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

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Summary & General Discussion

Brief background

Plant-microbiota are communities of microorganisms that live together in a complex system of dynamic interactions [1]. They exert a significant influence on their environment and on the host plant with which they are associated. This close relationship has evolved to such an extent that the absence of specific host organisms or key microbiota members impacts the resilience of the community. It can even affect the viability of the plant host and its associated microbiota under environmental stress conditions [4]. To explore this close relationship and decipher plant–microbiota interactions, various approaches have been used, including culture-, molecular, and omics-based methods. However, these approaches provide limited insight into interactions occurring at the cellular and topological structural level. Understanding these structural aspects is crucial for interpreting dynamic interactions in their biological context.

To address these structural aspects, microscopy-based approaches have been applied. These techniques enabled the identification of distinct colonization patterns and plant morphological changes during colonization [5, 184]. These observations suggest that the plant host actively shapes tissue-specific niche conditions to guide and regulate microbial colonization. However, both fluorescence and light microscopy are fundamentally limited by optical diffraction (~200 nm), which restricts their resolution. As a result, cellular interactions and structural changes occurring on a much smaller scale cannot be captured by light microscopy alone. Thus, an approach is required that overcomes this limit, as offered by cryo-electron microscopy (cryo-EM). In addition, cryo-EM enables the observation of cellular structures in their native hydrated state.

Research aim

The aim of this thesis is to contribute to the understanding of nanoscale interactions between endophytic microbiota and their host plant in a near-native state, using cryo-EM. To achieve this, it was necessary to develop a workflow for the preparation of large-volume samples to make plant tissue accessible for cryo-EM.

In addition, the study investigated whether endophytic microbes also undergo morphological changes during colonization, as cellular structural changes had already been observed in plant tissue [184]. For this purpose, the potential key microbiota members *C. pinensis* and arbuscular mycorrhizal fungi (AMF) were included in the structural analysis. As the study progressed, phages emerged as an integral part of plant-associated microbiota and were therefore included in the investigation.

Methodology – Endophytic morphological structure

First, structural reference data had to be obtained from axenic culture of endophytes to assess possible morphological changes during plant tissue colonisation. For this purpose, *C. pinensis* was analysed by cryo-ET, and two different cell morphologies were detected: long filamentous and small spherical cell morphologies (**Chapter 3**). In this context, it was found that the small spherical cells did not correspond to the typical characteristics of dormant cells, such as the presence of condensed DNA or structural differentiation of the outer layer from the filamentous cells [108]. These spherical cells had previously been referred to as spores or cysts in the literature, based solely on their appearance in light

microscopy [97–99]. However, the observation by cryo-ET raised the question of whether these spherical cells truly correspond to the characteristics of a dormant cell stage.

In order to distinguish the small spherical cells from dormant cell stages, both cell forms were examined comparatively to assess their resistance and growth behaviour as well as their transcriptomic activity.

To further investigate potential functional differences between the two cell morphologies, hitchhiking assays were conducted using *B. subtilis*. These experiments revealed that only the small spherical cells were able to co-migrate with *B. subtilis*. To identify potential key factors mediating the observed interaction, a series of *B. subtilis* motility mutants was used, following the approach of Muok *et al.* (2020) [65]. In that study, *Streptomyces* spores were shown to hitchhike via flagellar attachment to a specialized surface layer. In contrast, cryo-EM observations in this study revealed no comparable mechanisms in *C. pinensis*. In particular, no co-migration occurred on swimming plates, suggesting that hitchhiking requires surface spreading and may involve surfactin-dependent mechanisms.

This led to the hypothesis of surfactin cheating behaviour, which was subsequently tested and confirmed. However, *C. pinensis* colonies were consistently found only along the movement direction of *B. subtilis*.

To further examine this phenomenon, a crowd-movement assay was conducted. The results showed that *C. pinensis* was unable to spread against the movement front, even in the presence of surfactin, indicating that passive co-migration is directionally constrained.

Attempts, were also made to visualise the interaction using fluorescence light microscopy (FLM) and cryo-EM. However, no clear attachment could be observed by FLM, and only two cryo-EM images showed loosely associated *C. pinensis* cells that had already begun to transition from spherical to slightly rod-shaped morphology. This observation highlighted the need to better understand the timing and progression of the morphological switch.

To determine the time of transformation and better track its progression, an attempt was made to synchronise the cell cycle and to fuse *gfp* into the chromosomal *fts*Z. This would have enabled real-time monitoring of colonisation and helped to determine which cell form occurs *in planta*. However, the methods used proved to be unsuitable and did not lead to a stable integration of *gfp* into the chromosome of *C. pinensis*.

Simultaneously, an attempt was also made to identify a possible trigger for transformation using the quorum sensing assay. The following conditions were considered potential triggers: nutrient concentration, cell density, cell form-specific quorum sensing signals, and the morphological state of the cells. As these are difficult to test individually, an attempt was made to vary their concentrations. However, the experiment turned out to be too complex, and a trigger could not be determined.

Methodology – Prophage among endophytic Bacteroidota

As previous studies have linked morphological changes in bacteria to the presence of phages [12, 168, 169], we investigated whether such a mechanism could play a role in the morphological transition of *C. pinensis* (**Chapter 6**). Since morphological plasticity was observed in the absence of externally added phages, we focused our investigation on the potential role of prophages encoded within the bacterial genome. Considering the possibil-

ity that prophages may have a broad host range, other endophytic Bacteroidota strains were included in the screening. Prophage prediction revealed that all tested endophytic Bacteroidota strains contain prophage sequences of varying predicted activity. In particular, 11 out of the 12 tested strains were predicted to harbour active prophages. Further culture based analyses confirmed the presence of inducible and active prophages in all strains tested, including both the wild-type and laboratory strains of *C. pinensis*.

To identify suitable strains for further phage enrichment and isolation, host-range assays were performed. The assay revealed that all extracts showed lytic activity against more than one bacterial strain. In some cases, strains from different Bacteroidota groups were susceptible to the same phage extract. However, attempts to isolate and purify individual phages were unsuccessful, as the phage concentration in the extracts remained too low. Neither enrichment using different host strains nor standard purification methods proved effective.

To confirm the presence of phage particles, extracts were analysed by TEM, which revealed fully assembled phages and phage-like particles in some extracts. Still, the limited yield and technical constraints prevented a more detailed investigation into their role in the morphological transformation of *C. pinensis*.

Methodology – large-volume sample preparation for cryo-EM

Alongside the structural characterisation of endophytic Bacteroidota and their phages, dedicated workflows were established to enable cryo-EM imaging of AMF (**Chapter 4**) and plant tissue (**Chapter 5**). For both sample types, the core workflow combined high-pressure freezing (HPF), fluorescence-guided targeting, cryo-lift-out and volume reduction as well as lamella preparation using cryo-plasma focussed ion beam scanning electron microscopy (PFIB/SEM).

Sample preparation was tailored to the specific requirements of each sample type, including the use of a novel planchette design and an assembly procedure adapted to the respective biological context. Due to the specific characteristics of AMF and plant root samples, the preparation procedure prior to HPF had to be adapted accordingly. For AMF, this involved further improvements of the waffle method [150] and the introduction of a new planchettes design, the so-called snap-button planchettes (**Chapter 4**). For plant tissue, a modified planchette assembly procedure – carried out while immersed in n-hexadecene –and the selection of a suitable model organism proved essential. Further details of these adaptations are provided in **Chapter 4 & 5**.

In addition to these adaptations, further refinements were made to improve the localisation of HPF samples during downstream processing. For plant tissue, histological stains allowed early visual evaluation of sample presence and positioning within the planchette, improving selection efficiency prior to PFIB/SEM processing. Fluorescent dyes, applied to both plant and AMF samples, subsequently enabled more precise localisation via integrated FLM. Details of the staining strategies and their implementation are described the respective chapters (**Chapter 4 & 5**).

Key findings

These methodological developments enabled the morphological and structural analysis of previously inaccessible volumes of intact AMF and root tissue and laid the foundation

for future investigations. Given the limited time frame and the technical complexity of cryo-EM for large-volume samples, the focus of **Chapter 4 & 5** was on the development and validation of a robust and reproducible sample preparation procedure.

Beyond the technical development, the structural investigation of *C. pinensis* in **Chapter 3** revealed unexpected morphological plasticity. While the transcriptomic activity of the spherical cells indicated a slow-down, they did not exhibit the typical characteristics of dormant cells and were shown to engage in hitchhiking behaviour.

In **Chapter 6**, this investigation was extended to explore whether phages could act as regulatory trigger of the observed morphological change, and it was shown that active prophages are widespread among endophytic Bacteroidota and have a broad host range.

Synthesis of the results

Taken together, the results of this thesis reveal structural and functional characteristics of endophytic microbiota and raise new questions about their dynamic adaptive behaviour in plant-associated environments. Although no clear regulatory mechanisms could be identified, the results suggest a broader regulatory network. Morphological plasticity, prophage activity, and microbial interactions could contribute to maintaining the dynamic stability and balance of the endophytic microbiota.

This is particularly relevant as plants have been shown to actively modulate their microbiota through creating spatially and chemically distinct niches. In addition, plants have been shown to selectively shape their microbial communities and actively suppress certain members when they are no longer beneficial [185, 186].

Endophytic microorganisms must be able to adapt to changing conditions. This requires functional integration into the host environment while maintaining their ecological role within the endophytic microbiota. In this context, the morphological plasticity of *C. pinensis*, together with the associated transcriptomic shift, may represent an adaptive strategy. It could also help reduce competition among the endophytic microbiota and support persistence under resource-limited conditions.

In addition, the small spherical cells of *C. pinensis* offer distinct advantages for dissemination. Their ability to hitchhike and exploit surfactin-mediated motility allows them to passively follow microbial community movements. In this way, they could also exploit the chemotactic behaviour of motile members to reach favourable environments with minimal energy costs.

Moreover, their small size could permit passive transport through the plant's vascular system, potentially allowing relocation to sites of pathogenic attack. However, this remains hypothetical, since *in planta* tracking was not possible within the time-frame of this study.

A further hypothesis related to the role of AMF as potential mediators of microbiota dissemination. AMF networks are known to connect the roots of different plants and facilitate the exchange of nutrients and chemical signals, including warning cues when under pathogenic attack. It is conceivable that small spherical cells of *C. pinensis* could be recruited and disseminated via the AMF hyphal network. This would allow for the targeted delivery of beneficial microbes to sites of pathogen invasion, even across different host plants.

Such mechanisms could represent an efficient strategy for pre-emptive defence, limiting further pathogen spread. Whether this process is actively mediated by the plant, the AMF itself, or through cooperative signalling between both, remains an open question. It is also possible that AMF may exert selective control over which microbial partners are shared (or withheld) depending on the host's compatibility and cooperation. This raises broader ecological implications on how microbial resources might be differentially distributed in multi-partner networks.

The extent to which free phages are spread via AMF networks is still unclear. It is also conceivable that the prophages use their bacterial hosts as microbial 'Trojan horse' to further spread themself. This mechanism could have positive effects by spreading metabolic traits that enhance endophytic behaviour and promote mutualistic interaction with the plant. However, when triggered, prophages may alter microbial composition and activity, potentially affecting host–microbiota interactions.

These findings highlight the structural and ecological complexity of plant-associated microbiota, while also reflecting methodological and time limitations that shaped the scope and depth of this study. Limited access to key equipment further constrained certain experimental approaches, particularly in the cryo-EM workflow.

Challenges, limitations and future perspective

Each chapter was characterised by its own set of methodological and experimental constraints, which in turn shaped the scope of the data and the available avenues for interpretation.

In **Chapter 3**, one such challenge was the inability to synchronise the cell cycle of *C. pinensis*, despite repeated attempts using standard protocols and time-controlled reinoculation. As a result, transitional stages between the two morphologies could not be temporally resolved, which limited the interpretation of comparative transcriptional activity. The observed changes in growth and morphology occurred inconsistently across replicates, which limited the reproducibility of experiments involving the wild-type strain.

One possible solution could be to test alternatives such as nalidixic acid, which have been shown to reversible arrest the cell cycle in *Escherichia coli* [187, 188]. The effect may be further enhanced by combining it with chloramphenicol [188]. Whether this approach would yield similar effects in *C. pinensis* is currently unknown, but its potential merit further investigation. Since nalidixic acid primarily affects metabolically active cells, its effect could be tested first on the spherical cells. Given their reduced transcriptional activity observed in this study, synchronisation might be more reliable in this state. Establishing a more consistent synchronisation protocol for *C. pinensis* would also enable phase-specific comparative analyses at the transcriptomic, proteomic, and metabolic levels. This could help clarify whether the observed slow-down in transcription is merely size-related or reflects a functional differentiation between the two cell morphologies. It may also contribute to identifying intrinsic regulatory factors that trigger the morphological switch.

A major experimental limitation in **Chapter 3** was the transformation of *C. pinensis* with a green fluorescence protein (GFP) fusion construct. This was the objective of a collaboration with Le Zhang (van Wezel group, LU, NL) however, its aim was unsuccessful. This prevented live tracking of the morphological states and *in planta* visualisation of bacterial

localisation. Although the plasmid had previously been successfully applied in *Flavobacterium*, its instability in *C. pinensis* may have been caused by promoter incompatibility or active defence mechanisms against foreign DNA.

To overcome the challenges in transformation in the wild-type strain, future experiments could explore the use of the laboratory strain of *C. pinensis* (DSM 2588). Preliminary observations indicate that this strain also undergoes morphological switching, although the characteristics of the transition differ slightly from those in the wild type. These differences were not examined, as this was outside the scope of the present study. However, based on the comparative prophage analysis in **Chapter 6**, the laboratory strain may exhibit a reduced defensive response to foreign DNA, which could make it more amenable to genetic manipulation. Alternatively, *Flavobacterium anhuiense*, which exhibits similar morphological shifts as *C. pinensis*, could be used as a tractable model organism. It could be used to further investigate the underlying mechanisms of the morphological shifts and the potential influence of prophages on this process.

Another challenge was the difficulty in reliable quantifying cell numbers and colony-forming units (CFUs) at specific growth stages of *C. pinensis*. Early colonies were extremely small, colourless, and only detectable under a stereomicroscope. Attempts to count cells directly using counting chambers were unsuccessful, as the small cells were difficult to focus within a single optical plane but also hard to distinguish from debris. A more efficient strategy could involve spot-plating of dilution series and fluorescence staining, combined with automated imaging using a stereo-fluorescence microscope. CFUs could then be quantified using image analysis software such as ImageJ [101].

In **Chapter 6**, the main limitation was the inability to isolate and purify individual phages in sufficient quantity for sequencing and further characterisation. Although fully assembled phage particles were detected and confirmed by TEM in some extracts, the overall phage yield was too low to enable downstream analysis. Standard concentration methods, including salt precipitation, and ultracentrifugation, proved too harsh and led to substantial loss of phage particles. Similarly, the use of filtration systems did not yield sufficient phage concentrations. Future efforts should focus on development of purification and concentration strategies with higher yield, as well as using host strains that are more susceptible to propagation of the target phages.

If sufficient quantities of phages can be recovered, this would open the door to functional studies exploring their potential contribution to morphological plasticity in *C. pinensis* and potentially other endophytic Bacteroidota. Moreover, it would allow investigation of specific traits they may carry and confer to their bacterial hosts. This may provide insights into how these bacteria evolved an endophytic lifestyle.

In **Chapter 4 & 5**, the central objective was to develop a workflow for cryo-EM analysis of large-volume biological samples, that include AMF and plant root tissue. While both AMF and plant tissue required sample specific adaptations, the overall development was shaped by similar technical constraints. Early experiments were hindered by limited access to cryo-EM equipment, in particular cryo-shuttles suitable for planchette-based sample transfer. As a result, vitrified samples had to be manually recovered from the planchette after HPF and mounted onto EM grids, which led to substantial sample loss and reduced vitrification quality.

These limitations could only be overcome through a collaboration with J. Nováček and his team at CEITEC (Brno, CZ), who provided access to PFIB/SEM systems with integrated manipulator for cryo-lift-out procedures and florescence guided targeting via integrated FLM. The use of cryo-shuttles compatible with planchettes enabled bypassing the manual mounting steps and allowed direct transfer of vitrified samples into the PFIB/SEM for downstream processing. Furthermore, the snap-button planchette system developed by J. Nováček (**Chapter 4, section 4.3.3**) significantly improved sample integrity, recovery rates after HPF, and overall workflow efficiency, while minimising sample loss.

Additional support came from the team of Toby Kiers and Vasilis Kokkoris (VU, Amsterdam, NL), who provided pre-inoculated root organs with AMF for cryo-preservation. Based on these samples, a suitable model organism could be identified for large-volume cryo-EM sample preparation. Its use enabled more consistent vitrification, better sample integrity after HPF, and increased procedural efficiency. In particular, the root organs facilitated easier handling and allowed the collection of multiple root tips from a single plate.

However, due to time constraints, it was not possible to complete downstream processing and cryo-EM data acquisition for all vitrified samples. Several AMF preparations, including early developmental stages, remain cryogenically stored at CEITEC and may be processed in future studies.

Despite these advances, sample preservation during transfer and storage remained a challenge. In particular, ice crystal contamination frequently occurred during transfer from the PFIB/SEM to the cryo-EM. This issue was further exacerbated by long waiting times prior to cryo-EM imaging. These limitations highlight the need for improved transfer procedures, and faster cryo-EM processing capabilities in future applications.

The workflows developed in this study form the basis for future investigations of the nanoscale structure of interaction between plants and their endophytic microbiota. This includes the analysis of hyphal development and intracellular organisation of AMF. It also enables the precise localisation of endophytic bacteria in plant tissue, including *C. pinensis*, provided that stable GFP integration can be achieved. This could help clarify which of the two cell morphologies of *C. pinensis* occurs in which part of the plant tissue and contribute to a better understanding of their respective functional roles. In addition, the method enables investigation of potential interaction interfaces *in planta* between endophytic bacteria and AMF at the nanoscale.

Concluding remarks

This thesis provides both biological insight and methodological progress in the structural analysis of plant–microbiota interactions. Despite significant experimental limitations, key results were achieved that lay the foundation for further nanoscale investigations. These outcomes underscore the importance of collaboration and methodological innovation in overcoming technical barriers.

The developed cryo-EM workflows now allow high-resolution studies of endophytes and AMF directly in vitrified plant tissue. By linking microbial structures to ecological function, this work contributes to a deeper understanding of host–microbiota systems and their regulatory complexity.

As automation in cryo-lift-out and lamella preparation continues to advance, the

processing of large-volume samples will become more standardised and allow for higher throughput. Altogether, the findings and workflows presented here offer a solid basis for future research into the structural dynamics of plant–microbiota interactions.