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1 ***In situ* ESEM using 3-D printed and adapted accessories to observe living plantlets and their**
2 **interaction with enzyme and fungus**

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29 **Abstract:**

30 This paper describes an innovative way of using environmental scanning electron microscopy
31 (ESEM) and the development of a suitable accessory to perform *in situ* observation of living
32 seedlings in the ESEM. We provide details on fabrication of an accessory that proved to be
33 essential for such experiments but inexpensive and easy to build in the laboratory, and present
34 our *in situ* observations of the tissue and cell surfaces. Sample-specific configurations and
35 optimized tuning of the ESEM were defined to maintain *Arabidopsis* and flax seedlings viable
36 throughout repetitive exposure to the imaging conditions in the microscope chamber. This
37 method permitted us to identify cells and tissues of the live plantlets and characterize their
38 surface morphology during their early stage of growth and development. We could extend the
39 application of this technique, to visualize the response of living cells and tissues to exogenous
40 enzymatic treatments with polygalacturonase in *Arabidopsis*, and their interaction with
41 hyphae of the wilt fungus *Verticillium dahliae* during artificial infection in flax plantlets. Our
42 results provide an incentive to the use of the ESEM for *in situ* studies in plant science and a
43 guide for researchers to optimize their electron microscopy observation in the relevant fields.

44 Keywords: ESEM accessories, *In Situ* observation, *Arabidopsis thaliana*, *Linum usitatissimum*,
45 Polygalacturonase, *Verticillium dahliae*

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57 **1. Introduction:**

58 Many macroscopic properties and qualitative characteristics of plants and biomaterials
59 originate from their cell wall composition and organization (Holland et al., 2020). Observation
60 of cells, tissues and organs and subtle alterations of their morphology often requires high-
61 resolution imaging (Talbot and White, 2013). Therefore, specialized microscopy techniques
62 have been developed to reveal structural features such as distribution of different cell types,
63 and to characterize cell wall components at micron and submicron-scale (Mayo et al., 2010;
64 Sozzani et al., 2014). Application of high-resolution microscopy in plant studies, however, is
65 not always straightforward. The crucial limitations can arise from the nature of the samples
66 and difficulties in their preparation and conservation, as well as requirements imposed by the
67 microscopy techniques, such as specific imaging environment and complex image
68 reconstitution (Costa and Plazanet, 2016; Wu and Becker, 2012). Scanning electron
69 microscopy (SEM), ever since the early days of its application by biologists, has been widely
70 used to observe plant cells, tissues and their surface morphology (Carr and Carr, 1975; Stant,
71 1973). SEM can provide high-resolution pseudo-three dimensional (3D) images of the surfaces
72 at a broad magnification range. These images are acquired more rapidly and comprehended
73 more easily than those obtained by more complex techniques used in two or three-
74 dimensional microscopic analyses of plant cells (Barbier de Reuille et al., 2005; Costa and
75 Plazanet, 2016). Nevertheless, the application of conventional SEM to study the surface of
76 hydrated tissues or living cells has some limitations. Hydrated samples can lose their water in
77 an uncontrolled dehydration process when exposed to vacuum of the microscope chamber,
78 often resulting in deformation or destruction of their native structure (Bogner et al., 2007;
79 Bozzola and Russell, 1999). Plant materials have usually a very high water content and are
80 strongly dependent on turgor pressure for their structural integrity (Ensikat et al., 2010; Sorbo
81 et al., 2008). Therefore, sample preparation is crucial in order to conserve structural features
82 of plants tissues and cells for observation under the high vacuum of an electron microscope
83 (Pathan et al., 2010). Accordingly, specialized sample preparation protocols have been
84 developed to minimize the artefacts and artificial changes that may occur in the original
85 structure of the hydrated samples during their conservation process (Ensikat et al., 2010; He
86 et al., 2019; Kirk et al., 2009; Popielarska-Konieczna et al., 2010). These methods however, are
87 not suitable for *in situ* observation and analyzing the live plant tissues in their native state

88 (Tihlaříková et al., 2019). Development of environmental SEM (ESEM), first commercialized
89 by ElectroScan and later by Philips/FEI (Amsterdam, the Netherlands) in the 1980s, introduced
90 the possibilities of imaging wet objects in a gaseous environment with no prior specimen
91 preparation (Donald et al., 2003; Gilpin, 1997; Muscariello et al., 2005). In an ESEM, the
92 hydration state of the specimen is maintained by simultaneously controlling its temperature
93 and the water vapor pressure in the chamber (Donald, 2003; Stokes, 2008). The ESEM,
94 therefore, has been widely used in biological research including plant science to study the
95 ultrastructure and the morphology of hydrated samples (Stabentheiner et al., 2010), and the
96 use of ESEM in comparative studies of static morphology of biological materials has received
97 considerable attention. Nevertheless, the observation of dynamic processes in biology has
98 been largely confined to the *in situ* mechanical testing of tissues or physical interactions with
99 biological surfaces such as wetting behaviors (Dauwe et al., 2021; McGregor and Donald,
100 2010; Stabentheiner et al., 2010; Wigzell et al., 2016). Attempts have been made to test the
101 viability of plant cells and view dynamic natural processes in living tissue using ESEM. Zheng
102 et al. (2009) could maintain the cells of the upper epidermal tissue of *Allium cepa* (onion)
103 hydrated and viable inside the ESEM chamber. McGregor and Donald (2010) successfully
104 recorded the stomatal movements in freshly-cut leaf tips of *Tradescantia andersonia*
105 (Spiderwort) which are regarded as robust tissues with suitable stomatal movements for ESEM
106 imaging. Such ESEM observations were realized by controlling the physiological temperature
107 and adjusting the water vapor pressure with the thermodynamic constraints in the chamber
108 and minimizing the electron beam damage (McGregor and Donald, 2010). In fact, achieving a
109 precise and careful control of chamber's conditions to maintain the sample in its natural state
110 is complex and often counted as the main concern for performing *in situ* observation in the
111 ESEM (Kirk et al., 2009). Furthermore, the optimal working conditions for biological
112 applications are always sample-specific and determined empirically, which prevents the
113 standardization of ESEM methods (Tihlaříková et al., 2019). Consequently, despite the strong
114 interests in using ESEM for studying the plants cells and tissues, application of ESEM by the
115 plant science community has not yet reached its full capacity. For example, the use of ESEM
116 for monitoring the time-course of changes in plant cell walls and tissues during growth has
117 not been reported. Limitations in maintaining the plant sample viable during examination and
118 lack of commercial accessories dedicated to such analyses have been considered as the major
119 hindering factors for these applications (Kwon et al., 2019; Zhang et al., 2020). In this study,

120 we modified a commercially available sample holder of an ESEM and optimized the chamber
121 condition and imaging parameters for *in situ* observation of living plant cells and tissues. We
122 describe how we obtained electron microscopic images during early growth stages of living
123 model plants i.e. *Arabidopsis thaliana* and flax (*Linum usitatissimum*). We also show the effect
124 of polygalacturonase (PG), a biopolymer-modifying enzyme, on cell wall structure of
125 *Arabidopsis*, and illustrate the fungal colonization of roots of flax seedlings and invasion of the
126 epidermal cells by the hyphae on the living plant surfaces.

127 **2. Materials and methods**

128 2.1. Environmental scanning electron microscopy (ESEM) and accessories

129 2.1.1. ESEM and Low vacuum modes

130 We used a Quanta 200 field emission gun environmental scanning electron microscope (ESEM,
131 FEI Company, USA). Environmental (ESEM) and low vacuum (LoVac) modes of this SEM allow
132 the users to image samples in a gaseous environment using Pressure Limiting Apertures (PLA)
133 and cones. The standard configuration of the environmental mode requires installing a
134 gaseous secondary electron detector (GSED) and a thermoelectric cooling (Peltier) stage that
135 can operate in a temperature range from -25°C to 55°C. The desired relative humidity (RH),
136 when using water vapor, is obtained by controlling the pressure of the ESEM chamber from
137 10 Pa (0.075 Torr) to 2700 Pa (20 Torr) and the temperature of the sample by the
138 thermoelectric controller of the Peltier stage (FEI Company, 2010). These adjustments are
139 made using liquid/gas phase border according to the relative humidity isobar. The phase
140 diagram of pure water and the isobar chart are presented in supplemental Figure S1. In the
141 low vacuum mode a large field detector (LFD) is used and the pressure ranges from 10 Pa
142 (0.075 Torr) to 130 Pa (0.97 Torr).

143 2.1.2. Cooling stage

144 The Peltier cooling/heating stage in the ESEM is used to control the sample's temperature
145 within 20 °C around the ambient, ranging from - 25 °C to + 55 °C. This stage can be used to
146 create water condensation on the sample surface and achieve 100% relative humidity (RH)
147 with respect to the water vapor pressure of the chamber (FEI Company, 2010). The original
148 cooling stage provides a support for a standard cylindrical ESEM sample holder with diameter

149 of 10 mm (Figure 1a and 1b). This emplacement is sufficient for holding small-sized samples
150 such as *Arabidopsis* and flax seeds, however, it does not provide enough surface to
151 accommodate growing seedlings of these species. Upon germination of the seeds, the
152 seedlings grow rapidly and within a few days can reach the edge of the sample holder (Figure
153 1c). Therefore, we aimed to assemble a new module with increased surface area that could
154 hold a bigger ESEM mount to accommodate the growing seedlings. We first dismantled the
155 original cooling stage and specified the dimension and technical details of its components.
156 Based on the original design and according to the microscope manufacturer's requirements,
157 new pieces were redesigned and fabricated. Autodesk Fusion 360 software (Autodesk, Inc.,
158 San Rafael, CA, USA) was used to design all the components and develop 3D models. The
159 designed pieces were saved in stl. format and machined from stainless steel and aluminium
160 or fabricated with PVA filament using a Prusa MK3 3D-printer (Prusa Research, Czech
161 Republic). Electronic parts and wires were purchased from the local stores. Technical details
162 of the original and new designed accessories are provided in supplementary materials
163 (supplemental Figure S2).

164 2.1.3. Bio-transfer module

165 We designed a simple specimen holder (hereinafter referred to as "bio-transfer module") that
166 could fit the modified cooling stage. Two prototypes of a bio-transfer module were assembled.
167 The first prototype included a base composed of a 3-dimensional printed plastic ring and an
168 aluminium cylinder with a diameter of 25 mm that provided $\approx 5 \text{ cm}^2$ metal contact surface with
169 the Peltier module (supplemental Figure S3). In this model, the plastic part can be replaced by
170 other materials for specific applications, for example, glass or alternatively shatter-resistant
171 acrylic glass to increase the exposed surface to light as required for germination of some plants
172 seeds (Toole et al., 1955). In the second prototype, the whole base of the bio-transfer module
173 was machined from aluminium providing $\approx 10 \text{ cm}^2$ of metal surface. The larger metal surface
174 in the latter model, which was then used throughout this study, provides a high electronic
175 conductivity enabling the analyses of larger specimens and using higher accelerating voltages
176 during observation. A transparent cover composed of polycarbonate permitted light
177 transmission to the seedlings and their protection against contamination during their growth
178 and transport (supplemental Figure S3).

179 2.2. Biological samples

180 2.2.1. *Arabidopsis thaliana* and Polygalacturonase enzyme

181 *Arabidopsis thaliana* (*A. thaliana*) is a model plant with a fully-sequenced genome and many
182 interesting characteristics such as a short life cycle, prolific seed production, and numerous
183 mutant lines (The *Arabidopsis* Genome Initiative, 2000). We used the ESEM to study the cells
184 of elongated hypocotyls in etiolated seedlings and their modification after exogenous
185 application of heterologously expressed enzymes. Pectin depolymerizing enzymes such as
186 polygalacturonases (PG) can induce pectin remodeling and affect cell elongation and integrity
187 (Hocq et al., 2020). Here we added an *Arabidopsis* polygalacturonase (AtPG (endo-PG, EC
188 3.2.1.15)) into the growth medium of *Arabidopsis* to observe the induced modifications on the
189 cells on the hypocotyl surfaces. The active enzyme was expressed heterologously in the yeast
190 *Pichia pastoris* X-33 strain and purified as previously described (Hocq et al., 2020). *Arabidopsis*
191 seeds were surface sterilized, soaked in 1mL water and placed for 2 days in a cold chamber
192 for stratification. Seeds were then placed to germinate in sterilized 24-microwell plate
193 containing a ½MS semi-solid medium plus either active or inactivated (control) enzymz (8µg
194 AtPG /600µL/well). Microplate containing the seeds were first exposed to 6 hours of flash light
195 (120µmoles/m²/s), then positioned vertically in the dark for 48-72 hours to favor the straight
196 elongation of the hypocotyls (Desnos et al., 1996). The 2-days old seedlings were carefully
197 transferred into the sample holder for ESEM observations.

198 2.2.2. Flaxseeds and seedlings

199 Flax (*Linum usitatissimum* L.) is an important agricultural plant with an increasing application
200 in nutraceutical and bio-pharmaceutical industries, fibers, human food and animal feed. Many
201 interesting characteristics of flax are influenced by its seeds germination and seedlings
202 properties (Dauwe et al., 2021; Jhala and Hall, 2010; Wang et al., 2015; Wang et al., 2016).
203 The seeds of brown flax (cv. Baladin), kindly provided by the seed company Laboulet (Airaines,
204 France), were visually screened and those with similar sizes and weights were selected as the
205 experimental material. Flax seeds were germinated on wet filter paper in 90-mm-diameter
206 Petri dishes as described previously (Dauwe et al. 2021) or directly in the bio-transfer module.
207 After 48h growth in the dark, they were then transferred to a phytotron chamber and grown
208 under controlled conditions (at 23°C and 80% humidity) using a light-dark cycle (16h:8h) with

209 a light intensity of 3350 lux to 3500 lux. These seedlings were then transferred into the ESEM
210 using the bio-transfer module for microscopic observation.

211 2.2.3. *Verticillium dahliae*

212 *Verticillium dahliae* is a fungus that causes *Verticillium* wilt in many crops plant. This fungus
213 predominantly infests the fiber flax cultures in northern France causing significant yield losses
214 (Blum et al., 2018). We have artificially infected the flax seedlings using an undisclosed
215 protocol developed by the company “Linéa Semences” (Grandvilliers, France). Briefly, this
216 protocol consists of inoculation of roots of two-week old flax plantlets grown under controlled
217 conditions (as above) with a spore suspension of *V. dahliae* grown on a potato dextrose agar
218 (PDA) medium for 7 days. Using ESEM, we observed the native fungal structure and
219 morphology in their hydrated culture medium, and studied the interaction of fungus mycelium
220 with plantlets by imaging the epidermal and cortical cells of artificially infested plants.

221 3. Results and discussion

222 3.1. Modification of accessories

223 *In situ* observation of samples such as hypocotyls of *Arabidopsis* and germinated flaxseeds and
224 their growing plantlets necessitated modification of the standard thermoelectric cooling
225 stage. The hydration of a specimen in the ESEM was controlled by the temperature and the
226 pressure of water vapor in the microscope chamber. The pressure control of the ESEM
227 chamber is achieved by the use of differential pumping system and pressure-limiting apertures
228 (PLAs), and the temperature of the sample is adjusted by a cooling stage operating by Peltier
229 effect (Stokes et al. 2008). For an accurate control and reading of the sample’s temperature,
230 we aimed to maximize the contact of the samples with the holder by redesign of the cooling
231 stage. The holder is in contact with a thermoelectric (Peltier) cooling device that is typically
232 manufactured from two thin ceramic wafers which sandwich a series of p and n doped
233 bismuth telluride (Bi_2Te_3) semiconductor materials. The ceramic material on both sides of the
234 thermoelectric device adds rigidity and the necessary electrical insulation. When current
235 passes through these elements, one side of the wafer heats and the opposite one cools, and
236 reversing the polarity switches cold and hot sides (Supplemental Figure S2) (FEI Company,
237 2010; Shourideh et al., 2018). In our redesigned module, we have replaced the original Peltier
238 device ($10 \times 10 \text{ mm}^2$) with a larger one ($30 \times 30 \text{ mm}^2$) that could consequently receive a larger

239 mount recipient with the diameter of 25 mm. The mount recipient is a plate into which the
240 sample holder is inserted. A thermistor (PT 100, RS components, UK) was placed inside of the
241 new mount recipient to give an accurate temperature reading in close vicinity under the
242 sample. The mount recipient was installed on the new Peltier using a thermal grease. The
243 inner cable and water hoses were then connected to the base of the stage to pass the current
244 and remove excess heat efficiently as required by the microscope manufacturer. The details
245 on the configuration and dimensions of the new module are presented in supplementary data
246 (Supplemental Figure S2). The functionality of the new assembly was confirmed by
247 comparison of temperature and water condensation and evaporation rate of this module with
248 that of the standard module in an identical condition. Figure 2 shows the original cooling stage
249 module and the modified design allowing comparison of the surface areas of the two modules
250 regarding their provided surface areas (Figure 2). The higher surface contact and temperature
251 exchange area of the new cooling stage module, enabled us to assemble a compatible
252 specimen bio-transfer mount to transfer the germinating seeds and growing seedlings to the
253 SEM chamber with minimal change in their natural posture. In such way, samples in the bio-
254 transfer module could be transported into and out of the microscope with minimal
255 dislodgement or loss of their contact with the holder. As discussed later in the following
256 sections of this paper, such a configuration enabled us to maintain the sample in their native
257 state during the ESEM observation and analyze the structure and organization of the cells and
258 the effect of the enzyme and fungus on their morphological aspects during the early growth
259 stages of the seedlings.

260 3.2. Observation of live plantlets

261 3.2.1. Optimization and imaging

262 We first optimized the imaging parameters and the chamber condition of the microscope to
263 observe the native structure of very small seedlings of *Arabidopsis*. In dicotyledonous
264 seedlings, the hypocotyl is part of the stem between the root and the cotyledons and is widely
265 used as a model for the physiological study of the mechanism of cell elongation and variety of
266 other biological events (Gendreau et al., 1997). The young etiolated hypocotyls with a
267 diameter smaller than 250 μm and a length varying between 2 to 20 mm are very fragile and
268 known to be highly susceptible to changes of relative humidity (Burgert and Keplinger, 2013).

269 As a reference, we therefore began by analyzing young hydrated etiolated *Arabidopsis*
270 seedlings (2-days old dark-grown) to obtain images of their surfaces and cellular structure.
271 Theoretically, hydration of the samples in the ESEM is achieved by setting the microscope to
272 a specific water vapor pressure and cooling the specimen to the relevant temperature
273 indicated by the phase diagram of water (as described above, supplemental Figure S1). In
274 practice, however, the young plantlets were greatly impacted before reaching the desired
275 relative humidity, because of dehydration during the pump-down and evacuation of the air
276 from the chamber. This resulted in deformation of cells in hypocotyls and other organs such
277 as roots and testa (Figure 3a-d). We could mitigate this issue by cooling the sample down to
278 the target temperature and adding several drops of ultrapure water to the stage base before
279 closing and pumping down of the chamber. Furthermore, purging water vapor into the
280 specimen chamber (five cycles) during pump-down could improve saturating the sample's
281 surroundings to prevent dehydration before reaching the designated chamber pressure.
282 These steps have previously shown to be effective in preventing dehydration of desiccation-
283 sensitive samples in the ESEM (Jenkins and Donald, 2000). We observed that the overall
284 structure of the live seedlings was preserved in the ESEM chamber when the relative humidity
285 was maintained around 85% (Figure 3e), conforming to their structural characteristics
286 previously revealed in the fixed samples (Gendreau et al., 1997). At lower RHs the hypocotyl
287 structure was extensively deformed (Figure 3f). We could achieve high levels of RH, by
288 maintaining the temperature at 20 C° and adjusting the pressure to 14 torr. Higher vapor
289 pressures could reduce the charging effect and proved to be essential for preserving the
290 tissues of the fragile seedlings, however, further increase in the relative humidity
291 compromised the sharpness of the image with significant loss of resolution. Moreover,
292 condensation of vapor formed droplets and films of water on the surface of the specimens
293 (Figure 3g), which obscured the underlying structure and reduced contrast as previously
294 reported for ESEM observations (Jansson et al., 2013; Jenkins and Donald, 2000). Details on
295 optimal image acquisition parameters of *Arabidopsis* seedlings are summarized in Table 1.
296 Optimization of imaging parameters for ESEM analyses of the sensitive hypocotyl cells
297 provided reference points to observe other cells and tissues of the seeds and small seedlings
298 of *Arabidopsis*. Figure 4 shows examples of ESEM images of diverse organs and cells including
299 germinating seed surfaces and collumella of their mucilage secreting cells and cotyledons, root
300 and shoot apical meristems, trichomes, stomata, rhizoderm cells and extremely delicate root

301 hairs in the growing seedlings. These ESEM images of *Arabidopsis* organs and cells conform
302 to the previously reported SEM images that were obtained from the chemically-fixed tissues
303 (Byrne, 2006; Pillitteri and Dong, 2013; Tsukaya, 2013). Our ESEM results not only show the
304 disposing of sample preparation steps that are required for classical SEM observations, but
305 also the ability of this approach to accurately study the surface morphology and anatomical
306 development of the cells in their native state in the live growing seedlings.

307 3.2.2. Time course *in situ* ESEM and repetitive observation of growing plantlets

308 To observe the early stages of seedling growth and development on a daily basis by *in situ*
309 ESEM, we combined our home-designed cooling stage and bio-transfer module. The flax seeds
310 germinated readily in the bio-transfer module in a phytotron. The module containing
311 germinated seeds could be easily transferred to the ESEM chamber and fitted on the newly
312 designed cooling stage. We could subsequently image diverse cells and tissues and follow their
313 time-course of modification in the germinating seeds and seedlings (Figure 5), then return the
314 module back to the phytotron after observation. Similar to our ESEM observation of
315 *Arabidopsis* seedlings, imaging of flax seedlings also required an adjustment of the acquisition
316 parameters for different tissues and cells. The combination of the optimal parameters used
317 for ESEM imaging of different parts of the living seedlings of *Arabidopsis* and flax are outlined
318 in Tables 1. Natural resistance of plant tissues to dehydration and beam damage varies by
319 their types and upon their growth (Stabentheiner et al., 2010). Structures such as seeds or
320 pollens are usually tolerant to desiccation in most plants, however, there is a great variation
321 in the resistance and deformation of vegetative tissues upon dehydration (Oliver, 1996; Wakui
322 et al., 1999). We found that, the *Arabidopsis* hypocotyls were more resistant to partial vacuum
323 in older plantlets and could be observed under low vacuum (pressure not exceeding ~1 torr)
324 after a week of germination without showing significant structural deformation. The increase
325 of the cell wall thickness following the expansion of the thin primary cell walls in growing
326 plants, could play a role in the resistance of the older plant to the vacuum and dehydration
327 (Kumar et al., 2016; Vicré et al., 2004; Vogler et al., 2015), and therefore to the partial vacuum
328 or oscillation of the humidity in the microscope chamber during analyses (Burgert and
329 Keplinger, 2013; Treitel, 1949). Furthermore, cuticle that is a complex structure mainly
330 composed of a lipid polymer, i.e., cutin and waxes, coats the outermost layer of periclinal cell
331 walls of all aerial organ epidermal cells. The cuticle layer provides a barrier to prevent non-

332 stomatal water-loss, and plays various roles in response to environmental stresses (Jacq et al.,
333 2017; Yeats and Rose, 2013). This could also explain why it is possible to observe older plant
334 tissues and cells by SEM at lower pressure i.e. low vacuum mode. We observed that the roots,
335 even in older seedlings, suffered more easily from dehydration caused by the partial vacuum
336 in the microscope. We could prevent this dehydration by wrapping the roots with blotting
337 paper soaked in water. The lack of a strong diffusion barrier, such as cuticle, at the surfaces of
338 epidermis cells in the primary growth stage could explain the higher sensitivity of the roots to
339 the variation of humidity of the ESEM chamber compared to aerial plant organs (Berhin et al.,
340 2019). Beside appropriate operation parameters to maintain the tissue fully hydrated, the
341 viability of cells depend on the beam intensity, particularly in relation to the beam accelerating
342 voltage and the total electron dose (Zheng et al., 2009). During the imaging process, the image
343 acquisition parameters such as working distance, acceleration voltage, scan speed and spot
344 size were optimized to minimize beam damage to the living cells (Table T1). The images were
345 generally recorded at acceleration voltages between 5 and 20 kv and a working distance
346 between 5 to 10 mm. The beam was blanked during imaging intervals to reduce the beam
347 damage to the samples and visible artifacts that could occur during ESEM observation as
348 previously reported (Dobberstein et al., 2005). Our post-ESEM surveying of the seedlings that
349 underwent such repetitive exposure to the vacuum and electron beam showed that they
350 maintained their vitality and vigor and continued their growth and development
351 (Supplemental Figure S4). Nevertheless, the growth rate of these plantlets was found to be
352 slightly lower compared to those that had not been observed in the ESEM. Furthermore,
353 during our repetitive observations, we occasionally noticed the occurrence of epidermal
354 meristems on the hypocotyls (Figure 5, D9 b). The presence of such meristems has been
355 previously attributed to the exposure of seedlings to drought or radiation stresses (Tafforeau
356 et al., 2004). These findings could indicate the presence of stresses in the underlying cells that
357 remain undetectable at the surface. Such damage can occur, for example by an increase in the
358 reactive oxygen species (ROS) or the rupture of plasma membranes (Bandurska et al., 2012;
359 Muscariello et al., 2005; Zheng et al., 2009), which may be triggered in the ESEM chamber due
360 to the formation of free radicals through electron beam interactions or pressure oscillation.
361 Faster image acquisition could prevent possible beam and thermal damage to the samples,
362 however, it compromised the signal to noise ratio and lowered the quality of the images. It
363 should be noted that the insertion of pressure limiting aperture (PLA, 500 μm) on the pole

364 piece, essential for ESEM operation, limited the field of view and restricted the lowest
365 magnification to around 200x. Increasing the working distance and subsequently acceleration
366 voltage to compensate the beam scattering and the weak signals, could provide a larger view,
367 albeit at the expense of optimization time for imaging in the wet conditions of the chamber
368 (Muscariello et al., 2005).

369 3.2.3. Effect of enzyme application on hypocotyl structure

370 ESEM permitted us to observe the overall structure of hypocotyls and, in greater detail,
371 identify the individual cells and their boundaries in the live seedlings without any sample
372 preparation (Figure 4). The etiolated (dark-grown) seedlings of living *Arabidopsis* (wild type
373 Col-0) featured typical morphological phenotypes such as apical hook, hypocotyls,
374 unexpanded cotyledons and short root (figure 4d-f) in accord with the results of previous SEM
375 studies on cryogenically-prepared samples (Desnos et al., 1996; Gendreau et al., 1997). The
376 hypocotyls' cells in etiolated seedlings of *Arabidopsis* are known to have a simple anatomy
377 and a postembryonic growth that is exclusively the result of a controlled series of cell-
378 elongation events (Desnos et al., 1996; Gendreau et al., 1997). Such characteristics could make
379 this organ an apt model to study the effects of diverse growth factors on cell elongation. We
380 used heterologously expressed AtPG, a PG (EC 3.2.1.15) that induces pectin remodeling (Hocq
381 et al. 2020) to illustrate structural modification of hypocotyls cells. Our ESEM analyses
382 revealed that the presence of active AtPG, modified the integrity of the hypocotyls by
383 impairing the longitudinal and ultimately transversal adhesion of cell walls in treated seedlings
384 (Figure 6b and c), more apparently in epidermis cells and occasionally in their underlying
385 cortex cells (Figure 6 c). The hypocotyls of the control seedling grown with inactivated AtPG
386 (denaturated protein) did not show any separation of the adjacent cells (Figure 6 c). These
387 ESEM observations are comparable to the observation we made on cryo-prepared samples
388 using low-vacuum SEM and light microscopy as shown in Figure 6 (d-i) and accord with the
389 results of prior studies on the relation between the role of polygalacturonases in modification
390 of cell wall rigidity and elongation (Sénéchal et al., 2014). Polygalacturonase is a pectin lytic
391 enzyme implicated in cell expansion and elongation by degrading pectin in plants cell walls.
392 This enzyme hydrolyses the α -1,4-linked-D-galacturonic acid units which compose the
393 homogalacturonans (HG), the major polymer of pectins (Babu et al., 2013; Hadfield and
394 Bennett, 1998; Xiao et al., 2014). Our finding on separation of the cells could be attributed to

395 the softening and decomposition of pectin in HG-rich middle lamella of the cells by PGs,
396 however, the exact function of these enzymes during cell elongation is not known (Zhang et
397 al., 2021). During our observation, we noted that the AtPG-treated hypocotyls were more
398 sensitive to the vacuum of the ESEM compared to the untreated ones, probably because of
399 the enzyme's effect on the loss of integrity of the epidermal tissue and weakening of their
400 mechanical properties. The vulnerability of such organs in the ESEM environment, could be
401 mitigated by increasing purge cycles during the pump-down of the chamber and faster image
402 acquisition during observations as described above. Our method of observation shows
403 promise as a means of analyzing and illustrating the structural modification of living cell walls
404 of plantlets upon the application of biopolymer-modifying enzymes and this may lead to
405 broader use of this technique with the increasing interest in the *in situ* enzymatic treatment
406 of plant cells.

407 3.2.4. *In situ* plant-fungus interaction

408 We could observe a laboratory culture of *Verticillium dahliae* mycelium in its native state in
409 the ESEM. *In situ* observation of the structure of mycelium networks of this pathogen in the
410 culture medium and then its identification during its development in infected plantlets (Figure
411 7a-i) was performed by optimization of imaging parameters at a relative humidity of 75%
412 (Table 1). *V. dahliae* is a soil borne pathogen and belongs to the fungal class Deuteromycetes
413 (Fungi Imperfecti), a group of fungi with an unknown sexual stage. We imaged the vegetative
414 part of this fungus, by which it spreads in plants, and could identify different hyphal structures
415 such as conidium, conidiophore and phialide in the fungal colony in the medium (Figure 7b
416 and c). The structural and anatomical information on the fresh fungus provided by the ESEM
417 observation in their native state accord with those previously reported on the chemically-fixed
418 samples from isolated *V. dahliae* obtained by optical or scanning electron microscopy (Babu
419 et al., 2013; Inderbitzin et al., 2011). ESEM therefore could avoid the time-consuming sample
420 preparation and thus had advantages over other imaging techniques for *in situ* studies.
421 Attention should be paid, however, to the high sensitivity of fungal cells to dehydration and
422 their low intrinsic contrast during their ESEM analyses (Kaminskyj and Dahms, 2008). Our
423 observation of the infected seedlings showed that the fungus surrounded the roots with a
424 thick mantle of closely interlaced hyphae (Figure 7d-f). The hyphae expanded to the higher
425 parts of the plantlets and colonized stem (hypocotyl) and leaves (cotyledon) three days after

426 infection covering the epidermal cells in these zones (Figure 7g-i). To observe the presence of
427 mycelium underneath the epidermis, we transversally cut the roots and stems using a sharp
428 single-edged razor blade and immediately observed the freshly cut surface using the ESEM.
429 The external structures of the transversally-cut roots, including cortex and epidermis cells did
430 not withstand the vacuum and collapsed, with the exception of the central cylinder retaining
431 its structure (supplemental Figure S5). To overcome the deformation of the freshly-cut
432 sections and observe the sub-epidermal tissues and the fungus hyphae, the infected seedlings
433 were plunge-frozen in liquid nitrogen, and then fractured in the root and hypocotyl regions as
434 previously described (Wakui et al., 1999). The fractured surfaces of roots and hypocotyls then
435 were instantly observed in low vacuum mode at ≈ 1 torr to elucidate the presence of fungus in
436 the internal structure of plantlets. Preparation of these tissues by freeze-fracturing could
437 preserve their overall structure and components such as the epidermal and cortex cells and
438 vascular cylinder, and allowed detection of fungal mycelium in the internal surfaces of roots
439 (Figure 7j-l). These images showed that the hyphae could penetrate into the cortical cells, that
440 could later colonize the vascular tissue of the plants as previously shown by bright-field and
441 fluorescent images by Blum et al. (2018). Obviously, freeze-fracturing and low vacuum
442 observation of the plant samples, did not preserve their vitality, but it was unavoidable to
443 detect details of internal structure of tissues and to complement the ESEM observation of the
444 external surfaces. These results, indeed, can be always supplemented by the conventional
445 SEM analyses following classical sample preparations.

446 **4. Conclusion**

447 In this work, we modified a commercially available ESEM sample holder to observe and image
448 live growing plantlets *in situ*. The use of newly designed and 3-D printed accessories in
449 combination with optimized control of the relative humidity and tuning of imaging parameters
450 permitted maintaining the plant seedlings viable during observation and acquiring quality
451 ESEM images. This approach allowed us to observe structural modification of cells in living
452 plantlets upon the application of a biopolymer-modifying enzyme or interaction with a fungus.
453 These novel *in situ* ESEM observations are largely comparable to those made on cryo-prepared
454 samples using low-vacuum SEM and light microscopy. Therefore, we conclude that our
455 adopted approach in using ESEM for *in situ* analysis of the surfaces of living plantlets is a
456 reliable means of assessing their evolution and growth, modification by enzymes, or

457 degradation by fungi at cellular levels. There is clearly considerable scope to use this method
458 to examine the interaction of various physical, chemical and biological agencies with surfaces
459 of living plants or other biological samples. However, maintaining the humidity and viability
460 of samples during the ESEM observations, imposed some limitations such as smaller field of
461 view and shorter observation timespan compared to conventional SEM. Further research will
462 address the technical limitations of this approach by employing technologies currently
463 commercially available, such as micromanipulators and in-chamber optical cameras for faster
464 sample navigation or novel detection systems, and developing more sample-specific
465 accessories to expand the application of *in situ* ESEM into the relevant fields.

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478

479

480 **Reference**

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653 **Figure and Table captions**

654 **Figure 1:** Standard cooling stage module: (a) cooling stage (Peltier/Seebeck) assembly
655 containing a standard ESEM sample holder, installed on the stage of the Quanta 200 ESEM
656 chamber, (b) the image and dimensions of a standard ESEM sample holder, and (c) a 3-days
657 old *Arabidopsis* plantlet (arrowed in upper image) and a flaxseed (arrowed in lower image)
658 showing their size relative to the standard holder's surfaces. The yellow arrows in b and c
659 indicate the width of the edge of the holder

660

661 **Figure 2:** Comparison of the standard cooling stage with the new-designed module and their
662 sample holders. Modification of the standard cooling stage (Peltier/Seebeck) (a) increased the
663 mount-receiving surface (emplacement of sample holder) from 1 cm² to 5 cm² (b). Technical
664 details on this modification are provided in Supplemental Figures of this article

665

666 **Figure 3:** The effect of chamber pump-down and relative humidity on the structure of a very
667 young (2-days old) hydrated *Arabidopsis* seedling: (a) a low magnification image of an entire
668 seedling taken at a long working distance showing the dehydrated cells, (b-d) higher
669 magnification images of different organs of the same plant, showing the deformation of the
670 cell walls in testa, root and hypocotyl, (e-g) images of the hypocotyls showing the effect of
671 chamber's relative humidity in conservation of their structure during the ESEM observation;
672 note the structural deformation at RH lower than 75% (f) and water film formation and
673 submersion of the tissue at 100 % RH (g) (images were acquired at 5Torr and between 4 to
674 6°C)

675

676 **Figure 4:** ESEM observation of tissues and cells of seeds and young seedlings of *Arabidopsis*
677 following optimization of the chamber and imaging parameters: (a) observation of a seed, (b)
678 cullumola of the mucileagous cells, (c) endothelial cells at the micropylar region in a 2-day
679 germinating seed, (d) overall view of an entire plantlet (e) cotyledon (leaves within the embryo
680 of the seed), (f) apical hook, (g) higher magnification image of a cotyledon showing a jigsaw
681 puzzle arrangement stomatal and non stomatal cells, (h) meristematic and transition zones of

682 the primary root apical meristem, (i) rhizoderm cells and extremely delicate root hairs (tubular
683 elongations of the root epidermis (rhizodermis)), (j & k) the shoot apical meristem positioned
684 between the two cotyledons, (l) marginal elongated epidermal cells seen parallel to the
685 cotyledon edges, (m, n and o) vegetative apex showing the adaxial side of the leaf adjacent to
686 the central shoot apical meristem and the presence of trichomes indicating dorsoventrality
687

688 **Figure 5:** ESEM observation of flax plantlets during their early stage of growth starting from
689 second day after the seed germination in the “bio-chamber”. Each row includes a photograph
690 and examples of ESEM micrographs acquired from different parts of the growing plantlets at
691 a specific day (D) after germination; D2: hypocotyl (a) and remaining of a single cell
692 endosperm layer (b and c); D5: cotyledon (a), hypocotyl (b) and individual angular cells of
693 endosperm layer (c); D7: epidermal cells at apical hook zone of a hypocotyl showing the lines
694 containing protruding cells (a), abaxial epidermis of cotyledon with the pavement cells and
695 stomata (b), and a higher magnification image of a stomata showing its guard cells (c); D9:
696 epidermal cells and stomata on hypocotyl (a), zone of the hypocotyl showing the view of an
697 epidermal meristem (b), epidermal cells of cotyledons (c); D12: parallel lines of protruding
698 epidermal cells on the hypocotyl (a), the shoot apical meristem (b), and abaxial epidermis with
699 the pavement cells and stomata (c)

700

701 **Figure 6:** Structural modification of 4 day-old dark-grown *Arabidopsis* hypocotyls resulting
702 from the application of AtPGLR enzyme. ESEM images (first row) of hypocotyls treated with
703 either inactivated enzyme showing no structural modification (a) or an active enzyme showing
704 deformation and longitudinal separation of the walls of epidermal and underlying cortex cells
705 (b and c). Second and third rows are images acquired by low vacuum SEM of cryo-prepared
706 samples and light microscopy, respectively, showing hypocotyls treated either with
707 inactivated (d and g) or active enzymes (e, f, h and i); note their high comparability with ESEM
708 images

709

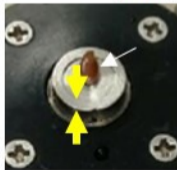
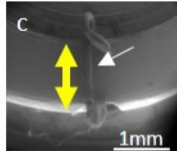
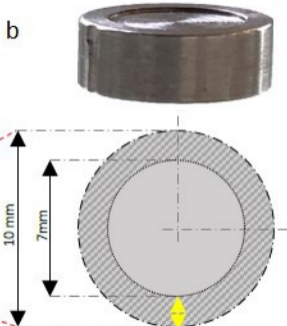
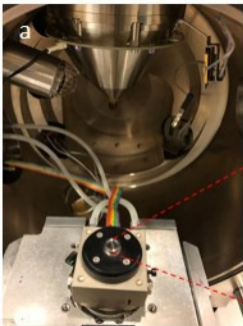
710 **Figure 7:** Fungus *Verticillium dahliae* in the culture medium and colonization of the artificially
711 infected flax plantlets; *In situ* ESEM observation of vegetative mycelium of *V. dahliae* in the

712 culture medium showing the mycelium network (microsclerotia) (a) and different hyphal
713 structures such as conidia, conidiophores and phialides in the fungal colony (b and c);
714 observation of the roots in artificially infected plants in day one after infection showing a thick
715 mantle of closely interlaced hyphae covering the Rhizodermis cells (d, e and f); hypocotyl of
716 an infected flax plantlet showing the fungal hyphae covering the epidermis cells (g), stomata
717 (arrowed in h) and remaining of endosperm layer 3days after infection (i); freeze-fracturing of
718 a 21 days-old flax plantlet root, 3days after their artificial infection by *V. dahliae* (j-l), low
719 vacuum SEM revealing the presence of fungi mycelium on the external surfaces (j and k) and
720 its penetration into the sub-epidermal cells (arrowed in l)

721

722 **Table 1:** Summary of the optimal parameters for Low vacuum (LoVac) and Environmental SEM
723 (ESEM) observations of tissues of living plantlets and fungus

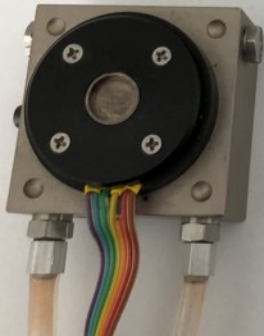
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a

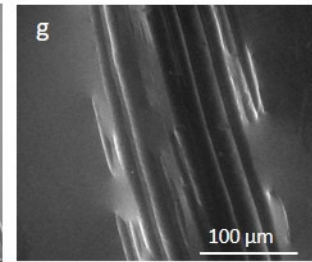
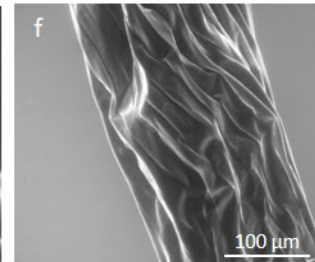
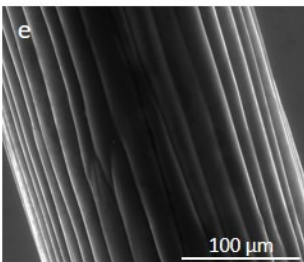
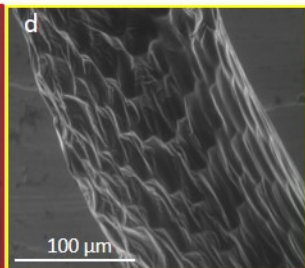
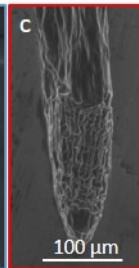
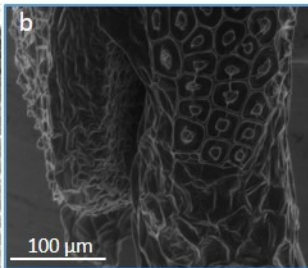
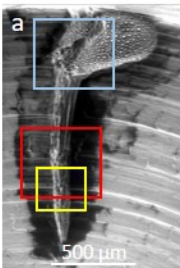


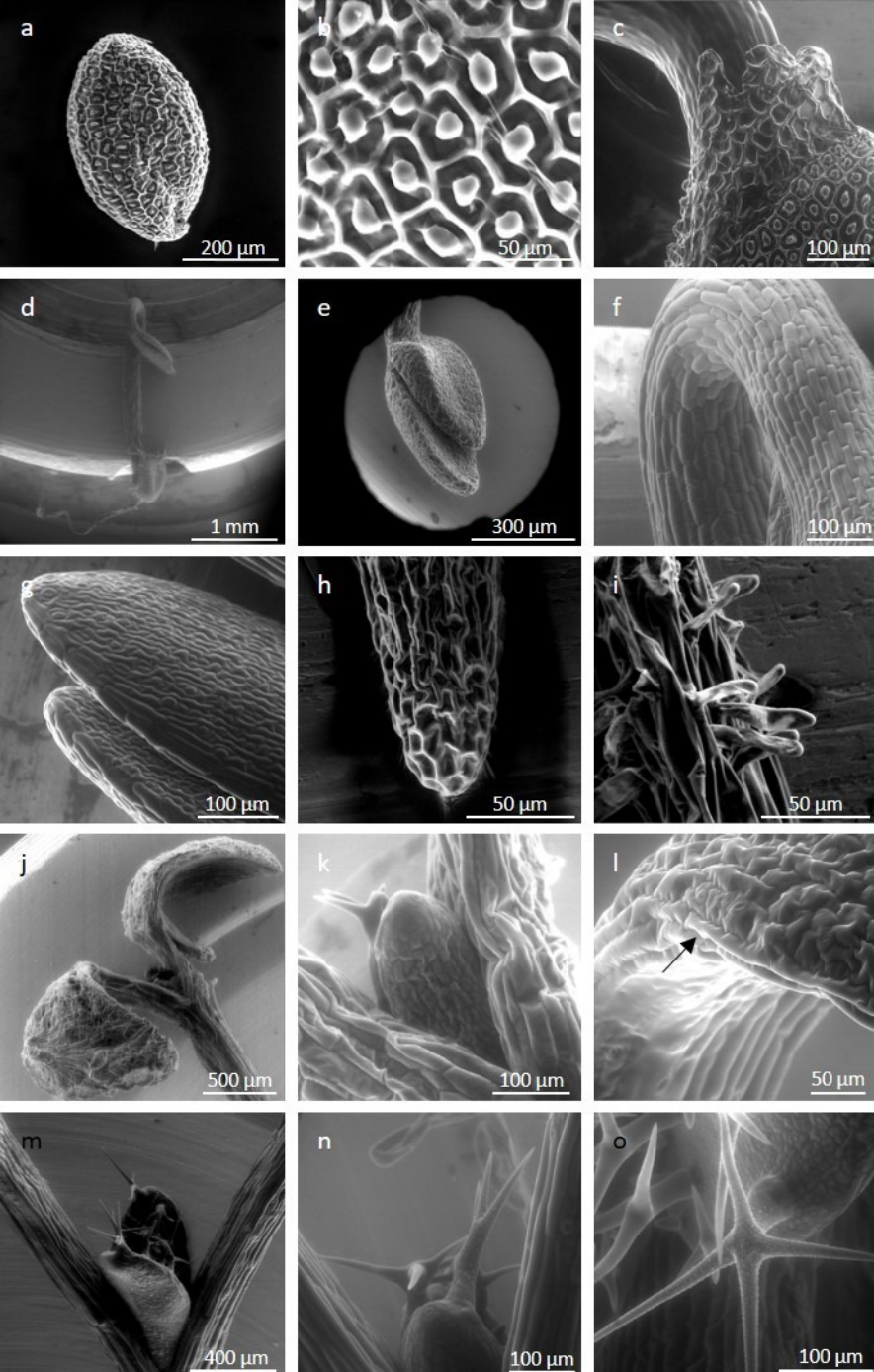
b

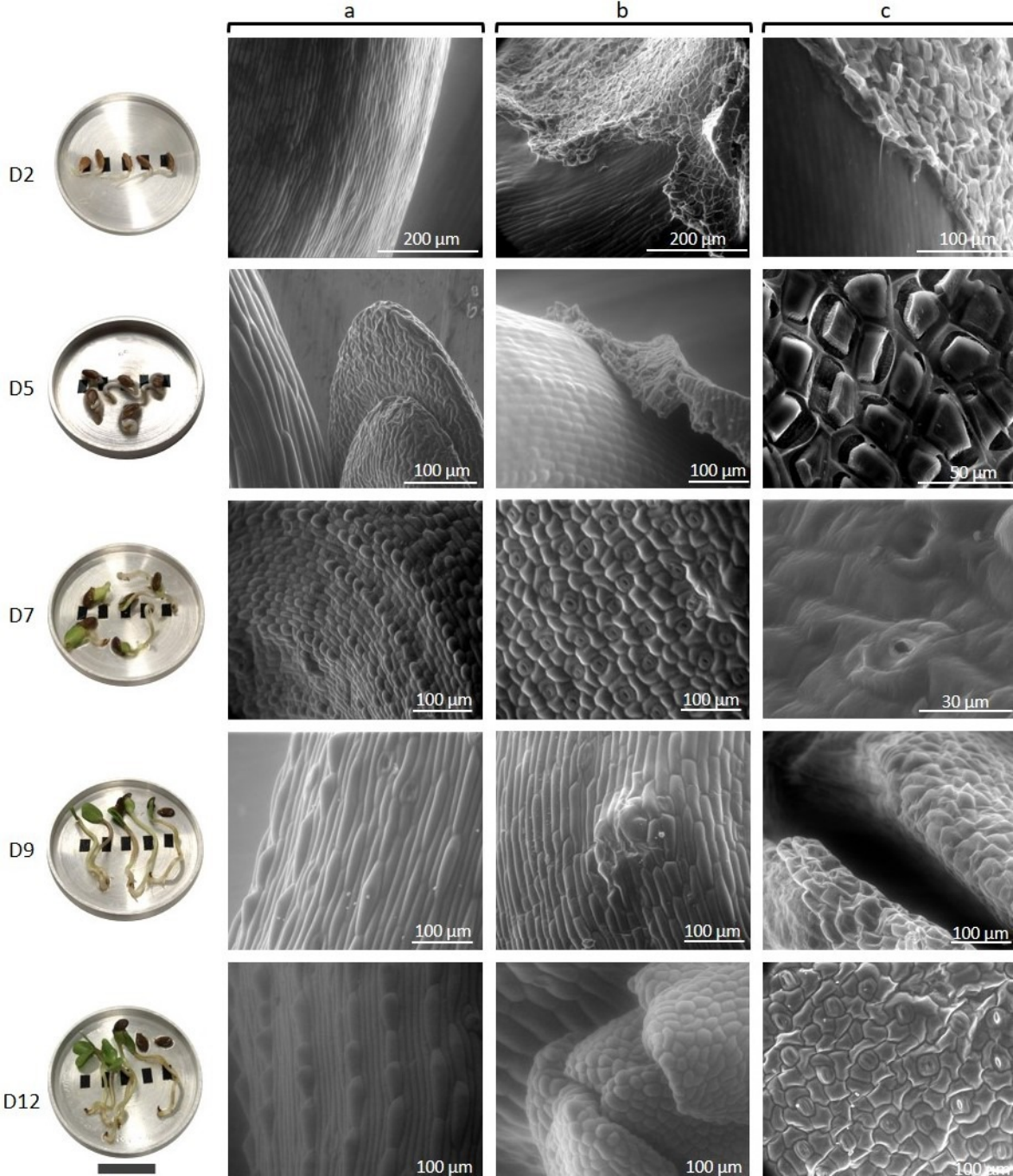


10 mm





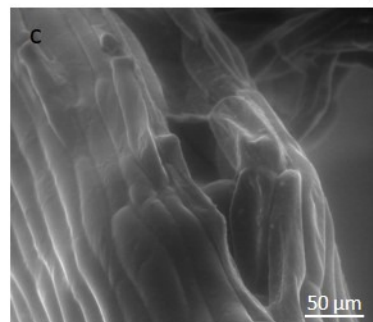
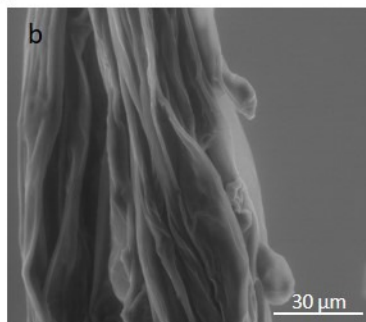
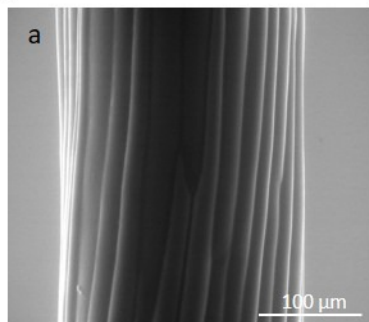




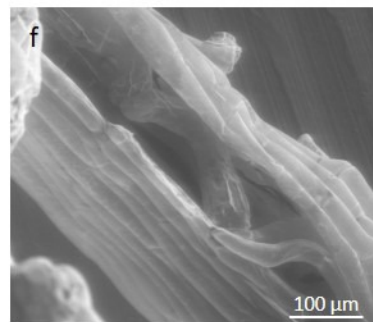
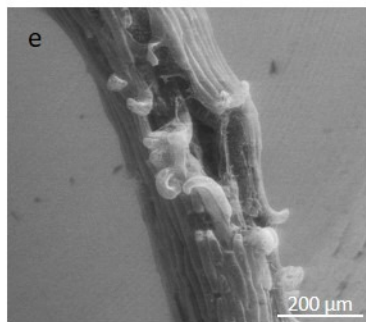
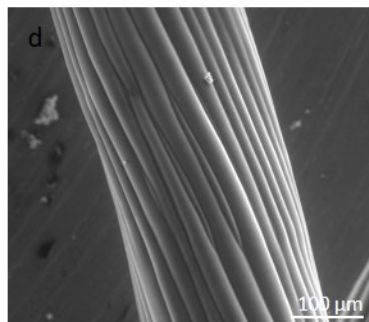
Inactivated enzyme

Active enzyme

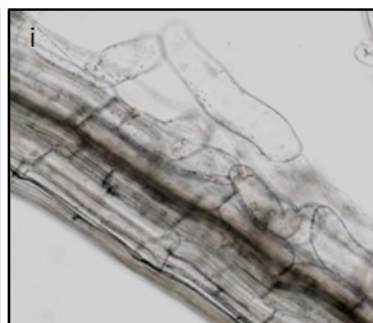
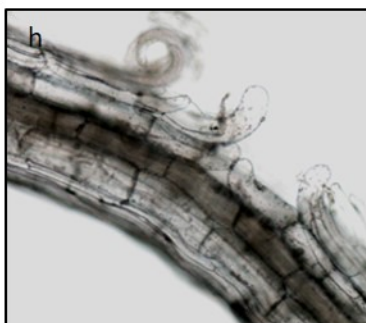
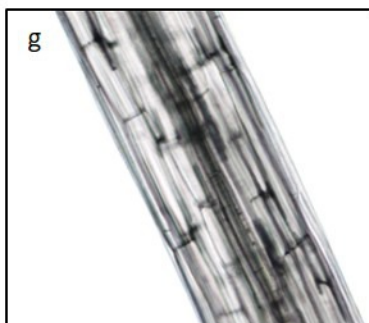
ESEM



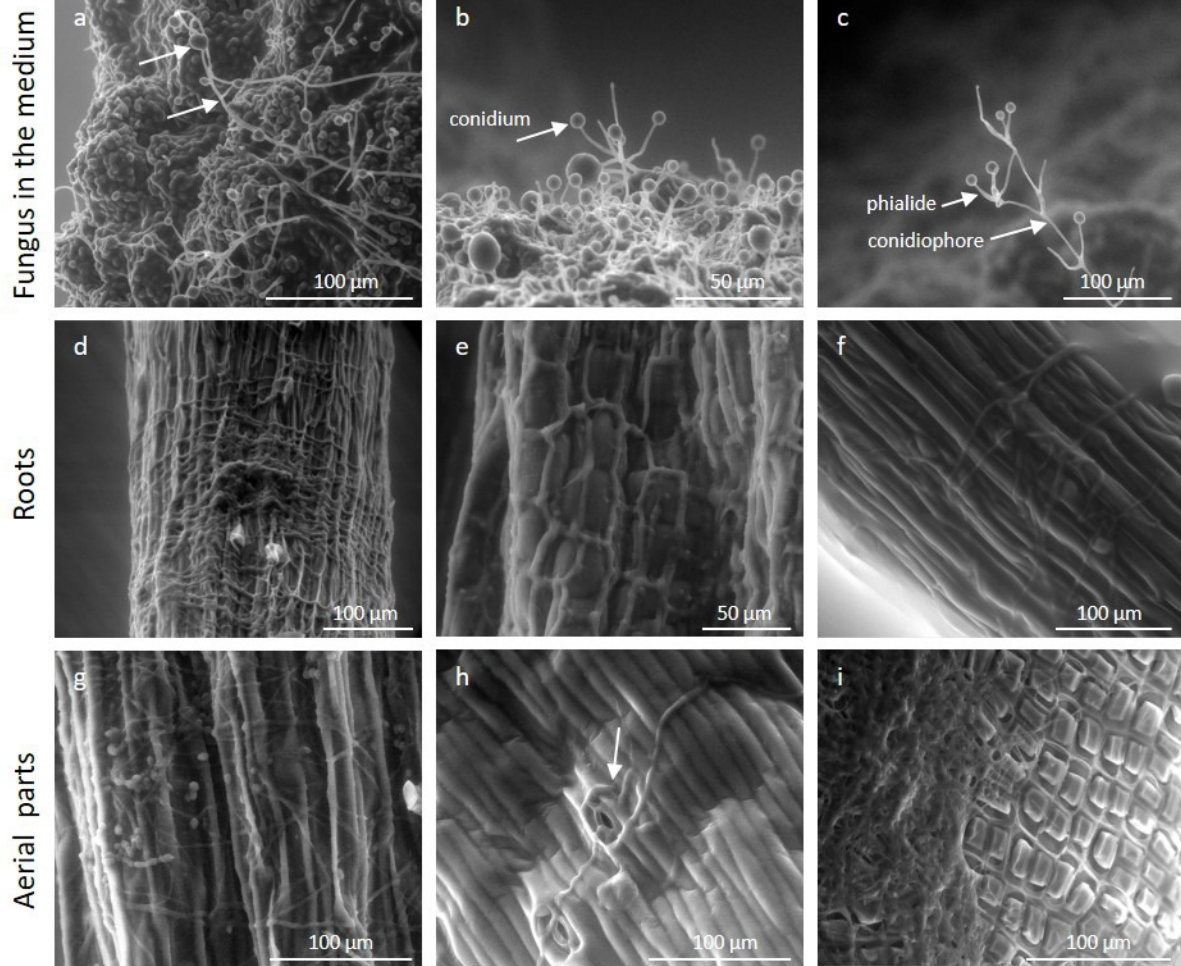
LV SEM



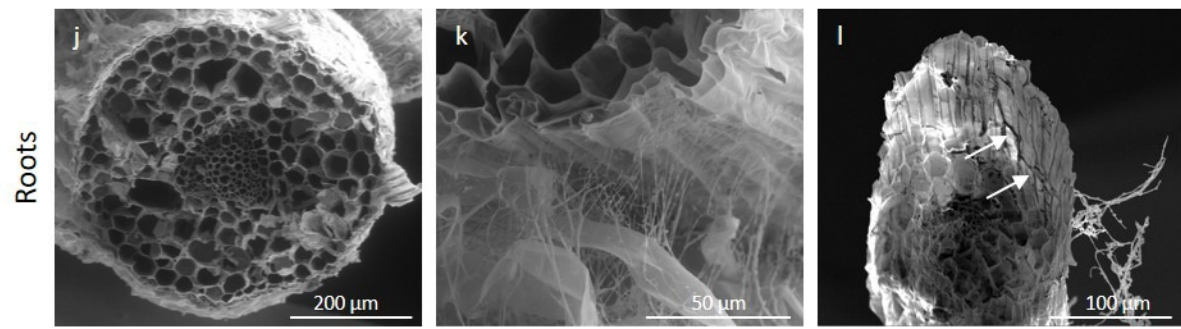
Light Microscopy



ESEM



LV SEM



Samples	Operational mode	Accelerating voltage, kV	Magnification	Working Distance, mm	Vapor pressure, Torr (Pa)	Spot size	Temperature, C°/relative Humidity %	
<i>Arabidopsis</i> [†]	Seeds	LoVac	5	500-1000	5	0,75 (100)	3	-
	Hypocotyls	ESEM	5-10	350 - 4000	4-5 mm	13-15 (1733-2000)	3.5	18-20/85-100
	Leaves	ESEM	15-20	500	5,5	14 (1867)	4,5	20/80
	Trichomes	ESEM	5	500	6	15 (2000)	3,5	20/75
Fungus ^{††}	ESEM	15	500-800	5,5	12 (1600)	4	19/70-75	
Flax ^{†††}	Roots	ESEM	10-15	500-1000	6,5	6,5 (867)	3,5	8/80
	Infected plantlets	ESEM	20	200-500	7,5	9 (1200)	4	18/60
	Freeze-fractured Tissues	LoVac	10	400-800	6-8	1 (133)	3	-

[†]*Arabidopsis thaliana* (2 to 5-days old); ^{††}*Verticillium dahlia* (grown for 5 to 7 days); ^{†††}*Linum usitatissimum* (2 to 5-days old)

