmental chemistry. The articles deal with the research of morphological changes of ice containing salt impurities in dynamically changing conditions of the ESEM specimen chamber, e.g. during melting, sublimation and temperature cycling. The published results make it possible to understand and explain the important processes associated with the formation of sea ice, its importance as a reaction medium and the release of salts back into the atmosphere in the form of aerosols and reactive halogens depleting the ozone layer.

The doctoral thesis summarizes author's results from many years of research and development of unique methods and instrumentation in the field of environmental scanning

electron microscopy. These results broaden the boundaries of possibilities and applicability of ESEM for interdisciplinary and internationally excellent research. The author focused on several areas: 1) development and implementation of prototypes of unique ultrasensitive detectors of signal electrons which, unlike commercially available detectors, are able to image sensitive samples under low energy and very low current of electron beam, hence with minimal radiation damage; 2) development of own Monte Carlo simulation programs to understand the physical processes accompanying signal generation and its amplification in gas for image formation in ESEM, which is crucial for the optimization of the efficiency of our detectors; 3) thermodynamics simulations for accurate setting of optimal conditions (values of gas pressure, temperature and relative humidity) in the proximity to the observed sample to prevent its drying, or for the implementation of advanced in-situ studies of samples in dynamically changing conditions in the ESEM specimen chamber; 4) advanced and dynamical in-situ experiments in defined environmental conditions, which are beyond the capabilities of commercially available electron microscopes. Thanks to the achieved results, new methods, unique instrumentation and advanced modifications of electron microscopes, significant progress has been made not only in environmental scanning electron microscopy, research of wet susceptible biological and polymer samples, but also in many other disciplines.

PODĚKOVÁNÍ

Chtěl bych především poděkovat mým kolegům, členům vědecké skupiny Environmentální elektronová mikroskopie ÚPT AV ČR, v.v.i. v Brně za spolupráci a pomoc v oblasti výzkumu a vývoje Pokročilé environmentální rastrovací elektronové mikroskopie a také spoluautorům mých vědeckých článků, které prezentují možnosti a využití této metody napříč vědními obory.

Vilém Neděla

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Vilém Neděla

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RESUMÉ

Doktorská práce dokumentuje vědecké výsledky autora dosažené v oblasti Environmetální rastrovací elektronové mikroskopie za posledních dvacet let. Úvodní část práce obsahuje komentovaný přehled těchto výsledků v kontextu mezinárodního výzkumu a definuje přínos autora pro rozvoj vědního oboru. Současně popisuje klíčové výhody a nevýhody výše uvedené metody a dále se zaměřuje na výzvy pro současný a budoucí rozvoj tzv. pokročilé environmentální rastrovací elektronové mikroskopie. Ta umožní zobrazovat povrchy velmi citlivých, často vlhkých biologických nebo polymerních vzorků v nativním stavu bez poškození. Vzorky budou studovány ve statických nebo dynamicky se měnících podmínkách, nebo při působení různých fyzikálních či chemických vlivů. Pro zobrazování povrchů s velkým rozlišením a hloubkou ostrosti budou použity velmi nízké proudy a energie elektronového svazku minimalizující radiační poškození vzorků. Systematický vývoj pokročilé environmentální rastrovací elektronové mikroskopie od prototypu elektronového mikroskopu AQUASEM II až po současný, modifikovaný mikroskop QUANTA 650 FEG včetně výsledků použití této metody v různých vědních oborech popisuje druhá část práce, tvořená souborem vybraných vědeckých článků.

RESUME

The doctoral thesis presents the scientific results of the author in the field of Environmental Scanning Electron Microscopy in the last twenty years. The first part provides a commented overview of the international research results and defines the author's contribution to the development within the scientific field. It also describes the key advantages and disadvantages of the above-mentioned method and focuses on the challenges of current and future development of so-called Advanced Environmental Scanning Electron Microscopy. This should allow surfaces of very sensitive, often wet biological or polymeric samples to be imaged in their native state without damage. Samples will be studied under static or dynamic conditions or under various physical and chemical influences. Very low currents and electron beam energy minimizing radiation damage to the samples will be used for imaging of high resolution and depth of field observation of surfaces. The second part describes the systematic development of advanced environmental scanning electron microscopy from the prototype of the AQUASEM II electron microscope to the current modified QUANTA 650 FEG microscope, including the results of application of this method in various scientific fields.