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Johnny Bui April 10, 2024

Biofilms on a Bead: A Novel Model System to Probe Plant-Microbe Interactions

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

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Abstract

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The rhizosphere is a complex ecosystem where various plants, fungi, viruses, and bacteria interact. *Striga asiatica* has been shown to have a spatiotemporal dependence on hydroquinone for germination. However, the precise reactions of different bacterial species to root exudate in the rhizosphere are not yet fully understood. Biofilms are architecturally complex communities of bacteria that provide mutualistic benefits. The defined diffusion range of hydroquinone, a major component of root exudate, makes it difficult to study the interaction between bacterial biofilms and root exudate using simple streaking techniques. In addition, spatiotemporally controlling a biofilm akin to manipulating a *Striga* seed would be near impossible without a new way of utilizing biofilms.

In this study, we developed a novel biofilm model system of *Pseudomonas chlororaphis* and *Agrobacterium tumefaciens* on a bead to investigate how they react to root exudate. This biofilm model system successfully propagated and showed robustness and adaptability to extreme environmental stress. We used *P. chlororaphis*' phenazine production and *A. tumefaciens*' GFP fluorescence in reaction to reactive oxidative species to observe the biofilm's and individual species' reactions.

Our results showed that *P. chlororaphis* wild-type grown with *A. tumefaciens* with a *mexE* gene deletion (more sensitive to pyocyanin: *P. chlororaphis*'s phenazine) exhibited higher fluorescence intensity than the combination with P*. chlororaphis* without phenazine production, demonstrating the potential of the biofilm-on-a-bead model. While conducting the studies for the model, we learned of the redundancy of the phenazine pathway in *Pseudomonas* chlororaphis, emphasizing its importance in the bacteria and encouraging us in the future to take a different approach to its pathway deletion.

To expand our findings, we plan to perform additional assays to gather more definitive data and test the bead with sorghum roots to investigate how the bacteria react to reactive oxidative species caused by the presence of both hydroquinone and phenazines. Successful completion of the biofilm-on-a-bead model will aid in understanding the complex interactions that occur between bacterial biofilms and root exudate in the rhizosphere.

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I also want to express my deepest thanks to Dr. Lynn for being a fantastic mentor and showing me that research is not only about running experiments but thinking why, too. Had it not been for Dr. Lynn, my research career would've continued in the BCDB, but it was fate to approach biochemistry from the chemistry department's view. Science isn't always about the ideal and what we want to see. The beauty in science should be the results and the questions we form to observe those results. No one would have taught me to see science that way without you.

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Lastly, thank you to my family and loved ones for being my rock during my four years at college. At my lowest points during my undergraduate career, I could always give a call and feel like everything was okay. That, to me, is worth more than anything I could ever dream of. Thank you.

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Chapter 1: Introduction

1.1 Biofilms and Their Nature

Despite their presence in much of the world, biofilms are seldom acknowledged for their true value. While biofilms have been a sore point for the medical sphere, for rhizosphere research, biofilms are as important as the plant roots that inhabit the same ecosystem, hence the necessity to begin deeper studies on their roles in the rhizosphere[1]. Biofilms are colonies of microorganisms that form architecturally complex communities[2]. Biofilms seemingly occur everywhere throughout nature, and from medical devices to industrial machines[2]. Some infections in the body are also linked to biofilm formation on human surfaces like the teeth, skin, or urinary tract[2]. Biofilms can also form on the hulls of ships and inside pipes, causing severe issues[2]. However, not all biofilms are harmful[2]. For example, dental plaques with biofilms of beneficial species deter colonization by competitive species[2]. Biofilms in nature often form mutualistic symbioses. *Actinobacteria* often grow on ants, which allows them to curate pathogen-free fungal gardens[2]. Given biofilms' versatility and resilience to thrive in numerous circumstances, the desire to study and better understand them becomes increasingly important in the face of climate change.

In ecology, competition and synergies between species define community structure and activity[3]. The stable coexistence of numerous organisms in communities is balanced by individual tradeoffs and optimization of strategies for limited resources[3]. Outside of a laboratory setting, microorganisms typically coexist in multicellular communities[3]. The coexistence of the microorganisms means they are optimizing access to nutritional resources similar to those of other organisms[3]. Competitive fitness is achieved by focusing on a suitable or specialized dietary niche[3]. Motility provides a system where microbes can continually reposition themselves and adapt to changing nutritional and physical conditions[3].

Bacteria can also secrete antimicrobial compounds that kill or impair other species in the same niche[3]. The competition mainly occurs when local microbial densities are high, like biofilm communities[3]. At such high densities, quorum sensing controls the coordination and reinforcement of community behaviors in many bacterial species[3]. Biofilm formation and quorum sensing are two bacterial community behaviors that have significant potential to influence multispecies interactions[3]. Motility, quorum sensing, and biofilm formation are mechanisms bacteria use to compete and exist within microbial communities[3].

Bacteria that form biofilms obtain numerous benefits. One benefit is resistance to many antimicrobials, protection from protozoan grazing, and protection against host defenses[4]. A

theorized reason for the increased resistance to the environmental stress observed in biofilm cells is the increase in the portion of persister cells within the biofilm[2]. Persister cells are resistant to many antibiotics and are nondividing[2]. These persister cells have been proposed to be protected from antibiotics because they express toxin-antitoxin systems where the toxin modules block the target of antibiotics[2].). In addition to persister cells, an extracellular matrix protects constituent cells from external pressure[2]. Extracellular matrices act as a diffusion barrier to small molecules[4]. The diffusion of nutrients, vitamins, or cofactors is slower, resulting in a bacterial community in which some cells are metabolically inactive[2]. The bacterial growth rate is influenced by the cells within a biofilm confined to a limited space[4]. This condition is like the stationary phase observed in laboratory growth conditions[2]. Biofilm formation can then be seen as representing the natural stationary phase of bacterial growth[2].

During the stationary phase, bacteria change their physiology by increasing the production of secondary metabolites like antibiotics, pigments, and other small molecules[2]. These secondary metabolites function as signaling molecules that start the process of biofilm formation or inhibit biofilm formation by other organisms sharing the same habitat[2]. The secondary metabolites and their role as signaling molecules during biofilm formation are very similar to the macro-scale implementation of the project. *Agrobacterium tumefaciens* and *Pseudomonas chlororaphis* interact with plant roots in a spatio-temporal manner, and we want to study them by using a biofilm containing each of these species.

1.2 Biofilms as a Model for Natural Coexisting Bacteria in the Rhizosphere and Related Plants

As stated before, biofilms are architecturally complex communities of bacteria. For the project, *A. tumefaciens* and *P. chlororaphis* will represent bacteria interacting with plant roots in the rhizosphere, thus enabling an electron transport chain otherwise impossible without the delicate system formed by the three species[5]. However, our first part of the project will not include probing plant roots as the goal is to develop a reliable model using the biofilm. The rhizosphere is the soil region where the plant roots reside[5]. In this layer of soil, many bacteria and fungi coexist to enable numerous chemical reactions that allow for the combined survival of the plant, mutualistic bacteria, and fungi[5]. We ultimately aim to explore the chemical gradient released by grasses, specifically the roots of *Sorghum bicolor* (Sorghum), as a model organism previously used to explore *Striga asiatica's* germination capabilities. Dr. Taran from the Lynn Lab made a graphic to illustrate the potential redox reactions between benzoquinones and phenazines (Figure 1)[5]. But before we can probe the three-species system, developing the biofilm on a bead model is paramount to studying the biofilm's behavior. Without it, exploring the specifics of the spatial orientation at a given time point would be extremely difficult.

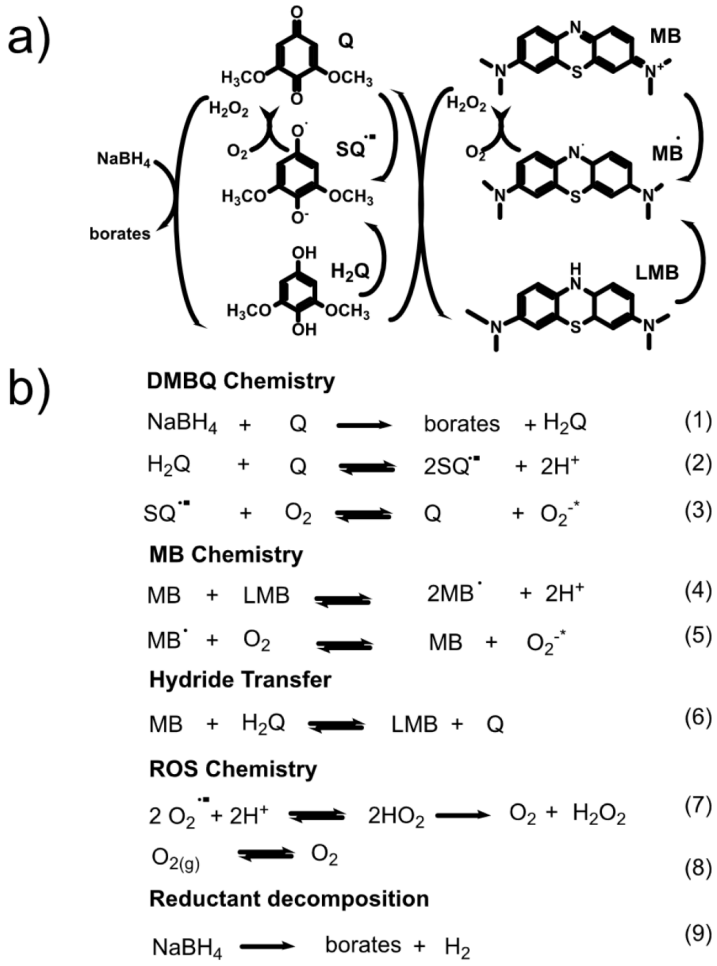

Figure 1. (*A*) Phenazines and benzoquinones form the redox reaction network in the presence of $NaBH_4$ and O_2. (*B*) The simplified reaction mechanism between the two molecules.

S. asiatica is a hemiparasite requiring host root attachment for survival[6]. In agar, no germination was observed at distances greater than one centimeter from the sorghum host root surface[7]. Dr. Taran reasoned this to be because of Sorghum Xenognosin (SXSg), which autooxidizes into 2-hydroxy-5-methoxy-3-p-hydroquinone, being exuded by sorghum roots[5]. It is also known that the electron-rich hydroquinones accumulate at high concentrations along the monocot host's root surfaces[6]. The compound was chemically imaged with the pigment methylene blue[5]. The reaction-diffusion phenomena with the phenazine and benzoquinone system can be seen in Dr. Taran's images (Figure 2)[5].

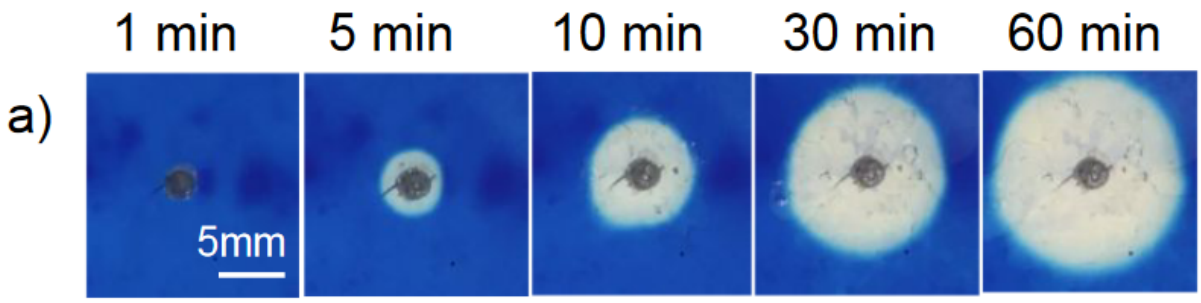

Figure 2. (*A*) Time-dependent propagation of the reducing font (no color) in DMBQ; the methylene blue mixture (blue) is in a thin layer of the agarose gel.

The roots also produce oxygen, allowing the resulting autoxidation to create a reaction-diffusion gradient of the hydroquinone as the resulting quinone has no germination activity[7]. Striga seeds required a minimal concentration (ED_{50}) and an exposure time of $t^{1/2}$ to the active hydroquinone, defining the area where the seeds germinate for adequate growth and attachment to the host through a process known as xenognosis[7].

The exudation and the inherent instability of the compound established an apparent steady-state concentration gradient of the germination stimulant around the sorghum root[7]. This requirement for long-term exposure to a steady state concentration of the compound led to insight into signal detection and host commitment in *S. asiatica's* parasitism[7].

We propose that the same process is spatiotemporally activating the soil's hot spots (biofilms). The activation event for bacteria may be deoxygenation caused by electron transfer from the hydroquinone via the phenazine (naturally released by bacteria) to oxygen or something more complex that couples the bacteria with neighboring fungi. We are working on possible mechanisms to test, but we know that oxygen is produced by photosynthesis and then released from the roots. It is important to remember that the germination and haustoria development outlined here are separate. However, they may be at least functionally connected and provide distinct mechanisms and developmental transitions.

P. chlororaphis colonies or hotspots produce phenazines that open one-electron chemistry to reduce O_2 to complex reactive oxygen species (ROS)[8]. This electron transport chain deoxygenates the local environment, diverting electrons to other terminal acceptors and activating the hot spots as it activates Striga seed germination[8]. Dr. Fuller made a figure demonstrating another semagenesis aspect of this complex electron transport chain (Figure 3[7].

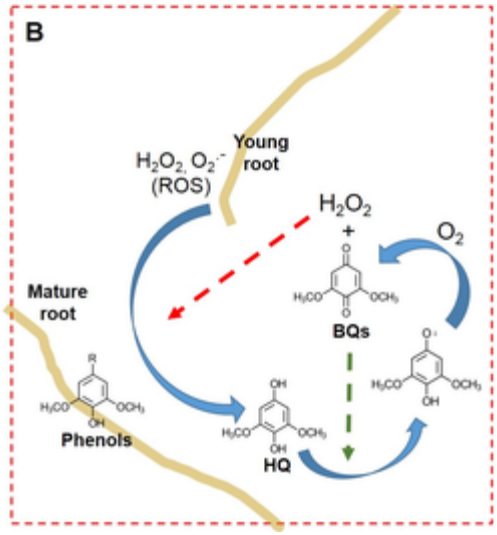

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Figure 3. (*B*) At a certain distance, ROS produced by the tip of a growing seedling promotes oxidation of cell wall-associated phenols to yield benzoquinones. DMBQ is shown as an example. The benzoquinones saturate and contribute to the autocatalytic production of different ROS intermediates.

1.3 Exploring Germination and Semagenesis to Model the Reaction-Diffusion Gradient

Para-quinones are naturally produced by plants[7]. *S. asiatica* needs exposure and the proper concentration of para-quinones for activation[7]. The gradient of para-quinones allows *S. asiatica* to germinate when presented with ideal conditions[7]. Hydroquinones induce haustoria formation for *S. asiatica*[7]. In the concentration gradient, reductions activate germination, an activation process[7]. Oxidations induce haustoria formation through semagenesis, an active environmental screening process by the parasite[7]. *P. chlororaphis* produces phenazine, which reacts with hydroquinones in a way that deoxygenates the environment[7]. We hypothesize that this, too, is an active screening process by *P. chlororaphis*. Pseudomonas hot spots in the soil may use these reduced phenazines to activate growth in response to plant-derived resources. The previously described concentration gradient has a figure produced by Dr. Fuller (Figure 4)[7]. In it, the proposed role of Ca^{2+} in root recognition is explored.

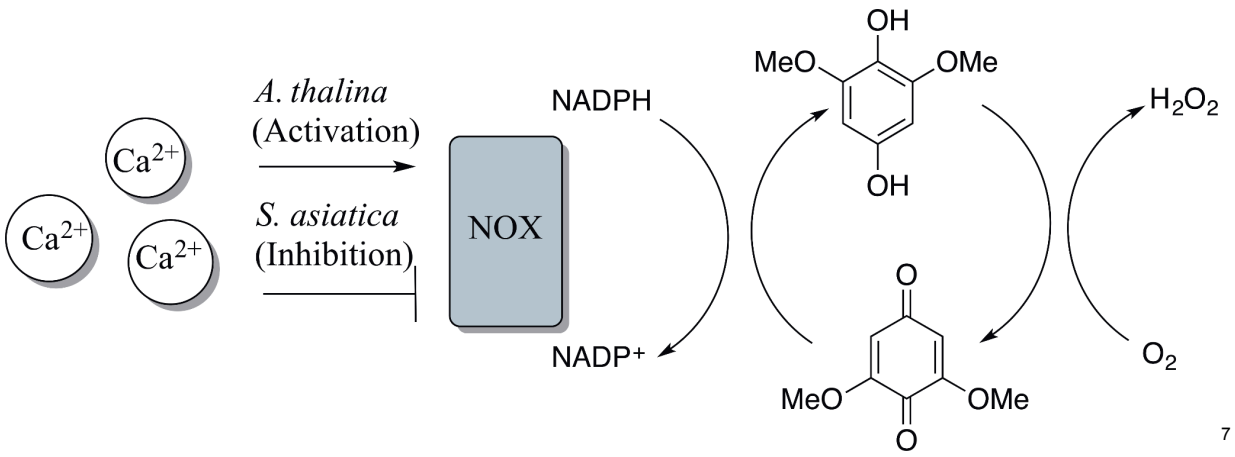

Figure 4. DMBQ reduction by a plant's NADPH-oxidase is necessary for H_2O_2 production via redox cycling. Ca^{2+}-assisted NADPH down-regulation of NADPH oxidase in *Striga* can lead to decreased H_2O_2 production. In contrast, up-regulation of the enzyme in *A. thaliana* leads to a rise in ROS concentrations, ultimately perceived as a signal to shut down root growth.

The plant also seeks nutrient hotspots, and there is evidence that hydroquinones serve as quorum-sensing agents for other plants[7]. This dynamic systems chemistry seems to play a critical role in the rhizosphere.

1.4 The History of *Agrobacterium tumefaciens* and *Pseudomonas chlororaphis* and Their Scientific Uses

A. tumefaciens was chosen for this study because it is a prokaryotic bacterial pathogen that infects dicotyledonous plant hosts. It is a facultative pathogen and is only virulent when carrying the tumor-inducing (pTi) plasmid[6]. The virulence of the Gram-negative *A. tumefaciens* relies on transforming eukaryotic host cells into crown gall tumors[6]. Dr. Liu from the Lynn Lab produced a graphic showing how *A. tumefaciens* would activate and begin tumorigenesis (Figure 5)[8]. In addition to its natural abilities, a construct of it was created by Dr. Andy Binns that makes *A. tumefaciens* more sensitive to ROS and would be integral to *A. tumefacien's* role in the project as a reporter.

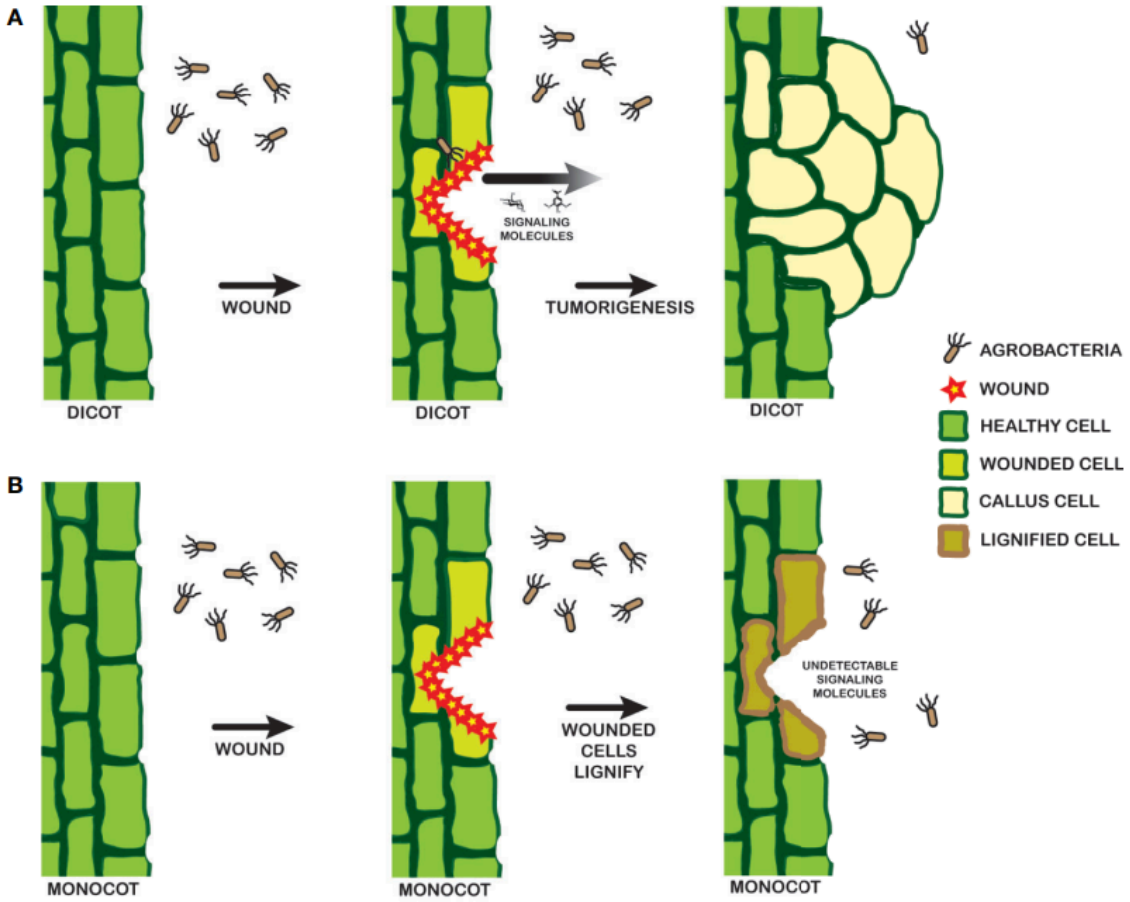

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Figure 5. (*A*) For dicots, wound events release signals into the rhizosphere recognized by *Agrobacterium*. *Agrobacterium* then begins chemotaxis, leading to DNA transfer and tumorigenesis. (*B*) In monocots, wounds lead to lignification in the cell wall and a lack of released strong signals to be recognized by *Agrobacterium*.

P. chlororaphis has been under particular study because of its capability of quorum sensing[9]. Quorum sensing (QS) is a prominent mechanism of intercellular signaling among bacteria and is often discussed when biofilms are mentioned[9]. Gram-negative and Gram-positive bacteria use small molecular weight QS signals to control target genes' transcription based on the signal amount present[9]. This communication is known as “quorum sensing” because the signal strength is related to signal producers' density or “quorum.”[9]

QS influences different aspects of biofilm development and maturation[9]. In scientific studies, certain QS-regulated factors have been shown to play critical roles in surface adherence, aggregation, and dispersal[9]. The dispersal includes structural components of flagella, type IV pili, polysaccharide biosynthesis, protease, and rhamnolipids[9]. QS is a global regulatory system affecting the expression of multiple genes, and the precise nature of the role of QS in biofilm cultivation is still being studied[9]. *P. chlororaphis* has defined QS regulation of phenazine

production[9]. Because of this defined QS regulation, *P. chlororaphis* provides the potential for exploring the spatio-temporal aspects of the delicate electron transport chain formed by Sorghum bicolor, *P. chlororaphis*, and *A. tumefaciens*.

1.5 Forming Biofilms With Co-cultivated Species

External factors influencing bacterial biofilm formation have been demonstrated through the dual-species system of *P. aeruginosa* and *A. tumefaciens*[3]. *P. aeruginosa* (and *P. chlororaphis*) and *A. tumefaciens* can be isolated from the same environment since they coexist in freshwater, bulk soil, and the rhizosphere[3].

The main concern is that *P. aeruginosa* (and perhaps *P. chlororaphis*) manifests a significant competitive advantage over *A. tumefaciens* through its rapid growth rate in laboratory conditions[3]. *P. aeruginosa* forms flat, tightly packed biofilms with complete homogeneity in succinate-based minimal media[3]. *A. tumefaciens* forms loosely packed biofilms with significant architectural heterogeneity compared with *P. aeruginosa* biofilms[3]. The two different biofilms lead to a "blanketing" effect in which *P. aeruginosa* eventually covers *A. tumefaciens* when cultivated in a biofilm-promoting environment[3]. The "blanket" effect leads to *P. aeruginosa* forming throughout the biofilm and small amounts of *A. tumefaciens* forming on the glass of the tubes[3]. It's worth noting that before blanketing, the amount of *A. tumefaciens* biofilm biomass was similar to that in pure cultures at equivalent stages of cloth[3]. Blanketing occurs around 48-72 hours[3]. However, *P. aeruginosa* added to pure *A. tumefaciens* leads to significant *P. aeruginosa* colonization but not complete blanketing[3].

1.6 Hypothesis

Plant root tips produce hydrogen peroxide, and *A. tumefaciens* targets wound sites in dicot plants that also produce hydrogen peroxide[8]. Given this ability to survive in ROS-producing environments, *A. tumefaciens* was engineered to report on ROS production by cloning GFP behind the sodBII promoter. *P. chlororaphis* wild-type was also engineered with a knockout of a key amine addition step in phenazine-production to create a non-phenazine-producing *P. chlororaphis* strain, Δ*phzE*.

The current goal is to produce a biofilm on a bead of *P. chlororaphis and A. tumefaciens*. Inspired by the parasitic plant *S. asiatica* that lies dormant until it senses a host and begins germination, the biofilm will be used to mimic the behavior of *S. asiatica* to probe the spatiotemporal response of the reaction-diffusion system[5].

Looking at the long-term benefits of the research, we know there must be some benefit for the plants or grasses in general. A critical feedback possibility is that reactive oxygen species have

been shown to activate lateral root formation in plants[8]. The phenazines may be critical for their generation of ROS as evidence for hot spots. The anaerobic environment may liberate other elements from the soil (nitrogen, sulfur, phosphorus, iron, copper, etc.). This systems chemistry develops into a beautiful model for understanding the complex plant and microbial community that allows for spatiotemporal ordering in the external organ known as the rhizosphere. We are working on potential mechanisms to propose alongside the co-cultivation of *P. chlororaphis* and *A. tumefaciens* to map this chemical signaling network.

We hypothesize that by having a *P. chlororaphis* WT and *P. chlororaphis* Δ*phzE* (no phenazine production), we can have *A. tumefaciens* report the presence of reactive oxidative species as its GFP fluoresces when in the presence of pyocyanin (*P. chlororaphis*'s phenazine). In addition, there should be a quantitative difference between the Δ*phzE* and WT, where WT should have a higher mean fluorescence intensity (GFP activated). After producing a successful biofilm, the biofilm bead would be placed in a pattern around various plant roots like maize or sorghum to study and model the redox system produced by the interactions between *P. chlororaphis* and the plant root. This would create a ROS-rich environment, trigger *A. tumefaciens,* and allow us to follow the behavior of the bioluminescent *P. chlororaphis* WT and *P. chlororaphis* Δ*phzE*. We predict that *P. chlororaphis* will either migrate toward the plant root and/or change its metabolic profile in search of alternative terminal electron acceptors.

We have learned the importance of biofilms in nature by reviewing the prior literature and the potential benefits of their use as a model system. Because of this, there lies potential in pairing biofilms in a study with plant root exudate or its synthetic analogs to understand further the complex relationship between plants and bacteria in the rhizosphere. *Agrobacterium* and *Pseudomonas* are fantastic model organisms, given their natural origins, roles within the rhizosphere, and ability to coexist. Because of their potential together, research has already shown the limits of the growth when both are used in a co-cultivated biofilm. A workaround was proposed in the same paper and implemented into our experiments going forward. We also learned the importance of hotspots, rich with redox activity, in the rhizosphere.

Co-cultivating the two species and forming a biofilm would give a variable closer to natural settings and allow for more accurate observations. With hotspots in mind, the need to control the spatiotemporal element of the biofilm informs a critical ordering element in nature. Cultivating mixed assemblies on a bead and being able to choose its starting point in the experiment removes the element of chance a random streak on a plate might introduce. Therefore, this project aims to produce a biofilm of *P. chlororaphis* and *A. tumefaciens* to develop a biofilm on a bead to have a model capable of probing spatiotemporal intricacies and pave the path for defining the systems chemistry dynamics.

Chapter 2: Materials and Methods

2.1 *Agrobacterium*'s Plasmid Construction and *Pseudomonas*'s Construct Design

One bacterial species used is *Agrobacterium tumefaciens*, which has a mexE gene deletion but still carries the pTiA6 plasmid[10]. The Δ*mexE* (deletion of the *mexE* component of the *mexE*/*mexF* ABC transporter) makes *A. tumefaciens* more sensitive to pyocyanin[11]. The other species is *Pseudomonas chlororaphis*. *P. chlororaphis* had a lux gene inserted into its genome via transposons downstream of *glmS*. More specifically, *pTN7PA143Lux* was inserted. TN7 is a bacterial transposon, and Tn7L and Tn7R borders (ends of the transposon) were integrated downstream of the *P. chlororaphis glmS* gene.

2.2 Making a Biofilm on a Bead

Much of the following protocol is inspired by the paper made by Dr. Müsken[12]. The evening before the inoculation of the 96-well plate, 5 mL overnights of the two species were grown in Luria Broth with Kanamycin (50 mg/mL) at 28°C and put on a shaker (180 rpm) for 18 hours. The following morning, a sterile 96-well plate had its wells labeled for control wells (no bacteria), WT *P. chlororaphis*-only wells, Δ*phzE P. chlororaphis*-only, *A. tumefaciens*-only wells, PCWT+AT wells, and PCΔ*phzE*+AT wells. *P. chlororaphis* was diluted to an OD_{600} of 0.02. *A. tumefaciens* was diluted to an OD_{600} of 0.20 for their respective wells. Each well had a final volume of 200 µL.

Beads and tweezers were sterilized with ethanol before use. The beads were then placed into a well and repeated until all wells had a bead. To ensure local humidity within the 96-well plate, sterile water was added to vacant wells. Once the plate was prepared, the lid was reseated and transferred to an incubator to let the cultures grow for three days (72 hours) at 28°C. A tub of water was also put into the incubator to ensure overall humidity in the environment. After three days of growth, the 96-well plate was removed from the incubator to prepare Day 0 plates. First, two LB+KAN (50 mg/mL) plates and 2 Agar+Water+KAN (50 mg/mL) plates were placed near a Bunsen burner to remove excess condensation. After labeling the plates, beads were dipped into sterile water using a sterilized tweezer to wash them briefly[13]. This ensures that the bacteria on the bead are in a biofilm instead of free-floating bacteria. Beads were transferred from the well onto their plates until all beads were transferred. The Day 0 plates were incubated at 28°C and allowed to grow for 24 hours. The Agar+Water+KAN plates were put into an incubator at 28°C and allowed to grow for one day, two days, and seven days to observe biofilm resiliency.

2.3 Imaging the Biofilm Beads

Initially, plates were imaged with an iPhone 14 Pro Max camera. Then, the plates were taken to the Emory ICI core to image the plates with their BioTek Lionheart FX Inverted Widefield microscope. The GFP filter cube used automatically filters out luminescence. Lux emission peaks at 490nm while the GFP filter cube observes at 505-550 nm. After acquiring pictures with the microscope, the images were processed through ImageJ for further analysis.

Chapter 3: Results and Discussion

The first part of the project was to figure out how to make a biofilm on a bead. The initial experiments began with figuring out the conditions to co-cultivate the *P. chlororaphis* and *A. tumefaciens,* as both have specific conditions for optimal growth. After studying previous works from the Lynn Lab and from Dr. Andy Binns, it was found that 28°C would likely be the best temperature for both species. After this confirmation, standard inoculation of the overnights began, and the protocol outlined in the “methods” section was performed. The following results were gathered.

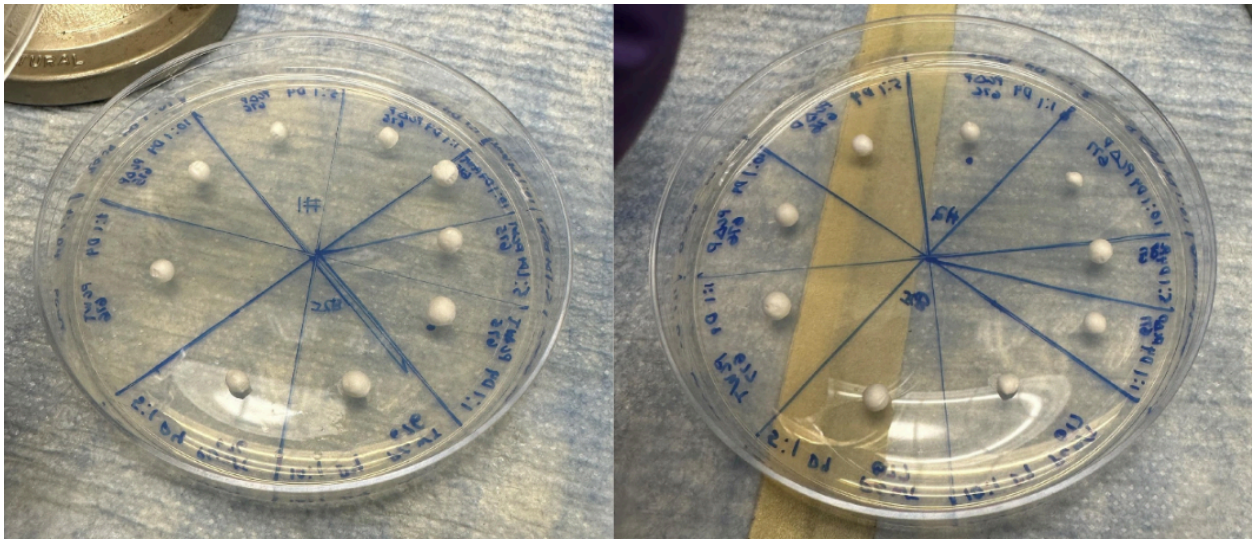

Figure 6. Day 4 Dormant Beads (No nutrients in the agar)

It was observed that after up to a week, the biofilm beads were remarkably able to maintain a dormant state and not starve, similar to their inspiration: *S. asiatica*. The image above exemplifies our labeling strategy and how they looked on Day 4 of biofilm induction through starvation (Figure 6).

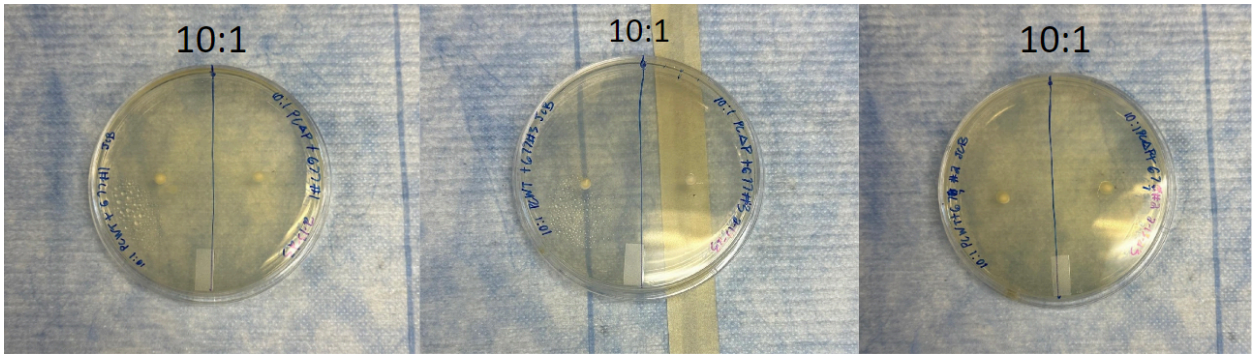

Figure 7. Day 0 Triplicate Beads

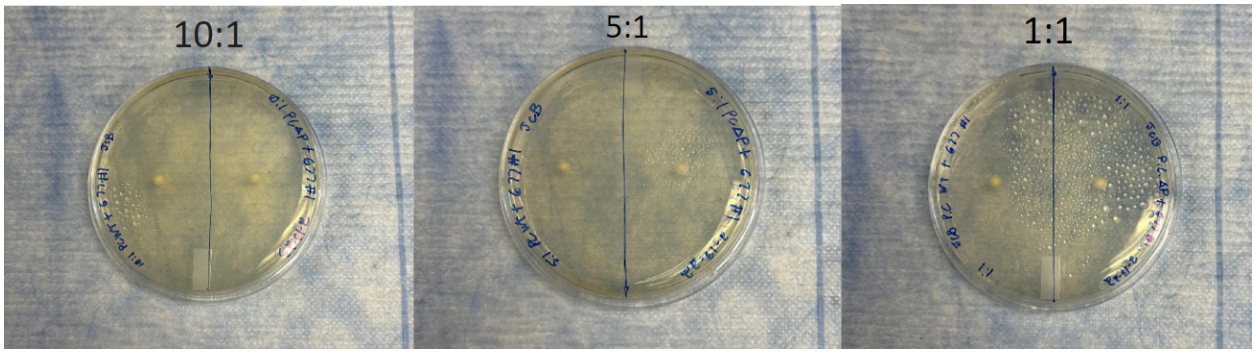

Figure 8. Day 0 Dilution Beads

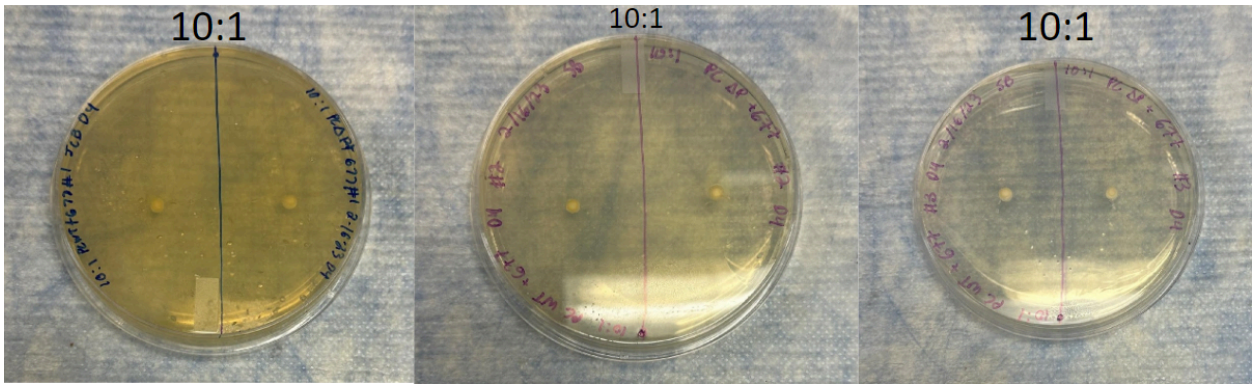

Figure 9. Day 4 Triplicate Beads

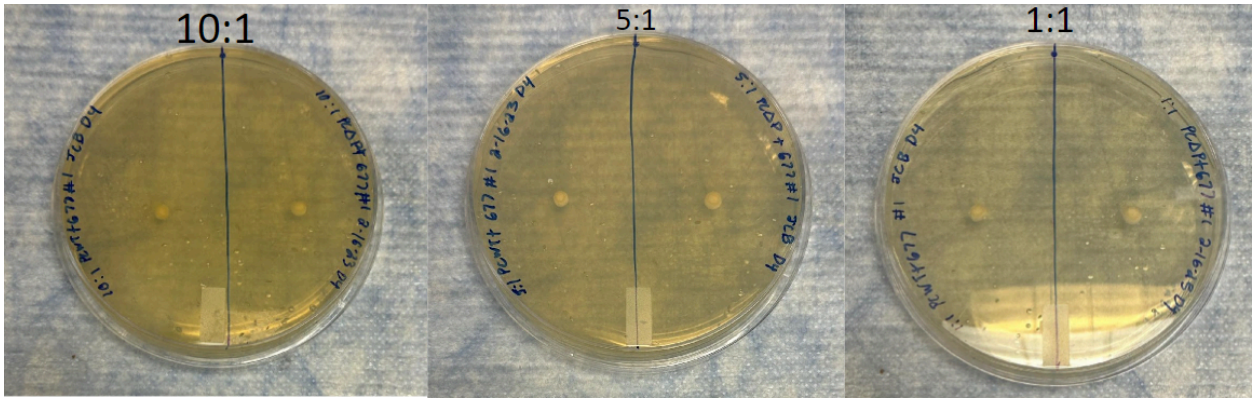

Figure 10. Day 4 Dilution Beads

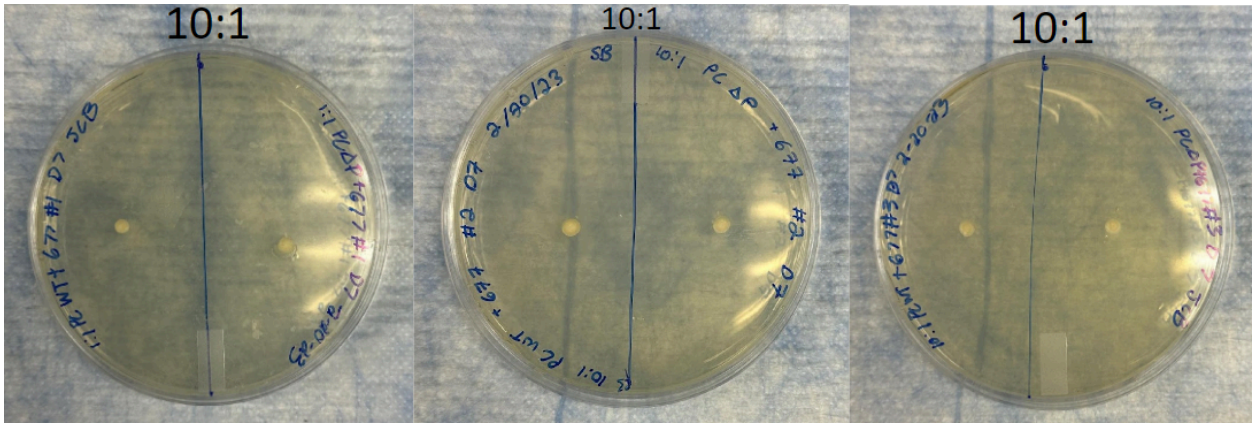

Figure 11. Day 7 Triplicate Beads

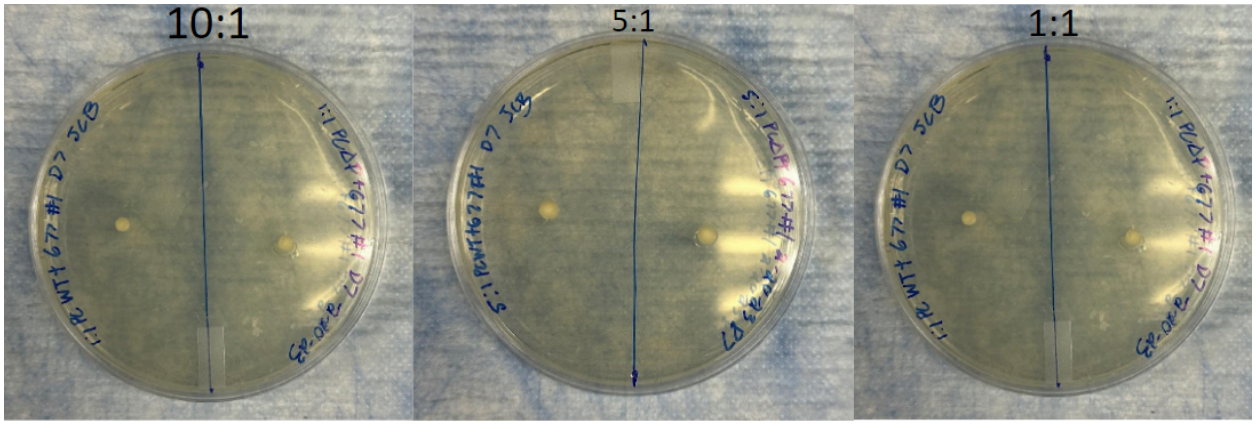

Figure 12. Day 7 Dilution Beads

Figures 7-12 yield invaluable insight into the co-cultivation of the two species. We performed different dilutions to see which would be best for growth and a triplicate of the supposed ideal (10:1) to ensure our results were reproducible. Without fail, on Day 0 (immediate transfer from 96-well plates), the colonies propagated without a problem. More times were tested, like Day 4

and Day 7, to ensure that biofilms that underwent starvation for biofilm induction could still "activate" in response to a change in environmental conditions (reintroduction to nutrients). Again, without fail, even a week after starvation, the beads repopulated LB+KAN plates without issue. Because of the wash step, we were confident that what repopulated the plates were primarily bacteria associated with a biofilm, as most free-floating bacteria would have been removed during the wash step. With these results, we moved on to preparing samples to take to the Emory ICI for imaging up a fluorescence microscope.

D4 PCΔP+AT			
	Glass #1	Glass #2	Sty #1
Min	3424.000	4032.000	5920.000
Max	16128.000	19712.000	33568.000
Mean	11417.478	13206.227	20547.660
STDEV	2650.151	3696.327	5310.468

Dormant D4 PCΔP+AT				
	Glass #1	Glass #2	Sty #1	Sty #2
Min	848.000	928.000	944.000	864.000
Max	8800.000	6048.000	8208.000	7872.000
Mean	2132.919	2125.909	2527.091	2347.992
STDEV	387.908	311.600	703.162	589.344

D4 PCWT+AT			
	Glass #1	Sty #1	Sty #2
Min	6304.000	7056.000	12272.000
Max	31248.000	46224.000	40768.000
Mean	22282.674	28555.594	30563.140
STDEV	6248.013	7582.099	6587.426

Dormant D4 PCWT+AT			
	Glass #1	Sty #1	Sty #2
Min	784.000	1024.000	960.000
Max	9344.000	7280.000	7440.000
Mean	2121.318	2545.824	2746.020
STDEV	364.571	741.261	851.330

Figure 13. The biofilms' minimum, maximum, mean, and STDEV fluorescent intensity values.

AT ONLY			
	Glass #1	Glass #2	Sty #1
Min	2448.000	2816.000	2656.000
Max	9152.000	14832.000	13472.000
Mean	5607.068	8046.312	7766.842
STDEV	1047.223	1623.147	2284.699

Nothing on Bead		
	Glass #1	Sty #1
Min	10416.000	22400.000
Max	65520.000	65520.000
Mean	21130.352	31065.207
STDEV	3137.821	4481.324

PCWT Only		
	Glass #1	Sty #1
Min	5568.000	3584.000
Max	22400.000	49616.000
Mean	10182.404	23413.395
STDEV	2000.437	10325.132

PCΔP Only		
	Glass #1	Sty #1
Min	4144.000	4656.000
Max	21312.000	38976.000
Mean	14472.320	21225.016
STDEV	3029.073	7188.529

Figure 14. The controls' minimum, maximum, mean, and STDEV fluorescent intensity values.

The numbers represent the fluorescence intensity observed by the Lionheart Inverted Widefield Microscope. They come directly from the unaltered TIF files, and the values from the histograms cannot be modified in any way. The first observation was that the propagated biofilms are much more fluorescent than the dormant biofilms. However, it is important to note that a majority of

the cells in the dormant biofilm had likely died off. As for the controls, we see that *P. chlororaphis* has a higher range of autofluorescence than *A. tumefaciens*. The beads with no bacteria proved hard to interpret due to how much their signal varied, so for now, I cannot make any conclusions about them.

I also want to highlight the difference between PCΔphzE + AT and PCWT + AT. Due to the difficulty in getting all the images on one standardized scale, I adjusted the images to the point where the background was black, and the fluorescence would follow. In practical terms, the images had their brightness and contrast changed to make viewing easier, but they are not proportional to the histogram values. That is why I used the histograms to pull the prior objective values for my conclusions, and the images reflect more of what we can see with the inverted widefield microscope. It proved impossible to have a perfect minimum and maximum value to serve as a standard for the images, given the values varied too widely between the dormant biofilm beads, controls, and beads with bacteria on them.

The fluorescence intensity for one glass bead PCΔ*phzE* + AT was 11,417.478, while PCWT + AT had a glass bead with a value of 22,282.674, a significant difference numerically (Figure 13). In addition, the mean of a glass PCΔ*phzE* + AT is very close to the glass controls of PCWT and PCΔ*phzE* only, which were 10,182.404 and 14,472.320, respectively (Figure 14).

Because chemiluminescence is filtered out with the GFP filter on the inverted widefield microscope, my theory is that we are observing the autofluorescence of PC due to PCWT + AT having a higher value, which signals more GFP activation compared to PCΔ*phzE* + AT, which should not activate GFP without phenazine production. That said, we can begin to look at the images captured of the beads.

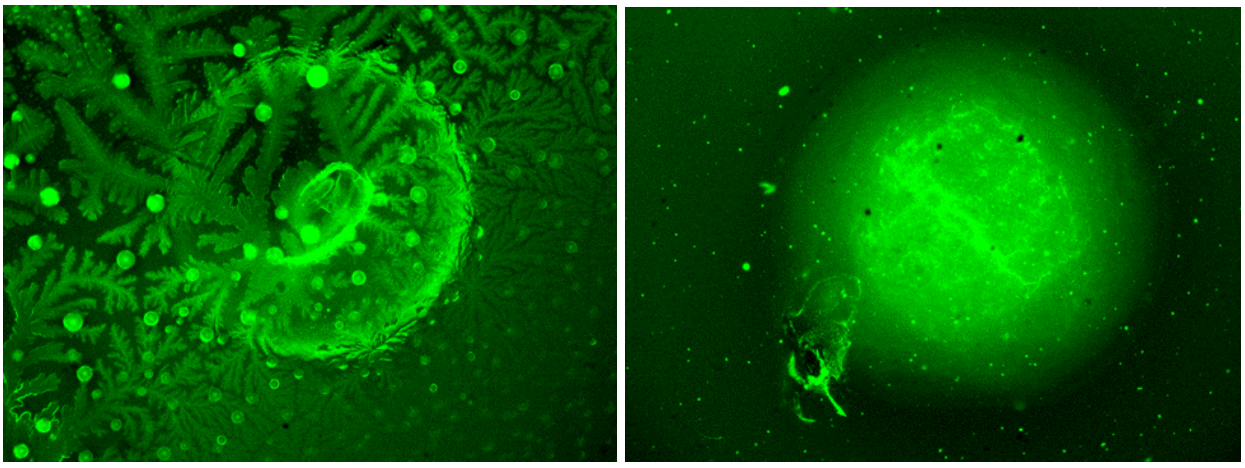

Figure 15. The left is a glass bead with no species on it placed on LB+KAN, and the right is a Styrofoam bead with the same variables.

Remember that both images are on different scales (brightness and contrast) due to their varying maximum and minimum values. That means the main purpose of showing the figure is to see what's on the plate. The main takeaway is that both images display autofluorescence despite having nothing on them (Figure 15). Why? For the glass bead, we attribute the autofluorescence to the potential dried agar and silicate purity (Left of Figure 15). The styrofoam bead is likely because styrofoam has autofluorescence due to styrene containing aromatic rings[14] (Right of Figure 15).

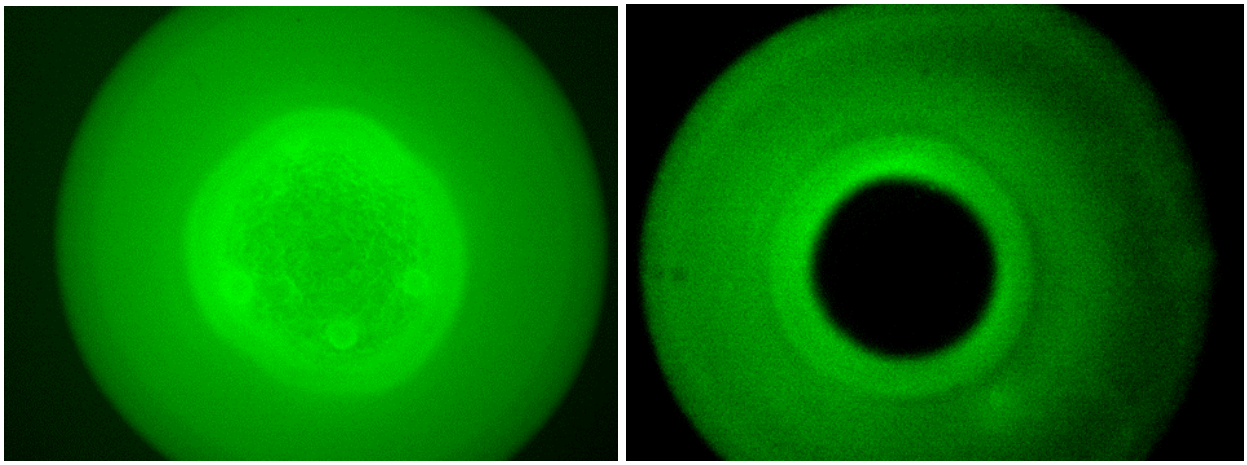

Figure 16. The left is a Styrofoam bead with only *A. tumefaciens* and incubated on LB+KAN, while the right is a glass bead with the same variables.

We can see that the glass bead has a much clearer resolution and less noise compared to the Styrofoam bead. This, among the other controls, led to using glass beads for cleaner pictures. Autofluorescence from Styrofoam makes it hard to determine what bacteria are and are not.

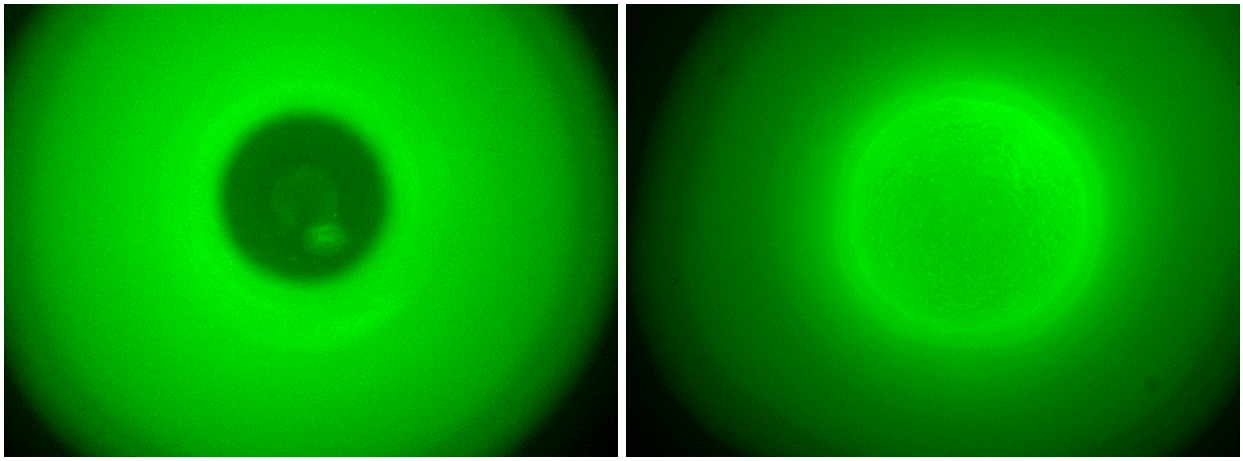

Figure 17. The left is a glass bead with only PCΔ*phzE* and incubated on LB+KAN, while the right is a Styrofoam bead with the same variables.

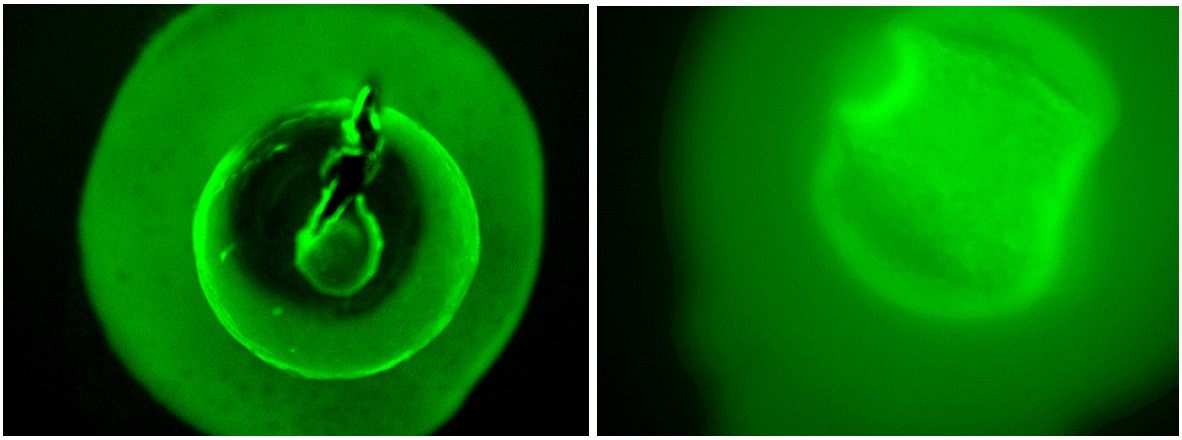

Figure 18. The left is a glass bead with only PCWT incubated on LB+KAN, while the right is a Styrofoam bead with the same variables.

Looking at the *P. chlororaphis* beads, we observed much autofluorescence, which differed from AT (Figure 14). The fluorescence intensity value of a glass AT control is 5,607.068 versus PCWT's 10,182.404 and PCΔ*phzE*'s 14,472.320. The biggest takeaway from these is their fluorescence values and to compare them against the D4 PCΔ*phzE* + AT biofilm to see if the controls have similar fluorescence values as co-cultivated biofilms since these represent the amount of autofluorescence from *P. chlororaphis* controls. The values are proved to be similar. Again, the numerical values noted from *P. chlororaphis* control plates mirrored those of co-cultivated biofilms using the PCΔ*phzE* + AT combination, leading to the running theory that what's being observed is autofluorescence.

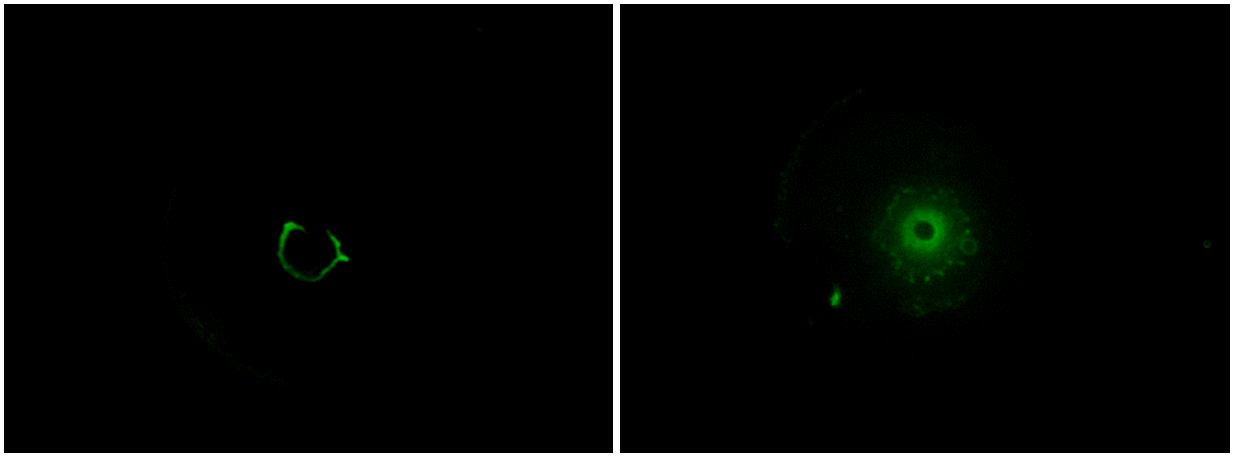

Figure 19. The left is a glass bead with PCΔ*phzE* + AT grown dormant (no nutrients on the plate), while the right is a Styrofoam bead with the same variables.

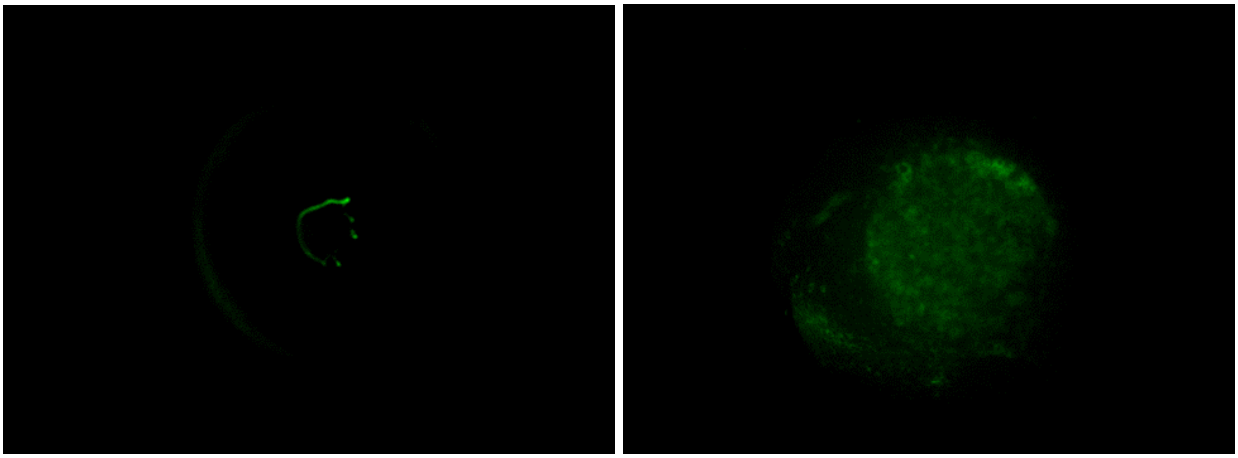

Figure 20. The left is a glass bead with PCWT + AT on it that has grown dormant (no nutrients on the plate), while the right is a Styrofoam bead with the same variables.

The most significant thing to note for the dormant beads is that the fluorescence values are significantly lower than the active biofilm beads. This may correlate with the bacteria being dormant and surviving in a very minimal state. The remaining signal picked up is likely autofluorescence from the cells remaining. To further clarify, the bead may have autofluorescence, and the bacteria can still exhibit fluorescence but to a much lesser extent.

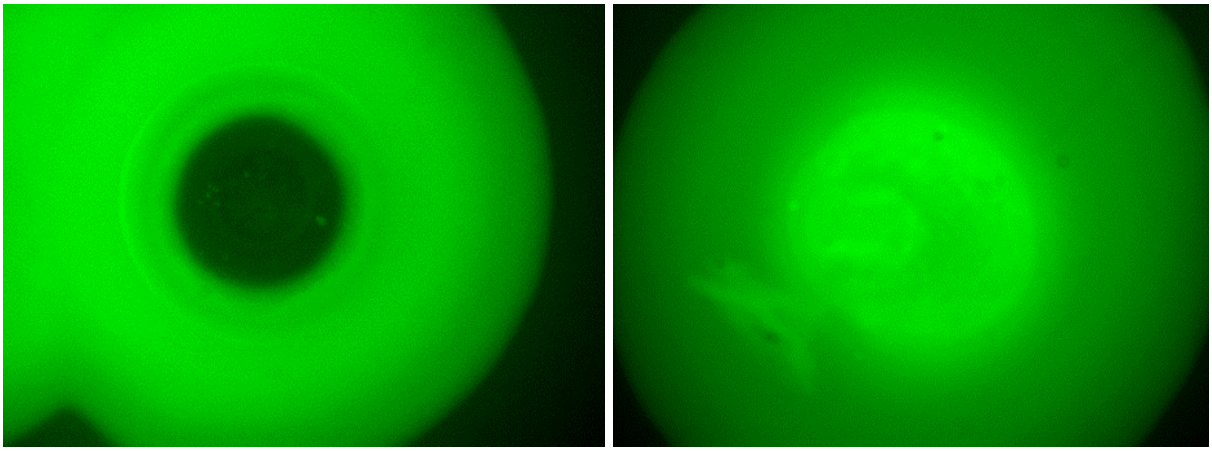

Figure 21. The left is a glass bead with PCΔ*phzE* + AT grown on LB+KAN after four days of dormancy, while the right is a Styrofoam bead with the same variables.

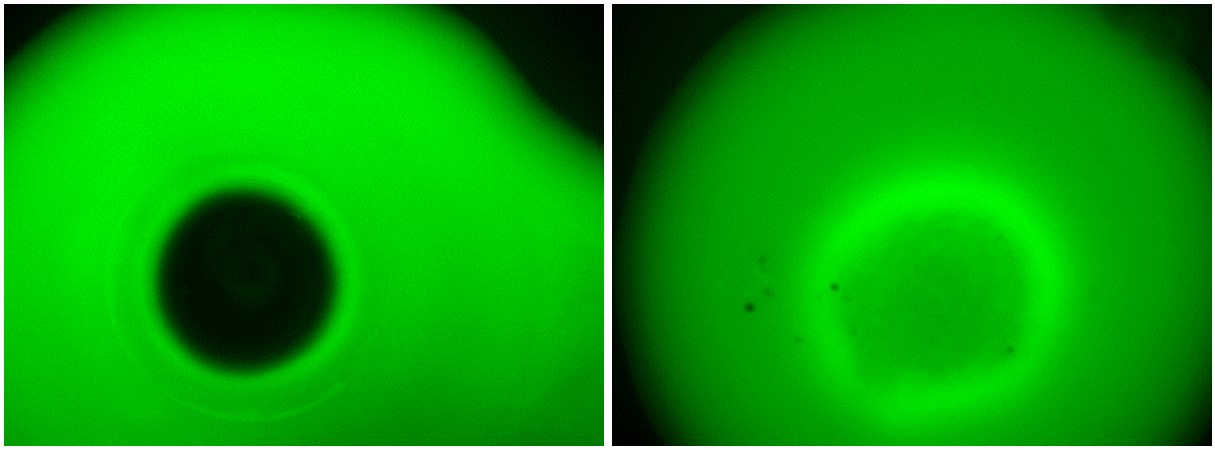

Figure 22. The left is a glass bead with PCWT + AT grown on LB+KAN after four days of dormancy, while the right is a Styrofoam bead with the same variables.

I noted the numerical difference between the two for the PCΔphzE + AT and PCWT + AT images. I interpreted the correlation between PCΔ*phzE* + AT, PCΔ*phzE* only, and PCWT, meaning that the mean fluorescence we observe from them would be the *P. chlororaphis's* autofluorescence. The conclusion means the much larger mean fluorescence intensity of PCWT + AT would be the *A. tumefaciens* activating due to GFP being activated due to *A. tumefaciens* in the presence of phenazines. At least, this would have been the case until we discovered that the phenazine knockout in *P. chlororaphis*'s construct did not work as intended.

In 2023, a member of the Lynn Lab, Ansley Felix, performed a TLC run of phenazine extracts of both PCWT and PCΔ*phzE*. The constructs were designed under the guidance of previous literature, which suggested that the engineering of a knockout through removing a key amine addition step in production would stop its production[15,16]. Theoretically, the PCΔ*phzE* should not have bands where we expected phenazines for PCWT. This is because the PCΔ*phzE* should have no capability to produce phenazines. To the lab's surprise, a band appeared on both PCWT and PCΔ*phzE,* which brought immediate concern to the experiments performed and called into question the legitimacy of our experiments run over the past two years. Even after receiving new strains to retest, the TLC results remained the same, and, as a result, none of the results gathered can lead to any confident conclusions. Unfortunately, the negative control cannot serve as a negative control because of this, but the general idea of the project still stands. If the project could be redone, we'd have to find a way to ensure the deletion of a pathway that assures no phenazine production in the construct.

Chapter 4: Conclusions

The findings presented here illustrate a potential breakthrough in the capabilities to study biofilms with spatiotemporal control. First, the beads were shown to survive starvation for up to

seven days in our efforts to induce biofilm formation further. In addition, following other papers that include a rinse step to ensure that the bacteria on the bead is primarily the biofilm meant that we could feel more confident in our results. What was initially even more promising were the results taken from the Lionheart Inverted Widefield Microscope. The PCWT + AT biofilm beads were noticeably different in fluorescent intensity values from the PCΔ*phzE* + AT beads. This difference might have indicated that *A. tumefaciens* reports on *P. chlororaphis's* phenazine production. Because *A. tumefaciens* has GFP production behind the sodBII operon responsible for being the bacteria's superoxide dismutase, the reactive oxygen species-rich environment could have activated the GFP production of *A. tumefaciens*. When comparing the PCΔ*phzE* + AT against the *P. chlororaphis* controls, it was evident that the P. chlororaphis's inherent autofluorescence was likely being picked up as noise.

However, it must again be stated that upon discovering that our PCΔ*phzE* construct wasn't functioning and that it was producing phenazines or a very similar compound, we could not draw any real conclusions from the data gathered. That isn't to say that the proposed project would not work. It's more of an error in not detecting and addressing the PCΔphzE's defect sooner. Suppose the project can find a way to make the negative control work as intended. In that case, the project will begin to function as a proper model for using biofilms in spatiotemporal studies.

From our studies, the biofilm on a bead project shows a potential avenue to study biofilm and plant interactions, given that the biofilm aspect can now be controlled in a spatiotemporal sense. Currently, there is no straightforward way to study the interactions between the two with definitive results that you would want from an in-vivo study. The biofilm on a bead could be the answer, as the ability to control space and time has been the main issue behind many of the studies where the space of the biofilm would be somewhat random. Because of the discovery of the negative control not working properly, the imminent direction is to find a way to properly disable *P. chlororaphis*'s ability to produce phenazines so the new results gathered can have conclusions made in confidence. The best lead is to delete the phenazine pathway entirely.

After the project's foundation is established, future research can progress towards recreating potential natural chemical environments to use the biofilm on a bead to study how biofilms interact with a plant root's chemical redox cascade. Then, it would be a good idea to see what genes are overexpressed and underexpressed. Lastly, and most ambitiously, it is theoretically possible to use the biofilm on the bead model to grow two co-cultivated biofilms and place them near each other to see how bacteria might interact in a certain environment. Fungi could be included since the spatiotemporal aspect of the biofilm is controlled. The potential research that can come now that the bead's competence has been proven provides an optimistic future in rhizosphere biofilm research.

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