

Custom-engineered micro-habitats for characterizing rhizosphere interactions

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*I dedicate this thesis to my parents for their love and dedication, and for
encouraging me to pursue math and science,
to my partner, Jeff, who has supported me from the beginning of graduate school,
and to all the female scientists preceding me, whose perseverance empowered me to
also pursue the highest degree in education.*

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Abstract

The interactions amongst plants and microorganisms within the rhizosphere have a profound influence on global biogeochemical cycles, and a better understanding of these interactions will benefit society through improved climate change prediction, increased food security, and enhanced bioenergy production. However, the rhizosphere is one of the most complex and bio-diverse ecosystems on earth, making it difficult to parse apart specific interactions between species. This difficulty is compounded by the inability to directly visualize rhizosphere interactions through the soil. Additionally, conventional laboratory techniques do not offer real-time, high-resolution visualization or the proper environmental control to isolate and probe these interactions. A knowledge gap persists in how to design appropriate culturing platforms that allow researchers to collect spatially and temporally sensitive information about physical and chemical interactions in the rhizosphere. This dissertation addresses that gap by demonstrating the design and use of several custom-engineered micro-habitats in characterizing plant-microbe interactions. Specifically this thesis introduces novel protocols for culturing plants and microorganisms together in microfluidic platforms, pairing platforms to multi-modal imaging techniques with organelle scale resolution, and recreating the structural complexity of the rhizosphere in a microfluidic habitat. Not only does this thesis introduce novel engineered systems, but the work contained herein also goes beyond proof-of-concept experiments and demonstrates the ability of these platforms to generate hypotheses and answer outstanding biological questions.

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Chapter 1

Introduction

The rhizosphere, defined as the underground ecological zone influenced by the proximity of plant roots, is one of the most complex and biodiverse ecosystems on earth [1]. Compared to the microorganisms in bulk soil, rhizosphere microbial communities are distinct, both in overall Operational Taxonomic Unit (OTU) richness (α -diversity) and in the number of unique species between the two environments (β -diversity)[2]. Network analysis has shown that rhizosphere microorganisms also have higher levels of interaction than microorganisms in bulk soil [3]. Within the rhizosphere, specialized microorganisms share and compete for resources, forming a dynamic trophic web with the host plant. The physical and chemical characteristics of the soil environment can influence these biological interactions by moderating the nutrients and space available to rhizosphere organisms [4]. In return, rhizosphere organisms shape the composition of their environment through biogeochemical processes and soil particle aggregation [5, 6, 7]. The variations on these intricate feedback cycles ensure that rarely are any two plant rhizospheres exactly alike. This complexity, compounded by spatiotemporal heterogeneity and the opacity of soil, has made rhizosphere ecology difficult to study holistically [8].

Despite being difficult to study, rhizosphere ecology is a priority research area; its interactions sustain global nutrient cycles and, consequentially, human life. Studying plant-microbe interactions can advance the basic science of inter-taxonomic kingdom communication, co-evolution, and immune system response [9, 10, 11, 12]. Likewise, applying beneficial microorganisms to plants may supplement climate change adaptation, bioenergy production, and food security [13, 14, 15]. The benefits of studying plant-microbe interactions outweigh the challenges, and, as scientists develop new ways to parse apart rhizosphere complexities, new basic science and applied discoveries continue to emerge.

The following sections briefly introduce the current state of knowledge surrounding the rhizosphere including who the key actors are, variables influencing community phenotype, and rhizosphere spatial organization.

1.1 Rhizosphere Microbiomics

1.1.1 Community Metagenomics

Among the many taxonomic kingdoms represented by organisms within the rhizosphere, the scientific community has been especially interested in the host plant’s interactions with its microbiome members, including bacterial kingdoms and fungi [16, 17]. The microbiome of a single plant’s rhizosphere can constitute thousands of unique bacterial and fungal species [18]. It is common for rhizosphere-sampled rarefaction curves (a measure of microbial OTU richness) to not reach saturation, which means that many more species have yet to be, and are continuously, discovered [19, 20, 21]. Of the microorganisms that have been identified, less than 5% of bacteria and less than 5% of fungi are culturable, which greatly reduces the ability of rhizosphere ecologists to characterize these species [22]. Similarly, modifying the genome of culturable species (e.g. to introduce fluorescent markers for imaging or mutations to study traits) is not always straightforward, which can also limit the amenable species suitable for certain experiments. Using comparative genomics to closely compare related taxonomic species is, increasingly, aiding the prediction of species’ functions when culturing the organisms is not possible [23].

Due to some of these evolutionary-conserved traits, it can also be informative to characterize the rhizosphere composition, not by every individual species, but by grouped taxonomic phyla or even lower classifications (i.e. genera). Overall abundance studies have, indeed, demonstrated that certain phyla dominate the rhizosphere and are conserved across multiple plant rhizospheres. For example, in three separate experiments focused on *Populus deltoides* root microbiomes, dominant and conserved phyla included *Actinobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Acidobacteria*, *Verrucomicrobia*, and *Planctomycetes* [24, 20, 25].

The microbial composition of a given rhizosphere can vary widely with several environmental parameters. In agricultural settings and other managed land systems,

the structure of the microbiome is heavily influenced by environmental parameters like soil type, tillage, and pesticide additions [20, 26]. In natural land systems the host plant genotype can have a strong correlation to its microbiome’s metagenome because of the long-term effects of co-evolution between the organisms [27]. This is not always the case in natural systems, however, as there is high variation in microbiome composition even among host plants of the same species depending on factors such as geography, seasonality, and CO₂ levels [25, 28]. For example, 196 geographically diverse natural accessions of *Arabidopsis thaliana* were shown to have a wide range in their ability to associate with the same bacterial strain [29]. To form robust relationships between microbiome composition and the environment, scientists are currently cataloging the geographical diversity of the entire earth’s soil microbiome [30].

1.1.2 Community Function

Metagenomics is not always the most informative measurement of a rhizosphere community. For instance, relic DNA from dead microbial cells in the soil can obscure a metagenomic understanding of which bacteria or fungi are actively contributing to the microbiome’s overall function. It was also recently discovered that microbial species with high genetic abundance in the soil (including *Verrucomicrobia*) were not directly correlated to the overall metatranscriptomic activity of the soil [31]. In other words, low abundant species may be contributing more to the overall microbiome activity and function than previously thought. For this reason, experts are proposing a shift away from thinking only about a microbiome’s metagenome to, instead, considering the microbiome’s "metaphenome", an all encompassing term that considers how environmental parameters act on the metagenome to determine the microbiome’s overall function [32].

In considering rhizosphere microorganisms by their metaphenome rather than individual species, it can be helpful to characterize microbiome structure in terms

of functional guilds or core microbiomes [33]. Core microbiome members, sometimes referred to as "keystone taxa", are defined as those species which exert the most influence over the microbiome due to their ability to recruit members with complementary functions, reinforce other core members, and to ward off pathogens [34]. Core microbiomes have been shown to emerge from various environmental conditions. For example, a plant's core root microbiome may re-equilibrate after the introduction of a stressor [35]. Microbial interaction network models are helping predict which members of a microbiome may be the core members [36].

Understanding the metapenome, and even the phenotype of individual microbiome members, is essential for deciphering rhizosphere interactions. There are many different types of plant-microbe interactions which range from being beneficial to both species, commensal (i.e. only benefiting one of the organisms but not harming the other), or pathogenic. Still other interaction types are more fluid. Opportunistic microorganisms may benefit the plant under certain environments but turn pathogenic when conditions change [37, 38].

For these reasons, it is ideal to study plant-microbe interactions in their natural habitat (i.e. an intact holistic rhizosphere) in order to determine how naturally-occurring environmental conditions influence the organisms' functions and relationship. However, *in situ* rhizosphere studies are not conducive to every type of experiment, especially those that require high environmental control. In these situations, field studies can be complemented by greenhouse and laboratory bench-top scale experiments. Both greenhouse and field scale approaches use soil as a substrate for rhizosphere organisms, while laboratory scale approaches, often conducted on an agar substrate with a characterized chemical composition, are more reductionist [39, 40]. Although benchtop experiments preclude a holistic understanding of the rhizosphere, they are effective at isolating and characterizing specific plant-microbe interactions. However, care must be taken to not over extrapolate microbial characteristics determined from experiments conducted in enriched, homogenized media to phenotypes that may be induced in a more natural system. Taken together,

these three experimental approaches have helped scientists sample and characterize rhizosphere members, reproduce community phenotypes, and isolate biochemical signaling pathways [41, 42, 43, 44].

1.1.3 Root Exudation

Differences in microbiome community metagenome and metapenome are believed to arise, in part, from changes in the composition of root exudates produced by the plant [45]. Of its total photosynthetically-fixed carbon, plants re-release a significant quantity back into the environment in the form of root exudates [6, 46]. Root exudates are typically characterized either as high molecular weight compounds such as proteins and polysaccharides or soluble, low molecular weight compounds (e.g. sugars, organic acids, phenolic compounds, secondary metabolites) [47]. The latter are highly labile within the rhizosphere, and can be transformed by certain microorganisms into usable sources for other neighboring microorganisms [48]. Not only are these compounds food sources, but they can also act as specific signalling molecules [47]. For example, the plant can exude tryptophan, an amino acid, which certain bacteria can then convert into indole-3-acetic acid, a plant growth hormone [49, 50].

Root exudation is a dynamic process; exudate profiles change during a plant's development, but also with the introduction of new species or stressors [51, 52, 53]. For example, early in its development *Arabidopsis thaliana* seedlings exude many simple carbon compounds which can be converted to energy by generalist microorganisms, and it is hypothesized that, by doing so, the plant is recruiting microbial members from surrounding bulk soil to its microbiome [54, 55, 56]. During vegetative growth, exudate profiles can shift to produce phenolic compounds which may only be consumed by specialist microbiome members. Some of these exudates, such as salicylic acid, act as antibiotics to modulate the colonization of certain bacterial species [57]. Whether the plant is actively or passively selecting microbiome members, however, remains unknown [58]. Recently, pre-programmed changes in

root exudation due to a plant’s development was shown to interact with microbial substrate preference to predict community assembly [59]. However, current methods for characterizing root exudates rely on unnatural environments like hydroponic systems, and cannot capture microbial feedback dynamics in exudate profile changes.

1.1.4 Spatial Organization within the Rhizosphere

The spatial organization of organisms in the rhizosphere is an often overlooked dimension in experimental designs due to the difficulty in reconciling the disparate length scales of microorganisms and plants, but it is no less important in understanding rhizosphere interactions. Spatial variability can arise in the distribution of microorganisms and plants, as well as in abiotic factors like moisture, minerals, exudates and other organic substrates. Variability across field or larger geographic scales is easier to measure, but the amount of environmental variability that a single microorganism may be subjected to underground has been less characterized.

Partitioned soil samples show that, although ubiquitous in the rhizosphere, microorganisms are not uniformly distributed by species or with respect to the plant roots [60, 61]. This spatial variability of microorganisms is also reflected in the microenvironment. It was recently discovered that soil microaggregates ($<250\text{ }\mu\text{m}$ in size) harbored different and more diverse taxa than soil macroaggregates ($>2000\text{ }\mu\text{m}$) which were different still from bulk soil [62].

Along with hosting unique taxa, soil aggregates can also change community function and microbial phenotype through spatial confinement. Across aggregate structures, pore spaces may range from $10\text{-}30\mu\text{m}$ in inter-aggregate pore spaces (i.e. the surfaces of aggregates) to $1\text{-}2\mu\text{m}$ within intra-aggregate pores (Table 1.1) [63]. At these length scales, diffusion of microbial signaling molecules is severely limited [64]. For quorum sensing bacteria, signaling molecules can accumulate past a critical threshold, changing microbial pathogenesis, biofilm capabilities, motility, and production of secondary metabolites [65]. Because of this, these

Table 1.1: *Relevant length scales of microorganisms and soil features, and their interactions at these scales*

μm	Biological Relevance	Soil Relevance	Interaction
<1	viral particle size [66], E. coli cells deform (300nm) [67]	particle surface roughness promotes selective adhesion of specific bacterial species (10-100 nm) [68]	lisogeny and gene transfer [69], bacterial shape deformation [67], surface attachment [68]
1-2	bacteria cell size [67]	pores within soil microaggregates [70]	Nitrogen fixation [71] fungal mycelia reinforce aggregate tensile strength [74], bacterial biofilm extra-cellular polysaccharide production binds soil particles together [5]
1-15	fungal hyphae diameter [72], bacteria biofilm thickness in sand [73]		
10-30	distance at which the vast majority of bacteria cell interactions occur (20 μm) [75]	pores between soil microaggregates can retain water against gravity for multiple days [70]	denitrification[76], quorum sensing bacteria exhibit inhibited cell division [65]

microscale interactions exert important controls on rates of nutrient cycling in natural environments and are key to understanding metabolic pathways within the broader soil matrix.

Outside of soil aggregates, but still on a micrometer scale, interactions amongst microorganisms continue to be dictated by distance. A single gram of soil contains millions of microbial cells, yet nearest neighbor analysis has suggested that bacteria may not interact with other cells outside of a 20 μm radius [77, 75]. Within soil pores, available open space is confined and surface areas are high, promoting bacterial-surface attachment and biofilm formation [78, 79]. Bacterial cells contained within biofilms have even less flexibility in movement and exchange with neighboring microorganisms, and rely on percolating flows to replenish their nutrients.

Plant-microbe interactions are currently understood to occur as far out as millimeter scale distances. For example, studies have estimated that plant exudates can diffuse up to 5mm from the roots, limiting the area of influence that a plant has over the surrounding soil [8, 60]. The plant also exhibits spatial variability in its exudation patterns. For example, *A. thaliana* root tips exude higher concentrations of sugars while other parts of the root exude higher tryptophan concentrations [49]. These chemical gradients can influence where microbial species are recruited to on the root [58, 80]. However, more research is needed to confirm specific exudate distances and gradients in natural media as solutes can adsorb to grain surfaces and diffusion distances are inhibited by soil grains.

Capturing the spatial and temporal heterogeneity of micron-scale rhizosphere interactions would be further enabled by real-time high-resolution imaging. However, the natural opacity and auto fluorescence of soils hinder *in situ* visualization. Current laboratory-scale rhizosphere imaging methods are either low-resolution, such as agar plate assays, or they cannot capture the dynamics of the interaction, as with chemical fixation methods [81, 82].

1.2 Microfluidics

Recently, microfluidics has enabled researchers to design custom, laboratory-scale culturing platforms to visualize biological interactions [83, 84, 85].

These microfluidic platforms are traditionally fabricated using a combination of photolithography and soft-lithography molding in a transparent polymer [86]. With this process, researchers can create designs in the x-y plane that are then extruded in the z dimension, resulting in a pseudo-3D design. Additional complexity in the z-direction can be created by stacking multiple layers of channels. The combined photo- and soft-lithography process can be used to create sub-micron scale design elements. 3D-printing can complement photolithography by creating true 3D designs and at scales that are amenable to larger organisms [87].

The transparency of microfluidic platforms creates a major opportunity for imaging biological systems in real time. Often, the organisms can be cultured directly in the platform, eliminating the need to transfer or chemically fix the organisms prior to imaging. Similarly, the polymers frequently used for both soft lithography (poly-methylsiloxane) and 3D printing (poly-methylmethacrylate blends) are inherently bio-compatible and pose no risk to adversely affecting the organisms [88].

Not only can these platforms be used for real-time imaging of biological interactions, but they also afford an extremely high level of environmental control within a fully defined system. This allows the researcher to isolate organism behaviors while minimizing unspecified experimental variation [89]. The ability to faithfully

replicate the same design for each experiment also permits the user to precisely simulate multi-physics within the platform using finite element analysis [90, 91].

Within the past decade, microfluidic and 3D platforms have been adapted to accommodate whole organisms such as plants [92]. A popular feature of these platforms is that they can be used for high-throughput and precise experimental replication. This has allowed biologists to rapidly identify plant phenotype changes due to the introduction of various treatments [93].

Beyond basic plant research, microfluidic platforms have promising potential in helping elucidate specific interactions between plants and their associated microorganisms. The high environmental control of these platforms can allow researchers to manipulate the organisms' environment to probe community responses to environmental perturbations. For rhizosphere purposes, microfluidic platforms can also be designed with increasing complexity to reconstruct the natural plant-microbe environment in a well-defined system [94].

1.3 Research Aims

This thesis provides several examples of how microfluidic platforms can be incorporated into rhizosphere research to illuminate the dynamic spatial relationships between plants and their associated microbiome members. While these systems do not fully encompass the complexity of a holistic rhizosphere, they can, in a parameterized fashion, be customized to retain more features of the natural environment. Here I demonstrate how microfluidic platforms can isolate plant characteristics (Chapter 2) and I work toward creating a more realistic rhizosphere-on-a-chip by adding microbiome members (Chapter 3) and the physical structure of natural porous media (Chapter 4). Figure 1.1 provides a graphical representation of this thesis outline.

The first aim of this dissertation is to extend the current capabilities of microfluidic plant culturing platforms by designing features that enable fine root isolation and by

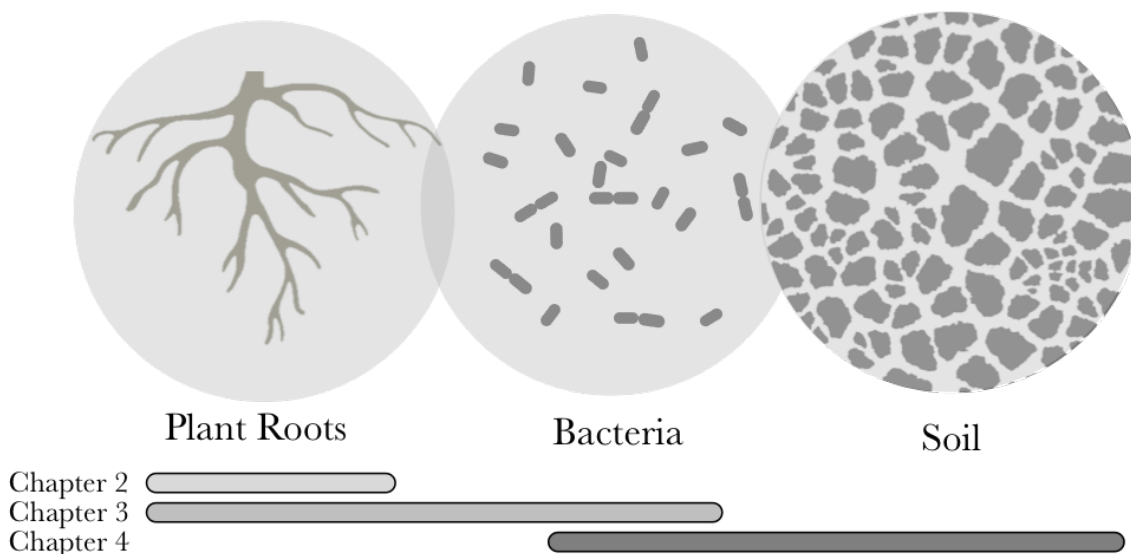


Figure 1.1: Plants, bacteria, and the soil are key actors in the rhizosphere environment. Chapters 2, 3, and 4 will provide methods and proof-of-principle experiments to study the interactions between these organisms.

pairing the platform with multi-modal imaging instrumentation. For these proof-of-concept experiments, I have identified *Arabidopsis thaliana* as a suitable host plant given its characterized genome and root exudate profile, and proven compatibility with microfluidic systems [56, 95, 96]. This work will help expand the length scales and measurement capabilities that researchers have access to with microfluidic platforms.

The second aim of this thesis is to introduce root associated microorganisms to a plant in a microfluidic platform in order to visualize the dynamics of root colonization in real time. For microbial community members, I chose two strains isolated either from the rhizosphere, *Pantoea* sp. YR343, or endosphere, *Variovorax* sp. CF313, of *Populus deltoides*. These bacteria strains were modified to fluoresce with gFP and mCherry markers, respectively, making them easy to identify on plant roots [18, 97]. Both bacteria species have fully sequenced genomes that house genes for plant hormone production, qualifying them as plant-growth promoting bacteria (PGPB) [98]. Using the capabilities of a microfluidic platform, our intention was to answer long-standing questions about plant-bacteria interactions that traditional methods have failed to fully address, including:

1. How quickly do bacteria colonize plant roots?
2. What part of the root do certain bacteria species prefer?
3. How does the presence of a multiple bacteria species change individual colonization rates and spatial distributions [11]?

The final aim of my dissertation is to design a microfluidic platform that recreates the heterogeneous structure of natural soil to determine how the soil matrix and subsequent nutrient flow affect the spatial distribution of bacterial biofilm formation underground. This work will begin to illuminate how the structure of a porous media environment dictates the spatial location and subsequent chemical exchange between organisms within the rhizosphere. By creating a benchtop model that replicates the physical characteristics of porous media, I can retain more parameters of a holistic rhizosphere while culturing the organisms in a highly controlled and fully defined environment. Because these platforms go beyond traditional open-chamber designs, and begin to replicate the organisms' natural environment, I have referred to them as "micro-habitats" throughout this thesis.

Chapter 2

Expanding Plant-on-a-chip

Capabilities to Enable Multi-Modal

Imaging of *A. thaliana* Fine Roots

with Subcellular Resolution

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2.1 Abstract

Root hairs increase root surface area for better water uptake and nutrient absorption by the plant. Because they are small in size and often obscured by their natural environment, root hair morphology and function are difficult to study and often excluded from plant research. In recent years, microfluidic platforms have offered a way to visualize root systems at high resolution without disturbing the roots during transfer to an imaging system. The microfluidic platform presented here builds on previous plant-on-a-chip research by incorporating a two-layer device to confine the *Arabidopsis thaliana* main root to the same optical plane as the root hairs. This design enables the quantification of root hairs on a cellular and organelle level and also prevents z-axis drifting during the addition of experimental treatments. We describe how to store the devices in a contained and hydrated environment, without the need for fluidic pumps, while maintaining a gnotobiotic environment for the seedling. After the optical imaging experiment, the device may be disassembled and used as a substrate for AFM or SEM imaging while keeping fine root structures intact.

2.2 Introduction

Fine root features increase water and nutrient acquisition for the plant, exploring new soil spaces and increasing the total root surface area. The turnover of these fine root features plays a major role in stimulating the underground food chain and the number

of fine roots in certain plant species is expected to double under elevated atmospheric carbon dioxide [99, 100]. Fine roots are generally defined as those smaller than 2 mm in diameter, although new definitions advocate for characterizing fine roots by their function [101]. Like many fine roots, root hairs provide the function of uptake and absorption but occupy a much smaller space with diameters on the order of microns. Because of their small size, root hairs are difficult to image *in situ* and are often overlooked as a part of the overall root architecture in field scale experiments and models.

Ex terra root hair studies, such as from seedlings grown on agar plates, have provided the scientific community with valuable information on cellular growth and transport [102, 103]. While agar plates allow roots systems to be imaged non-destructively and in real time, they do not offer high environmental control for the addition of experimental treatments such as nutrients, plant hormones, or bacteria. An emerging solution to facilitating high resolution imaging while also affording dynamic environmental control has been the advent of microfluidic platforms for plant studies. Although microfluidic platforms are a relatively new method for studying plant systems, many laboratory groups have contributed to several generations of designs, each armed with unique capabilities to address specific plant research questions. Some of the very first designs, such as the RootChip and RootArray, served as proof-of-principle experiments in extending the reach of microfluidic systems to whole organism studies [92, 104]. These platforms characterized the growth of roots in a microfluidic environment and demonstrated high throughput potential for rapidly phenotyping replicate organisms. Building on these systems, other authors added flow focusing capabilities for localized treatment and mechanisms for measuring the mechanical force of growing root tissue [105, 85]. Microfluidic platform designs have focused on the use of single open space fluidic layers in which the roots may propagate, permitting the root hairs to drift in and out of optical focus during growth or treatment.

Here we extend the current capabilities of microfluidic plant habitats to include designs that enable fine root feature isolation, multi-modal imaging, and subcellular resolution. We provide representative results for the quantification and visualization of root hair morphology in this platform by optical, scanning electron microscopy (SEM), and atomic force microscopy (AFM) techniques. We present a procedure (see Appendix A) for developing a two-layer microfluidic platform using photo and soft-lithography methods that builds upon previous plant-on-a-chip designs by confining the seedling root hairs to the same imaging plane as the main root. This allows us to track root hair development in real time, at high resolution, and throughout the experimental treatment process. Our culturing methods allow *Arabidopsis thaliana* seedlings to be germinated from seed within the platform and cultured for up to a week in a hydrated and sterile environment that doesn't require the use of syringe pump equipment. Once the time-lapse imaging experiment has concluded, the platform presented here can be opened without disturbing the position of the finer root features. This allows the use of other high resolution imaging methods.

2.3 Representative Results

The two-layer PDMS microfluidic devices described here have a 200 μm high channel for the main *Arabidopsis* root and a 20 μm high chamber to confine laterally growing root hairs (Figure 2.1A). This design may be used for plant species with similar root diameters as *Arabidopsis thaliana* and can be readily modified to accommodate species of different sizes. The design incorporates an inlet for the plant as well as 8 side inlets for any desired chemical or biological treatment. Prefilling the devices with media and pouring agar around the device keeps the root environment hydrated for the duration of the experiment without the need for external fluidic equipment. (Figure 2.1B) The agar also stabilizes the device within the Petri dish, allowing the seedlings to be grown vertically to encourage root growth down the main channel.

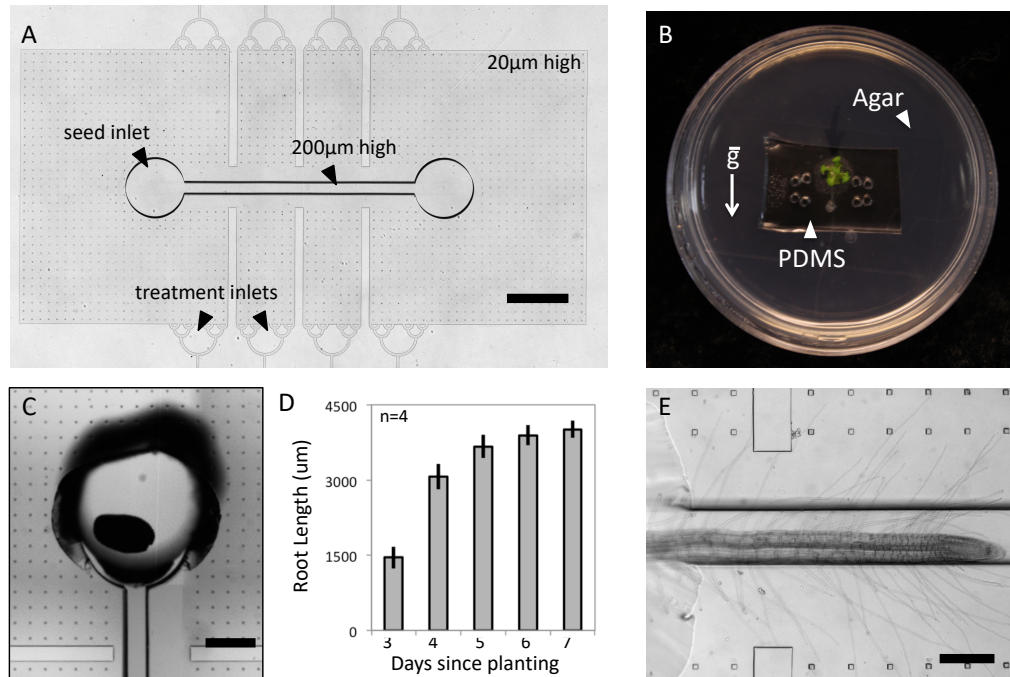


Figure 2.1: Growth of *Arabidopsis thaliana* in Two-layer Microfluidic Platform. (A) Device consists of two layers to confine root hairs to the same imaging plane as the main root (scale 1 mm). *A. thaliana* seedlings may be (B) contained in a gnotobiotic environment, (C) germinated from seed within the device, Scale bar = 400 µm, (D) and monitored for growth over the course of a week (error bars are standard deviation, SD). (E) A representative root is imaged in the platform, Scale bar = 200 µm.

The Petri dish keeps the seedlings contained in a gnotobiotic environment and allows the root system to be imaged through the Petri dish to magnifications up to 20X. Another option would be to directly seal the PDMS device to a glass-bottom Petri dish for even higher optical magnifications.

Arabidopsis thaliana seeds can be directly planted into the device inlet that is punched at a 45-degree angle to facilitate root growth into the main channel. (Figure 2.1c) The height of the PDMS device should not exceed a few millimeters, as the leaves must be able to grow out of the top of the platform. Because of their small size, *Arabidopsis* seeds are very difficult to orient so that the emerging root radical faces the channel upon germination. Therefore, approximately half of the seeds planted will germinate with their leaves in the channels and cannot be used for root visualization experiments. Devices from these seedlings may be re-autoclaved and used again beginning from step 2.2. To encourage phototrophic growth of *Arabidopsis thaliana* shoots, the viewing chamber of the device may be covered in aluminum foil to block light and improve success rate. *Arabidopsis thaliana* roots have grown steadily in this platform for a week, at which point growth becomes directed toward lateral root formation. (Figure 2.1d) The growth rate of roots in this platform are comparable to growth rates of *Arabidopsis thaliana* roots in other microfluidic platforms [106]. The two-layer design successfully confines the root hairs to the same imaging plane as the main root. (Figure 2.1e) However, some root hairs may continue growing in the main channel out of optical focus and future designs should aim to more gradually guide root hairs into the root hair chamber.

Within the microfluidic platform, root hairs can be quantified on a cellular level in terms of their length, density, and the angularity of their growth from the root. (Figure 2.2A) Root hair length and density of *A. thaliana* wild type seedlings grown in our platform are within range of seedlings grown vertically on agar plates [107]. The angle of root hair growth is typically perpendicular to the surface of the root. In our platform, the angularity of root hair growth toward the tip may be an artifact of the confinement of the root hairs to a 2-dimensional plane. Mechanical stimulation,

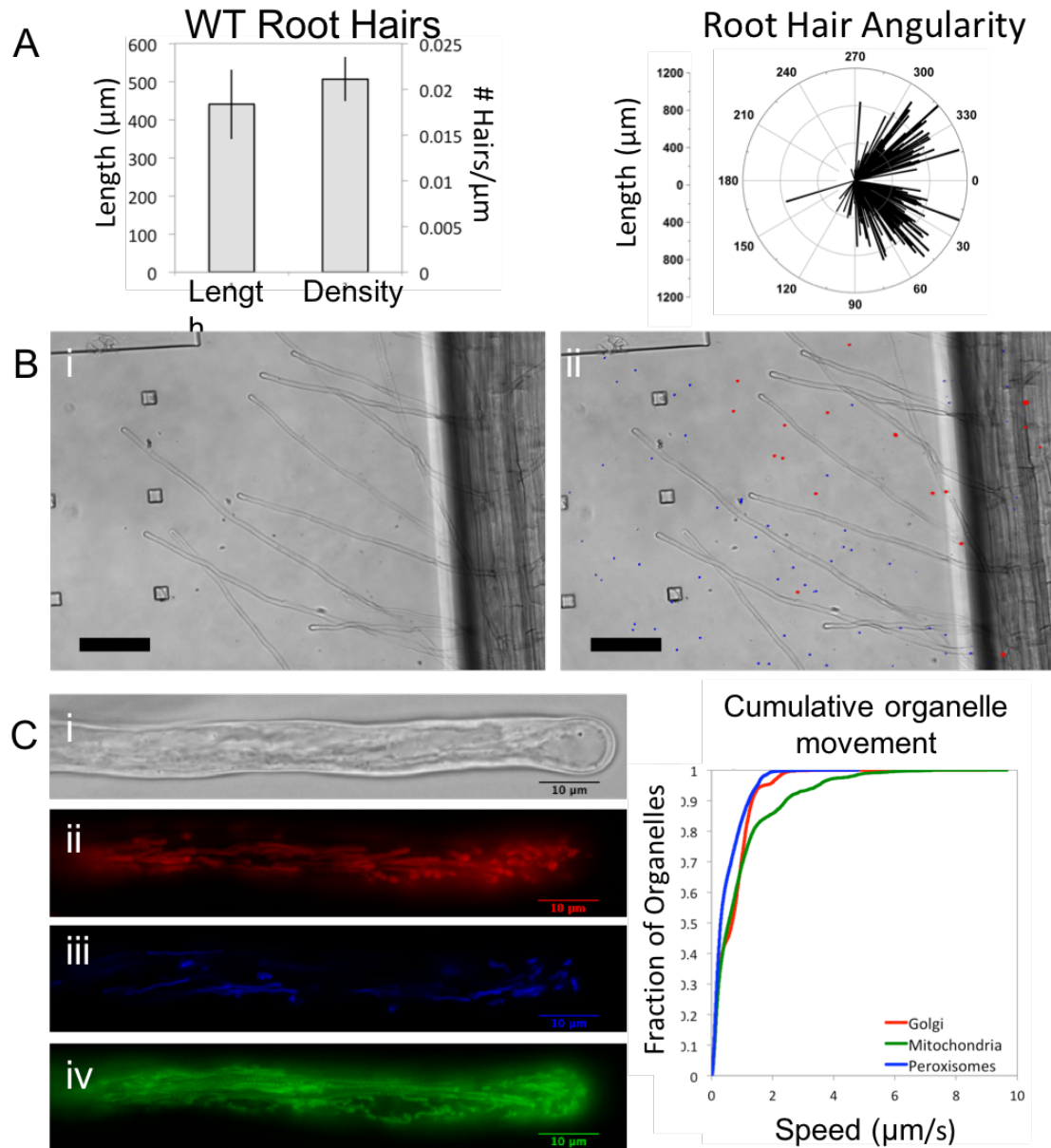


Figure 2.2: Root Hair Characterization and Treatment. Root hairs were quantified on a cellular level by (A) length, density, and angularity for $n=4$ wild type (WT) seedlings (error bars are SD). Angularity is determined as the angle between the main root tip and root hair tip with the main root tip defined as the 0 degree mark. (B) The optical focus of the root hairs remains unchanged (i) before and (ii) after treating them with polystyrene fluorescent beads (Scale bar = 100 μm). (C) Organelle level quantification is demonstrated by imaging the (i) cytoplasmic streaming from a differential interference contrast image and, in a separate root hair, the fluorescence of three organelles: (ii) Golgi (iii) peroxisomes, and (iv) mitochondria (Scale bar = 10 μm). Organelle trajectories were visualized by merging fluorescent images taken every 23 - 32 s for 20 s. The movements of the three organelles were tracked automatically and cumulative speed distributions plotted on the right.

in this case by the walls of the microfluidic device, has been shown to induce changes in root growth and orientation which may explain the discrepancy between a confined microfluidic platform and an open agar plate [108]. It is unlikely that this phenotype is the result of a nutrient stress in our platform as *A. thaliana* seedlings utilize stored food reserves from the seed in the first few days of growth. Although hypoxia may be a concern in fully saturated root systems, the oxygen permeability of PDMS has previously demonstrated the biocompatibility of the polymer with oxygen dependent tissues [109, 110].

One of the strongest advantages of microfluidic platforms over agar plate methods is the ability to uniformly add precise concentrations of chemical treatments to the organisms. In single channel microfluidic platforms, the addition of treatments can potentially displace fine root hairs from optical focus. Here we demonstrate the maintenance of root hair optical focus in our two-layer microfluidic platform during the addition of fluorescent beads; a seedling is imaged (i) before and (ii) after the addition of fluorescent carboxylated polystyrene beads (blue and red) Figure 2.2B. The addition of this abiotic treatment did not disrupt the orientation or optical focus of the seedling’s root hairs.

Higher magnification imaging and fluorescent markers may be used to image and quantify organelle level changes in root hair development (Figure 2.2C). Cytoplasmic streaming can be seen in a root hair from a (i) differential interference contrast image and, in another root hair from a seedling containing a triple organelle marker, the trajectory and spatial distribution of three organelles ii) Golgi (mCherry), (iii) peroxisomes (CFP), and (iv) mitochondria (YFP) can be seen from the maximum intensity projection in each respective panel [111]. The movement of these three organelles are captured in a cumulative distribution plot in Figure 2.2C.

The PDMS device presented here has not been chemically bonded to a glass coverslip but rather forms a weaker physical bond that is established during the autoclaving step. This allows the device to be disassembled after the optical imaging experiment is complete. The PDMS device may be peeled from the glass,

inverted, and used as a substrate for higher resolution non-optical imaging of the root morphology (Figure 2.3A). The platform conveniently holds the root in place during contact with a cantilever during atomic force microscopy (Figure 2.3B). Further, if care is taken during the disassembly process, root hairs will remain in position on the PDMS substrate. This is demonstrated from the optical image of the root before the disassembly (Figure 2.3C) and after disassembly of the platform and coating the seedling in a 20 nm conductive chromium layer for imaging with a scanning electron microscope (Figure 2.3D). To further preserve the seedling before deconstructing the platform, an aldehyde-based fixative may be injected into the platform to crosslink the plant tissue proteins in place.

2.4 Discussion

The method described in this article for creating a plant-on-a-chip platform is unique in that the two-layer design confines the root hairs to a single imaging plane and the platform may be deconstructed and used as a substrate for high resolution non-optical imaging. Using high- resolution non-optical imaging can provide valuable information about the plant tissue that could not be obtained from optical imaging alone. For example, AFM imaging can provide force measurements to calculate the elasticity of root tissues during development or after a specific chemical or biological treatment. Similarly, SEM imaging can provide high-resolution details of surface topography of the root tissue and, when coupled with chemical imaging, can provide information on elemental composition of the tissues [112]. Future generations of this microfluidic platform will include optimization for compatibility with other chemical imaging systems such as matrix-assisted laser desorption/ionization- mass spectroscopy (MALDI-MS) and Coherent anti-Stokes Raman spectroscopy (CARS).

Critical steps in this method involve using agar to secure the device and provide hydration to the plant without the need for complicated fluid flow procedures. Care must also be taken when deconstructing the PDMS device from the glass substrate

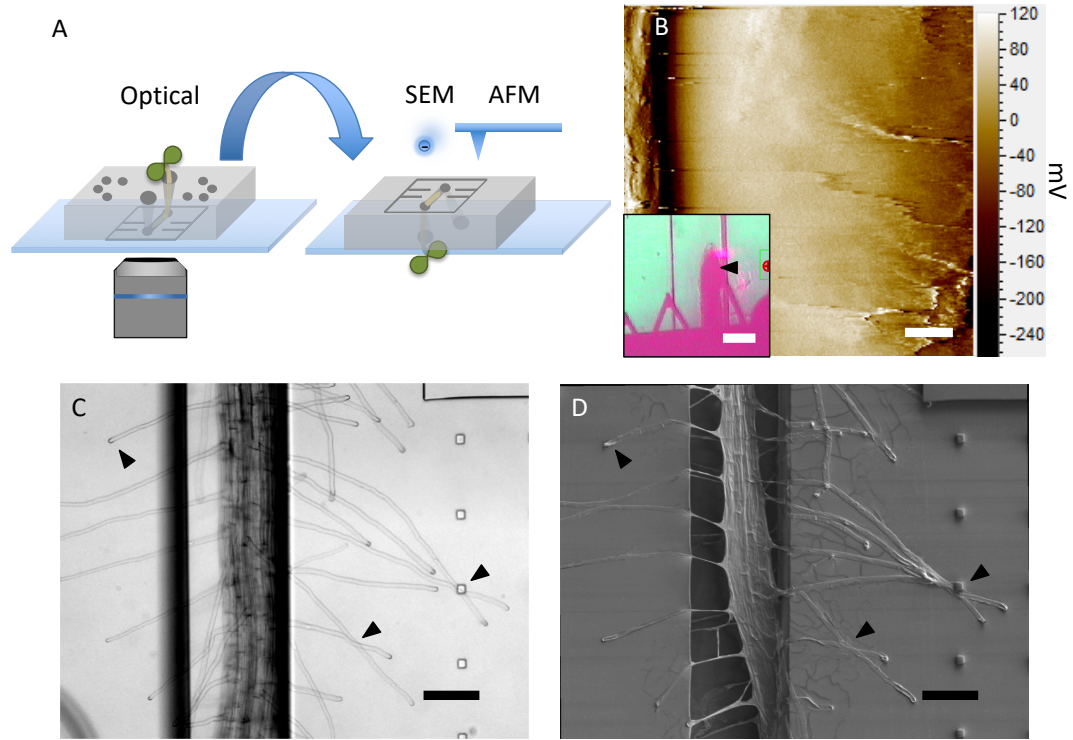


Figure 2.3: *Device Deconstruction and Non-optical Imaging.* (A) The microfluidic platform can be disassembled and used as a substrate for high-resolution non-optical imaging methods. (B) The surface topography (diffraction image) of an *Arabidopsis thaliana* root tip is imaged using contact mode atomic force microscopy, Scale bar = 2 μm . The location of the cantilever on the root is indicated by a black arrow in the inset, Scale bar = 100 μm . (C) Optical image and (D) corresponding scanning electron micrograph of the same seedling before and after device is disassembled. Arrows emphasize root hairs that remain undisturbed, Scale bar = 100 μm .

in order to keep the root morphology intact. If the experimental goal is solely for low to medium resolution optical imaging without the need for device deconstruction, the procedure may be modified to chemically bond the device to the underlying glass substrate using oxygen plasma. Higher resolution optical images can be obtained without removing the device from its gnotobiotic environment by chemically bonding the PDMS directly to a glass bottomed Petri dish and then pouring agar around the PDMS as previously described.

The design of 8 treatment side channels was an attempt to confine treatments to certain regions of the root. However, this design was unsuccessful in preventing the diffusion of the treatment to other areas of the root due to the conductance of the open area in the main root channel. In order to locally treat the root, the device architecture will either need to be redesigned to confine treatments or the treatment will need to be introduced via a viscous media to temporarily slow diffusion throughout the device.

If it is desirable for experimental treatments to be added via flow, modifications will also be required for this platform. Currently the addition of flow to any of the platform inlets results in the expulsion of the seed from its inlet and difficulty in controlling the location of the fluidic treatments. This method works well for seedlings up to one week of age. It is limited in how long seedlings may continue to grow in the platform, because of the length of the main channel. Future modifications will involve elongating the main channel and incorporating more 200 μm high channels for lateral roots while retaining the 20 μm tall chamber for root hair confinement. This modification will require knowledge on the anticipated location of lateral root emergence in order to design an appropriate device.

2.5 Acknowledgements

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Chapter 3

Quantifying the Spatiotemporal Dynamics of Plant Root Colonization by Beneficial Bacteria in a Microfluidic Habitat

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3.1 Abstract

Plant-microbe interactions underpin processes related to soil ecology, plant function, and global carbon cycling. However, quantifying the spatial dynamics of these interactions has proven challenging in natural systems. Currently, microfluidic platforms are at the forefront of innovation for culturing, imaging, and manipulating plants in controlled environments. Using a microfluidic platform to culture plants with beneficial bacteria, visualization and quantification of the spatial dynamics of these interactions during the early stages of plant development is possible. For two plant growth-promoting bacterial isolates, the population of bacterial cells reaches a coverage density of 1-2% of the root's surface at the end of a 4d observation period regardless of bacterial species or inoculum concentration. The two bacterial species form distinct associations with root tissue through a mechanism that appears to be independent of the presence of the other bacterial species, despite evidence for their competition. Root development changes associated with these bacterial treatments depend on the initial concentrations and species of the bacterial population present. This microfluidic approach provides context for understanding plant-microbe interactions during the early stages of plant development and can be used to generate new hypotheses about physical and biochemical exchanges between plants and their associated microbial communities.

3.2 Introduction

Plants live symbiotically with highly specialized and signature microbial communities with which they are constantly exchanging physical and chemical signals [113, 41]. Each plant selects for the members of its microbiome, either actively or passively, providing carbon compounds that support microbial species able to compete in this environment [114]. In return, some bacterial species, known as plant growth-promoting bacteria (PGPB), contribute to this mutualistic relationship by providing bioavailable minerals, nutrients, and plant hormones that can alleviate plant stress from drought, pathogens, and nutrient deficiency [115, 116]. This chemical exchange is highly dynamic and dependent on both abiotic and biotic environmental factors such as climate, soil type, and plant species [117, 118]. Increasingly, new insights regarding the chemical relationship between plants and PGPB come from community survey approaches and meta-omic analyses [119, 80]. However, information on the dynamic spatial-temporal organization of microbes within these systems is lacking and could offer deeper understanding into the interactions that drive host-microbe and microbe-microbe associations. Further, the beneficial and competitive relationships among microbial community members, the impact of chemical heterogeneity in the environment, and the resulting changes in host phenotype require improved definition.

Diverse bacterial species create a spectrum of microscale niche habitats in the soil [120, 75]. These habitats can dictate plant root growth, and competition between different bacterial niches can lead to changes in community composition. Physical colonization of roots by PGPB is believed to be an important precursor to establishing symbiotic relationships [13, 121]. However, physical relationships such as the attachment and spatial distribution of PGPB near, along, and within plant roots have been inherently difficult to study *in situ* given the opacity of soil and disparity between root and microbial spatial scales [60]. Conventional laboratory culture systems, such as agar plates, provide a means of bringing these systems into a laboratory environment but often preclude imaging at high resolution [81, 122]. When

high-resolution imaging tools are applied, they typically require chemically fixing the sample prior to imaging, compromising the ability to study real-time, dynamic physical changes.

Transparent microfluidic platforms offer the opportunity to culture and image plant seedlings in real time and at high resolution [85]. These platforms have been used to screen plant phenotypes in a high-throughput manner, to dose seedlings with chemical treatments, and to image the physical interactions between roots and pathogenic eukaryotes [106, 123, 104, 92, 124, 84, 125]. Microfluidic platforms, therefore, have significant utility in resolving questions regarding the spatiotemporal dynamics of physical plant-microbe interactions [84]. Recently, Massalha et al. demonstrated the application of microfluidic platforms to explore the interactions of plants and multiple microbial species, a first in highlighting the value of high resolution imaging for capturing these complex interactions [125]. These authors applied treatments of *Bacillus subtilis* and *Escherichia coli* to *Arabidopsis thaliana* WT and mutant plant roots and observed chemotactic motility of *B. subtilis* cells toward the root elongation zone as well as competition between the two bacterial strains. In these experiments, however, bacteria treatments were applied to 7-9 day old seedlings and the plant-microbe interaction was only imaged for the first 12h. Under these conditions, colonization in the beginning stages of root development is overlooked and the plant-microbe interaction may not have had adequate time to induce changes to the root morphology.

Here, we employ a microfluidic platform to co-culture *A. thaliana* seedlings for 4 d with two PGPB species isolated from the endosphere and rhizosphere of *Populus deltoides* [18]. By tracking and quantifying the colonization kinetics and spatial distribution of each bacterial species on the plant roots, individually and combined, we reveal specific spatio-temporal bacterial colonization patterns. Correlated changes in root morphology over the course of the experiment confirm that the associated plant phenotype depends on the bacterial strain and inoculum concentration. By visualizing the physical interactions between defined microbial isolates and the

plant roots, hypotheses regarding microbial colonization kinetics and preferential niche colonization can be formulated and used to inform future meta-omic analyses and interaction models. This exploratory work highlights the effectiveness of a microfluidic habitat for quantifying features of plant root-microbe relationships and their associated influence on plant phenotype during early development (Figure 3.1)

3.3 Experimental Methods

3.3.1 Microfluidic Platform Design and Operation

The microfluidic device was molded in poly-dimethylsiloxane (PDMS) in a 1:10 ratio (curing agent to silicon elastomer) using standard photo and soft lithography techniques. The design consists of two layers: a center 'taproot' channel for main root growth (200 μm wide x 150 μm high and 3.8 mm long) and a surrounding treatment chamber (20 μm high). The device is sectioned into four areas on either side of the main root channel, each with a bifurcating injection channel (8 total treatment ports) to uniformly administer treatment to the root (Figure 3.2). Regularly spaced support beams (20 μm x 20 μm) prevent the microfluidic chamber from collapsing during fabrication. Devices were plasma bonded to a glass coverslip then autoclave sterilized. Details of the device design and fabrication are described in previous work (see Chapter 2)[126].

3.3.2 Seed Planting and Hydration

In order to maintain complete hydration within the channels, the PDMS devices were vacuum degassed for 30 min to remove excess air and then filled with 1/4x Murashige and Skoog (MS) plant-based media at a pH of 5.7 [127]. Devices were individually placed in a culture dish and freshly autoclaved agar was poured around the device until the agar was flush with the top of the PDMS. The agar was allowed to solidify, and culture dishes were stored until experimentation.

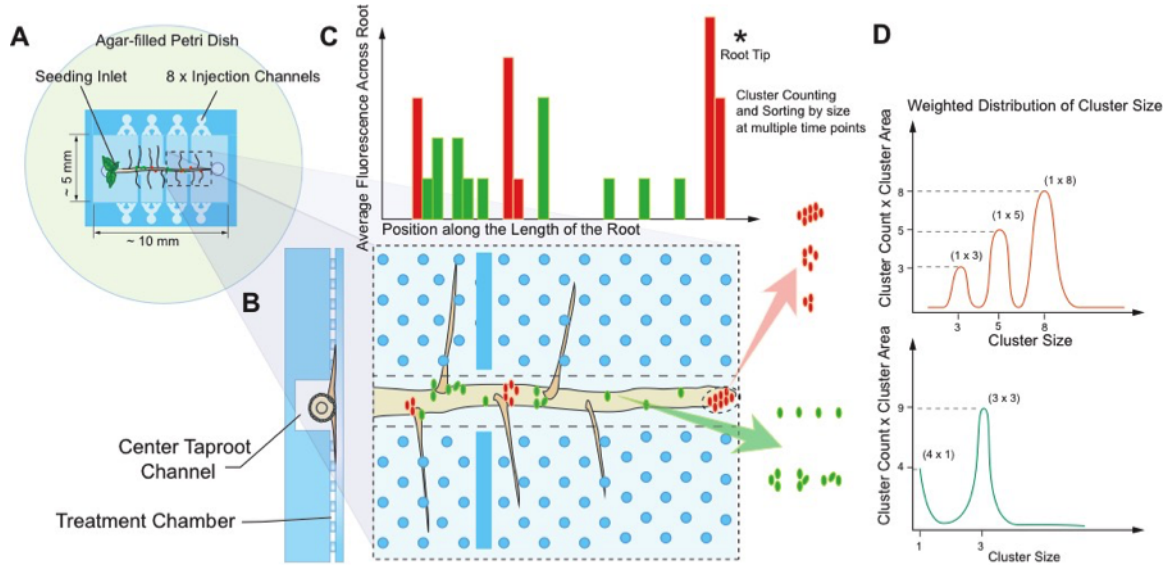


Figure 3.1: Spatial and temporal information about plant growth and beneficial microbial community development is captured and quantified to understand the early stages of growth and organization within these dynamic systems. A) The microfluidic growth platform, fabricated from PDMS, is embedded in an agar matrix held inside of a Petri dish to prevent drying of the platform during experiments. After stratification, seeds are added to the seeding inlet and roots are allowed to grow into the channel for 3 d before bacteria are added. Eight injection ports allow the introduction of bacteria uniformly within the raised treatment chamber area. The distribution and growth of these bacteria is monitored over the next 4d. B) A cross-section schematic of the device highlights control of the primary taproot growth within a center taproot channel (3.8 mm long x 200 μ m wide x 200 μ m tall). Root hairs are able to extend into the raised treatment chamber (20 μ m tall). C) Fluorescence imaging and analysis of microbial populations within the primary channel provide an understanding of how bacteria are distributed along the length of the root. D) Analysis of bacterial community development by monitoring cluster size can provide insights into how microbes associate and grow over time.

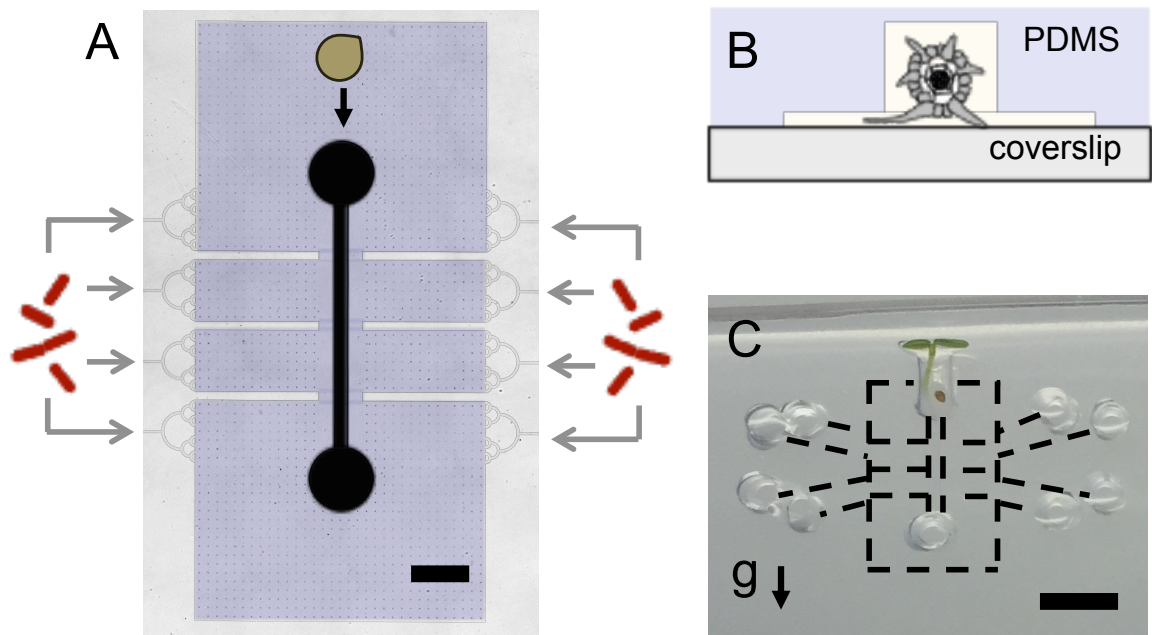


Figure 3.2: The microfluidic platform consists of two layers. A) The channel (shown in black) guides the main root through the platform (200 μm wide by 150 μm high, scale bar=1mm). A treatment chamber (shown in grey) is 20 μm tall and confines root hairs to the same imaging plane as illustrated in the schematic in panel B). C) An *Arabidopsis thaliana* seedling grows vertically into the platform with underlying microfluidic channels outlined (scale bar 3 mm).

A. thaliana Columbia wild-type seeds were surface sterilized in a 1.5 mL microcentrifuge tube with a solution consisting of 30% commercial bleach and 0.1% Triton X for 10 min and rinsed with sterile water 4 times. Seeds were wrapped in aluminum foil and stratified at 4°C for at least 2 d but no longer than 7 d to synchronize germination. The stratified seeds were transferred directly to the microfluidic device port using a pipette tip. The culture dishes were covered with parafilm and transferred to a 16 h light/dark cycling growth chamber at 23°C where they were placed in a vertical position to encourage root gravitropism growth into the microfluidic channel. After 3 d, roots that had begun to enter the microfluidic channel were kept as a control or treated with bacteria. Detailed methods for *A. thaliana* culturing within this system are described in previous work [126].

3.3.3 Bacterial Strain Description and Growth

Two bacterial strains, *Pantoea* sp. YR343 and *Variovorax* sp. CF313 isolated from *P. deltooides* root rhizosphere and endosphere, respectively, were used throughout this study [18]. Strains constitutively expressing fluorescent proteins were constructed. *Pantoea* sp. YR343:GFP has been recently described [97]. a *Variovorax* sp. CF313 was isolated from the endosphere of *P. deltooides* and a strain expressing mCherry (CF313-mCherry) was constructed by chromosomal insertion of mCherry using pBT270 (pUC18-miniTh7T2- PA1/04/03-mCherry, a gift from B. S. Tseng, University of Washington). Both bacterial strains were grown overnight in R2A media, the cells were harvested by centrifugation, and resuspended in carbon-free 1/4x MS media.

For the monoculture treatments, either the CF313 strain or the YR343 strain was added in an 8 μ L aliquot with an optical density at 600 nm (OD600) of 1.0, 0.1, or 0.01, which corresponded to a log10 cell counts of 6.3, 5.2, or 4.3, respectively, for both bacteria (Figure 3.3). For the mixed culture treatments, the CF313 strain and YR343 strains were individually resuspended to an OD600 of 0.5 then added together

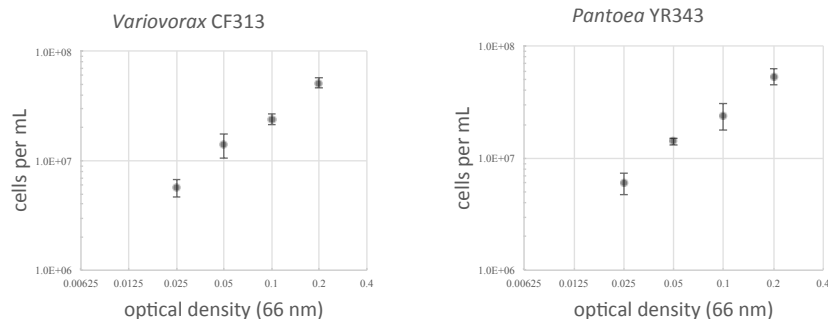


Figure 3.3: Cell counts are related to optical density measurements. Overnight cultures were diluted to $OD_{600} = 0.2$ then serially diluted in 2-fold dilutions. Each dilution was counted 3x on a Reichert hemacytometer.

in equal volume and mixed. An 8 μ L aliquot of mixed culture was then added to the microfluidic platform via pipette (1 μ L per treatment port).

3.3.4 Image Acquisition and Analyses

Bacteria fluorescence was imaged using an Olympus IX51 inverted microscope with a 10x objective and 1 s exposures. Upon acquisition, z-direction focus was adjusted to maximize the amount of bacterial fluorescence on the root. Images were processed prior to analysis by subtracting background noise (sliding paraboloid, 200 px) and correcting for uneven lamp illumination. Images were then thresholded automatically in Fiji and fluorescence was quantified using the particle analyzer [128]. The distribution of the bacteria along the root was determined by cropping the main microfluidic channel containing the root and averaging the fluorescence in the y-axis along the x-axis from the beginning of the channel to the tip of the root.

3.4 Results and Discussion

3.4.1 Kinetics of Root Colonization

Using a custom microfluidic platform (Figure 3.2) pre-filled with carbon-free 1/4x Murashige-Skoog media, we imaged fluorescently tagged bacteria on the same

seedlings every 24 h for 4 d of co-culture [126]. Two bacterial strains were chosen for this study based on the location of their isolation from the *P. deltooides* microbiome either as a root endophyte (*Variovorax* sp. CF313, hereafter referred to as CF313) or a root rhizosphere species (*Pantoea* sp. YR343, hereafter referred to as YR343) [18, 97, 20]. Surface-sterilized *A. thaliana* wild-type seeds were individually sown into each device inlet, and after 3 d, seedlings with roots that had grown into the main channel were kept and treated with defined quantities of bacterial cells: OD600 = 1.0, OD600 = 0.1, or OD600 = 0.01, corresponding to 106.3, 105.2, and 104.3 cells, respectively, for each strain (Figure 3.3).

For the seedlings inoculated with the YR343 strain, the per- cent of root area covered in bacterial fluorescence on day 4 of co-culture (seedlings were 1 week old) approached 1.0-2.0% and was independent of the initial concentration of the bacterial inoculum (Figure 3.4A). This convergence of total fluorescence coverage (i.e., total bacterial population) suggests that further growth of the bacterial population is limited, perhaps by nutrient availability or attenuated by a signaling molecule. Other authors have reported a similar threshold for the amount of bacteria that are able to colonize plant roots [129, 130]. It is possible that the plant produces exudate at a defined rate and is capable of supporting a limited population of microorganisms. In this study, plants inoculated with the highest concentration of rhizosphere bacteria approach this threshold after 1 d of co-culture.

Interestingly, the seedlings inoculated with the upper and middle concentrations of the CF313 strain also exhibited 1.0-2.0% area coverage by the bacteria over the course of 4 d (Figure 3.4B). Despite the difference in their known association with the root (endophyte vs rhizosphere), these two bacterial strains reach a similar coverage density. Changes in the root coverage within each of the CF313 treatments were not significant when comparing growth across 24 h intervals throughout the 4 d co-culture experiment (Student’s t-test, $p > 0.05$). This may be because, as a root endophyte, the CF313 strain enters the cell vasculature and systemically moves to aboveground areas of the plant not visualized in this experiment. Quantification of cells on the root

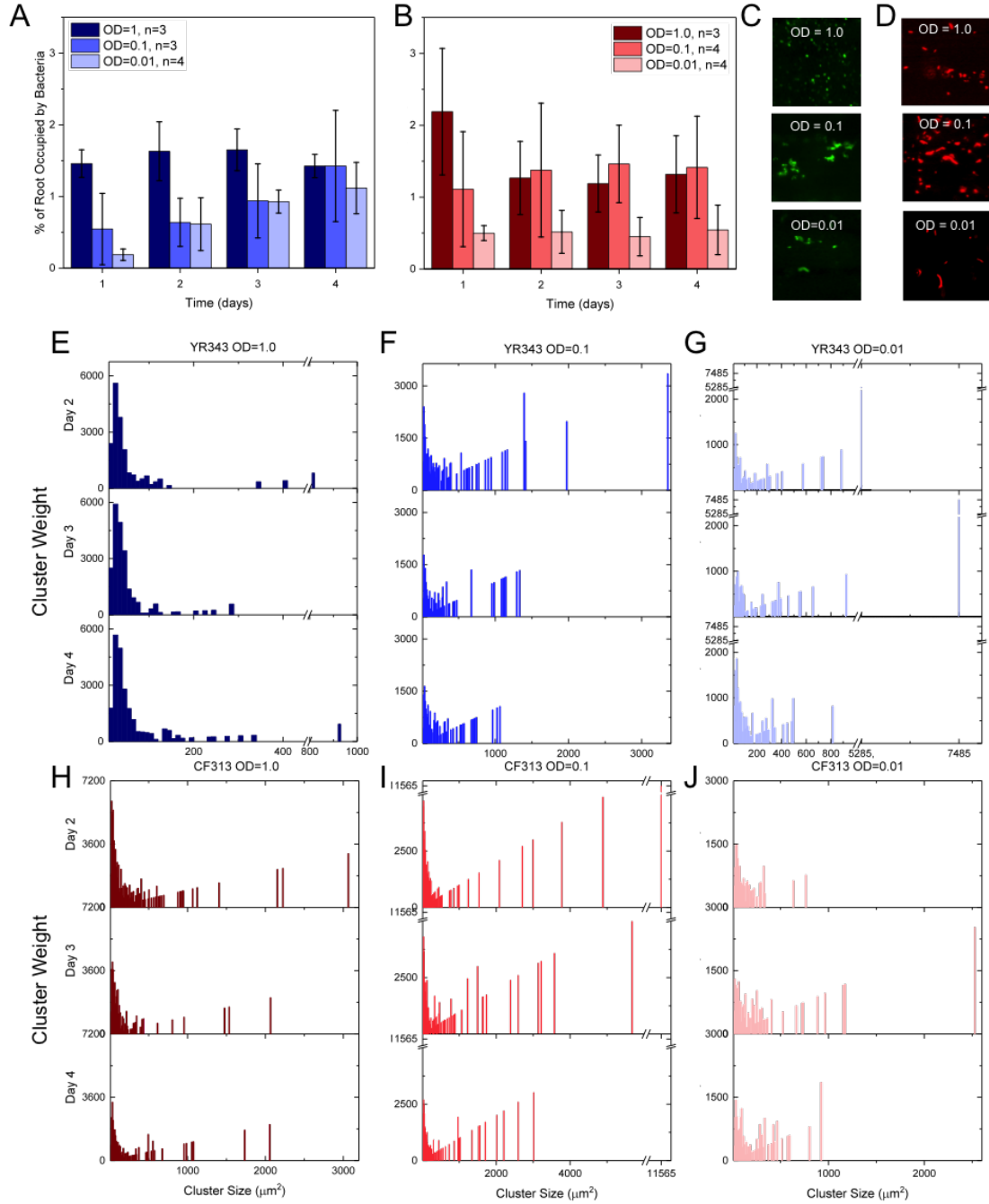


Figure 3.4: Bacterial growth and cluster formation on *A. thaliana* roots is quantified over a 4 d experiment. A) The % of the root surface area covered in YR343 and B) CF313 cell clusters was quantified over the course of 4 d for three concentrations of inoculum. Error bars represent one standard deviation and the number of replicates is listed in the figure legend. C) Representative fluorescent images of YR343 and D) CF313 clusters on the main roots after 4 d of co-culture (images are $200 \mu\text{m} \times 200 \mu\text{m}$). E) A weighted histogram (cluster size multiplied by the bin number on the y-axis) shows the distribution of cluster sizes during days 2-4 of the experiment for YR343 OD = 1.0, F) YR343 OD = 0.1, G) YR343 OD = 0.01, H) CF313 OD = 1.0, I) CF313 OD = 0.1, and J) CF313 OD = 0.01. Panels (E-J) have three replicates each.

show that 1% root coverage by CF313 ($OD = 1.0$) is achieved soon after inoculation, within 3 h of co-culture is achieved (Figure 3.5). Although it is difficult to distinguish if these cells are closely associated with the root or still mobile, the location of several fluorescent peaks along the root remain do not change position over the next 4 h suggesting that these bacteria are closely associated with the root. Another possible explanation for the lack of CF313 growth over the 4 d experiment is that the CF313 strain may induce an immune response in the plant that quickly attenuate growth of the bacterial species [80, 131].

The total population of bacteria on the root provides information about the number of bacterial cells that a plant can sustain in its microbiome. However, this quantitative measure does not provide information on the phenotype of associated bacterial species. Previous work has proposed that colonization is an important precursor to establishing robust plant-microbe relationships [61]. Because bacteria may form aggregates by both growth and attachment, we chose to define groups of bacteria larger than 10 px^2 ($17.7 \text{ }\mu\text{m}^2$) as clusters rather than colonies. To determine the robustness of bacterial association on the main root, bacterial cluster size was quantified after 4 d of co-culture by the area of their fluorescence, using the particle analyzer feature in Fiji [128]. The cell clusters were categorized into $10 \text{ }\mu\text{m}$ bins and plotted in a weighted histogram (bin size multiplied by the number of clusters in each bin on the y-axis). (Figure 3.4E-J).

For the highest concentration of YR343, the total cluster weight comprised hundreds of small clusters in a distribution that did not significantly change from day 2 of co-culture to day 4 (unweighted distribution, two-sample KS test, $p < 0.05$) suggesting that these clusters did not grow in size. In contrast, the medium and low concentrations of YR343 produced multiple clusters with areas approximate to the weight of all the smallest clusters combined. The lowest concentration of YR343 ($OD = 0.1$) was the only treatment that exhibited a significant change in the unweighted cluster size distribution between experimental days. This treatment saw

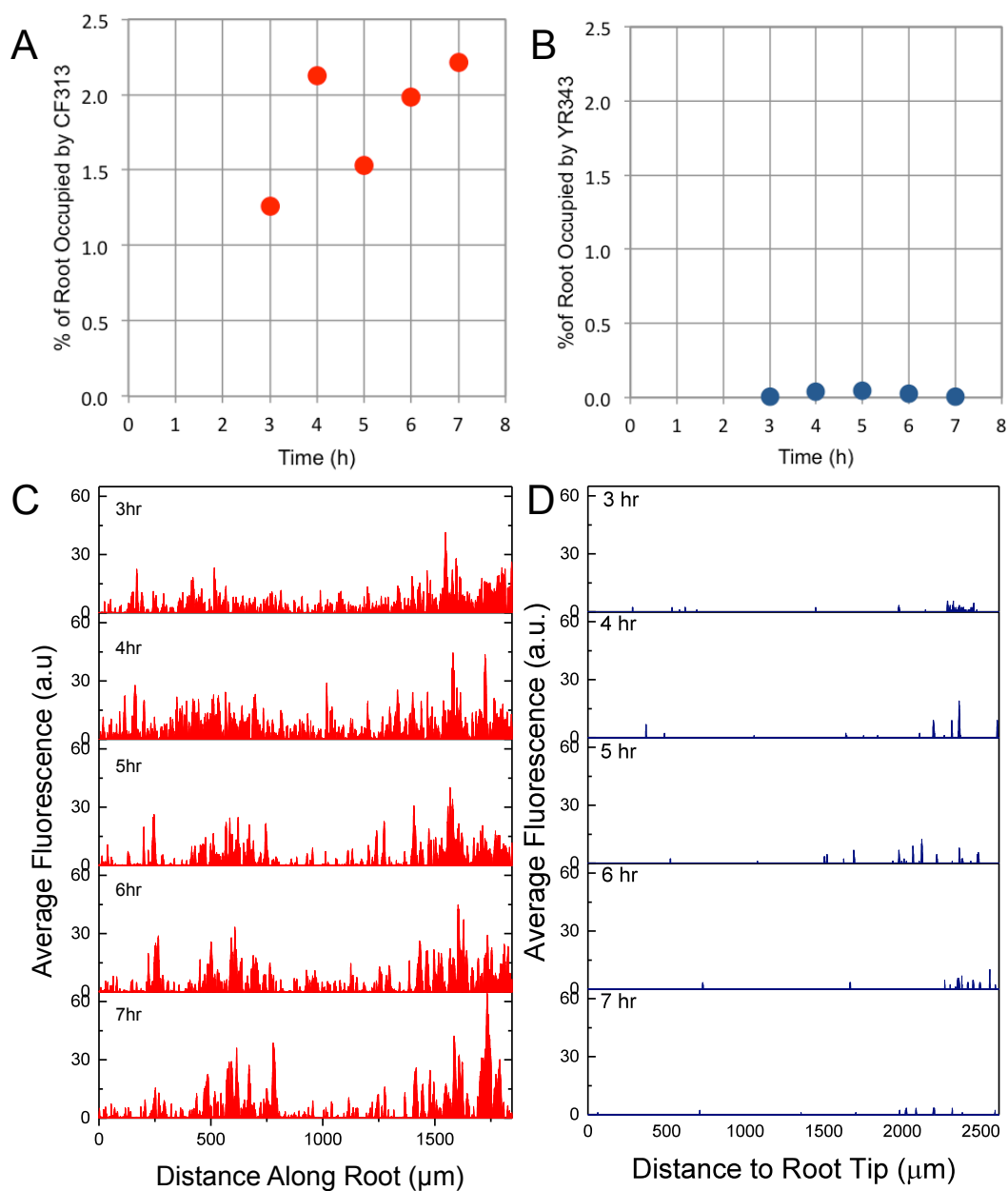


Figure 3.5: Difference in colonization rates for the two bacterial species during the first 7 hours of co-culture (single seedling per treatment). The density of (a) CF313 ($OD=1.0$) surpasses 1.0% of the root after 3 hours of co-culture while association by (b) YR343 ($OD=1.0$) is much slower. There are several peaks in the (c) CF313 fluorescence spatial distribution that remain stationary over the first 7 hours suggesting association with the root, while (d) YR343 peaks change with time suggesting taxing cells.

an increase in the number of small clusters, which may indicate that more bacteria became associated with the root over time.

Within the CF313 treatments, the unweighted cluster size distributions were not significantly different between days 2-3 or days 3-4. The highest and medium concentrations of CF313 exhibited a decrease in the total number of clusters over time while the total number of clusters in the lowest concentration treatment remained unchanged. The medium concentration of CF313 had several large clusters that disappeared by day 4, possibly due to biofilm dispersal, a bacterial phenotype that has been shown to correlate to plant virulence [132].

To determine if the root tissue as a substrate influenced clusters on the main root, the size of bacterial clusters 400 μm from the main root axis were also measured. For both strains and every concentration, the mean cluster size was larger for clusters on the main root than clusters that were not associated with the root (Figure 3.6, Mann-Whitney test, $p < 0.05$). This result provides evidence that these bacterial groupings are more than just aggregates of cells. They are likely microcolonies supported by the release of carbon from the plant.

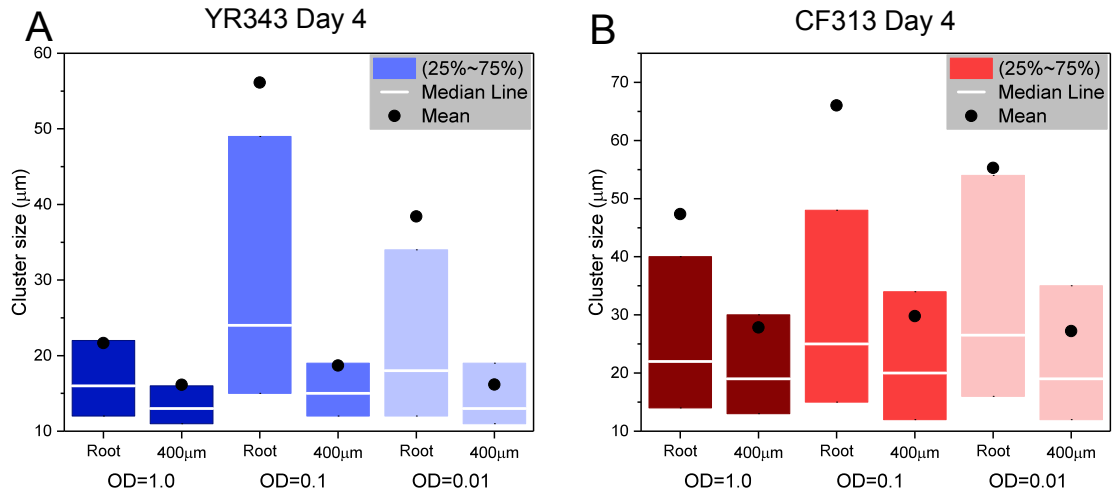


Figure 3.6: The mean cluster sizes on the main root are significantly higher for all inoculum concentrations of both (a) YR343 and (b) CF313 strains on day 4 of co-culture than clusters found 400 μm away from the root's longitudinal axis (Mann-Whitney test, $p < 0.05$).

The bacterial density coverage implies that plants have an upper limit of bacterial cells that they can support. As shown in previous work, exudate composition and secretion rates can vary significantly along the length of the developing root [49]. Thus, it is essential to consider not only the overall population of community members, but also their distribution along the root to understand their association with the plant.

3.4.2 Spatial Distribution of Bacteria

During the course of the 4 d co-culture experiment, *Arabidopsis* seedlings had not begun to form lateral branches; therefore, the spatial distribution of bacterial cells was evaluated as the average fluorescence value across the root diameter for every micrometer along the main root's axis.

The rhizosphere isolate, YR343, exhibited a polarized distribution ($D_{max} = 0.423$, $D_{crit} = 0.188$, $p < 0.05$) along the x-axis, the position along the root length, as determined by a two- sample Kolmogorov-Smirnov (KS) test, with the majority of bacteria colonizing toward the tip of the root and little to no bacteria forming clusters proximally to the stem (Figure 3.7). The spatial distribution of YR343 cells from a representative seedling over the course of the 4 d co-culture shows that the location of bacterial clusters in mature root tissue do not change over time (Figure 3.8). However, clusters formed near the root tip briefly move forward with root elongation before remaining stationary on the root's main axis. This finding follows "Scenario 2" proposed by Watt et al. in which the bacteria colonize by anchoring to the root tip and are carried forward with the tip as it extends [4]. As new root tissue develops, more area is available for the bacterial cells to establish themselves, stretching their distribution accordingly. Interestingly, the stark demarcation on the root between the presence and absence of YR343 bacteria coincides with the location of the root tip when the bacteria were first introduced to the system. This supports the theory that

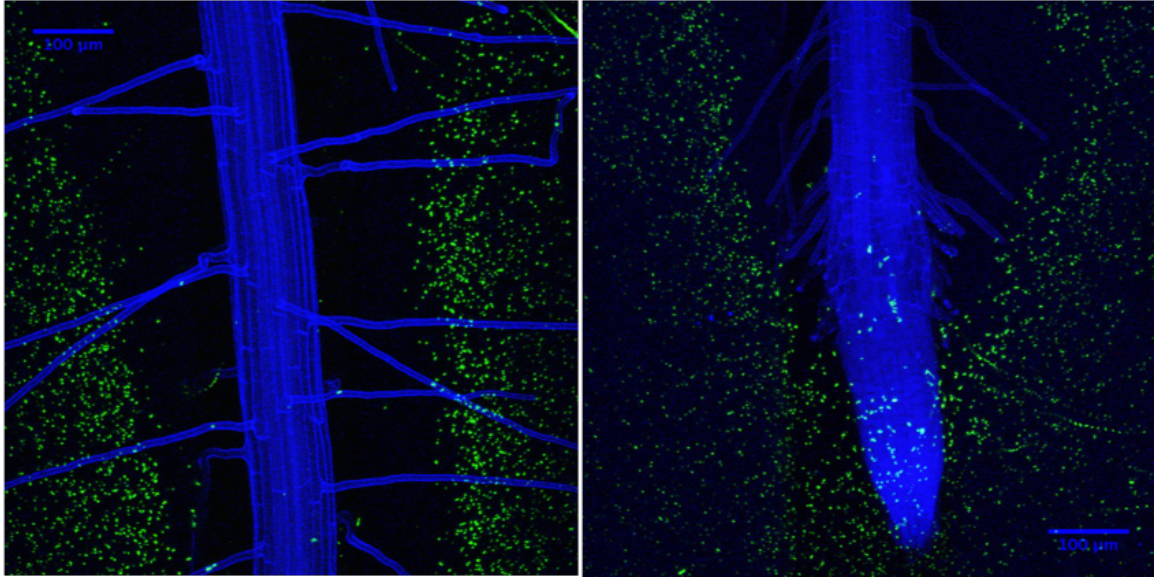


Figure 3.7: Maximum intensity projections of YR343 ($OD=1.0$) cells (green) after 3 days of co-culture show avoidance of the root shaft (left) and preferential colonization near the root tip (right)

the cells colonize the root by attaching to newly elongated root tissues and continue to survive for a period of days, while older root tissue is not preferred for colonization.

The nonuniform attraction of rhizosphere bacteria to specific root zones has been demonstrated previously using fixed field samples, and recently through real-time imaging [125, 70]. This has been attributed to a spatial variation in root exudation, which has been demonstrated previously with notably higher sugar exudation near the root tip and increasing tryptophan exudation toward the lateral root forming regions [49]. Spatial variations in the degree of lignification and suberin production along the *Arabidopsis* root have also been shown using fluorescent dyes with the majority of phenolic compounds being produced proximal to the root tip [133]. Phenolic compound exudates are often used as plant defense mechanisms to regulate the microbiome composition, which may explain why this strain of *Pantoea* is absent in the basal region of the root [56, 134].

The endophyte isolate, CF313, did not exhibit any preference for a specific region along the long axis of the root, but rather formed discrete clusters of cells uniformly along the length of the root ($D_{max} = 0.098$, $D_{crit} = 0.188$). These clusters became

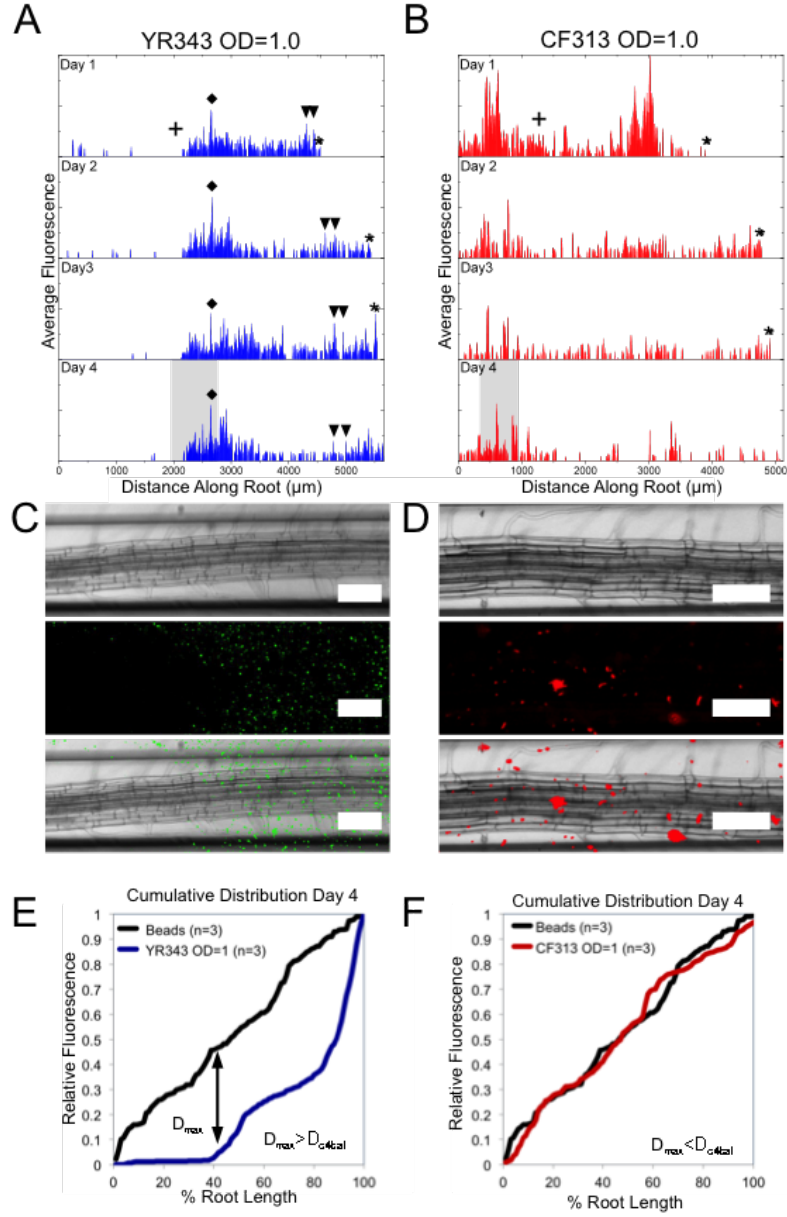


Figure 3.8: The spatial distributions of YR343 ($OD = 1.0$) and CF313 ($OD = 1.0$) cells along the main *A. thaliana* root were quantified over the course of the experiment. A representative seedling from the A) YR343 and B) CF313 treatment is shown. The location of the root tip is indicated by an asterisk (*). The plus symbol (+) shows the location of the root tip on day 0 before bacteria cultures were added. For the YR343 treatment, arrows indicate the fluorescent peaks that move with the root tip while the diamonds show fluorescent peaks associated with older plant tissue that do not change position over time. The grey highlighted section of the day 4 spatial distribution corresponds to the section of the root shown in the brightfield (top), fluorescence (middle), and overlay (bottom) images in panels C) and D) for YR343 ($OD = 1.0$) and CF313 ($OD = 1.0$), respectively (scale bars = 100 μm). The distribution of E) YR343 was significantly different than abiotic beads (two-sample Kolmogorov-Smirnov test: $D_{\text{max}} = 0.515$, $D_{\text{crit}} = 0.188$, $p = 0.05$) while F) CF313 was not significantly different than the abiotic control ($D_{\text{max}} = 0.098$, $D_{\text{crit}} = 0.188$, $p = 0.05$).

patchier over the course of the 4d experiment as their distribution shifted from continuous to discrete along the main axis of the root. New root growth was also quickly colonized by the CF313 strain. Endophyte cells often gain entrance to the inside of the root via small cracks, and some species can also access different areas of the root by systemic colonization within vascular tissues [129]. Once inside the root, endosphere dwelling bacteria are much less susceptible to changes in chemical gradients induced by the root’s exudation, which could explain the broad distribution of CF313 along the root’s main axis. We did not distinguish between bacterial fluorescence inside the root or on the surface of the root. The CF313 distribution may also be explained by its potential use of a uniformly exuded carbon source.

Because the microfluidic platforms were pre-filled with liquid media instead of actively perfused, there was no influence of convective transport in the system. To confirm this, seedlings were cultured with an abiotic control of 2 μm fluorescent carboxylate polystyrene beads. After 4 d, the beads exhibited a uniform distribution ($D_{max} = 0.094$, $D_{crit} = 0.188$, $p < 0.05$) along the main axis of the root. This result confirms that any bacterial deviation from a uniform bead distribution can be attributed to biological factors such as cell attachment, growth, or motility.

3.4.3 Bacterial Species Interactions

The addition of multiple bacterial species to plants can provide a tractable model of natural microbiome interactions for well-defined experimentation [43]. Providing living plant tissue as a natural substrate for these isolates may induce emergent behavior and bacteria-bacteria interactions that may otherwise be omitted in a purely synthetic medium. To determine how the colonization kinetics and spatial distribution of the chosen bacterial isolates change in the presence of one another, we added both YR343 and CF313 to the seedlings in equal concentration (each at $\text{OD} = 0.5$).

The physical niches established by each of the species were not exclusive, with overlapping colonization occurring in many areas axially along the root (Figure 3.9).



Figure 3.9: Maximum intensity projection of mixed culture confocal z stack showing the location of both CF313 (green) and YR343 (red) on a seedling's root tip.

Due to their occupation of overlapping physical spaces, it is likely that there is some chemical exchange, between the CF313 and YR343 strains [75]. The presence of a second bacterial species, however, did not affect the individually established spatial distributions of either the CF313 or YR343 strain after 4 d of co-culture (Figure 3.10, D_{max} (CF313) = 0.131 D_{max} (YR343) = 0.118, D_{crit} , 0.1884, $p < 0.05$). Our results contrast with another study of two-member bacteria and plant co-cultures, wherein it was discovered that the addition of *B. subtilis* significantly altered the location of *E. coli* with respect to *A. thaliana* roots [125].

Quantifying the density of both bacterial species together, an optimal 1-2% root surface area coverage was once again established. This result is consistent with previous work that determined that the percent of surface area covered by microbes was generally less than 11% for field-grown seedlings [135, 136]. Naturally, this leads to a different absolute amount of each bacterial strain on the root when compared to seedlings cultured with only one of the species. Unlike the total bacterial coverage, the ratio of YR343:CF313 did not approach a steady state value after 4 d of co-culture, but varied depending on the degree of bacterial colonization after 24 h. In all

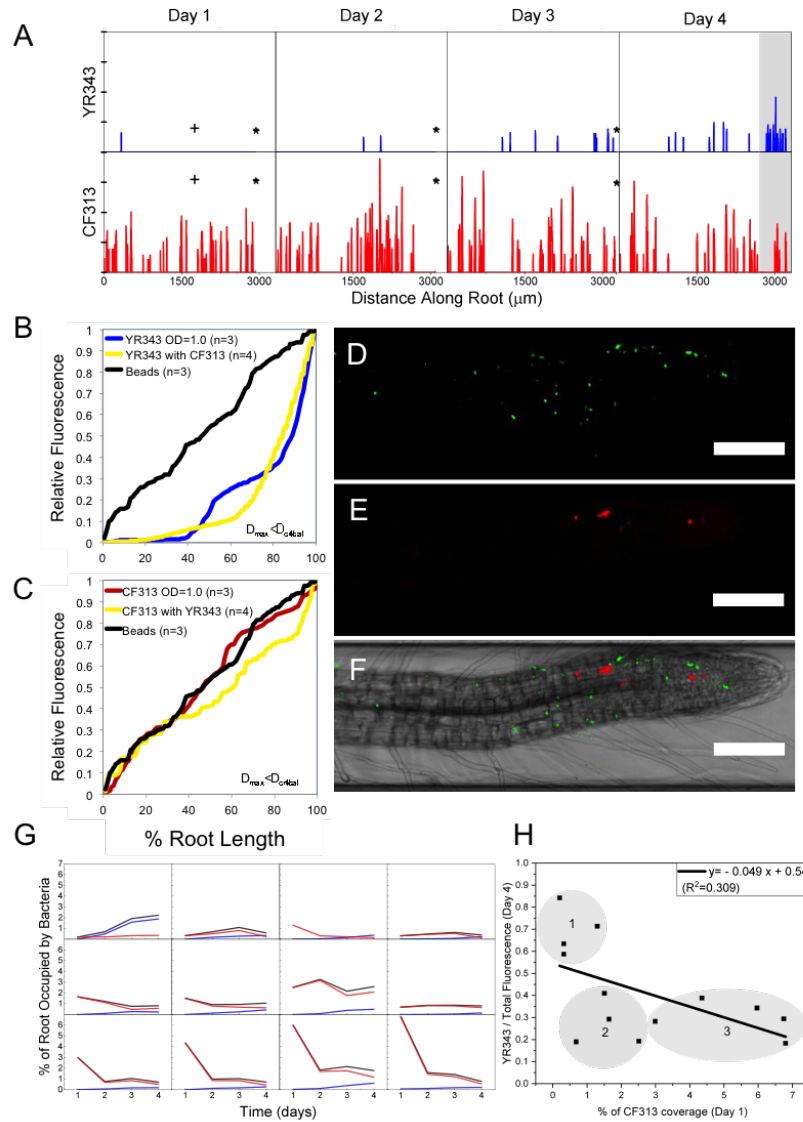


Figure 3.10: In a mixed culture, root colonization kinetics change but the spatial distribution of each species does not. A) The spatial distribution of both YR343 and CF313 cells on a representative seedling over the course of a 4 d co-culture experiment with both bacteria. The asterisk (*) indicates the location of the root tip while the plus symbol (+) shows the location of the root tip on day 0 before bacteria cultures were added. The region highlighted in grey corresponds to the fluorescence images of D) YR343 E) CF313 and to the F) overlay image of the root (all scale bars 100 μm). The addition of another bacteria species had no effect on the spatial distribution of either B) YR343 or C) CF313 (Kolmogorov-Smirnov test, $p < 0.05$). G) The % root coverage by CF313 (red), YR343 (blue), and total bacteria fluorescence area (black) over time for 12 replicates shows scenarios where 1. The YR343 fluorescence exceeds that of CF313 by day 4 (top row), 2. Both bacteria demonstrate small changes in density over the 4 d (middle row), and 3. The CF313 population has a strong initial association but quickly decreases in intensity (bottom). H) The ratio of YR343 to the total bacterial fluorescence on day 4 of co-culture is inversely correlated to the density of CF313 after 24 h of co-culture. The points cluster according to one of the three scenarios from (G) (shown in grey circles with corresponding scenario number).

13 replicates, CF313 was the first to associate with the roots and, the colonization of YR343 was correspondingly slowed compared to the rate at which YR343 populated the roots alone (Figure 3.10H). The root colonization kinetics in the mixed cultures followed one of three scenarios depending on the magnitude of CF313 colonization exhibited on the first day of co-culture (Figure 3.10G). In four of the replicates, CF313 did not reach a coverage density of 1.5% after 24 h, which enabled YR343 to become the dominant bacteria on the root after 4 d of co-culture. The delayed growth of YR343 in the mixed culture suggests that there is some competition between the bacterial species for resources, which favors the isolate that more quickly establishes on the root (CF313) on day 1. However, on day 4, the cluster size distribution of the YR343 in dual culture was not significantly different from the distribution of YR343 OD = 0.1 clusters from the monoculture treatment, nor was the cluster size distribution of CF313 in dual culture significantly different than that of CF313 OD = 0.1 in monoculture (two-sample KS tests, $p < 0.05$, Figure 3.11).

The ability of both bacterial strains to use various carbon substrates was tested using a Biolog PM1 and PM2A MicroPlate as previously described [122]. Out of 190 compounds, both strains grew on 81 carbon sources, with 55 in common and 26 unique to both strains (Table 3.1). Previous studies have found differences in C-source utilization by endophyte and rhizosphere bacterial isolates with a bias for phenolic compounds being used by endophyte strains [122]. Experiments conducted over a longer time period may experience several shifts in the dominant bacterial species as the composition of root exudates, and therefore microbial food supply, changes with the age of the seedling [51, 137]. Early in root development, *A. thaliana* exudates are high in sugars, a nutrient pool common to many bacterial species, while exudate composition shifts to amino acids and phenol-based compounds after 3 weeks in a seedling's development [56]. The diversification in exudate composition may lead to specialized chemical, as well as physical, niches for these two bacterial species.

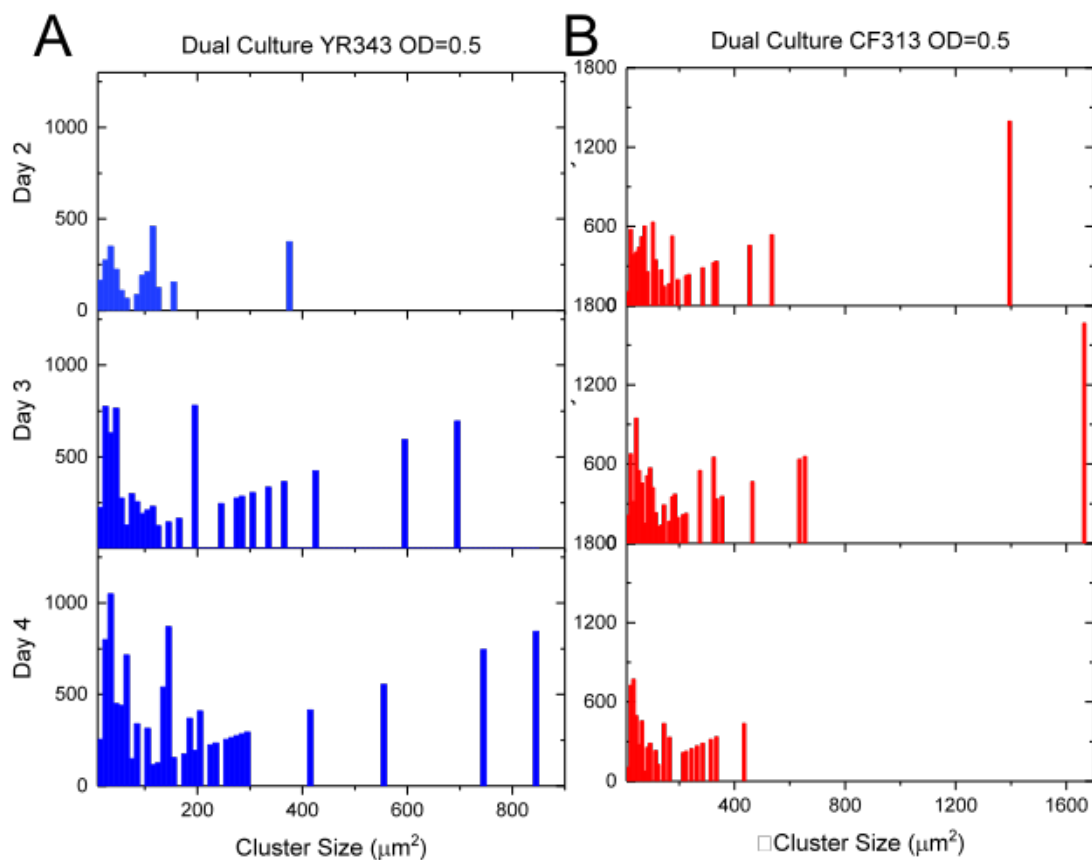


Figure 3.11: The weighted cluster size histograms for A) YR343 and B) CF313. The unweighted distributions of these clusters in dual culture were not significantly different than from their OD=0.1 monoculture counterpart distributions (two-sample KS test, $p < 0.05$)

Table 3.1: Carbon substrates utilized by each bacterial strain

Common	Unique to CF313	Unique to YR343
L-Arabinose	D-Sorbitol	N-Acetyl-D-Glucosamine
Succinic Acid	L-Lactic Acid	D-Saccharic Acid
D-Galactose	D-Melibiose	D-Alanine
L-Aspartic Acid	D-Aspartic Acid	Sucrose
L-Proline	a-Keto-Glutaric Acid	D-Glucose-1-Phosphate
D-Trehalose	Lactulose	Adonitol
D-Mannose	a-Hydroxy Butyric Acid	2-Deoxy Adenosine
Glycerol	p-Hydroxy Phenyl Acetic Acid	Adenosine
L-Fucose	m-Hydroxy Phenyl Acetic Acid	D-Threonine
D-Glucuronic Acid	Clucuronamide	Mucic Acid
D-Gluconic Acid	b-D-Allose	Glycyl-L-Glutamic Acid
D-Xylose	D-Arabinose	L-Serine
D-Mannitol	D-Fucose	L-Alanine
L-Glutamic Acid	Maltitol	N-Acetyl-b-D-Mannosamine
D-Glucose-6-Phosphate	D-Raffinose	Glycyl-L-Proline
D-Galactonic Acid-g-Lactone	g-Amino Butyric Acid	D-Psicose
D,L-Malic Acid	Butyric Acid	L-Lyxose
D-Ribose	4-Hydroxy Benzoic Acid	D-Galacturonic Acid
Tween 20	b-Hydroxy Butyric Acid	Salicin
L-Rhamnose	Quinic Acid	2-Deoxy-D-Ribose
D-Fructose	Sebacic Acid	i-Erythritol
Acetic Acid	Succinamic Acid	b-Methyl-D-Galactoside
a-D-Glucose	D-Tartaric Acid	3-Methyl Glucose
Maltose	L-Alaninamide	b-Methyl-D-Xyloside
L-Asparagine	Hydroxy-L-Proline	Palatinose
D-Glucosaminic Acid	L-Phenylalanine	D-Glucosamine
Tween 40	L-PyroGlutamic Acid	L-Tartaric Acid
a-D-Lactose		
L-Glutamine		
m- Tartaric Acid		
D-Fructose-6-Phosphate		
Tween 80		
b-Methyl-D-Glucoside		
Maltotriose		
Citric Acid		
m-Inositol		
Fumaric Acid		
Bromo Succinic Acid		
D-Cellobiose		
Inosine		
L-Threonine		
L-Alanyl-Glycine		
Mono Methyl Succinate		
Methyl Pyruvate		
D-Malic Acid		
L-Malic Acid		
Pyruvic Acid		
L-Galactonic Acid-g-Lactone		
Dextrin		
D-Arabitol		
Arbutin		
Gentiobiose		
Lactitol		
Melibionnic Acid		
N-Acetyl-L-Glutamic Acid		
L-Histidine		

3.4.4 Changes in Root Morphology Depend on Bacterial Species and Concentration

A plant's response to its microbiome may manifest itself in several phenotypic ways. Previous authors have noted changes in lateral root branching density, photosynthetic rates, root to shoot ratios, and root area of the plant depending on the associated microbial community [43, 138]. The root imaging capabilities enabled by the microfluidic platform allowed us to observe changes in root area due to the addition of bacterial treatments. Root growth is highly time dependent, focusing initially on the extension of the main root and then shifting to lateral root formation [139]. For this reason, and because root length measurements began on the third day after germination, any asynchronization in germination rates would produce significant error in the measure of root length on day 4 of co-culture (7d from planting the seeds). Therefore, we normalized the daily root length by the amount that each seedling grew during the first 24 h of the experiment (Figure 3.12A).

A decrease in overall root length was observed from both CF313 and YR343 bacterial treatments when compared to the uninoculated control (Figure 3.12B). The normalized root length varied with inoculum concentration for each of the bacterial strain treatments, a response that was evident after only 24h of co-culture and became more pronounced as the experiment progressed. This result suggests that the plant's response to its microbiome is not just a binary function of bacterial presence but depends on the magnitude of chemical signals exchanged between the plant and PGPB. However, the root length reduction depended differently on concentration for the two species of the bacteria. For example, the seedlings cultured with the highest concentration of CF313 exhibited the strongest deviation from the control seedlings while for YR343 the most significant root phenotypes occurred from the addition of the lowest concentration of bacteria.

Shorter roots and an increase in lateral branching is a plant phenotype that is often seen in the presence of PGPB [43, 140, 11]. Because the seedlings in this experiment

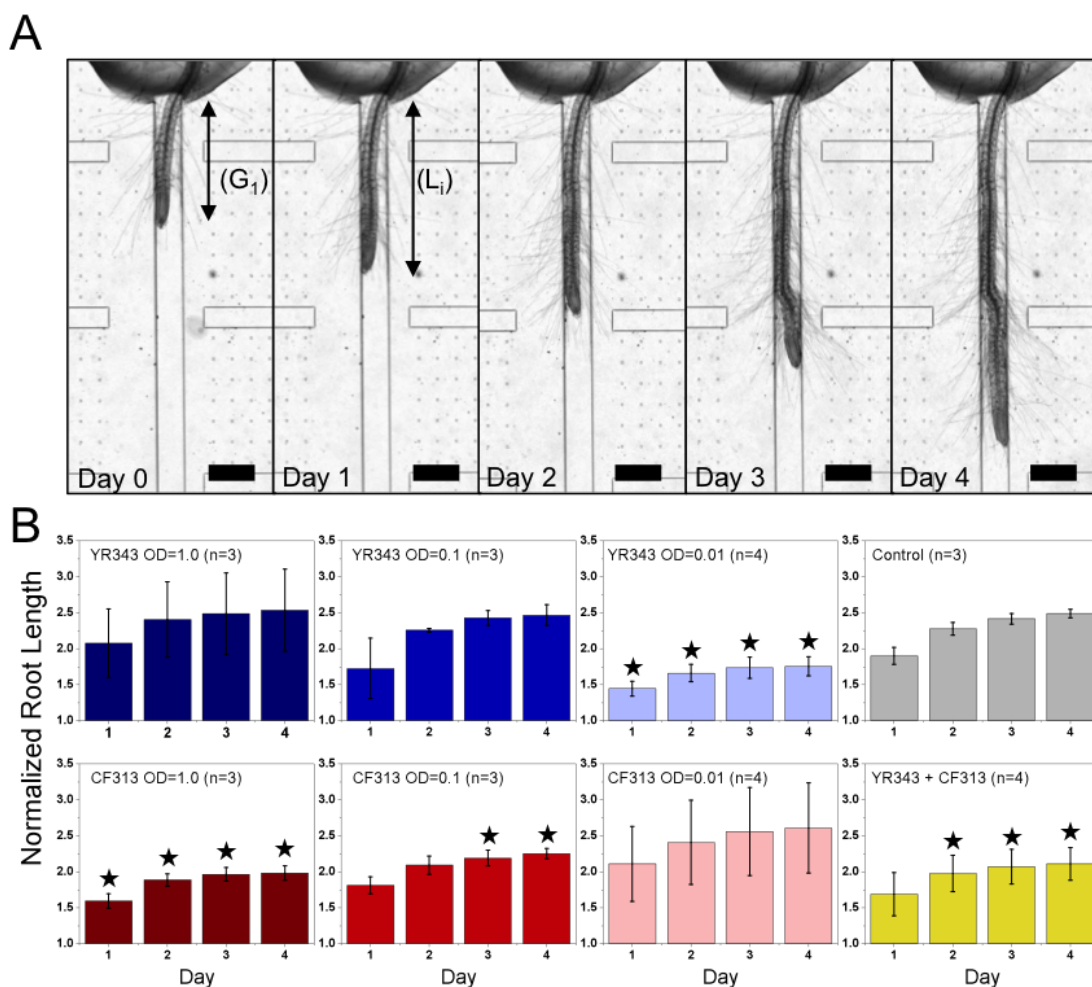


Figure 3.12: A) A control seedling grows in the platform for the duration of a typical co-culture experiment (scale bar = 250 μm). Root length on each day (L_i) was normalized by the initial growth (G_1 = growth on day 1) during the first 24 h of the experiment. B) The normalized root length phenotype depended on the microbial species and inoculum concentration (error bars are standard deviation). Stars indicate a significant difference (Student's t -test, $p > 0.05$) from the control on the same day.

were only allowed to grow for one week, phenotypes for lateral branching were not observed. The difference between the lengths of the main root for the two bacterial species arises from their potential molecular interactions with the plant.

Pantoea sp. YR343 has been shown to produce indole-3- acetic acid (IAA), a plant hormone (auxin) that, when produced by the plant, orchestrates plant cell elongation, division, and differentiation [97]. Bacterially produced IAA can interfere with the plant's own hormone regulation and, depending on the concentration, can have positive or negative effects on plant physiology [113, 141]. *Arabidopsis* seedlings that have been treated with exogenously applied auxins display a decrease in the main root length and increase in lateral root growth [142, 143]. *Pseudomonas thivervalensis* sp. MGL45, an IAA producing bacteria, has produced the same phenotype in *A. thaliana*, with root length inversely correlated to the concentration of the inoculum up to 106 cells per mL, which produced irreversible damage to the seedlings [144]. Besides being a signal molecule in plant-microbe interactions, IAA has also been connected to bacterial quorum signaling, with quorum sensing genes down regulating the amount of IAA produced in rhizosphere bacterium *Serratia plymuthica* HRO-C48 [145]. This involvement suggests that IAA production may not be linearly correlated to population density depending on the quorum sensing capabilities of the species in question.

The *Variovorax* genus contains species, notably *Variovorax paradoxus*, capable of utilizing plant produced 1-amino- cyclo- propane-1-carboxylate (ACC), the precursor to the plant senescence hormone ethylene, as a sole carbon and nitrogen source by hydrolyzing the molecule into NH₃ and α -ketobutyrate with a ACC deaminase [146]. Ethylene is a hormone that helps plants adapt to stress and, when exogenously applied to *A. thaliana*, can affect root architecture by shortening the primary root length and increasing root hair density, *Variovorax* sp. CF313 contains a gene that encodes for ACC deaminase and the hydrolysis of ACC by CF313 has been demonstrated [147, 148]. Microbial ACC deaminase activity usually elicits an increased root length phenotype [149]. The stunted elongation of the main root as a result of

co-culture with CF313 may be a consequence of other yet unidentified interactive chemical cross talk between the organisms.

When added in equal concentration (total OD = 1.0), the IAA producing bacteria YR343 and the ACC-deaminase bacteria CF313 produced a root phenotype that was between the YR343 (OD = 1) and CF313 (OD = 1) treatments. By itself, an optical density of 0.5 for YR343 may exhibit a phenotype that is not different from a control seedling following the YR343 trend in Figure 3.5. The resulting plant phenotype from the mixed bacteria culture, therefore, appears to be more dependent on the CF313 strain, with root length phenotypes between what was induced by the highest and middle concentrations of CF313.

3.5 Conclusions

Visualizing the interactions between plants and microorganisms can provide mechanistic insight into the physical and biochemical cues that shape their development. Our results show that for two specific microbiome bacterial isolates, the colonization kinetics and spatial distribution along root tissue is dependent upon the species of bacteria and the composition of the community as a whole. Further studies with additional rhizosphere and endophyte isolates will be needed to determine if some of these specific responses are universal for all endosphere or rhizosphere colonizing bacteria, or if the characteristics seen here are unique to these genotypes.

Microfluidic platforms allow for control over the experimental environment that, in this work, allowed *A. thaliana* roots to be uniformly treated with a predefined concentration of bacterial cells [150]. From these data, we observed that the population of bacterial cells associated with the main root after 4 d of co-culture was independent of the initial inoculum concentration. This suggests that the plant is limiting the amount of bacteria in its microbiome passively by producing a fixed amount of carbon, or actively by attenuating the quantity and type of root exudates that are available [12]. Despite an apparent concentration-independent steady state reached

by the bacterial populations after 4 d, the concentration of bacterial inoculum had a significant impact on the result of the plant-microbe interaction. The inoculum concentration dictated the size of the bacterial clusters formed on or within the root, with higher inoculum concentrations forming overall smaller and more uniform microcolonies in the rhizosphere bacteria treatment, which may indicate that high density inoculation inhibits growth and attachment on the root. This result could have practical application when choosing a quantity of beneficial micro-organisms with which to augment soil in the field.

Culturing *A. thaliana* with bacteria over 4 d using a direct sow method in our microfluidic platform provided a framework in which it was possible to quantify changes to root phenotype during the seedlings' first week of development. We observed a decrease in the normalized root length for both bacterial treatments but the degree of deviation from the control depended on the concentration of inoculum and bacterial species present. The plant's response, taken together with the bacterial side of the relationship, indicates that plant-microbe interactions depend on the concentration, composition, and phenotype of organisms present.

Quantifying the spatial distribution of each bacteria species over the course of the 4 d co-culture experiment demonstrated that YR343 cells prefer to associate with newly developed root tissue, while CF313 cells, displaying a uniform distribution, are less affected by potential spatial variations in chemical signals around the plant root system. Interestingly, the spatial distributions of these two species did not significantly change when both bacteria were added simultaneously to the plant despite evidence that suggests that the two were competing for a similar resource pool. This result may imply that these two species, when up against external biotic pressures, do not have an alternative mechanism to recur to for association with root tissues.

The ability to watch and quantify the spatiotemporal organization of complex biological systems over extended times in a microfluidic habitat opens the door to formulating and answering questions about community development and organization

that cannot be addressed in natural or conventional mesocosm systems. These microfluidic tools, though unable to replicate all the nuances of natural systems, provide a tractable experimental platform for controlling the chemical and physical environment, monitoring time dependent processes and imaging community members and plant phenotypes. Future microfluidic platforms can be designed to address specific questions regarding root exudation during plant development and its effects on microbial association or to understand how microbes influence root morphologies such as lateral branching. More complex fluidic architectures can even be developed to emulate natural soil networks [151, 152]. Pairing chemical imaging techniques with this platform will provide a powerful tool for correlating microbial associations and root exudate spatial distributions [153, 154].

3.6 Acknowledgements

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Chapter 4

Pore-Scale Hydrodynamics Influence the Spatial Evolution of Bacteria Biofilms in a Microfluidic Porous Network

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4.1 Abstract

Bacteria occupy heterogeneous environments, attaching and growing within pores in materials, living hosts, and matrices like soil. Systems that permit high-resolution visualization of dynamic bacterial processes within the physical confines of a realistic and tractable porous media environment are rare. Here we use microfluidics to replicate the grain shape and packing density of natural sands in a 2D platform to study the flow-induced spatial evolution of bacterial biofilms underground. We discover that initial bacterial dispersal and grain attachment is influenced by bacterial transport across pore space velocity gradients, a phenomenon otherwise known as rheotaxis. We find that gravity-driven flow conditions activate different bacterial cell-clustering phenotypes depending on the strain's ability to product extracellular polymeric substances (EPS). A wildtype, biofilm-producing bacteria formed compact, multicellular patches while an EPS-defective mutant displayed a linked-cell phenotype in the presence of flow. These phenotypes subsequently influenced the overall spatial distribution of cells across the porous media network as colonies grew and altered the fluid dynamics of their microenvironment.

4.2 Introduction

Microbial communities are complex systems that shape, and are shaped by, their local microenvironments. Bacteria often inhabit heterogeneous microenvironments with hydrodynamic flows that influence local nutrient transport and chemical gradients, creating specialized niches for microorganisms [77, 73, 155]. bacteria can influence

emergent phenomena like quorum sensing, intercellular communication, and biofilm formation [156, 65, 157]. These microenvironment factors and complexities contribute to microbial community diversity and synergism, which hinders their isolation and culture in bulk laboratory conditions [158, 159].

Soil exemplifies a complex and heterogeneous microbial environment. Within soil, the physical and chemical structure of the porous network dictates water and nutrient flow, influencing the cells' spatial distribution, communal behaviour, evolution, and even cross-kingdom interactions [160, 75, 1]. Some bacterial species can produce extracellular polymeric substances (EPS), which improve a soil's moisture retention and act to aggregate soil grains together, further altering underground hydrodynamics [5, 161]. Thus, bacterial characteristics are tightly coupled with the dynamics of soil conditions. This bacteria-soil interplay has implications for bioremediation, water quality, nutrient cycling, and underground ecology. It is therefore necessary to study soil bacteria within the structural and hydrodynamic context of their natural environment and on length scales appropriate to cellular functions (i.e. the pore scale) to elicit emergent behaviours. Unfortunately, the opacity of soil presents a challenge for the direct visualization and measurement of bacterial traits at the pore-scale *in situ*.

Experiments in sand columns have enabled measurements of bulk bacterial transport through porous media and have even allowed some preliminary imaging of bacteria in pore-spaces [162, 163, 164, 165]. Nafion, a transparent fluoropolymer that is sometimes used as a sand substitute, can further increase the imaging compatibility of bacteria in flow cells [166, 167]. However, these systems do not have a defined structure and are often treated as 'black boxes', making it impossible to correlate pore-scale hydrodynamics with bacterial biofilm distribution. In other synthetic systems, microfluidic platforms have been used to visualize bacterial behaviour in flow through narrow channels and around tight corners [168, 169, 170]. These platforms reduce the physicochemical complexity of natural porous media while testing bacterial characteristics in highly parameterized and fully defined systems [83].

Microfluidic systems have the added benefit of retaining the same physical structure for each experimental replicate, allowing flow in the channels to be computationally simulated [90, 171]. These systems have elucidated bacterial chemotaxis through tortuous channels, bacterial streamer formation, and microbial competition [160, 172]. However, microfluidic platforms have not yet been employed to study how the coupling of flow and biofilm ability affect the spatial distribution of bacteria in heterogeneous porous media.

Microfluidic platforms can also be designed with increasing complexity to approximate the porous media structure of natural environmental systems. Many researchers have used homogeneous designs of circular features with varied packing densities, radii, and pitches to approximate porous structure [173, 174, 175, 176]. While the reduction of grain shape to equivalent spheres has been successfully used in the field of soil physics to describe averaged fluid space quantities such as porosity and permeability, the size of a single bacterial cell is on a length scale well below the representative pore space volume that justifies the spherical grain assumption [177, 178]. In order to inform individual based models of soil microbial community behaviours, an accurate grain shape is needed to reproduce the fluid dynamics and labyrinthine pore spaces that an individual microbe would experience underground.

Some microfluidic designs have taken the heterogeneity of natural porous media into consideration. Pore throat networks inspired by reservoir rock have been reconstructed through the use of Voronoi cell tessellations, and focused ion beam-scanning electron microscopy (FIB-SEM) image triangulation, but geometrically partitioning the pore space results in a loss of information from the original sample [152, 179]. Another team used an algorithm generator to create ellipsoid-shaped grains with a pore space reminiscent of natural granular media and, within their platform, demonstrated the influence of EPS on underground hydration processes [151]. The ellipsoidal shapes of the generated porous media, however, are not based on shape statistics collected from natural grains.

In this work, we replicate both the two-dimensional shape and packing distributions of natural sand in a 2D microfluidic platform. A reduction in dimension from natural 3D soil substrates, while retaining the porosity of 3D soils, enables a quick image acquisition time to capture the dynamics of bacteria-grain interactions. Our heterogeneous design retains the pore-scale physical structure of natural porous media while providing a fully defined, tractable model for characterizing pore space hydrodynamics. Using gravity fed flow, we characterize the spatial evolution of bacterial biofilms in the porous network and correlate biofilm attachment and growth to pore scale hydrodynamics. We find that rheotaxis, or directed bacterial movement across a velocity gradient, has the biggest influence on the initial spatial distribution of bacteria, but over time EPS production ability and biofilm expansion ultimately determines the spatial distribution of bacteria in this heterogeneous network.

4.3 Results and Discussion

4.3.1 Device design and Simulation of Pore Space Hydrodynamics

The microfluidic grain design was created using a granular media algorithm to randomly generate 500 grains from the size and shape distributions of natural sands [180, 181]. Sand was chosen as a physical model for soil, the latter consisting of variable amounts of sand, silt, clay, and humus that form micro- and macro-aggregate structures. The resulting porous media section of the microfluidic platform contains a heterogeneous network of pore sizes and shapes including closed junctions between grains that can strain cells to large pores with high connectivity (Fig. 4.1 A-D). The distribution of pore spaces closely resembles a lognormal function with a median width of $50\mu\text{m}$ (Fig. 4.2). A large variety of pore feature allows for many sub-experiments on biofilm growth in various hydrodynamic conditions to be conducted at once.

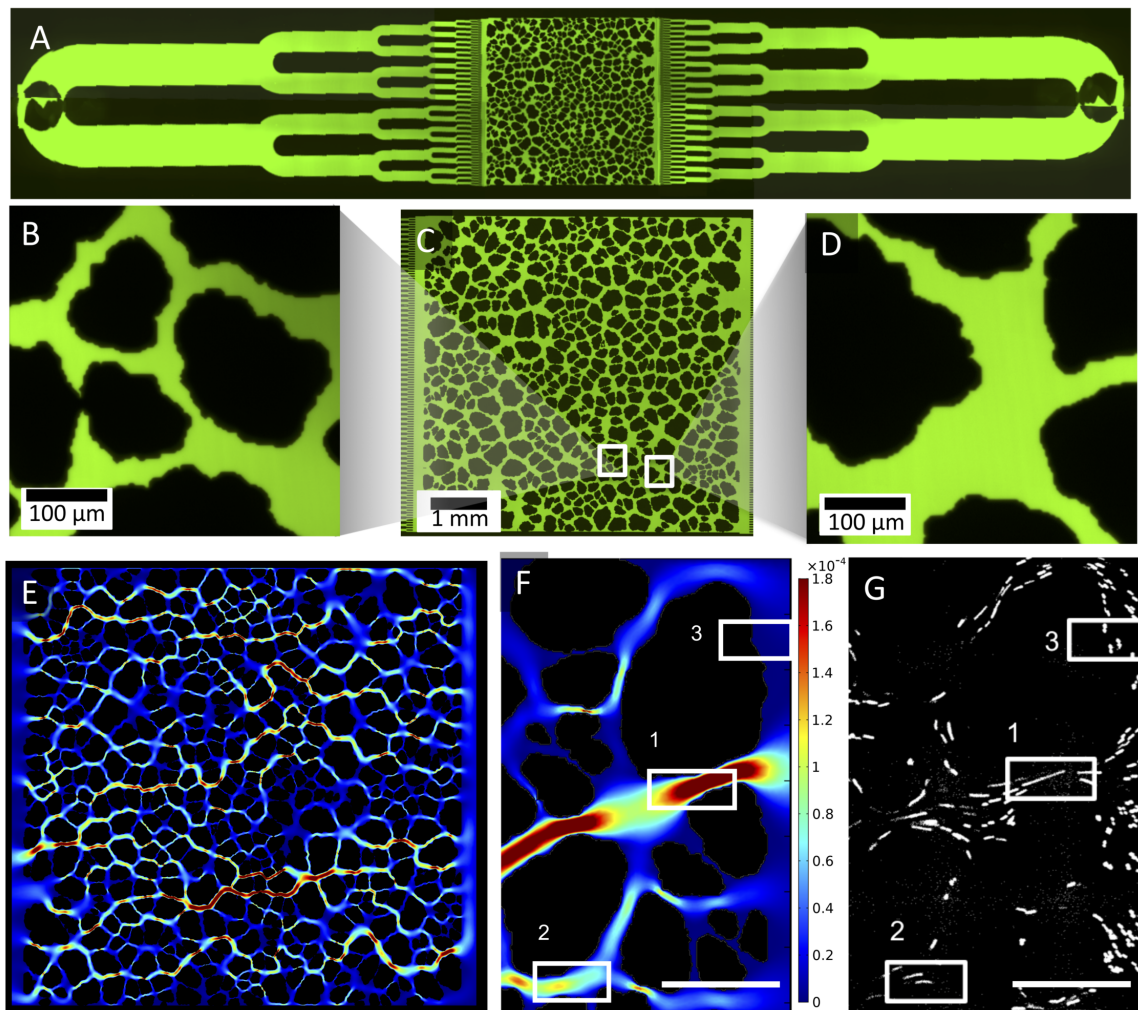


Figure 4.1: A microfluidic platform replicates the natural shape and layout of sand grains. (A) The device, filled with fluorescein to highlight its features, has a bifurcating inlet and outlet to uniformly distribute bacterial cells across the design (C) The porous network design (approximately 6,000 x 6,000 x 10 μm) consists of heterogeneous pore spaces that have (B) constriction points smaller than the size of a bacteria cell and (D) larger, highly connected pore spaces. (E) Velocities within the pore space were simulated using COMSOL Multiphysics, which illuminated multiple preferential flow paths across the system. (F,G) The pore space velocities were experimentally verified using particle image velocimetry and velocity magnitudes (F, regions 1, 2, 3, decreasing in magnitude) corresponded to bead speeds within the same pore (G, regions 1, 2, and 3)(scale bars = 100 μm , velocity units = m/s)

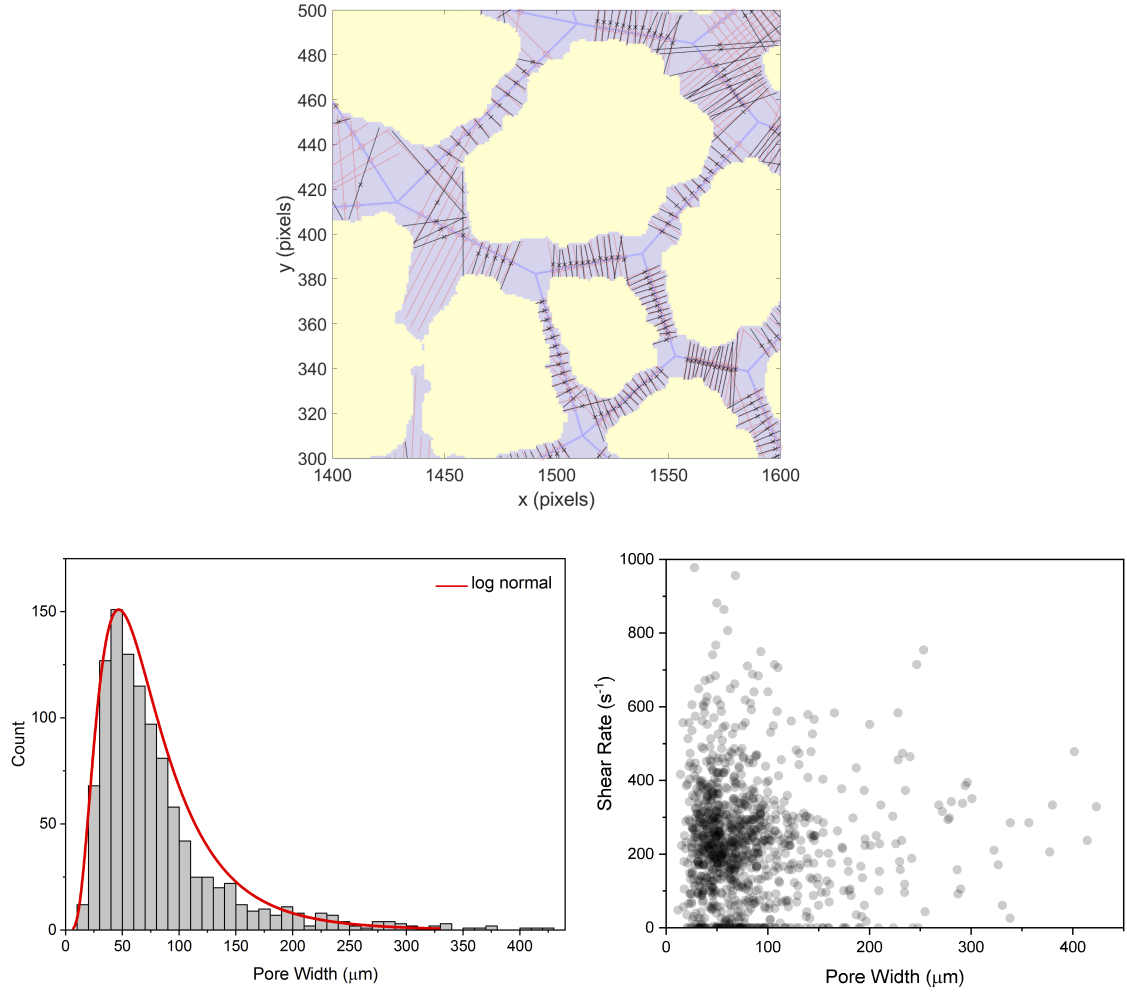


Figure 4.2: (top) Pore characterization program results shown in a 200 pixel $\times$ 200 pixel region-of-interest sampled from a larger 2D granular space of roughly 2000 pixels $\times$ 2000 pixels. Pores in the tessellation pattern with total path length less than 10 pixels were not included in the pore characterization statistics. (bottom left) A histogram of the pore widths shows a close resemblance to a lognormal function. (bottom right) Every pore is plotted by its average shear rate and width.

The microfluidic porous media maintains the same grain layout and characteristics for each experimental replicate, which provides a highly tractable method to correlate pore scale features with flow. Here the CAD file used to fabricate the microfluidic platform also created the geometry of a COMSOL Multiphysics laminar flow simulation to determine the hydrodynamic flow parameters at steady state for constant velocity inputs. A velocity magnitude heat map was generated to visualize flow between pores and illuminated several preferential flow paths across the system (Fig. 4.1E). To confirm the accuracy of the COMSOL model, particle image velocimetry was used to calculate average flow speeds within pores, which matched the velocity magnitudes simulated in the COMSOL model (Fig. 4.1F-G).

4.3.2 Bacteria Growth and Flow Rate are Coupled in a Pressure Driven System

To recreate the flow of percolating rainwater, a reservoir was suspended above the microfluidic platform and connected by tubing, generating gravity-driven flow. Prior to the experiment, either a wild type (WT) or EPS defective mutant (Δ UDP) of *Pantoea* sp. YR343, a rod-shaped, motile soil bacterium isolated from the rhizosphere of *Populus deltoides*, was grown overnight, diluted, and grown again to mid-exponential phase at an optical density (at 600nm) of 0.1 ($OD_{600} = 0.1$) [97]. The bacteria were then loaded into the reservoir and seeded into the device for one hour. After one hour, the reservoir was exchanged for pure R2A media, keeping a constant hydrostatic pressure, and the tubing was also replaced to prevent bacterial growth upstream of the device. The device was then imaged every hour for 21 hours. Additional tubing at the outlet carried effluent from the microfluidic platform to dish on a scale where it was weighed every 10 minutes to monitor the flow rate through the platform (Fig. 4.3). Prefilling and sealing the dish minimized weight variations due to evaporation.

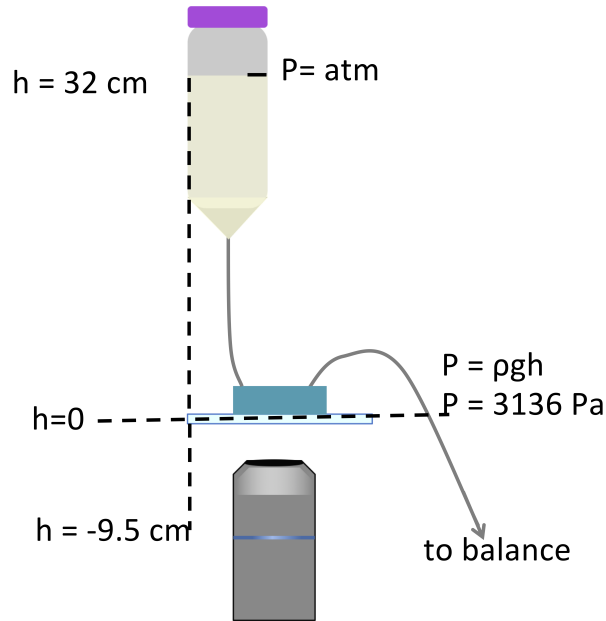


Figure 4.3: A schematic illustrating the experimental setup and the calculation of hydrostatic pressure. The lid on the reservoir tube was loosely fitted to assure atmospheric pressure above the liquid.

Suspending the reservoir 32 cm above the platform resulted in a constant control flow rate of approximately 5 $\mu\text{L}/\text{min}$. After cells were seeded in the platform, this flow rate steadily dropped off over time, approaching 1 $\mu\text{L}/\text{min}$ for both strains after 21 hours (Fig. 4.4A). Despite a reduced capacity to create EPS, a precursor to biofilm development, the ΔUDP strain showed no significant difference in flow rate change compared to the WT strain. However, change in flow rate over time in this heterogeneous porous network was drastically different than the abrupt, catastrophic clogging that other researchers have seen from similar experiments in a single tortuous channel [172]. Presumably, this is because, as bacteria obstruct one preferential flow path, other preferential flow paths open up and divert flow around the clog. This diversion of flow, or remodeling of the porous network, would have environmental implications for chemical transport in natural porous media. Other researchers have found that, as bacteria grow to clog pore spaces, flow redirects to provide nutrients to slower growing colonies [160]. This work suggests that slow growers have the

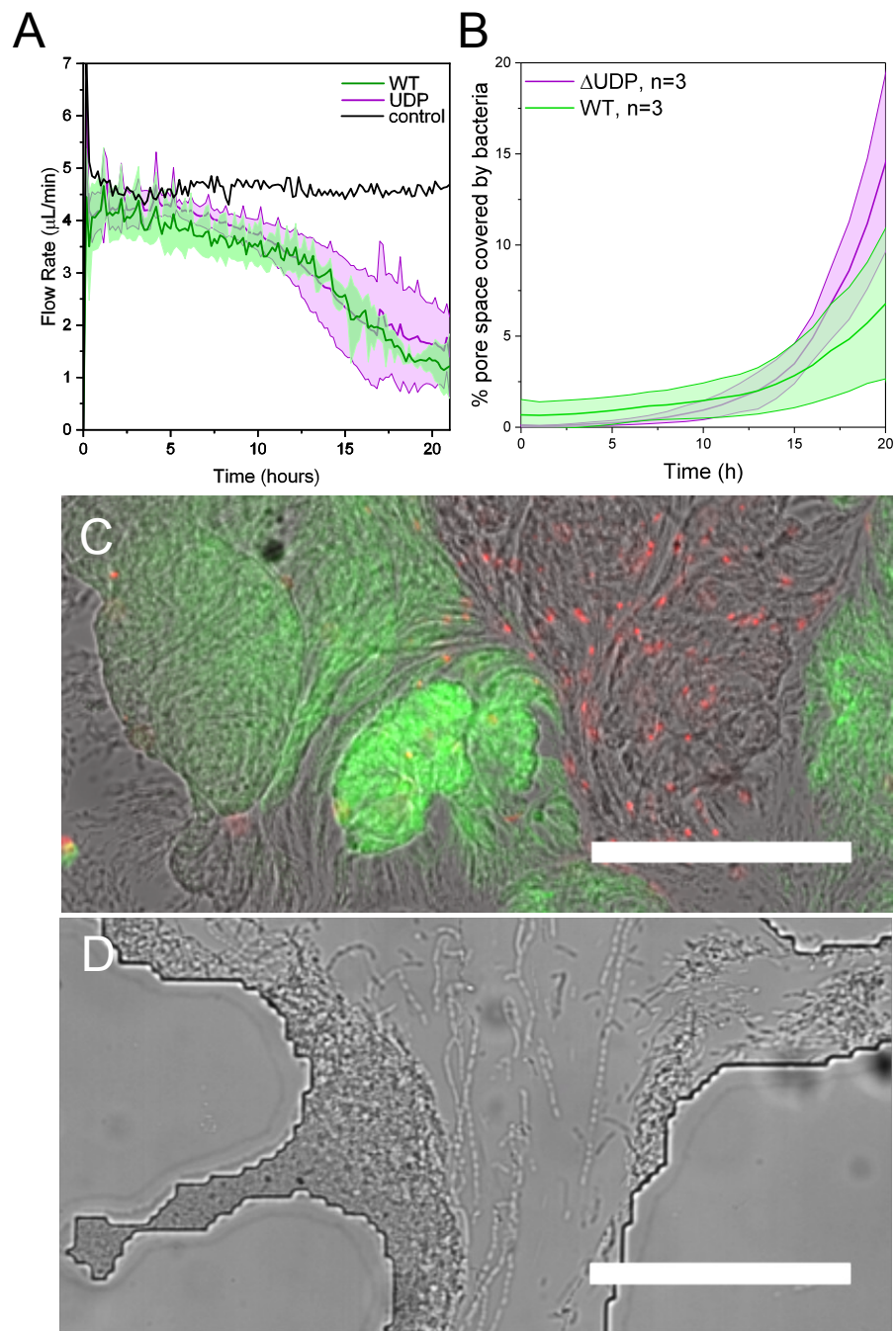


Figure 4.4: A WT strain and an EPS deficient mutant (Δ UDP) grow within the heterogeneous porous media platform. A) Both strains cut off flow at the same rate yet B) the Δ UDP strain grows to cover more area of the device. C) Flow induces a globular phenotype for the WT strain and a D) filamentous phenotype for the Δ UDP strain.

advantage in heterogeneous porous networks because they maintain a sustainable growth rate without cutting off their own nutrient supply.

The Δ UDP mutant cells also did not show a significant difference in pore space coverage by the end of the experiment compared to the WT strain (two-sample t-test, $p > 0.05$) (Fig. 4.4B). Given that the EPS defective strain cuts off flow at the same rate as the WT and grows to cover a similar area, the heterogeneous structure of the porous media does not seem to have interacted with bacterial genotype under these experimental conditions to produce different growth results. However, this system did not test bacterial competition, antibiotic susceptibility, or challenges to the community that would favor a robust biofilm producing strain [182].

Hydrodynamic conditions in the pore spaces did alter the phenotype of each strain compared to static bulk media laboratory conditions (i.e. agar plate assays). In the pore network, the WT strain formed compact, globular patches of living cells with a diameter approximating the size of the pore in which the bacteria were confined (Fig. 4.4C). The Δ UDP mutant exhibited phenotypes that depended upon the velocity within the pore space. In pores with flow the Δ UDP cells formed long chains of linked cells, while cells in static pores retained a single cell, rod-shaped structure (Fig. 4.4D). This Δ UDP phenotype was an important precursor for pore space clogging in lieu of EPS formation; strands of living and dead cells spanned multiple grains, forming a foundation on which more cells could attach and grow. It is unclear whether the flow-induced phenotypic changes in this study are purely a passive result of physical forces, or if the cells are sensing local hydrodynamic changes and regulating gene expression accordingly. There is evidence that at least some bacteria are able to transduce mechanical signals into the biochemical signals needed to promote cell attachment to surfaces and initiate biofilm formation [183] Increasing fluid shear has also been shown to increase the expression of genes responsible for virulence in enterohemorrhagic *E. coli* [184]

Although the Δ UDP mutant and wildtype bacteria exhibited similar influence over the overall flow through the porous media platform, their pore-scale growth

restructured the network flow profiles differently. The linked-cell phenotype of the Δ UDP mutant manifested itself across the pore network in a streamer-like distribution of cells that follows the general direction of flow (Fig. 4.5B). The WT strain, however, exhibited a much patchier distribution of growing microcolonies across the entire pore space (Fig. 4.5A). A patchy distribution of soil organisms is traditionally thought to be a direct result of heterogeneous resource allocation[185, 155]. In this system, however, nutrients are universally dispersed in the media and continually replenished.

The spatial distribution of both bacteria strains across the porous network after 21 hours appeared to evolve from the location of the initially attached bacteria (Fig. 4.5). Therefore, we next examined the simulated hydrodynamics within the pores where we saw the highest incidence of initial attachment, or seeding.

4.3.3 Correlating Initial Bacterial Attachment with Flow Hydrodynamics

Despite the reproducibility of the pore space layout between experiments, bacterial fluorescence was not always located in the same pores after one hour of seeding, neither between bacterial treatments (WT or Δ UDP) nor between replicates (n=3). While we attempted to control the initial conditions as much as possible, this result may be, in part, due to minor experimental variations that amplified across the network [186]. Although bacteria did not always initially clog the same pores between replicate experiments, there was a trend in the hydrodynamics of pores where cells were initially captured. In comparing the simulated hydrodynamic conditions of the pores where cells were captured, the probability of clog initiation was highest in two shear regimes: in pores with very low shear rates (approaching 0 s^{-1}) due to uniform velocity profiles across the pore space, or at the maximum shear rate in the device (1000 s^{-1})(Fig. 4.6).

Because shear rate is the differential of velocity, extremely low shear rates (approaching 0 s^{-1}) can occur whenever there is constant velocity across a pore.

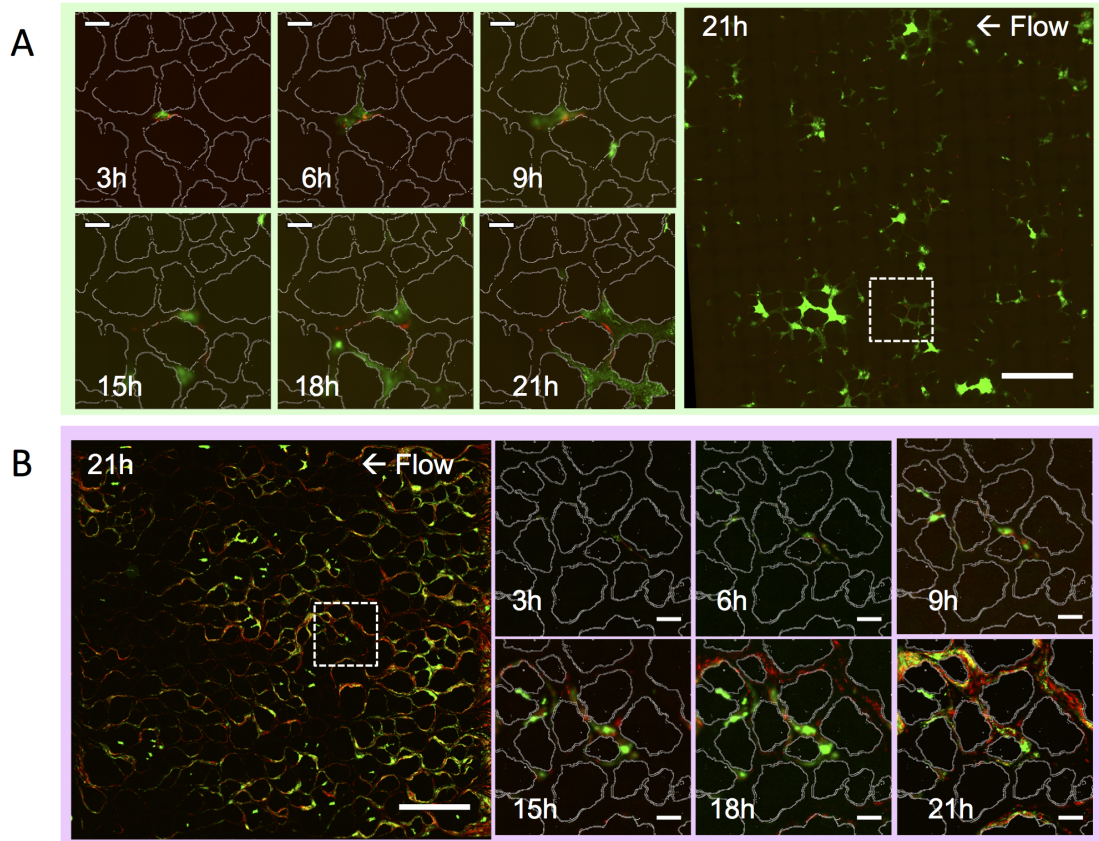


Figure 4.5: A) WT and B) Δ UDP cells in the platform at 21 hours (scale bars = 1mm) and in a subset (indicated by dashed box) of the device over time with the particle design overlaid (white) (scale bars = 100 μ m)

However, because of the no-slip boundary condition (zero velocity) at the grain surfaces, the only pores with constant velocity will be the pores with static fluid conditions. The clogging maximum in the pores with extremely low shear could therefore be occurring because the velocities within these pores are low enough for *Pantoea* sp. YR343 swimming speeds (mean swimming speed: 3.5 $\mu\text{m/s}$, max swimming speed: 7 $\mu\text{m/s}$) to overcome. Bacterial motility is therefore likely to be the dominant transport mechanism in these pores.

Aside from the pores with approximately zero shear, the probability of clogging steadily increased with shear rate up to the maximum shear rate in the device, 1000 s^{-1} . At these high shear rates cells are directed toward grain surfaces by a transport mechanism known as rheotaxis [89]. In bacteria, rheotaxis is passively directed movement across velocity gradients due to lift forces on the bacteria flagella and drag forces on the bacteria cell body [187]. Because velocity at the surface of a grain is zero, following the no-slip boundary condition, high shear rates tend to occur close to grain surfaces thus encouraging cell-grain interactions and, subsequently, biofilm initiation. Videography of cell trajectories confirmed that rheotaxis was directing cells across velocity gradients toward grain surfaces (Fig. 4.6F). This mechanism of microbial transport through soil is a significant departure from colloid filtration theory (CFT), which is sometimes used to estimate the transport of bacteria cells underground, because rheotaxis cannot act on spherical, non-motile colloids. [187, 188, 189].

We wanted to understand the relationship between shear rate and pore size within the network. An algorithm was created (see Methods section) to quantify the average width of each pore within the microfluidic platform. Each pore's average width was then compared to the average shear rate within the same pore (Fig. 4.2). Theoretically, if two pores of the same size were aligned in series, the shear rate (and subsequent pore clogging) would be the same for both pores, following the Hagen-Poiseuille equation [190]. However, because the pores experience different flow rates across the network, pore size was not strongly correlated to the shear rate across

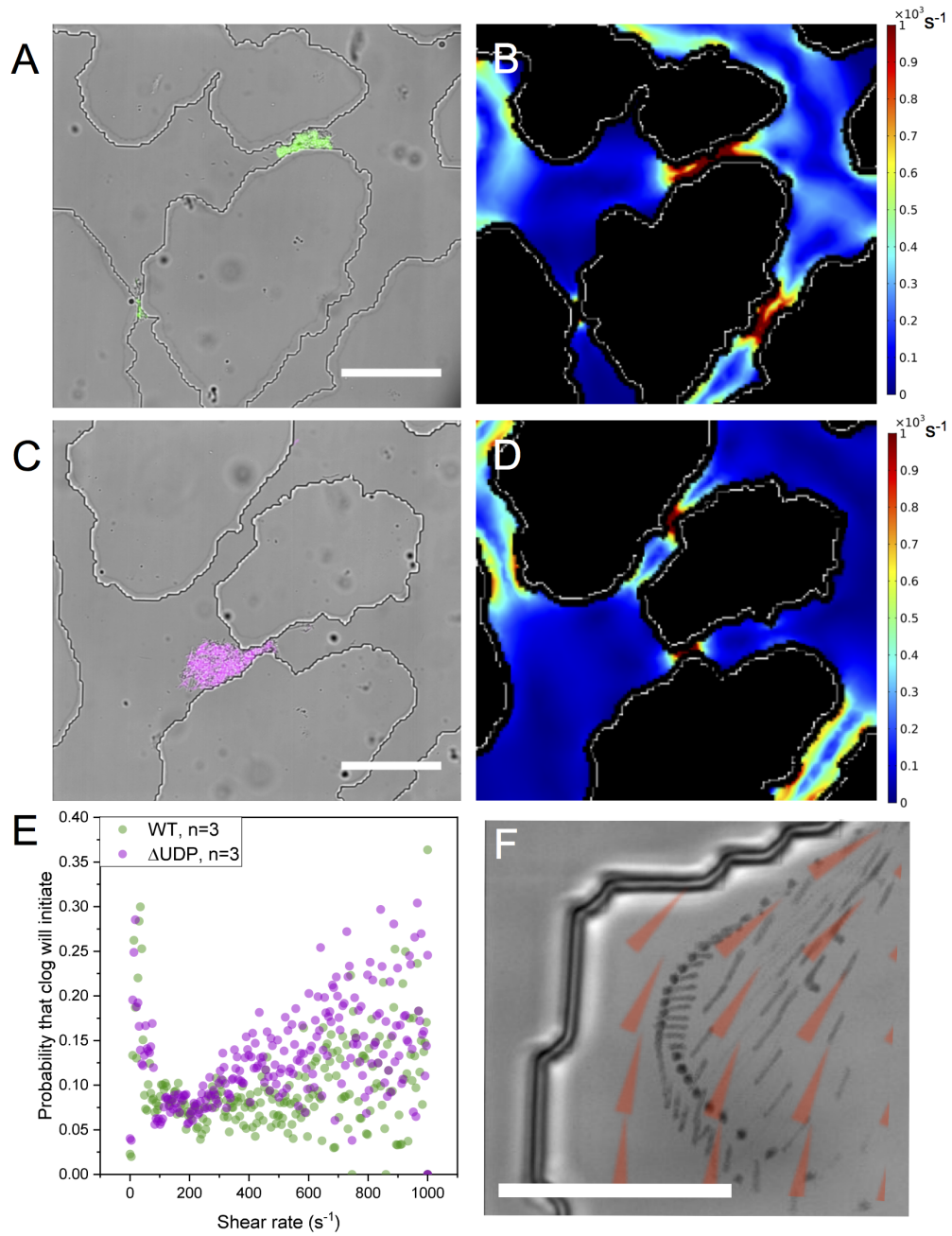


Figure 4.6: Differential interference contrast images overlaid with fluorescent images of A) WT and C) Δ UDP cells show that the location of initial bacteria seeding correlate to (B,C) areas of high shear from the COMSOL simulations. (scale bars = $100 \mu\text{m}$) E) The probability of a clog occurring in a pore is high at zero shear and increases again with shear rate. F) A minimum intensity projection over time of WT bacteria cells (black) moving across the velocity gradient (overlaid red arrows from COMSOL simulation) confirming rheotaxis in the pore spaces. (scale bar = $25 \mu\text{m}$)

the entire platform. Pores with the same size had different shear rates depending on the pore's location with respect to preferential velocity flow paths throughout the network. Therefore in porous media networks, transport due to rheotaxis cannot be predicted solely from pore size. In addition to being an important factor in trapping bacteria during the initial stages of biofilm seeding within the pore space, we next tried to elucidate the role of rheotaxis in shaping growing biofilms over time.

4.3.4 Predicting the Spatial Evolution of Bacterial Biofilms

Once bacteria have attached to the surface of a grain, their distribution is affected not only by flow, but also by growth [191, 192]. Daughter cells may detach and be carried downstream to attach in another location. They can also reattach upstream when cells have specialized characteristics like curved structure or twitching motility [193, 194]. Under these growth conditions, the distribution of live cells and pore scale hydrodynamics are coupled. As resistance in the system builds and overcomes the pressure at the device inlet, pore flow decreases locally and bacteria can use swimming motility as a dominant transport mechanism.

To determine if shear trapping via rheotaxis was dictating the spatial distribution of cells during the growth phase, we converted images of living cells in the device at an initial time (t_i) to CAD files and incorporated them into the geometry of the COMSOL simulation (Fig. 4.7)(see Materials and Methods). The shear profile was then recalculated and compared to the distribution of living cells one hour later (t_{i+1}) using object overlay analysis. The probability of colocalization between cells at t_{i+1} and shear simulated from t_i was relatively constant for $i=3, 9$, and 15 hours indicating that shear trapping is no longer a dominate mechanism determining the spatial distribution of a growing biofilm. However, this method of modeling biofilms is limited and might be improved by adding polymeric material properties to the biofilm geometry and incorporating a solid-liquid interaction into the simulation.

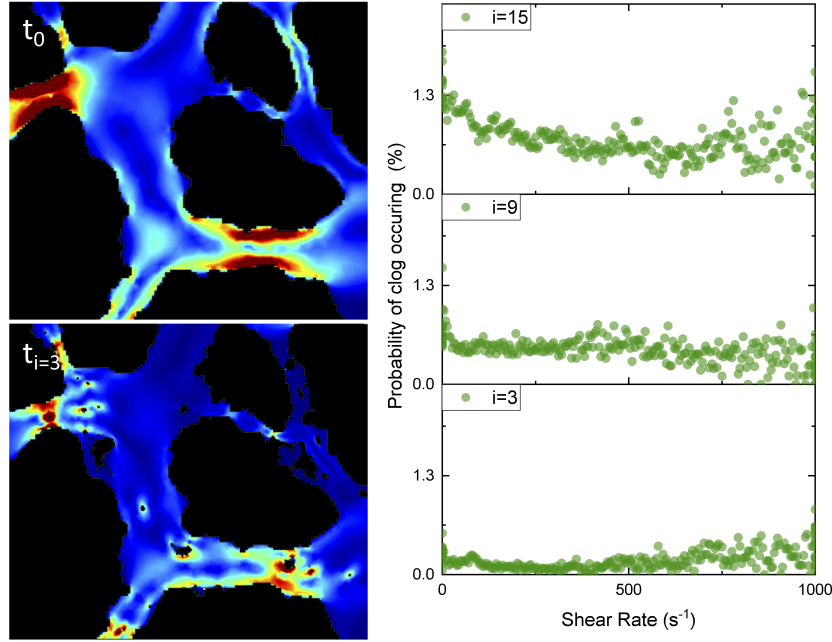


Figure 4.7: (left) An example of the simulated shear rate before (t_0) and after subtracting the biofilm from the geometry ($t_i=3$) shows that biofilm influences pore space shear rates. (right) The relationship between shear rate and the probability of a clog occurring is relatively constant over time.

Next we conducted a nearest neighbor interaction analysis for the wildtype bacteria to understand how the spatial distribution of bacterial biofilms changed over time. The bacterial fluorescence was compared in pairs of images (t_i and $t_{(i+1)-i}$ for $i = 3, 9$, and 15 hours) using a nearest neighbor interaction analysis plugin in Fiji [195, 196]. The probability of a cell cluster encountering a neighboring cluster peaked at the relatively close distance of 6, 15, and 20 μm for growing cells at $i = 3, 9, 15$ hours, respectively (Fig. 4.8). By looking at the change in bacterial fluorescence over time, changes indicate that the biofilms grew through pore spaces in a downstream direction. This result suggests that the spatial distribution of the growing biofilm over time depends largely on the historical location of the mother cells which, in turn, demonstrates how early events (i.e. seeding location) can amplify into large changes in flow and biofilm development across the pore network. However, other factors may also be contributing to the changes in fluorescence besides growth such as cell decay and biofilm dispersal.

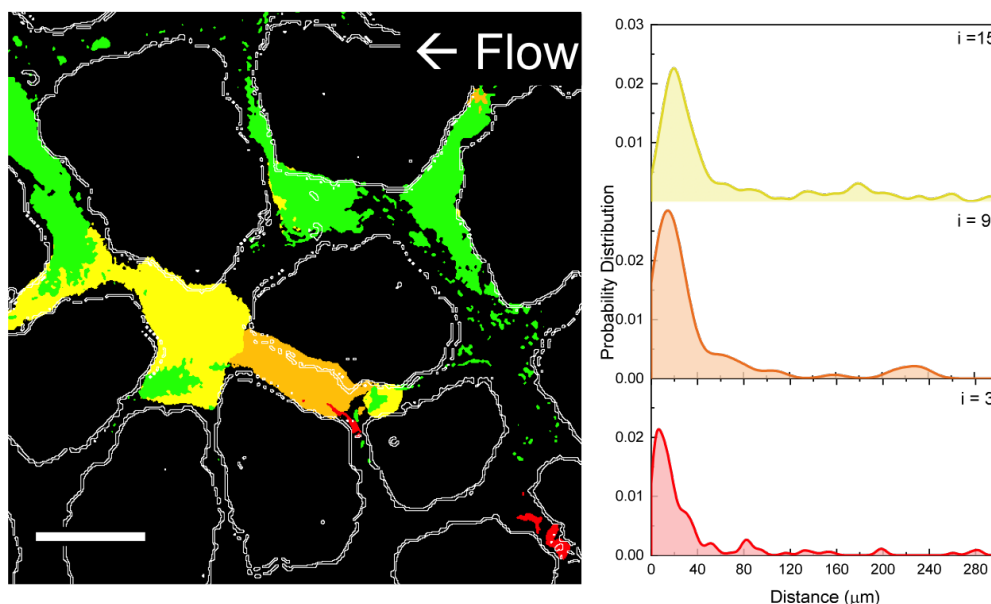


Figure 4.8: (left) The change in bacterial fluorescence over time from 0-3 hours (red), 3-9 hours (orange), 9-15 hours (yellow), and 15-21 hours (green) show that bacteria biofilms are growing steadily in a downstream direction. (right) The nearest neighbor probability for $i=3, 9$, and 15 hours shows that nearest neighbors are within a 20 μm radius (not considering pore space tortuosity).

Other authors have also determined through nearest neighbor analysis that most soil bacteria interactions occur within a 20 μm radius [75]. If the fluid dynamics of this system were static, this result would suggest that bacteria interactions are largely clonal, occurring mostly between descendants of the same ancestor cell. However, flow acts to disperse cells, metabolites, and signaling molecules to downstream microcolonies, increasing the extent of bacterial interactions, albeit in one direction. Therefore, biofilm growth and EPS production, although jeopardizing the incoming flow of nutrients by choking off flow, may provide a mechanism for increasing local communication and bi-directional exchange (e.g. horizontal gene transfer) between bacterial microcolonies in the soil.

This work demonstrates the value of studying microbes in complex systems like porous media in order to accurately capture natural microbial characteristics and community interactions. Here, the use of a faithfully replicated physical environment reveals that microbial dispersal and initial attachment in porous media is strongly

influenced by shear forces within pore spaces. Results from this work (i.e. cell phenotype changes, spatial distribution, and rheotactic transport) testify to the ability of heterogeneous matrices to influence emergent microbial properties compared to bulk laboratory methods, and these results can be used to validate individual based biofilm models. While this platform only replicates the 2D physical structure of natural sands, increasing complexity can be incorporated into the design, including chemical treatments and nutrient gradients, to recreate natural microbial habitats and emergent bacterial phenomena in a fully defined, parameterized approach.

4.4 Methods

4.4.1 Device Design and Fabrication

Using a published granular media algorithm with defined input parameters (Supplementary Table 1), the porous media design was created to replicate the shape distribution of sand particles from Tecate, Mexico [180, 181]. The algorithm generated a .bmp file of the particle layout that was vectorized in LayoutEditor to create the CAD design. The particle size of the CAD file, defined by the D50 gradation, was scaled to 0.13 mm to reflect smaller sand particles. The design's porosity (0.38), defined as the ratio of open space to solid space, approximated the porosity of sandy loam (0.25-0.35) [151]. A bifurcating inlet and outlet were added to the CAD to uniformly distribute bacteria across the design.. The smallest inlet and outlet dimensions were 22 μm in width. This size was chosen in order to uniformly deliver bacterial cells across the porous media platform, as any bias in bacterial introduction would amplify through the entire network.

The design was transferred onto a chrome mask using a Heidelberg DWL 66 mask writer and replicated onto a silicon wafer using standard photolithography techniques with an NFR photoresist and Bosch etch (Oxford RIE) for to give a final depth of 10 μm . This depth was chosen to confine bacteria to a single focal plane during the

Table 4.1: *Input parameters for the grain generating algorithm*

Parameter	Value
Number of Grains	500
SurfaceCOV	0.8
Orientation	0
Anisotrophy	1
itermax	1.00E+06
error	0.002
solid fraction	0.65
nvar0optim	6
covspectrum	0
typespectrum	1
file spectrum	tecate
Dmax	0.02
Rmin	0.02
pmax	0.05

course of the experiment. Trichloro(1H,1H,2H,2H-perfluoro-n-octyl)silane), 85°C, 60 min) was evaporated onto the surface of the silicon wafer in order to prevent adhesion during the polymer molding process. The microfluidic devices were replicated from the silicon master using poly-dimethylsiloxane (PDMS; 5:1 PDMS base to curing agent, wt/wt; Sylgard 184, Dow Corning) and cured overnight at 70°C [197]. Inlet and outlet holes were created using a 1.5 mm biopsy punch and the PDMS was bonded to a glass coverslip using air plasma treatment.

4.4.2 Bacterial Growth

Pantoea sp. YR343, a plant growth promoting rhizosphere isolate from the *Populus deltoides* microbiome, was chosen for its relevance to soil ecology and for its rod-shape and flagellar motility [97, 198, 18]. Fluorescent strains were constructed by integrating GFP into the chromosome of *Pantoea* sp. YR343 using the pBT270 and pBT277 plasmids as previously described [97]. The EPS mutant was isolated by screening a *Pantoea* sp. YR343 transposon library on Congo Red plates. The transposon insertion site for the EPS-defective mutant was confirmed to disrupt gene

PMI39_01848, which is located at the beginning of an operon with homology to EPS biosynthesis gene clusters found in *Pantoea stewartii* and *Erwinia amylovora*[199]. Fluorescence varieties of the WT and Δ UDP strains were grown overnight prior to the experiment on liquid R2A media (TEKnova, Inc.) at 37°C. Bacteria cultures were diluted 100x in fresh R2A the next morning and allowed to grow again to mid exponential phase at an optical density (600nm) of 0.1 , as measured by UV spectroscopy.

4.4.3 Experimental Setup

Large (for media) and small (for seeding bacteria) reservoirs were created by piercing a hole at the bottom of 50 mL and 15 mL centrifuge tubes and securing a 23 gauge blunt-tip needle to the tube with polyurethane glue (DUCO cement). Inlet and outlet Tygon tubing was measured to 25cm and 44cm, respectively. The microfluidic device was placed under vacuum for one minute to facilitate pre-filling with R2A media. Once filled, the device, inlet and outlet tubing, and the reservoirs were UV sterilized for 15 minutes in a UV Stratolinker oven. The materials were assembled on the microscope stage before the start of the experiment as shown in Fig. 4.3.

A bacteria culture (either WT or Δ UDP strain) was loaded into the smaller reservoir so that the top of the liquid was 32cm above the microfluidic platform inlet. This ensured a consistent pressure-driven flow at 3136 Pa following the hydrostatic pressure head equation ($P = \rho gh$) assuming that the cultures have the same density (ρ) as water. The tubing was attached to the microfluidic inlet and the cells were seeded into the device for 1 hour, after which the reservoir was exchanged for the larger R2A media reservoir taking care to keep the same hydrostatic pressure. The tubing connecting the reservoir to the platform was also changed at this time. Propidium iodide (PI) (0.025 μ M) was added to the R2A reservoir to stain dead cells over time. Effluent from the microfluidic platform was carried to a Petri dish on an analytical scale. The dish was sealed with Parafilm to limit evaporation and pre-filled with water

to quickly establish a steady-state vapor pressure in the dish. The scale was imaged every 10 minutes and the flow rate was calculated with the assumption that the liquid had the same density of water. After seeding, bacteria within the microfluidic platform were imaged every hour using an inverted microscope (Nikon Ti-Eclipse) with FITC and TRITC epifluorescence to capture both living and dead cells. Image acquisition was carried out with a 20x objective using Nikon Elements software and a back-illuminated iXonEM+ 897 camera (Andor).

4.4.4 COMSOL Simulations

The porous media CAD was incorporated as the pore space geometry in a 2D laminar flow (spf) COMSOL Multiphysics (version 5.2) simulation. The fluid space was assigned the material properties of water while the particle space was subtracted from the geometry. One side of the design was assigned as an inlet boundary with an entrance pressure of 313.6 Pa (3,136 Pa calculated from the hydrostatic pressure divided by 10 μm for the device height) and the other side of the design was assigned an outlet pressure condition of zero Pa. Due to anisotropy in particle layout, care was taken during experimental setup to match the experimental inlet with the simulated inlet. An extra-course free triangular mesh was used to partition the geometry into finite elements. The laminar flow study used a stationary GMRES solver (50 iterations) with a relative tolerance of 0.001.

An outlet flow rate from the simulation was calculated by multiplying the line integrated velocity magnitude at the outlet by the device depth (10 μm). This value (4.706 $\mu\text{L}/\text{min}$) closely matched the experimental control flow rate of only R2A media of 4.48 $\mu\text{L}/\text{min}$ ($n=1$). Experimental particle image velocimetry using fluorescent 2 μm carboxylated polystyrene beads confirmed that the simulated velocity matched experimental average speeds in the pore spaces.

For flow simulations with the biofilms, a fluorescent image of both live and dead cells at a given time (t_i) was converted to a CAD file (.dxf) and subtracted from

the COMSOL geometry. This method worked reasonable well at earlier time points (i=3, simulated flow rate= 4.56 $\mu\text{L}/\text{min}$ and experimental flow rate = 4.64 $\mu\text{L}/\text{min}$) but the experimental and simulated flow rates diverged at i=9 (simulated =4.57 $\mu\text{L}/\text{min}$, experimental= 3.91 $\mu\text{L}/\text{min}$). This method of modeling biofilms, is therefore limited, and might be improved by adding polymeric material properties to the biofilm geometry and incorporating a solid-liquid interaction into the simulation. However, the rheological properties of this particular bacterial biofilm remain uncharacterized.

4.4.5 Image Analysis

To remove out-of-focus autofluorescence from debris and background noise, images were processed using Fiji [128]. For each image, the background fluorescence was removed (sliding paraboloid, 700 μm) and then images were manually thresholded to further reduce noise. Total fluorescence area was analyzed using the particle analyzer feature.

4.4.6 Object Overlay Analyses

For object overlay analyses with the COMSOL simulations, experimental images were rotated, cropped, and downsampled to align with the same region of interest in the COMSOL shear rate profiles. A composite image was created from the experimental and COMSOL images using the AND Boolean operation. The histogram of greyscale pixel values from this composite image provided information on the shear rate values that were co-localized with each bacterial fluorescence pixel. To obtain the "probability that a clog will initiate" metric for each shear rate value, the shear rate associated with each bacterial fluorescence pixel at i=1 was normalized by the total number of pixels from the COMSOL simulation with the same shear rate value.

4.4.7 Nearest Neighbor Interaction Analyses

Change in bacterial fluorescence was calculated by subtracting an image at an initial time (t_i) from an image one hour later (t_{i+1}). The resulting image ($t_{(i+1)-i}$) was compared to t_i using the Mosaic Interaction Analysis Plugin to determine nearest neighbors for $i=3, 9$, and 15 [196].

4.4.8 Pore Space Characterization

A pore characterization program was created as an addendum to the 2D granular space simulation (GSS) reported by Mollon and Zhao [181]. The purpose of the complementary pore characterization program was to quantify the average width of each pore generated using the GSS. Pores are represented as linear segments in the GSS and the length of the linear segment is defined here as (L). The blue linear network shown in Fig. 4.2 (top panel) represents the Voronoi tessellation. An iterative algorithm is required to converge to an approximation of the real tortuous pore pathlength. The pore width is ultimately derived from this pore pathway reconstruction. Fig. S1 shows in the initial distribution of transverse pore lines in red (-) while the final transverse profile representing an approximation of the tortuous pore pathlength is shown by the population of black lines (-).

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Chapter 5

Conclusion

5.1 Research Summary

The interdisciplinary work contained within this thesis spans applied engineering and basic science research. The platforms that I designed herein, have extended the current capabilities of microfluidic platforms for studying underground biological systems. By incorporating structural heterogeneity into the microfluidic design, I have progressed toward reconstructing the natural complexity of the rhizosphere in order to induce natural microbial phenotypes in laboratory settings. These platforms go beyond traditional, open-channel microfluidics and, for that reason, are referred to as "micro-habitats" within this thesis. It is my hope that these micro-habitats will be promising tools that future researchers can use to answer their own outstanding biological questions.

This doctoral work did not stop at a proof-of-concept theory stage, however; I also employed these micro-habitats to generate hypotheses and to answer specific biological questions. The conclusions from this work and their broader implications for rhizosphere ecology are discussed further in the following sections.

5.2 Extended Capabilities for Plant-on-a-chip Research

The microfluidic design and subsequent plant culturing protocol described in Chapter 2 of this thesis is valuable to the field of rhizosphere ecology for several reasons. The platform's two-layer design confines the root hairs to a single imaging plane so that cells do not drift out of optical focus during treatment. This enabled us to image organelle (peroxisomes, mitochondria, and golgi) movement over time, before and after the addition of a treatment. Being able to visualize organelle movement is also beneficial for rhizosphere studies. Root cells have demonstrated cellular restructuring in reaction to chemical and mechanical stimuli produced by various

beneficial microorganisms [200]. Visualizing subcellular responses to root colonization will enable a more mechanistic understanding of beneficial plant-microbe interactions.

The seed planting method described in Chapter 2 is also unique among plant-in-chip studies in that it allows the seedling to germinate directly within the platform, eliminating potential damage to the root during transfer. Likewise, embedding the platform within a hydrogel matrix provided the plant with moisture and allowed the seedlings to be oriented vertically to encourage gravity directed growth. Low-resolution (10X magnification) imaging could also be conducted on the root directly through the Petri dish, without the need to remove the plant from its gnotobiotic environment and risk infection by potential ambient pathogens.

Finally, the procedure for deconstructing the microfluidic platform by unannealing the polymer from the glass coverslip demonstrated the multi-modal pairing capability of this platform. Using high-resolution non-optical imaging can provide valuable information about plant tissues that could not be obtained from optical imaging alone. Not only can this be used with SEM and AFM, as demonstrated herein, but for rhizosphere purposes, this capability can be used to probe chemical exchanges amongst organisms. For example, this deconstruction protocol can be used to sample root exudates and other exometabolites produced by the microorganisms through nano desorption electrospray ionization (nano-DESI) to map spatial chemical signals [201]. It could also be used for laser capture microdissection to harvest biological samples which can then be used for spatially-resolved proteomic or transcriptomic analysis [202].

5.3 Quantifying the Spatial Dynamics of Plant and Microbe Associations

The exploratory work of Chapter 3 highlights the effectiveness of a microfluidic habitat for quantifying features of plant root-microbe relationships and their associated

influence on plant phenotype during early development. By tracking and quantifying the colonization kinetics and spatial distribution of each bacterial species on the plant roots, individually and combined, we revealed specific spatio-temporal bacterial colonization patterns. We find that, regardless of inoculum concentration or species, bacterial cells cannot grow to cover more than 2% of the root's surface area, possibly because their growth is being limited, whether actively or passively, by the quantity of carbon exuded through the roots. Inoculum concentration did, however, affect the size of microcolonies formed on the root, with high-density inoculums forming the smallest microcolonies. This finding has practical applications in bioaugmentation; where adding high concentrations of bacteria to the soil may not lead to the largest colonies or strongest associations with the plant. Likewise, the "founder species" effect seen from adding both bacteria together to the roots could have practical applications for bioaugmentation and the study of invasive species. However, more research is necessary to determine when and why founder species obtain priority effects in the rhizosphere.

Correlated changes in root morphology over the course of the experiment confirm that the associated plant phenotype depends on the bacterial strain and inoculum concentration. By visualizing the physical interactions between defined microbial isolates and the plant roots, hypotheses regarding microbial colonization kinetics and preferential niche colonization were formulated.

5.4 Recreating the Structural Complexity of the Rhizosphere

Chapter 4 demonstrated that the natural heterogeneity of the rhizosphere can be incorporated into microfluidic designs and that this heterogeneity has implications for microbial phenotype in the laboratory. Not only is the platform heterogeneous, but we strove to retain realistic soil parameters as much as possible. For example,

we were able to use the shape statistics collected from natural sand grains and an anisotropic grain packing distribution that retains the connectivity of natural 3D soils. This micron-scale spatial heterogeneity, when coupled to gravity-driven flow, induced phenotypes in our bacterial strains that have not been otherwise observed in bulk liquid conditions. This evidence supports our argument that the heterogeneity a bacteria's natural environment needs to be retained in a laboratory setting.

By simulating the fluid hydrodynamics of the pore space using finite element analysis, we discover that the initial dispersal of bacterial species underground is strongly correlated to areas of high shear. This transport mechanism, also known as rheotaxis, has not previously been demonstrated to occur in a porous media environment and can help inform bacterial transport models which are currently based on colloid filtration theory. We also discovered that biofilm ability interacts with this pore-scale flow to influence the spatial distribution of cells across the entire network.

Although this chapter focused largely on bacterial transport through porous media in the rhizosphere and neglected to consider the presence of roots, it still has broader implications for rhizosphere ecology. For example, understanding the factors dictating bacterial transport (e.g. rheotaxis) will help inform the recruitment of microbial species to the plant's microbiome. It is currently understood that chemotaxis and rheotaxis are competing transport mechanisms, especially when the chemoattractant gradient is perpendicular to shear [187]. However, more research is necessary to determine how rheotaxis can affect microbial recruitment via chemotaxis in a porous media network. Similarly, understanding how the distribution of biofilm-producing cells emerges in the soil can inform how plants encounter these patches of clonal microorganisms as the roots elongate and grow.

5.5 Limitations of Microfluidics for Rhizosphere Research

While microfluidic platforms will never fully replace the need for *in situ* rhizosphere studies, micro-habitats do offer the rhizosphere ecology community a complementary suite of abilities, with more environmental control and definition than field or greenhouse studies but also more natural complexity than agar plates. These micro-habitats also offer a unique means to visualizing the dynamics and spatial organization of rhizosphere interactions at high-resolution.

However, micro-habitats also have their own set of limitations; an obvious limitation being that of size. Depending on the size and growth rate of the plant, microfluidic habitats can be used for no more than a few weeks to a month, after which the growth of the plant is severely constricted by the platform's dimensions. While this is an apparent limitation to studying full-sized plant systems, it does not prevent us from using micro-habitats to study the early stages of a plant's development. Because many important plant-microbe associations occur early on in a plant's life, such as founder species colonization, microfluidic size limitations are not as much of an issue for rhizosphere studies. In fact, since researchers have shown that the root's exudate composition changes with the plants development, we can use micro-habitats long enough to visualize the shift in exudate profiles from a seedling's germination to vegetative growth stage [51].

5.6 Areas for Future Study

This thesis demonstrated that the complexity of the rhizosphere can be added back to microfluidic habitats in a parameterize approach. Here, we were able to incorporate the structural heterogeneity of the rhizosphere, but many more factors can also be incorporated into microfluidic designs.

The chemical complexity of the soil, including mineral and soluble nutrients, has a strong influence over microbial "hot spots" underground [203]. Soluble chemical gradients could be incorporated into these micro-habitats with relative ease, by flowing the media through underlying gradient generator channels that are separated from the culturing channels by a membrane [204]. Similarly, advances in hydrogel 3D printing could create opportunity for creating solid-phase chemical heterogeneity. In this case, bacteria would be able to consume the organic soil grains themselves, creating a more realistic environment while still retaining transparency and imaging compatibility of the system. While the platform described here only replicates the 2D physical structure of natural sands, increasing dimensional complexity can be incorporated into the design using nanoscale 3D printing to fabricate a 3D soil replica.

With the toolkit that microfluidics and microfabrication offer, there are many opportunities to recreate the complexities of the underground environment to study rhizosphere interactions. It is my hope that this thesis will act as a foundation for rhizosphere-on-a-chip studies and that future work will continue to use cutting-edge technologies to probe biological interactions while maintaining a respect for the natural environment and its ability to shape ecosystem function.

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Appendix

Appendix A

Chapter 2 Protocol

A.1 Two-Layer Platform Fabrication

A.1.1 Fabrication of multilayer masters

1.1.1) Spin coat epoxy-based negative photoresist (63.45% solids, 1250 cSt) according to the manufacturer's specifications (2000 rpm for 45 seconds) onto a 4 inch diameter silicon wafer to obtain the desired height of 20 μm for the first design layer.

1.1.2) Soft-bake the resist coated wafers for 4 minutes at 95°C. Allow wafer to cool for 5 minutes. Expose the wafer to UV light for 15 seconds (150mJ/cm² at 365nm) through a photomask in a UV contact aligner to define the geometry of the bottom layer.

1.1.3) Post-exposure bake the wafer for 5 minutes at 95°C. Without developing, return the wafer to the spin coater and spin on a second layer of epoxy-based negative photoresist (76.75% solids, 80000 cSt) at 3000 rpm to achieve a second layer height of 150-200 μm .

1.1.4) Allow the wafer to rest for 5 minutes before transferring to a 95hotplate for 45 minutes. After baking on the hotplate, remove the wafer and allow the resist to cool and harden at room temperature for another 5 minutes on a flat level surface.

Align and expose the wafer to the second layer photomask for 30 seconds in a UV contact aligner (dose of approximately 300 mJ/cm²).

1.1.5) Perform a post-exposure bake by placing the wafer on a hotplate for 15 minutes at 95°C, then allow wafer to cool on a flat level surface for 5 minutes before developing.

1.1.6) Develop both resist layers simultaneously in appropriate developer (see table of materials) for 17 minutes in a plastic dish. Gently rock the dish occasionally to wash fresh developer over the wafer. After 17 minutes, rinse the wafer with isopropanol (IPA). If a white film appears, continue to iterate between rinsing the wafer with developer and IPA until the film disappears. Dry the patterned master with nitrogen.

A.1.2 Polydimethylsiloxane Soft-lithography

1.2.1) Expose the patterned master to an air plasma for 30 seconds in a plasma cleaner set on High (see table of materials) to clean. Silanize the wafer with trichloro(1H,1H,2H,2H-perfluoro-n-octyl)silane in a chemical hood on a hotplate below the flashpoint of the silane (85°C) for 2 hours.

1.2.2) Pour a 10:1 ratio of polydimethylsiloxane (PDMS) to curing agent onto the silicon master. Degas the mixed PDMS in a vacuum chamber then cure the polymer in a 70°C oven for one hour.

1.2.3) Using a scalpel, cut the PDMS devices and peel them from the master. Use a 1.5mm biopsy punch to create seed and treatment inlets, punching the seed inlet at a 45° angle to encourage root growth into the main channel.

1.2.4) Use clear adhesive tape to remove debris from the PDMS device and place the device design side down on a glass coverslip. Autoclave the assembled devices.

A.2 Planting Devices

A.2.1 *Arabidopsis thaliana* seed preparation

2.1.1) Surface sterilize *A. thaliana* seeds in a microfuge tube with a solution of 30% bleach and 0.1% TritonX detergent for 7 minutes. Wash seeds 4 times with sterile water.

2.1.2) Stratify seeds to synchronize germination by refrigerating microfuge tube overnight at 4°C . Note: Seeds may be kept at 4°C for up to a week.

A.2.2 Device Preparation

2.2.1) Place sterilized devices in a vacuum degassing chamber to remove air from the gas permeable PDMS and fluidic channels.

2.2.2) Remove the devices from the vacuum chamber and immediately submerge the devices in a Petri dish filled with liquid quarter strength Murashige-Skoog plant based media media at pH 5.7.

2.2.3) Using a pipette, pull liquid through the inlets to fill the device. Make sure no air bubbles remain within channels by visual inspection.

2.2.4) Transfer individual devices to new dry Petri dishes. Pour or pipette hot agar around the device until the agar level is almost flush with the top of the PDMS device. Allow agar to solidify, devices are now ready for planting seeds or can be stored at 4°C until needed.

A.2.3 Planting seeds within devices

2.3.1) In a sterile environment, transfer stratified seeds to the inlet of the devices using a small pipette.

2.3.2) Cover the Petri dish with wax film (see table of materials) and place in a light/dark cycling growth chamber or windowsill at room temperature. Be sure to

orient the Petri dish so that the devices are vertical and gravity will encourage the roots to grow through the channel.

A.3 Treatment

3.1 Experimental Treatments

3.1.1) At the desired time in the seedling's development, add the experimental treatment to the seedling by adding a prescribed amount of the treatment to each of the 8 side ports via pipette.

3.1.2) Reseal the Petri dish and return to growth chamber or windowsill.

3.1.3) Proceed with imaging samples at the desired time to capture individual time points or begin time lapse imaging.

A.4 Optical Imaging

A.4.1 Lower Resolution (4-20x) Imaging

4.1.1) Place Petri dish containing device and seedling under an inverted bright field microscope for lower resolution bright field imaging. After optimizing lighting conditions to elucidate key morphological features; seedling orientation, root growth and root hair morphology can be assessed.

4.1.2) Return Petri dish and seedlings to growth chamber or windowsill after imaging is complete.

A.4.2 Higher Resolution (20-63x) Imaging

4.2.1) Once the seedling has grown for a desired time, use forceps to remove coverslip and device from the agar. Clean off the bottom of the coverslip using an ethanol wetted laboratory tissue.

4.2.2) Lower magnification (10-20x) bright field and fluorescent images can be taken to analyze the root architecture and root hair morphology. Time lapse imaging can be performed by taking one image per minute to analyze root and root hair growth, as well as responses to treatments.

4.2.3) Image the root or root hairs at higher magnification with a reduced time delay between images using a 63x oil objective to analyze intracellular dynamics. For longer imaging times, use moist laboratory tissue or a cell culture chamber to keep the device and seedling hydrated.

A.5 Non-Optical Imaging

A.5.1 Device preparation

5.1.1) Once seedling has grown for desired time, remove the device and coverslip from the Petri dish.

5.1.2) Turn device upside down and gently peel away the coverslip. The root should stay within the PDMS channel

5.1.3) Proceed to use the PDMS device as a substrate for the root in higher resolution atomic force or scanning electron microscopy.

A.5.2 Scanning Electron Microscopy

5.2.1) Deposit a thin (20 nm) layer of chromium onto the root and surrounding PDMS using a Dual Gun Electron Beam Evaporation Chamber

5.2.2) Transfer the root and PDMS device to a scanning electron microscope chamber.

A.5.3 Atomic Force Microscopy (AFM)

5.3.1) Mount the PDMS device root side up on an AFM specimen holder. Note: To increase contrast in the camera and more easily distinguish the root from the PDMS,

the PDMS device may be placed on a piece of mica that has a reflective coating before mounting the device on the AFM specimen holder.

5.3.2) Secure a liquid well attachment on top of the PDMS device and fill with water to keep the roots hydrated during imaging.

5.3.3) Load the specimen holder into the AFM. Adjust the z-control for the thickness of the PDMS device. For best results with contact mode imaging, cantilevers with spring constants of 0.01 or 0.03 N/m were used to exert minimal force on the root during scanning.

Vita

Jayde Aufrecht was born in North Platte, Nebraska to Stacie and Ronald Aufrecht. Growing up, her parents strongly encouraged her to pursue STEM courses and extracurricular activities. Jayde was first introduced to scientific research in highschool through a summer research program for undergraduates in theoretical ecology (RUTE) at the University of Nebraska, Lincoln. She continued to engage in research in Dr. Rizia Bardhan's Nanophotonics and Nanomedicine Lab while obtaining her Bachelor's degree in the Chemical and Biomolecular Engineering department at Vanderbilt University. Before attending graduate school, Jayde interned with Dr. Frank Löffler at Oak Ridge National Laboratory, where she expanded her research breadth to include microbiology. She concurrently earned an M.S. degree in Environmental Engineering while working toward her Ph.D. in Energy Science and Engineering through the Bredesen Center for Interdisciplinary Research at the University of Tennessee. Jayde conducted her Ph.D. research at Oak Ridge National Laboratory in the Biological and Nanoscale Systems group under the mentorship of Dr. Scott Retterer. After graduation, Jayde will continue her passion for research as a Linus Pauling Distinguished Postdoctoral Fellow at Pacific Northwest National Laboratory.