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

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General Introduction and Thesis Outline

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Introduction

As Newton once said, "What we know is a drop, what we do not know is an ocean". Just as this applies to the universe it also holds true for our understanding of plant-associated microbiota. The microbiota refers to an assembly of living microorganisms within a spatially and temporally defined environment, including bacteria, archaea, fungi, algae, and small protists. According to Berg *et al.* (2020) [1], it also includes mobile genetic elements such as transposons, viruses, and bacteriophages. The diversity and function of microbial communities are crucial for almost all ecosystems, as they play a central role in geochemical cycles, drive nutrient dynamics, and help maintain ecological balance. Consequently, they also play a vital role in their host plant by enhancing nutrient uptake, mitigating the effects of abiotic stressors such as heat and drought, and providing protection against pathogens.

Rather than being passive recipients of microbial colonization and interactions, plants actively shape their microbiota through their physiology, root architecture, and a process known as the "cry-for-help" strategy [2, 3]. In this view, plants release specific compounds in the form of root exudates, which allow them to influence both the composition and functional properties of their microbiota while selectively attracting and enriching beneficial microorganisms. These interactions can extend to the point where they lead to the development of disease-suppressive soils, a phenomenon that has been shown to persist across plant generations through soil-borne legacy effects [4].

However, plant-microbiota interactions expand beyond the soil-root interface (the rhizosphere). In fact, they occur throughout the entire plant, including the internal tissues. Microorganisms that colonize plant tissues without causing harm or negatively affecting their host are referred to as endophytes. These endophytic communities are present in all plant compartments, but their composition varies between tissues. More specifically, plants show selective colonization patterns, favouring certain microbes while restricting others [5]. The microbiotas in different plant tissues are not isolated but interact and influence each other, including those on the plant surface and in the rhizosphere, leading to a complex network of interactions that shape the plant's microbial community. In recent years, the plant-associated microbiota has gained attention for its potential to enhance plant health and resilience, offering a sustainable alternative to chemical-based agricultural practices. Various strategies have been explored, including microbiome transplantation, microbial inoculants, and the use of microbial and plant extracts [6, 7]. In this context, studies have revealed that a subset of core microorganisms (also referred to as key microorganisms) is necessary for the long-term success of plant-microbiota treatments [1]. Core microorganisms are members of the microbiota that are consistently present and associated with specific host genotypes or environmental conditions. They play a critical role in maintaining both host and microbiota fitness. Thus, identifying these core microorganisms, as well as understanding their interactions with the host plant and the surrounding microbiota, is essential for optimizing plant health and productivity. Just as plants shape their microbiota through metabolites, microorganisms use their own metabolites to interact between species, influencing microbiota composition and function as well as host plant physiology.

Since bacteria dominate the plant-microbiota, they have become the main focus of research in the identification of potential core microbiota members. The most abundant bacterial phyla include Proteobacteria, Firmicutes, Bacteroidota (formerly known as Bac-

teroidetes), and Actinobacteria. Particularly, members of the Bacteroidota phylum have gained interest, as the majority of these bacteria tend to engage in mutualistic interactions, either directly supporting their host or indirectly benefiting other microbiota members [8]. For example, the strains *Chitinophaga pinensis* and *Flavobacterium anhuiense* are known to enhance plant resilience to fungal pathogens as well as abiotic stressors such as heat and drought. Besides its role in plant resilience, *C. pinensis* exhibits the ability to degrade complex polysaccharides, which may contribute to its ecological function within the plant-associated microbiota. However, the precise role of these metabolic capabilities in microbiota interactions and plant association remains unclear. Additionally, it is not yet known how these microorganisms establish stable associations within the plant microbiota despite environmental challenges. Further research is needed to determine whether, in addition to chemical signals, possible physical nanostructures facilitate host interactions and to what extent *C. pinensis* morphology influences its ability to interact with its microbiota and host plants.

The dynamic interactions within the microbiota, as well as between the host and its microbiota, are complex and influence each other's physiology. To maintain balance and ensure long-term persistence despite environmental challenges, regulatory mechanisms are essential to prevent the overgrowth of single species. In this context, bacteriophages play a crucial role, as proposed by the 'kill the winner' theory, which suggests that bacteriophages target bacterial populations that thrive under certain conditions [9]. Bacteriophages, or phages for short, are viruses that exclusively infect bacteria to hijack their replication system for propagation. They follow two distinct replication strategies, the lytic and the lysogenic replication cycle. In the lytic cycle, the phage attaches to the susceptible host bacteria to inject its genome into the cytoplasm and redirects the host's replication system to produce and assemble new virions. The cycle ends with the release of phages through cell lysis. In contrast, during the lysogenic cycle, the phage genome integrates into the host genome, and remains dormant as a prophage, replicating along with the host. Once triggered, the prophage switches to the lytic cycle, leading to host lysis and the release of new phages. Beyond their regulatory function within microbiotas, phages can also benefit their host by introducing additional metabolic capabilities, conferring antimicrobial resistance, or modulating bacterial morphology in ways that provide competitive advantages within the microbial communities [10–12]. However, little is known about phages that target Bacteroidota nor is it clear to what extent they play a role in the mutualistic endophytic lifestyle of Bacteroidota.

In addition to bacteria, fungi also play an important role in the plant-associated microbiota, with arbuscular mycorrhizal fungi (AMF) in particular considered core microorganisms. AMF have been closely associated with plants for more than 450 million years, forming symbiotic relationships with more than 72% of vascular plant species. This symbiosis is so intimate that the plant allows the fungus to invade its cells and form a specialized structure for nutrient exchange, known as an arbuscule [13]. AMFs help plants to overcome site-specific resource limitations through their extensive hyphal network that connects host plants across species. Interestingly, this fungal network not only facilitates resource exchange between hosts but may also serve as a potential transport route for phages and bacteria. However, little is known about how these exchanges of resources and transport processes are regulated, or which structures are involved.

Although microbial interactions play a crucial ecological role, their ultrastructural basis remains largely unknown. Cryo-electron microscopy (cryo-EM) has emerged as a powerful tool for visualizing the structural interaction between host and microbe in their native cellular environment. The technique offers the possibility to gain insight into the nanoscale features involved in microbial interactions in a near-native state and in three dimensions. However, to date, cryo-EM has been primarily used to study the ultrastructure of isolated proteins and single cells. Cryo-EM is based on freezing samples in vitreous ice [14]. To achieve this, samples are rapidly frozen using plunge freezing, where they are submerged in liquid ethane cooled by liquid nitrogen (-194 °C). This rapid process prevents the formation of crystalline ice, instead forming electron permeable amorphous (vitreous) ice that preserves the sample in its native hydrated state without structural alteration.

Initially, cryo-EM imaging was limited to samples with a maximum thickness of 500 nm due to electron beam penetration constraints. The development of focus ion beam scanning electron microscopy (FIB/SEM) overcame this limitation by utilizing an ion beam to selectively thin a predefined rectangular area (lamella) to 200 nm, making it electron permeable and thus accessible for high-resolution imaging. This advancement allowed cryo-EM imaging of samples with an initial thickness of up to 20 µm for the first time. Another breakthrough came with high-pressure freezing (HPF), which overcame the vitrification limitations of plunge freezing.

Due to water's poor thermal conductivity, plunge freezing is limited to sample thinner than ~10 µm, as heat cannot dissipate fast enough, leading to the formation of crystalline instead of vitrified ice. HPF addresses this issue by applying 210 MPa (~2100 bar) of pressure during vitrification, preventing water expansion, lowering the freezing point, and improving cooling efficiency. This allows the vitrification of thicker samples up to ~200 µm [15, 16].

However, increasing sample thickness introduced new challenges, particularly in lamella preparation, as prolonged thinning times increased the risk of de-vitrification, crystalline ice contamination, and beam-induced damage.

In recent years, these limitations have been overcome through advancements in cryo-lift-out techniques and the refinement of FIB/SEM into plasma-FIB/SEM (PFIB/SEM), significantly reducing thinning times and improving sample integrity. The combination of these methods has already enabled the acquisition of high-resolution cryo-EM images from metazoan tissue. While these developments represent a promising step forward, sample preparation for cryo-EM imaging remains an ongoing challenge. It is still unclear whether these techniques can be adapted to visualize the ultrastructure of plant tissue in a near-native state with nanoscale detail as well, along with the microbiota residing within, and their interactions.

Thesis Outline

This thesis was carried out as part of the NWO-GROOT project "Unwiring beneficial functions and regulatory networks in the plant endosphere" (OCENW.GROOT.2019.063), which investigates how endophytic microorganisms contribute to plant health and resilience.

The focus of this thesis is the structural investigation of key members of endophytic

microbiota and the development of a workflow for large-volume sample preparation, making plant tissue accessible for cryo-EM imaging and data collection. The structural analysis provided new insights into the morphology of *C. pinensis* and the cellular organization of AMF hyphae.

Chapter 2 reviews the technical developments in cryo-electron tomography and the novel insights they have provided into the structural organization of bacteria. In addition, the combination of methods and techniques in sample preparation, sample volume thinning and lamella preparation, along with automated data acquisition as well and advances in data processing, has enabled a deeper investigation of bacterial molecular machines regarding their structural organization and function.

In **Chapter 3**, the morphological structures in *C. pinensis* were explored in detail to identify potential adaptations that facilitate host-microbiota interactions. This revealed a drastic transition in morphology, from long filamentous to small spherical cells. However, the spherical cells did not exhibit characteristic features of spores, dormant cells, or persister cells, such as increased resistance or structural modifications of the cell wall. The potential advantages of this reduced cell size were analysed further and revealed that small spherical cells exhibit hitchhiking behaviour in the presence of motile bacteria.

Chapter 4 focuses on, optimization of the vitrification procedure for AMF samples and on the development of a workflow to prepare AMF hyphal network samples and spores for cryo-EM imaging. This study presents the first insights into vitrified AMF hyphae, revealing their structural organization in unprecedented detail. This workflow supports future research in elucidating the ultrastructure of AMF hyphal networks and investigating the mechanisms of their trading system, as well as its regulatory processes, at the nanoscale level.

Chapter 5 describes the development of a workflow for large-volume sample preparation of plant roots, making them accessible for cryo-EM imaging and tomography data collection. Additionally, root tissue from different plant species was tested, leading to the identification of root-organs as an optimal model system for this workflow. Notably, root-organs are already well-established models for studying plant-microbiota interactions. This workflow enables the vitrification of root tissue for cryo-EM imaging, providing the first insights into plant-microbiota interactions in root tissue at the nanoscale level. Future research will benefit from this workflow, as well as from the identification of root-organs as a suitable model system for nanoscale plant-microbiota interaction studies.

Chapter 6 describes the challenges in detecting and isolating lytic phages from soil samples, as well as the distribution of temperate phages (prophages) among endophytic Bacteroidota strains. This study revealed that all tested strains harboured active and inducible prophage sequences with a broad host range. The results provide a solid foundation for future research to investigate the potential role of active and cryptic prophage sequences in the endophytic lifestyle of Bacteroidota members.

Chapter 7, discusses and summarizes the outcomes of this thesis and their broader implications. Additionally, it addresses challenges in large-volume sample preparation for cryo-EM imaging and outlines future perspectives.