Towards a structural understanding of plant-microbiota interactions using cryo-EM techniques

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Large-volume sample preparation of plant-tissue for cryo-electron microscopy

Cryo-electron microscopy (cryo-EM) and tomography (cryo-ET) are increasingly applied to investigate structural interactions in host-microbiota systems at the nanoscale. While initial studies focused on single cells, recent technological advancements have expanded cryo-EM applications to large-volume (LV) samples, including tissue specimens. However, plant tissues pose unique challenges for vitrification due to structural barriers such as rigid cell walls, vacuoles, and intercellular gases, which hinder uniform cryo-fixation and leading to detrimental freezing artefacts. To address these limitations, we optimized the workflow for LV plant tissue vitrification and evaluated different plant tissues. Reproducible vitrification of plant tissue was achieved through the combined use of PFIB/SEM, cryo-lift-out techniques, methodological improvements (e.g., planchette modifications, enhanced sample handling), and root organs as a model system. These root organs are a well-established system for studying plant-microbiota interactions, and their thin diameter and lower risk of the formation of intercellular gas further improved vitrification outcomes. This approach enables cryo-EM studies of plant-microbiota interactions in a near-native hydrated state, allowing high-resolution imaging. The developed workflow provides a foundation for high-resolution imaging of plant tissue ultrastructure, expanding future applications in plant biology and microbiome research.

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5.1 Introduction

In recent studies, cryo-electron microscopy (cryo-EM) has increasingly been used to investigate host-microbiome interactions, as it allows the visualization of ultrastructural features in their native cellular context and enables the characterization of interactions at the nanoscale. While early efforts focused primarily on the cellular level using cell cultures, these techniques have recently been extended to tissue samples, thereby offering insight into more complex biological systems [45, 50]. Meanwhile, the term "large-volume" (LV) samples has appeared more frequently in the literature, often without further explanation or definition. In general, it is used to describe samples that are composed of multiple cell layers (e.g., biofilms) or multicellular specimens that exceed the dimensions of individual eukaryotic cells. However, a precise size threshold for LV samples has not yet been established and remains an open question in the field. Traditionally, samples for cryo-EM analysis with a volume of less than 10 µm were vitrified by plunge freezing, where the sample is applied onto a copper grid (3 mm) and rapidly plunged into liquid ethane, which is cooled by liquid nitrogen. Samples exceeding 10 µm require high-pressure freezing (HPF) for vitrification, where the sample is frozen within milliseconds under high pressure (~2100 bar) in liquid nitrogen [156]. Notably, most studies focus on metazoan tissue, whereas research on phyto-tissue remains limited. This is due to the challenges associated with vitrifying plant tissue. Unlike metazoan cells, plant cells possess a rigid cell wall, large vacuoles, and intercellular gases, which complicate uniform vitrification and contribute to freezing artefacts and structural damage.

The majority of postulated vitrification procedures of plant samples have been developed for single cells like algae [157], leaves, or peelings of the outer epidermal layers, which are thinner than 15 µm and can be vitrified directly on a grid via plunge freezing. Another often described approach is the vitrification of root tips of young Arabidopsis seedlings which were high-pressure frozen and subsequently subjected to freeze substitution. This technique combines cryofixation with sectioning at room temperature, whereby the water within the frozen sample is slowly replaced with organic solvents. The advantage of this method is that it allows the sample to be processed at room temperature, enabling the preparation of sections suitable for cryo-EM using microtomes and ultramicrotomes. However, this extensive sample processing can degrade resolution and image quality, and the potential impact of solvents on ultrastructures remains uncertain [41, 158]. For this reason, previous studies have attempted to cut vitrified samples using cryo-ultramicrotomy. While this approach eliminates solvent-related alterations, it remains highly time-consuming and requires extensive expertise and manual dexterity. Additionally, cryo-ultramicrotomy frequently results in artefacts and tissue damage, limiting its reliability [159]. Due to these drawbacks, as well as the specialized skills required, the method has seen little mention in the literature in recent years. The development of focused ion beam scanning electron microscopy (FIB/SEM) has provided an alternative to microtomy, reducing the complexity of sample preparation. FIB/SEM integrates an electron column for imaging and an ion column for milling, allowing real-time monitoring and fine-tuning of the milling process while precisely ablating material with gallium ions. In this process, a defined rectangular region is milled down to 100–200 nm, creating an electron-transparent area, referred to as a lamella, which is suitable for high-resolution cryo-EM imaging [160].

However, larger sample volumes significantly extend milling times and simultaneously increase the risk of localized partial de-vitrification and subsequent ice crystal formation due to prolonged beam exposure [160]. The advancement of the FIB/SEM led to the development of plasma-FIB/SEM (PFIB/SEM), which allows the use of multiple ion species (xenon, nitrogen, argon, oxygen), each with distinct milling properties. The use of xenon ions, enables significantly higher material sputtering rates at the same ion beam current as gallium, due to their greater ion mass [161]. The advantages of PFIB/SEM over FIB/SEM have already been demonstrated in various studies and can be summarized as follows: PFIB/SEM allows the processing of significantly larger volumes while reducing surface roughness and minimizing beam damage that negatively impacts high-resolution imaging. This not only increases efficiency substantially but also enables further optimization of the milling process for lamellae of LV samples [147, 162].

The simultaneous development of the cryo-lift-out method has provided a breakthrough in large-volume cryo-EM sample preparation. For the first time, this technique allows the extraction of small volumes from LV samples and their transfer to new grids. This minimizes the amount of volume that must be milled around the area of interest to obtain electron transparent lamellae, thereby reducing milling time and mitigating the associated challenges described above [160]. This improves both efficiency and the preservation of vitrified ultrastructures during the milling process. However, initially HPF samples had to be recovered and mounted on a grid, e.g., with cryo-glue, to enable processing via (P)FIB/SEM and cryo-lift-out [163]. This additional step increased complexity and reduced overall efficiency. The ongoing development of other methods, including the waffle method, enabled to HPF samples directly onto a grid. This significantly improved efficiency but remained limited to sample thicknesses of up to 50 µm [150]. Further technological advancements introduced new shuttles, which enabled the direct use of planchettes in (P)FIB/SEM, eliminating the time-consuming and inefficient recovery and mounting steps.

In this study, we aim to develop a workflow that integrates recent technological advancement to obtain lamellae from LV samples of plant tissue. To achieve this, we optimized the vitrification procedure and tested various plant tissues to identify a suitable model organism. This approach enabled the preparation of vitrified plant tissue samples for high-resolution cryo-EM imaging, allowing us to visualize host-microbiota interactions at an unprecedented ultrastructural level.

5.2 Material & Method

5.2.1 Sample types

Root tissue samples were obtained from various plant species: garden cress (*Lepidium sativum*), radish (*Raphanus sativus*), arabidopsis (*Arabidopsis thaliana*), and sugar beet (*Beta vulgaris*). Plants were obtained from a local market or grown on media plates, as described below.

Furthermore, (Ri) T-DNA transformed root organs (here after: root organs) of *Daucus carota* and *Lycopersicon esculentum* grown on standard MSR medium were provided by the group of Kokkoris & Kiers (Vrije Universiteit Amsterdam), who received the root organs from Goh *et al.* (2022) [148].

Plant seed sterilization

Seeds were sterilized with 70% ethanol and a 4% sodium hypochlorite solution (Glorix) and washed at least five times with sterilized water until no foam appeared. The treatment was repeated twice and between each step the treatment agent was discarded and the seeds collected with a fine sieve. The exposure time to each substance varied among seed types. Seeds of *Lepidium sativum* and *Arabidopsis thaliana* were treated in the same way, with an exposure time of 1 min in ethanol (70%) and 15 min in bleach (4%). The seeds of *Beta vulgaris* needed to be soaked and washed in water first to remove the seed coating prior to sterilization. Since the seeds of *Beta vulgaris* and *Raphanus sativus* are lager, the exposure time to each agent was set to 5 min in ethanol (70%) and 15 min in bleach (4%). All sterilized seeds were either directly used or stored at 4 °C for a maximum of 2 days.

Plant growth conditions

Sterilized seeds were germinated and grown on 1/2 Murashige & Skoog plates (MS) with 1% (w/v) agar (Daishin agar; gel strength 700–800 hg/cm^2; Duchefa Biochemie) and 1% (w/v) sucrose. After germination in the dark at 21 °C, the seedlings were further grown in a plant chamber with the following conditions: 21 °C/21 °C day/night temperatures; 180 µmol light m^{-2} s^{-1} at plant level, relative humidity 50%; day-night-cycle 16/8 h. After 5–7 days of growth post-germination, the young root tips were used for the cryo-EM large-volume sample preparation procedure.

Bacterial strain and plant root inoculation

A GFP-labelled strain of *Flavobacterium anhuiense* (unpublished), obtained from the Balazadeh lab (Leiden University, NL), was grown in LB medium with ampicillin (0.1 mg/ml) and erythromycin (0.1 mg/ml) at 25 °C and shaking at 200 rpm. Cultures were assessed for GFP expression prior further use, using a fluorescence light microscope (DMi8 M; Leica Microsystems GmbH, Austria). Overnight cultures of *F. anhuiense* were used for the plant root inoculation procedure without further quantification.

Plant roots grown on 1/2 MS for 5–7 days were inoculated at their root tip with 100 µl of an overnight culture of *F. anhuiense* and incubated in the dark for 30 min. After inoculation, the roots were washed twice with sterile PBS. Subsequently, the inoculated roots were further processed by high-pressure freezing and lamella preparation.

5.2.2 3D printed biopsy needles

To extract intact tissue samples of homogeneous size, a biopsy needle was developed in the Briegel laboratory, enabling precise sampling from different tissue regions, as described in Depelteau (2022) [163]. The needles were made from photoactive resin using a Nanoscribe Photonic Professional GT 3D printer (Nanoscripe GmbH & Co. KG, Karlsruhe, Germany). The biopsy needles were mounted onto the tip of pins that had been previously inserted into 200 µl pipette tips. This assembly was then inserted into a customized injection device. The biopsy needle was either inserted using the spring system of the injection device or manually. The stability of the biopsy needle was assessed on plant roots and stems of different developmental stages. Subsequently, biopsy needles containing the obtained samples were subjected to HPF.

5.2.3 Histological and fluorescence staining

Histological staining of plant roots facilitated the retrieval and sample assessment after high-pressure freezing. Histological staining of plant roots was first performed with bromothymol blue (64.1 µM) dissolved in sterile tap water for 4-5 minutes. After staining, the roots were washed twice with sterile tap water to remove excess stain. During workflow development, we replaced bromothymol blue with methylene blue (31.3 µM), due to its fluorescent properties [164], which allowed detection using the Helios 5 Hydra DualBeam (PFIB) with an integrated fluorescence microscope (iFLM). Methylene blue was directly applied to the plant growth media plate and incubated for 20 min at room temperature (RT) in the dark. After incubation, the roots were cut off and washed twice with sterile tap water and high-pressure frozen.

During the workflow development, we discovered that the fluorescence signal of methylene blue was too weak and faded too quickly. To improve visualization and enhance detection of area of interest, a combination of Nile red (NR, for lipid staining) and calcofluor white (CFW, for cellulose, BactiDrop™, Remel™, Thermo Scientific™) was used instead. Nile red (Nile blue A oxazone, Sigma-Aldrich) stock solution (50 mg/ml in DMSO) was diluted (1:100), and 100 µl was pipetted on the surface of a plant growth medium plate (⌀9 cm). After 60 minutes of incubation at RT in the dark the excess stain was removed, and 100 µl of a 1:46 diluted CFW solution was added. After another 45 minutes of incubation (RT, in the dark) the excess stain was removed, and the plate washed twice with PBS. Stained roots were cut off with a surgical spring-loaded scissor and used for the high-pressure freezing procedure.

Assessment of fluorescence stained root tissue and imaging was performed using a fluorescence microscope (DMi8 M; Leica Microsystems GmbH, Austria). Stained and washed root tissue were placed in an ibidi microscopy dish (35 mm, glass bottom, ibidi GmbH, Gräfeling, DE) with a small amount of PBS to prevent dehydration. Imaging was conducted at RT using the Leica microscope software. Images were acquired with 20x and 40x objectives, using the default exposure setting and the built-in filter sets for the respective fluorophores.

5.2.4 High-pressure freezing

Vitrification of biopsy needle

Biopsy needles containing samples were HPF with either 10 % Ficoll PM400 (Cytiva™) or n-hexadecene (Sigma-Aldrich) as cryoprotectants. As described in Depelteau (2022) [163], the needles were placed in 3 mm A-planchettes, positioned on the 200 µm deep side, filled with cryoprotectant and closed with a pre-filled 100 µm deep A-planchette side. HPF was performed using a Leica EM ICE. To avoid additional handling step post-freezing, a grid was incorporated in the assembly so enable direct freezing of the needle onto the grid. Following HPF, the biopsy needles containing samples were recovered and stored in cryoboxes in LN_2 until further use.

Vitrification of plant roots

For vitrification of plant root tissue, root tips were removed with a scalpel from roots submerged in cryoprotectant, either 10–30% Ficoll PM400 or n-hexadecene (Sigma-Aldrich), and placed in a 3 mm A-planchette (100–200 µm depth, Leica). To ensure proper sample

enclosure, the flat side of a 3 mm B-planchette (Leica), coated with a thin layer of soy-lecithin (1 mM in chloroform), was placed on top, resulting in a planchette assembly that was subsequently subjected to HPF using a Leica EM ICE. Subsequently, the samples were recovered and the planchette assembly was disassembled with either fine tweezers or the bevel of an injection needle. The remaining A-carrier with the attached root tip was transferred into a grid box and stored in LN_2 until further use. Prior to milling, the samples were examined under the cryogenic light microscope of an ultramicrotome equipped with a cryochamber (Leica EM UC7/FC7) to verify that the root tips remained attached to the planchettes.

5.2.5 Preliminary Sample Processing: Volume Trimming

Preliminary sample trimming was performed according to Depelteau (2022) [163]. To reduce the volume of HPF plant roots and biopsy needles containing plant tissue, samples were mounted on a grid using cryoglue (3:2; 2-propanol:100% ethanol) [163], utilizing the the cryogenic light microscope of an ultramicrotome with a cryochamber (Leica EM UC7/FC7). Subsequently, attempts were made to trim the sample volume using a cryo-trim knife (Diatome) at -160 °C. Fine-sectioning was further attempted using the cryo immuno diamond knife (Diatome). For the quality assessment of trimming, the trimmed samples were transferred to the Aquilos cryo-FIB/SEM (Thermo Fisher Scientific™), and initial milling was conducted.

5.2.6 Milling of plant planchettes, volume lift-out & imaging

Milling, SEM imaging and lift-out procedure were performed as described in chapter 5 and according to the protocol of Schiøtz *et al.* (2024) [152]. HPF plant planchettes (type A) and a clipped half-moon grid in an autogrid were inserted into a HPF-carrier shuttle (3 mm; pretilt 35°) and subsequently transferred to the cryo-stage of a Helios Hydra V Dual-Beam cryo-PFIB (Thermo Fisher Scientific™) equipped with iFLM (development version) and a cryo-lift-out manipulator. Prior to imaging, a conductive platinum layer was sputtered onto the planchette using an integrated micro-sputter (6 min, 99 nA, xenon plasma). Subsequently, SEM images were acquired (12.5 pA; 2 kV) using MAPS (v.3.25) and aligned with the images acquired with the iFLM.

After identification of target regions, an approx. 1 µm thick platinum precursor layer was deposited over a period of 1 min using cryo-deposition with the GIS. The stage was then rotated to 180° to create an imaging face. Rough milling was performed at an angle of 16° (stage tilt 13°) using a 15 nA ion beam current, followed by polishing at 4 nA (30 kV). Trench dimensions were adjusted based on the sample and volume size, with trenches milled around the target volume, using overlapping milling patterns. The extracted volumes ranged from 20 µm x 30 µm x 10 µm (L x W x H) to 40 µm x 60 µm x 30 µm (L x W x H).

Prior to lift-out, sample integrity was assessed by SEM imaging at 90° (stage tilt 51°; 25 pA; 1 kV), followed by manual milling at 16° (tilt angle 13°; ion-beam current 0.6 nA). Subsequently, an additional 1 µm platinum layer was applied to the sample surface and the block's leading face for 4 min to mitigate beam induced damage during further milling.

The stage was configured for perpendicular milling (stage tilt: 17°; rotation: 0°), and trenches surrounding the target volume were extended to a minimum width of 10 µm.

The lift-out needle with the attached copper block, was moved adjacent to the sample to be extracted. Subsequently, the extracted chunk was attached to the copper block by re-deposition using a single-pass pattern of regular cross-sections at the interface between the copper block and the aligned sample surface [152]. The lift-out was performed at a milling angle of 9° (stage tilt: 6°).

The extracted chunk was then transferred to a half-moon grid, and the volume was aligned by matching its edge to the prepared pin edge, then attached by re-deposition using a single-pass pattern of regular cross-sections (stage tilt: 38°; milling angle: 69°; 300 pA; 30 kV). The lift-out needle was released by milling away the interface between the copper block and the volume surface. After successful lift-out and attachment to the half-moon grid, the volume and plant tissue integrity were re-evaluated with iFLM and SEM imaging, as described above.

5.3 Results

5.3.1 Application of biopsy needles

The biopsy needle was designed to obtain samples from different locations within a tissue a tissue, as well as to obtain sample of a consistent size (Fig. 5.1). To facilitate the incision into the tissue, the needle was designed with a three-pronged tip with sharp edges. Furthermore, it contained a tripartite slit located underneath the tip region, allowing for the release of compressed air, as well as providing a predetermined breaking point of the needle. This is necessary ro effectively to release the lower needle segment containing the sample for further processing. The design is described in more detail in Depelteau (2022) [163]. While the needle design provided sufficient stability for obtaining root and stem tissue samples, small air gaps appeared during application and processing, hindering optimal vitrification. To resolve this issue, the biopsy needle needed to be prefilled with a cryoprotectant such as hexadecene prior to use (Fig. 5.1).

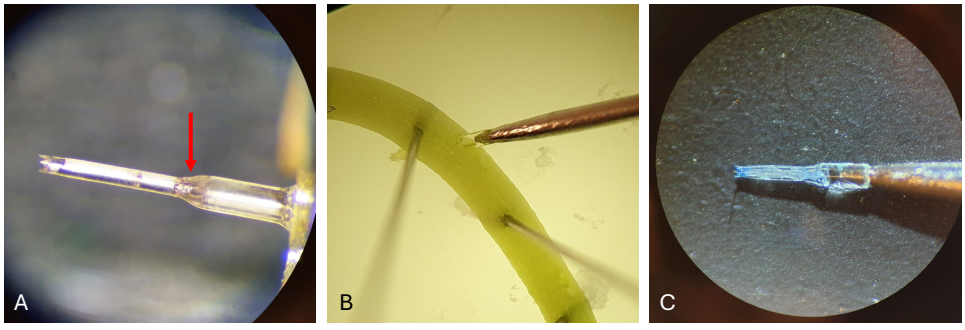

Figure 5.1: 3D-printed biopsy needle filled with cryoprotectant prior to use. The biopsy needle was designed with a three-prong tip and tripartite slits (red arrow) that serve as predetermined breaking point for segment release after sampling (A). Further details on the needle design can be found in Depelteau 2022 [163]. The stability of the biopsy needle was tested on various plant tissue types and developmental stages, including roots and stems (B). A biopsy needle containing root tissue shows small air gabs within the sample segment, which led to insufficient vitrification during high-pressure freezing (C).

During further testing of the needle for cryo-EM sample processing, the Briegel group discovered that the resin used for the 3D printed biopsy needle was not suitable for FIB/SEM or PFIB/SEM milling: the beam caused the plastic resin to melt, rendering this approach unsuitable for "in-needle milling". Consequently, its further application in this project was discontinued.

5.3.2 Staining enhances traceability of plant tissue

During the vitrification process of plant tissue and subsequent sample recovery, it became clear that the colourless root tissue was difficult to locate in liquid nitrogen. Additionally, visually assessing the extent to which the tissue remained attached to the planchette or grid after HPF proved challenging as well. To address this, bromothymol blue staining was initially used as tissue stain that provided the necessary contrast (Fig. 5.2). Later, this staining method was replaced with methylene blue due to its fluorescence properties. This also improved tissue traceability during the sample processing in the PFIB/SEM instruments. However, only weak fluorescence was detected with the available emission filters (L3385; L470; L565) in the PFIB/SEM.

Despite its weak fluorescence, the staining nonetheless greatly facilitated sample handling by enabling visualizing the tissue in the planchettes before the loading process into the PFIB/SEM (Fig. 5.2). Furthermore, the staining allowed precise positioning of the sample in the HPF-carrier shuttle for subsequent milling and cryo-lift-out procedures (Fig. 5.2).

The use of fluorescent staining further improved the traceability and localization of vitrified plant tissue during milling and lift-out procedures in the PFIB/SEM. Additionally, it enabled assessment of sample integrity after HPF and volume lift-out. Moreover, the iFLM in the PFIB/SEM facilitated detection of GFP-labelled endophytes within plant tissue, allowing precise section of the milling site for lamella preparation (Fig. 5.3).

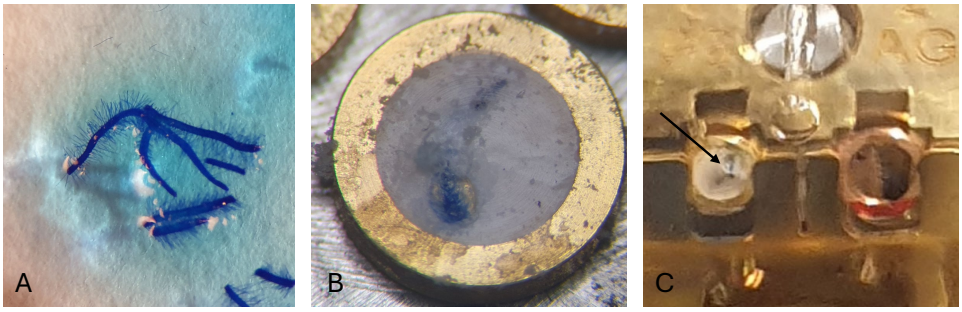

Figure 5.2: Plant roots stained with bromothymol blue (A) and methylene blue improved traceability of root samples and facilitated the assessment of root tissue presence in the planchettes after high-pressure freezing (B). Furthermore, staining assisted in the precise positioning of the root samples in the HPF-carrier shuttle, ensuring correct alignment perpendicular to the ion beam in the PFIB/SEM (C). Black arrow indicating the position of a root tissue sample within a HPF-carrier shuttle (Thermo Scientific^{TM}).

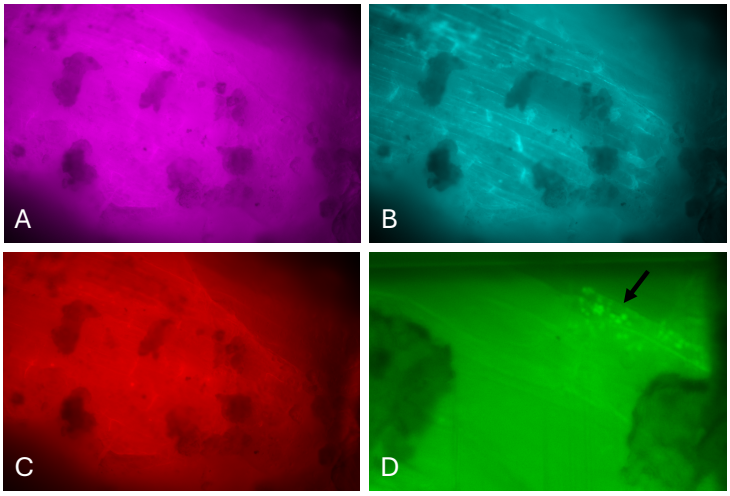

Figure 5.3: Fluorescence staining of root organ tissue using (A) methylene blue, (B) calcofluor white, and (C) Nile red. (D) GFP-labelled endophytes within root organ tissue facilitated precise milling positioning to obtain volume-samples and, later, lamellae from host-microbiota interaction areas. Images were acquired using the Helios 5 Hydra DualBeam (PFIB) with an integrated fluorescence microscope and the following emission filters: L385, L470, and L565 (Thermo Scientific™). The available emission filters enabled only weak detection of methylene blue fluorescence, which would yield a stronger signal with an excitation wavelength of 630 nm. However, despite the weak signal, staining still aided in the localization of the root tissue within the planchette in the PFIB/SEM. To enhance visualization, images were colour-edited in ImageJ [101].

5.3.3 Initial volume reduction

The initial plant tissue often exceeded 100 µm, significantly prolonging the milling process and increasing the risk of sample re-deposition and contamination. To mitigate this, cryo-ultramicrotomy was explored for preliminary volume reduction [163]. For this, the sample was first attached to a grid with cryo-glue [163]. Once attached, trimming was initiated. Initially, a flat surface was created as a starting point for further volume reduction. However, this process proved to be very time-intensive, and also increased the risk of de-vitrification and ice crystal contamination. After several trials, a radish root tip was successfully transferred to the Aquilos FIB/SEM, allowing for initial assessment of both volume reduction efficiency and sample integrity. In the FIB/SEM, the sample volume was only sightly reduce and exhibited numerous deep cracks. Further milling An attempts were highly time-intensive and reinforced the observation that larger sample volumes amplify re-deposition. These limitation prevented further sample processing, highlighting the need for alternative volume reduction strategies.

5.3.4 Volume sample preparation

Achieving successful vitrification of plant tissue proved challenging due to multiple influencing factors, including vitrification conditions (e.g., choice of cryoprotectant) and the developmental stage of the root tissue. Root tissue from older seedlings ($\geq$ 5 days) was either unsuitable or yielded poor results with existing methods. Therefore, root tissue and root tips from younger seedlings were used in further experiments. However, even in these cases, the success rate of vitrified samples remained low. This issue was further complicated

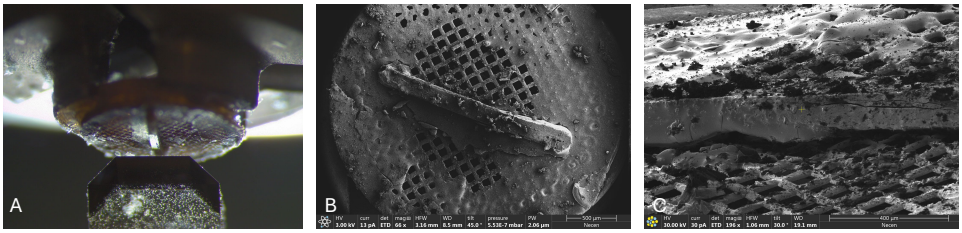

Figure 5.4: Initial trimming attempts of HPF root tissue to reduce sample volume for subsequent milling with a FIB/SEM ion-beam. (A) A HPF radish root tip was successfully attached to a grid using cryo-glue, and trimming was initiated with a diamond trim-knife in a cryo-ultramicrotome. (B) After initial trimming, the sample was transferred to a FIB/SEM (Aquilos, Thermo Scientific™) for assessment of vitrification quality and the trimming procedure. (C) During further milling with an ion-beam, significant contamination from redeposited excess material on the milled surface became apparent. Images were created in collaboration with Depelteau (2022) [163] as part of a joint study. These images were first published in Depelteau (2022) [163] and are included here with permission.

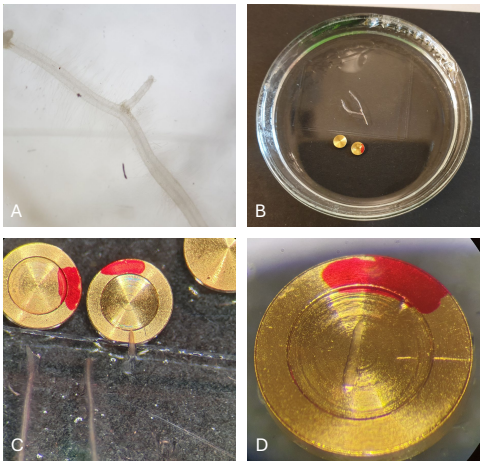

Figure 5.5: Optimized workflow for cryo-EM large-volume sample preparation using root organ tissue. (A) Root organs grow on media plates without requiring light or soil, offering the advantage that multiple root tips and lateral roots can be harvested a single plate. Additionally, root organs are thinner than young seedling roots, reducing the need for extensive volume-reduction steps such as sectioning prior to HPF. (B, C) Preparation of root organs for HPF, including planchette sandwich assembly while submerged in cryoprotectant (hexadecene). (C) Small root samples were cut while submerged in cryoprotectant at the edge of a glass slide, ensuring direct transfer into the prepared A-planchette. (D) Positioning of the root sample prior to planchette assembly and HPF. A red mark on the planchette edge facilitated sample-side identification after HPF and during downstream processing.

by the fact that poor vitrification quality only became evident during (P)FIB/SEM analysis, when tissue damage caused by HPF was observed. Partially vitrified tissue was used for initial milling trials in the PFIB/SEM. Despite the presence of numerous artefacts in the tissue, imaging provided the first structural insights into vitrified plant cells within the epidermis of the root.

The use of root organ tissue and root tips led to a notable improvement in vitrification success. Compared to younger seedling roots; root organs thinner, easier to maintain on plates without requiring light or soil and allow for the collection of multiple root tips from a single plate. Additional improvements in vitrification success were achieved by cutting the root tissue and assembling the HPF planchette sandwich while submerged in cryoprotectant, ensuring better preservation during freezing (Fig. 5.5). Furthermore, the HPF-carrier shuttle enabled the direct use of samples frozen onto planchettes, eliminating the need for sample transfer onto a grid or cryoglue. This reduction in manual handling minimized the risk of de-vitrification and ice crystal contamination. Additionally, the integration of the PFIB/SEM with a cryo-lift-out manipulator eliminated the need for volume trimming via cryo-ultramicrotomy, significantly reducing manual handling time and the associated risks.

The cryo-lift-out procedure was successfully performed on root tissue, allowing volume samples to be extracted extracted from different regions of the vitrified root, including deeper layers such as the root canal system. While most methods primarily enable sampling from outer root layers, this approach facilitated the extraction of vitrified volume samples from both the epidermis and the inner root tissue, demonstrating successful vitrification deep within the root structure. Furthermore, initial SEM images were obtained of HPF root tissue during the milling process, providing insight into both, vitrification quality and sample integrity. Samples processed using the optimized workflow demonstrated promising vitrification quality and high sample integrity. As a result, following successful cryo-lift-out and integrity confirmation, lamella preparation for cryo-EM imaging was initiated. Although only a small number of lamellae were successfully obtained, most were unsuitable for cryo-EM imaging due to ice crystal contamination during transfer. However, intensive milling induced partial de-vitrification in some samples, highlighting the need for refinements to minimize de-vitrification and re-deposition during the milling process. This study provides the first high-resolution structural insights into vitrified root tissue, revealing successful vitrification from the outer epidermis to the root canal system in unprecedented detail. These findings pave the way for more advanced investigations of plant tissue vitrification and cryo-EM sample preparation.

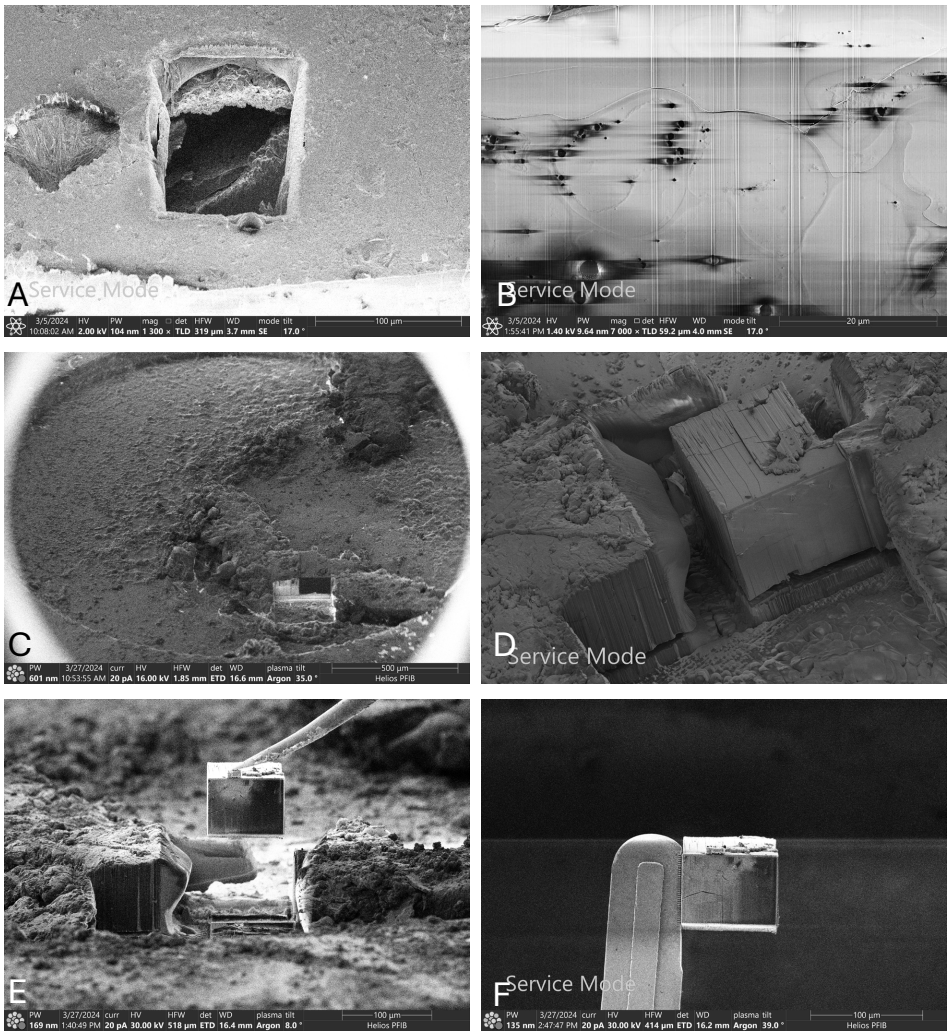

Figure 5.6: Evaluation of sample vitrification quality and cryo-lift-out workflow in PFIB/SEM. (A, B) Assessment of vitrification quality in seedling root samples. (A) Vitrification quality of seedling root samples was often poor, showing extensive structural damage, including imploded tissue. (B) Only the outermost cell layers (epidermis) of the root tissue achieved vitrification, exhibit better structural integrity. However, HPF artefacts persisted, preventing further processing. (C–F) Cryo-lift-out workflow using an improved sample preparation approach on tissue of root organs. (C) Localization of HPF root tissue without staining was difficult and surface features served as key orientation markers. (D) Once a potential target area of interest was identified, trench milling around the target region was initiated. (E) Following trench milling, the volume sample was attached to the lift-out needle, with a copper block as an intermediary for improved attachment. (F) The volume sample was then transferred to a half-moon grid and mounted to a pin for downstream processing, including volume imaging and lamella preparation for cryo-EM analysis.

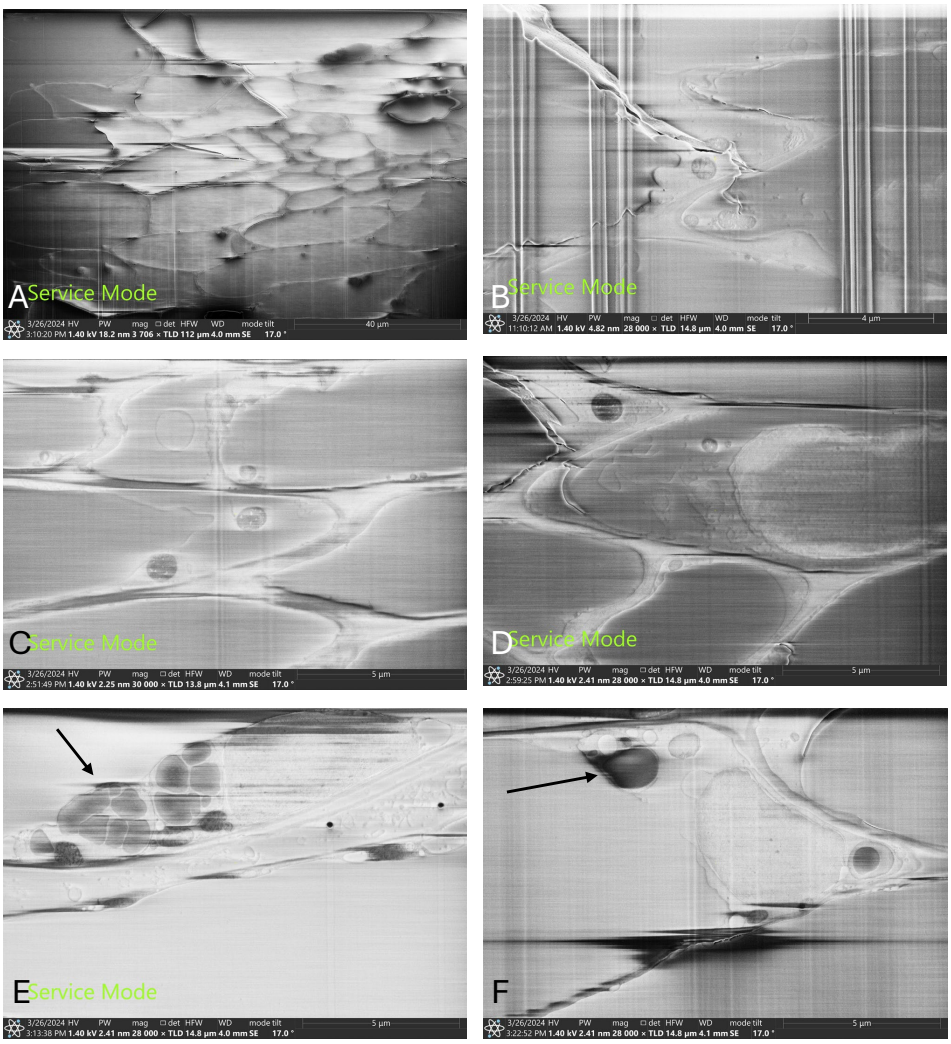

Figure 5.7: PFIB/SEM images of root organs with promising vitrification quality and high structural integrity. (A) Promising vitrification quality was achieved throughout the root tissue, ranging from the outer epidermal layer to the root canal system at the centre of the root. (B–F) The improved sample preparation procedure led to enhance vitrification, preserving structural integrity and providing the first high-resolution insight of vitrified plant cells and their ultrastructure. (E–F) Arbuscular mycorrhizal fungi within root organ tissue (black arrow) were successfully visualized, demonstrating the potential of the vitrification workflow for studying plant-microbe interactions at the ultrastructural level.

5.4 Discussion

To understand the interactions between plants and their microbiome, we need to investigate plant tissue samples at the nanoscale. To accomplish this, cryo-ET is the method of choice, as it allows imaging of near-native samples, in three dimensions and at macromolecular resolution. However, the workflows that enable the processing of such large-volume samples are still in its infancy and have only been successfully applied to a small set of biological samples. Here we aim to develop a robust workflow allowing to produce lamellae for cryo-EM imaging and cryo-ET data collection from native plant tissue samples. In our initial attempts, we employed 3D-printed biopsy needles, which enabled to consistently retrieved tissue of uniform size from various locations and tissue types. However, the resin used in the 3D-printed needles proved incompatible with further processing by FIB/SEM or PFIB/SEM. Nonetheless, the concept and design of these biopsy needles remain promising for future LV sample preparation. In future work, alternative resin types or novel materials should be explored for compatibility with (P)FIB/SEM processing.

Previous studies have demonstrated that one of the biggest hurdles and bottlenecks in analysing the ultrastructure of plants using cryo-EM is the vitrification of plant tissue. Vitrification by plunge freezing is limited to samples of ≥10 µm and has been successfully applied to algae and epidermal layers that can be directly applied onto a grid [157, 165, 166]. In contrast, the literature and protocols for vitrification of higher plant tissue are limited to the root tips of young Arabidopsis seedlings, which are less than ≥100 µm thick [165]. Samples exceeding 10 µm in thickness require HPF during which the sample is rapidly subjected under pressure (approximately 2100 bar) to liquid nitrogen. The application of high pressure during the cooling prevents sample expansion, an therefore, the nucleation of crystalline ice, and ideally, the sample is fixed in vitreous ice. Notably, the postulated limit for sample thickness at which vitrification can still be achieved by HPF is approximately 500 nm [158]. Yet to date only samples with a maximum thickness of 200 µm have been successfully vitrified by HPF [16, 50, 165]. This success is often dependent on the use and type of cryo-protectants, which significantly influence vitrification efficiency. Thus, the discrepancy between the postulated maximum sample thickness and the thickness actually achieved during vitrification can be attributed both to the inherent properties of the samples tissue and to the sample handling as well as freezing conditions. To improve vitrification outcomes, we applied modifications to the planchettes suggested in the literature [150].Prior to this modification, the planchette sandwich disassembly and recovery of vitrified sample were challenging, tedious, and occasionally lead to sample loss. By polishing and coating the flat side of B-planchettes with soy-lecithin, we improved the success of disassembly after HPF, which significantly enhanced sample recovery. Using this approach, we successfully vitrified root tips and root tissues with a sample thickness between 100–200 µm.

In parallel, cryoprotectants are used during planchette assembly to suppress ice formation. While agents such as sucrose and trehalose are common, their viscosity and electron density can introduce imaging artefacts. Alternatives like yeast paste, Ficoll, 2-methylpentane, proline, and hexadecene have been described [50, 166]. Among these, hexadecene proved particularly effective for plant tissue, likely due to improved tissue penetration. However, it increases stiffness after vitrification and complicates disassembly when combined with PBS (phosphate-buffered saline).

Furthermore, recent advancements in cryo-lift out now enable the use of planchettes for PFIB/SEM, thereby simplifying cryo-EM sample preparation. With this advancement, samples vitrified in planchettes can be directly transferred into the PFIB/SEM for milling, circumventing the tedious, low-yield process of recovering the vitrified sample for grid mounting via cryoglue [163]. This innovation also eliminates the need for sample trimming by the ultramicrotome, a demanding procedure prone to cutting artefacts and requiring substantial expertise [157]. In our study, we observed that as the sample volume increases, both the milling time and the risk of re-deposition of milled particles onto the milling area increases, too. In addition, prolonged milling time further increases the likelihood of beam damage, which necessitates the application of a conductive layer to create a smooth milling surface. The success rate improved significantly by switching from FIB/SEM to a PFIB/SEM system with an integrated lift-out manipulator. Not only did the PFIB/SEM system reduce the necessary milling time, but it also enabled the extraction of volumes from the LV sample that could be transferred to a new grid for further lamella preparation. Consequently, this approach minimized the volume of surrounding material and reduced the risk of re-deposition onto the milled area. However, re-deposition induced contamination could not be entirely avoided, leading to loss of lamellae. Additionally, the beam sensitivity also varies within sample such as the presence of fibres (e.g. lignin), which necessitates beam intensity adjustments during milling procedures [157].

Besides the common challenges of processing LV biological samples, plant tissue presents its own inherent obstacles for cryo-EM sample preparation. The variable tissue stiffness resulting from differing degrees of lignification depending on the developmental stage complicates pre-sectioning and the obtaining of samples with consistent size and volume. Moreover, unlike metazoan cells, plant cells possess an additional cell wall. This cell wall is a rigid structure, primarily determined by its cellulose content. It is composed mainly of chemical diverse polysaccharides, including cellulose, hemicellulose, and pectins. The cell wall serves as the first line of defence against intruders, and regulates molecular movement through a wide range of receptors, pores, and channels [167]. Despite its rigidity, the cell wall allows adaptation to different environmental conditions. In addition, intracellular compartments such as vacuoles, as well as tissue compartments like the xylem sap, exhibit chemical diversity that varies with environmental conditions and the developmental stage of the plant. This diversity combined with the barrier imposed by the cell wall, complicates the uniform diffusion of fixatives and cryoprotectants, leading to extended incubation times. Ideally, incubation times are kept short to avoid the adverse effects of cryoprotectants, which can exhibit toxicity or induce osmotic pressure, thereby causing artefacts.

The chemical diversity of plant tissue also affects the thermodynamic freezing point, which can differ between the cell contents and the surrounding intercellular matrix. Moreover, variations in turgor pressure within different cell compartments, particularly in the vacuole, which can constitute up to 90 % of the cell volume — present additional challenges during HPF. As a result, younger root tissue with less developed and less chemical diverse vacuoles, or plant cells with minimal vacuole development, are preferred for optimal vitrification. A significant issue in making plant tissue accessible for cryo-EM imaging is the presence of gases within the tissue matrix, both between cells and within the xylem sap. The presence of gases during vitrification result in incomplete vitrification and tissue rupture due to implosion. Therefore, minimizing or avoiding the formation of gas bubbles

and the presence of gases within the tissue is essential. This can be achieved by using cryoprotectants and reducing air exposure during plant root cultivation.

Root organs a suitable model system for LV cryo-EM of plant tissue

Root organs are well-established model systems for the investigation of host–microbiota interactions, in particular with arbuscular mycorrhizal fungi. Further root organs can be sourced from other agricultural important plants, broadening the scope for studying various microbiota members. The use of root organs, cultivated in media plates with the roots submerged, and without the need of light, helps preventing photosynthesis and potentially reducing the presence of gases within the tissue. When combined with HPF sample preparation under hexadecene — with both sample handling and planchette assembly conducted in a hexadecene-filled dish — these adjustments increased the likelihood of obtaining vitrified root tissue. Moreover, controlled and reproducible growth conditions enable the harvesting of root tissue with consistent chemical and physical properties, facilitating the development of standardized sample preparation protocols. In addition, the roots are thin and multiple root tips can be harvested from a single plate. Furthermore, using root organs permits rapid handling during pre-sectioning to obtain HPF suitable sample sizes while minimizing the risk of drying out prior to HPF.

The initial cryo-SEM imaging of vitrified plant tissue achieved in this study is a promising advancement, enhancing the potential for ultrastructural investigations of host-microbiota interactions. Nevertheless, the workflow is currently limited by low sample throughput and requires significant expertise, underscoring the need for further improvements to streamline the process. Furthermore, the presence of either single strains or a complex microbiota can alter the chemical and physical composition of the sample, potentially necessitating additional protocol adjustments.

Overall, the improvements in sample preparation, including modification in planchette assembly combined with recent advancements such as PFIB/SEM with iFLM and a cryo-lift out manipulator demonstrated in this study not only the success rate of root tissue vitrification but also enhanced the likelihood of obtaining lamellae suitable for cryo-EM. Furthermore, the use of root organs led to a significant improvement in both vitrification quality and sample throughput. These promising advancements indicate that current challenges will soon be overcome opening a new window into the ultrastructure of plant tissue and the structural interactions between plants and their microbiota.

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Author Contributions

J.L. developed the large-volume cryo-EM workflow for plant root samples. J.L. prepared all samples, performed vitrification, operated the cryo-PFIB/SEM system with J.M. and P.K., and wrote the manuscript. J.M. and P.K. provided training and technical support at CEITEC, performed lamella preparation, lift-out procedures and cryo-EM data acquisition, and contributed to the PFIB/SEM methods. After J.L.'s research visit, J.M. and P.K. continued working on the samples to obtain cryo-EM data. M.v.S. prepared and shipped the (Ri) T-DNA transformed root organ plates and provided additional material. J.N. provided technical advice. T.K. supervised the Amsterdam component and enabled access to the root organ system. A.B. supervised the project and provided feedback on the manuscript.