AN INVESTIGATION OF THE TRI-TROPHIC INTERACTIONS IN THE RHIZOSPHERE OF *MEDICAGO TRUNCATULA* USING A FUNCTIONAL MICROBIOME APPROACH

by

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As the first in my family to receive a Master's Degree, I would like to dedicate this to my mom, Doreen Pimentel, and my grandfather and grandmother, Manuel Richard Pimentel and Melba Pimentel, for their lifetimes of hard work and dedication to their children and grandchildren, allowing me to reach for more. I am tremendously lucky to have them, and I hope only to make all of my family proud; I love you all!

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ABSTRACT

The use of plant beneficial organisms derived from the plant microbiome (i.e. biologicals), is gaining interest in agriculture as a solution to decrease dependence on pesticide and fertilizer use. It is understood that the application of individual plant growth promoting rhizobacteria (PGPR) increases plant health. However, limited research efforts have investigated complex interactions occurring between multiple species of beneficial bacteria, and how the outcomes of those associations influence their ability to benefit the plant.

There currently is little research into the specific types of rhizosphere interspecies signaling communication that may affect how plant beneficial bacteria perform. Using a tri-trophic model system of the legume *Medicago truncatula* A17 Jemalong, its mutualistic symbiont *Sinorhizobium meliloti* strain Rm8530 (hereafter Rm8530), and the PGPR *Bacillus subtilis* UD1022 (hereafter UD1022), we show that interactions between the PGPRs may influence their individual associations and activities on the plant root. Expression and functional analysis of Rm8530 suggest that UD1022 produces extracellular compounds that impact the components involved in the Rm8530 quorum sensing (QS) system. At the same time, Rm8530 may be influencing the functional plant association strategies of UD1022. This interaction could have greater implications in altering the ability of the PGPRs to positively affect plant health.

Chapter 1

MICROBES INTERACT IN MULTIPLE WAYS TO DEFINE THE RHIZOSPHERE MICROBIOME AND EFFECT PLANT HEALTH

1.1 Plant Beneficial Bacteria in the Rhizosphere

1.1.1 Introduction

The bacterial diversity of the rhizosphere is highly enriched from that of the soil and has been attributed to the substantial input of plant root exudates (Nguyen, 2003). The primary attraction of plant exudates for microbes is that they are carbon-rich, especially compared to the environment found in bulk soil (Hartmann et al., 2008). It is only recently that the true extant of rhizosphere microbial diversity has been described (Weinert et al., 2011). Molecular genetic techniques have illuminated the magnitude of the many different groups of bacteria that reside in the rhizosphere, since a great number of these organisms remain un-culturable (Lebeis, 2015; Prashar et al., 2013).

Our knowledge of the realm of plant beneficial bacteria in the rhizosphere is rapidly expanding due to the intense interest in utilizing these types of microbes in agriculture (Schisler et al., 2004; Schlaeppi and Bulgarelli, 2015). Lifestyles of plant beneficial microbes include endophytic mutualists, root symbionts that have an intimate interaction with plant roots, bacteria that exist in biofilms on the root surface, and those that are closely associated with the plants rhizosphere (Gray and Smith,

2005). There is a large amount of diversity in terms of taxa, and in the different ways in which these bacteria are thought to be 'helping' the plant.

The most well described relationship is the mutualism between legumes and rhizobia, wherein members of the *Rhizobia* fix atmospheric N for the plant in exchange for carbon-rich photosynthates within specialized structures called nodules on the plants roots. Many other beneficial bacteria are grouped under the term 'plant growth promoting rhizobacteria' (PGPR), and serve the plant in multifaceted and, sometimes, not very well understood ways. Bacteria in the rhizosphere can provide direct and indirect benefits to the plant through niche competition with other microbe, production of antibiotics to inhibit pathogens, mineralization of organic macronutrients, chelation of micronutrients, and stimulation of the plant's own immune response systems to shield it from invading pathogens (Berg, 2009; Gamalero and Glick, 2011; Hayat et al., 2010; Mendes et al., 2013).

1.1.2 Nitrogen Fixing Bacteria in the Rhizosphere

The mutualistic symbiosis that occurs between rhizobia and their legume hosts is well described and many genetic and molecular determinants have been uncovered through various model legume-rhizobia systems (Oldroyd, 2013). The complex dynamics between the plant host and symbiont first began to be elucidated when a class of compounds known as flavonoids were isolated from legume seeds and seedling root extracts (Peters et. al., 1986). Flavonoids released by legumes are important signaling molecules, both serving to attract symbiotic bacteria (Dharmatilake and Bauer, 1992), and more significantly, are required to induce transcription of rhizobia Nod factors (NF) through interaction with the transcriptional regulatory protein NodD, which then binds to the nod box (Kondorosi et. al., 1989).

Peters et al. (1986) demonstrated that the legume-isolated flavone luteolin positively increased expression of the rhizobia *nodABC* operon, and thus began a flurry of research that has uncovered the incredibly specific molecular genetic details of this symbiotic interaction. It is now known that different strains of rhizobia are responsive to the specific flavonoid unique to their host plant (Peck et al., 2006). Motility via flagella in response to the flavonoid, and attachment *via* biofilms are also significant factors to position the rhizobia in the most ideal spot along the legume root hair to deliver their NFs to the plant (Bahlawane et. al., 2008; Downie, 2010; Sourjik et. al., 2000).

The genes responsible for production of rhizobia NFs required for nodule initiation were revealed through mutant analysis (Long et al., 1982). The NFs produced by rhizobia in response to flavonoids are lipo-chitooligosaccharides (LCO), and are specific to the species of bacteria due to various 'decorated' side chains on the LCO. The NF receptor in the legume root hair epidermal plasma membrane has an extracellular LysM receptor-like kinase that binds with its cognate NF. This binding sets off a cascade of signaling events, including calcium spiking, to initiate root hair curling, development of an infection thread and subsequent rhizobia infection (Oldroyd et al., 2011; Rose et al., 2012). The infection thread is a plant cell wall structure which funnels the bacteria into the root hair cell to transverse into the cortical cells where nodule organogenesis takes place (Gage, 2004). The bacteria divide and flourish within the nodule as bacteroids, separated from the plant cell cytoplasm by a plant derived symbiosome membrane (Gourion et al., 2015). It is in these structures that the rhizobia express nitrogenase enzyme to fix atmospheric N, which the plant can

utilize. The bacteria receive plant fixed C in the form of malate, as its metabolic substrate (Prell and Poole, 2006).

1.1.3 Plant Growth Promoting Rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are an un-confederated group of bacteria occurring on, within or near plant roots that provide a benefit to the plant. PGPRs can be from a wide variety of families, including *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Serratia*, *Streptomyces* and the most common being from *Pseudomonas* and *Bacillus* (Berg, 2009; Pieterse et al., 2014; Somers et al., 2004). There are many different mechanisms of growth promotion that rely on the close, interdependent communication between the plant and the microbe, as well as many complex means of indirect plant growth promotion in which PGPRs contribute. PGPRs are drawn to the rhizosphere for the same reason as the rest of the microbial consortia; the rich concoction of carbon, amino acids, organic acids, sugars and metabolites that come from the plants roots (Hartmann et al., 2008; de Weert et al., 2002: Rudrappa et al., 2008).

Chemotactic responses to plant root derived materials bring PGPRs to the rhizosphere where they can interact with the root at a variety of levels of intimacy. Bacteria may be several millimeters near the root and provide benefits such as nutrient mineralization (Finzi et al., 2015). Other microbes inhabit the root surface, often arranging themselves in patchy colonies within biofilms for attachment (Danhorn and Fuqua, 2007). The activities of these bacteria range from providing a competitive barrier against pathogens, to producing molecules that mimic plant hormones or otherwise stimulate the plant to increase nutrient acquisition or defense responses (Gamalero and Glick, 2011). Other PGPRs are endophytes that make their home

within the epidermis of the plant root and can fix nitrogen in a non-symbiotic relationship (Cocking, 2003; Gaiero et al., 2013; Rosenblueth and Martínez-Romero, 2006).

1.1.4 Conclusion and Future Perspectives

The sheer volume of microbial community members, and the immense diversity of their activities and functions that they enact in the rhizosphere presents a difficult challenge to building a comprehensive view of how the plants and microbes are influencing each other. While the plant can contribute a major role in these relationships through their root exudates, there can be no doubt as to the substantial impact that rhizosphere microbes have on the plant. Importantly, rhizosphere microbes are contributing significantly to shaping their own community through their interactions with other members of the rhizosphere microbial consortia.

The complex trophic and inter-species interactions in the rhizosphere contribute to plant health and define the functional characteristics of the rhizosphere as a system. Intrinsic qualities of the plant root itself can cause changes in the expression of bacterial products, which in turn, can have beneficial effects on the plant such as protection and nutrient acquisition (Debois et al., 2015). Both plants and rhizosphere microbes produce substances that inhibit, antagonize and attract each other intra-specifically and extrinsically.

Considering plants have always been exposed to microbes in their environment, the co-evolution of plants and microbes in the rhizosphere will have enabled sophisticated and unexpected interactions between species to occur. Some of these mechanisms are well described, such as symbiotic and pathogenic interactions involving specific recognition receptors in both parties, allowing them to participate in

'call and response' style of communication (Chinchilla et al., 2007; Stracke et al., 2002). With the discovery of new interactions involving specific compounds, further novel receptors and expression networks in both plants and bacteria is quite likely, which may have implications in how we may better develop new technologies based on these relationships.

Creating biotechnological solutions to plant health is one goal that is currently receiving great attention. The use of plant-associated microbes has been implemented in one form or another for quite some time, but with only a generalized understanding of the outcome when one party interacts with the other. This has been the case with bio-fertilizers such as rhizobia and with biocontrol organisms such as some *Bacillus* spp. and *Pseudomonas* spp. (Hayat et al., 2010). Attempts to create superior plant growth promoting activity with co-inoculations using multiple species of PGPRs with diverse beneficial functions have led to mixed results. Co-inoculation of *S. meliloti* U143 with the PGPR *Delftia* spp. strain JD2 increased alfalfa plant yield, possibly through enhanced nodulation (Morel et al., 2015). On the other hand, Kang et al. (2014) discovered that when the two otherwise compatible PGPRs *B. pumilus* WP8 and *Erwinia persicinus* RA2, were co-inoculated onto tomato challenged by *R. solanacearum* Rs 1115 (wilt), they had no greater beneficial effect than when inoculated alone. The authors propose that this could be due to the observed *B. pumilus* WP8 biofilm inhibition caused by *E. persicinus* RA2 (Kang et. al., 2014). The biofilm inhibition could be attributed to some antagonistic interaction; either through antibiosis, quorum quenching, or as the authors suggest, primary metabolites. Investigating and deriving a more detailed understanding of the contributions, feedback mechanisms, and specific components in the microbe-microbe and plant-

microbe dynamic in the rhizosphere may allow for more targeted approaches in developing novel, sustainable solutions to promoting plant health.

1.2 Rationale and Goals

1.2.1 Tri-trophic Model

Rhizobacteria are clearly a dominant component of plant health and ecology. Far from simply responding to plant factors in the rhizosphere, bacteria near the roots are participating in a species rich environment that impacts the plant in direct and indirect ways. In order to better elucidate the inter-species interactions at play, this work employs a model tri-trophic system consisting of the well-characterized legume *Medicago truncatula* A17 Jemalong, it's symbiotic mutualist *Sinorhizobium meliloti* strain Rm8530, and the gram positive PGPR *Bacillus subtilis* strain UD1022 (Fig. 1.2). This model is especially relevant in the context of the recent interest in utilizing multiple PGPRs in co-inoculation treatments of plants in order to enhance the plant improvement capabilities of the individual PGPRs. Specific qualities of both of these plant-associated bacteria are relevant to their interactions with their plant host, and to their interactions with each other; namely their production of quorum sensing (QS) controlled biofilms.

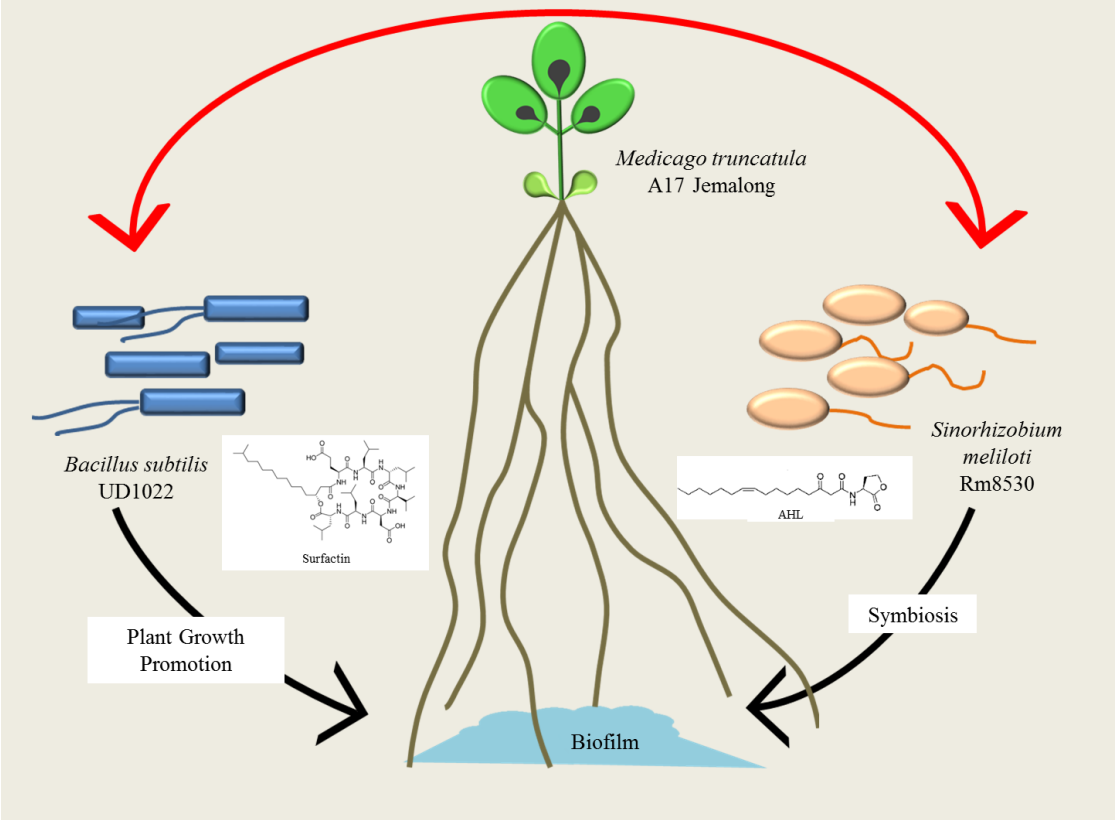

Figure 1.2 The Tri-trophic Model of Plant-Rhizobacteria Interactions. The interaction between UD1022 and Rm8530 may affect their plant growth and plant mutualistic functions. Both produce extracellular molecules; surfactin from UD1022 and N-Acyl-homoserine lactones (AHLs) from Rm8530. Additionally, both microbes require biofilm formation to appropriately interact with the plant.

1.2.2 Biofilm Formation by *Sinorhizobium meliloti*

Biofilm formation in *S. meliloti* has been extensively studied, and is under the direct regulation of the ExpR/Sin QS system which is analogous to the LuxR QS found in *Vibrio* spp. (Hoang et al., 2004; Marketon and Gonzalez, 2002). This QS system relies on the N-Acyl-homoserine lactone (AHL) receptor ExpR serving as a transcriptional response regulator that is involved in the expression of up to 500 different downstream genes (Gurich and González, 2009). SinI is the AHL synthase

which produces long-chain molecules unique to *S. meliloti* (Gao et al., 2005). *S. meliloti* biofilms consist of several major categories of extracellular polysaccharides (EPS), which are comprised of succinoglycan and high and low molecular weight molecules of galactoglucan termed 'EPS II' (Rinaudi and Gonzalez, 2009). These components of *S. meliloti* biofilms are widely accepted to play critical roles in the successful invasion and nodulation of the host plant *Medicago* spp. (Glenn et al., 2007; González et al., 1996; Pellock et al., 2000). In this regard, EPS II is commonly referred to as the 'symbiotically active component' of *S. meliloti* biofilms (Rinaudi and Gonzalez, 2009).

In order to observe biofilm and QS activity that more accurately reflects that of a true 'wild type' *Sinorhizobium* spp, *S. meliloti* Rm8530 (hereafter Rm8530) was selected as a model organism. Rm8530 is a result of a spontaneous excision of an insertion element in the genome of the common lab strain *S. meliloti* Rm1021. The insertion element disrupts the transcription and expression the QS response regulator gene *expR* (Pellock et al., 2002), and thus disrupted the production of the low molecular weight fraction of EPS II.

1.2.3 Biofilm Formation by *Bacillus subtilis*

Quorum sensing in *Bacillus* spp. and gram-positive bacteria in general are not similar to the classic systems of AHLs and transcriptional regulators. Instead, various small molecules have been implicated as 'autoinducers' to co-ordinate community level activities and, in *B. subtilis*, to define differential cell fates within biofilms (Bassler and Losick, 2006). The biofilms of *B. subtilis*, and of strain UD1022 (hereafter UD1022) specifically have been found to be critical to their interaction with the roots of plants (Bais et al., 2004; Rudrappa et al., 2008). Significantly, the cyclic

lipo-peptide (cLP) surfactin has been implicated as a small molecule that acts as an autoinducer that promotes *B. subtilis* biofilm formation. Lopez et al. (2009) found that transcription of the *yqxM-sipW-tasA* operon P_{yquM} which is responsible for the productions of biofilm matrix components was significantly increased with the application of exogenous surfactin. This may be occurring indirectly via the membrane sensor kinase KinC triggering the phosphorylation of the master transcriptional regulator Spo0A~P in response to potassium leakage caused by the formation of membrane pores by surfactin (López et al., 2009).

Bacillus spp. and *B. subtilis* specifically have been reported to produce quorum interference (QI) enzymes such as lactonase, which disrupt the biofilm formation of gram negative AHL producing bacteria. *B. subtillis* NCIB3610 has been reported to produce a lactonase homologous protein, YtnP (Schneider et al., 2012). This enzyme's QI activity was demonstrated through its inhibition of biofilm formation by *Pseudomonas aeruginosa*.

It is hypothesized that Rm8530 and UD1022 are influencing the plant growth promotional activities of each other when co-cultured. To test the hypothesis, the following research objectives were pursued:

1. Test the direct antagonism between the two bacteria.
2. Evaluate the specific activity of UD1022 on biofilm and quorum sensing of Rm8530.
3. Determine if growth with Rm8530 effects biofilm and surfactin production of UD1022.

Finding and describing specific mechanisms by which rhizosphere bacteria may be altering known plant growth promoting activities could lead to novels ways to

utilize or to improve the use of these organisms for biological plant protective applications.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial Growth

Primary cultures of all bacteria strains were grown and maintained on TYC media (TY media (Beringer, 1974) liquid or agar supplemented with 1 mM $CaCl_2$) with appropriate antibiotics. Subcultures of *Sinorhizobium meliloti* strain Rm8530 (hereafter 'Rm8530') and *Bacillus subtilis* strain UD1022 (hereafter 'UD1022) prepared for biofilm treatments were sub-cultured into minimal glutamate mannitol (MGM), low phosphate (0.1 mM), as described in (Marketon and Gonzalez, 2002).

AT medium for culturing pre-induced *A. tumefaciens* KYC55 was prepared as described in Joelsson and Zhu, 2005.

Table 2.1 Bacterial strains used in this study.

Strain	Genotype	Reference or Source
Rm1021	SU47 *str-21 expR102::ISRm2011-1*	(Meade et al., 1982)
Rm8530	*Sinorhizobium meliloti* Rm1021 *expR*$^+$	(Glazebrook and Walker, 1989)
Rm8530 SinI-gfp	with integrated pMG309	(Gao et al., 2012)
Rm8530 WggR-gfp	with integrated pMG310	(Gao et al., 2012)
KYC55	*Agrobacterium tumefaciens* (pJZ410)(pJZ384)(pJZ372)	(Zhu et al., 2003)
UD1022	*Bacillus subtilis*	(Bishnoi et al., 2015)

2.2 Cross-streak for Growth Inhibition Analysis

Rm8530 bacteria were grown to OD_{600} = 0.8 and UD1022 OD_{600} = 1.5. Both cultures were diluted to OD_{600} = 0.5 with sterile H_2O. Bacteria were streaked on TYC agar plates using a sterile loop in a cross pattern.

2.3 Plant Growth and Co-Inoculation

Seeds of *Medicago truncatula* [Gaertn.], line A17 of cv Jemalong, were acid scarified for 6 minutes, sterilized with 3% bleach for 3 minutes. Seeds were imbibed in sterile water at 4° C overnight, rinsed and placed in sterile petri dish and germinated covered overnight at room temperature (Liu et. al., 2006). Germinated seeds were placed in sterile Magenta® (Magenta Corp.) jars with Lullien's solution (Lullein et.al., 1987), sealed with 3M™ MicroPore™ surgical tape and grown in a controlled environmental chamber at 55% relative humidity and a 14-h, 22° C day/10 h, 18°C night cycle.

After 6 days of growth, plants were inoculated with bacteria treatments. Rm8530 was grown to OD_{600} = 0.8 and UD1022 at OD_{600} = 1.0. Bacteria were spun down, washed 3 times in sterile H_2O and re-suspended with 0.5X Lullein's solution with Rm8530 final OD_{600} = of 0.02 and UD1022 OD_{600} = 0.01 (in Magenta jar). Plants were harvested 7 weeks after inoculation.

2.4 Biofilm Assays

2.4.1 Preparation of Cell Free Supernatant (CFS) Derived from UD1022 for Biofilm Assays

UD1022 culture primary was inoculated from a single plate colony into 5 mL TYC and grown overnight (16 hr). The primary was sub-cultured 1:50 in 50 mL MGM in sterile 150 mL flask, and grown shaking for 8 hours. OD_{600} = 0.8 - 1.0. Cultures

were centrifuged 10 minutes, 4° C at 4,000 RPM. Culture supernatant was filter-sterilized with 0.22 µm membrane (Steriflip®, EMD Millipore) under gentle vacuum. Supernatant was centrifuged and filter sterilized once more. A sub-fraction was heat killed in water bath overnight at 65° C.

2.4.2 Preparation of Biofilm Treatments

Biofilm assays were based on methods found in (Rinaudi and Gonzalez, 2009) and (O'Toole et al., 1999). Rm8530 primary cultures were grown 48 hours in TYC to OD_{600} = 1.5-2.0. Cells were then 'pre-conditioned' by sub-culturing primaries 1:100 to MGM media and grown shaking 48 hours to OD_{600} = 0.8. Stock treatments were made by centrifuging and re-suspending cell pellets by adding fresh MGM, UD1022 CFS, or UD1022 'heat killed' CFL to a total of 5%. 100 µL of the treatments were then distributed to 96 well plates with 29 replicate wells per treatment. Plates were sealed with Parafilm® (Bemis Company, Inc.) and placed in shaker and measured at 24, 48 and 72 hours.

Plates were incubated, shaking, for 72 hours. Plates were then emptied and gently rinsed 3 times with sterile water, dried, and stained 20 minutes with 150 µL of 0.1% crystal violet. Plates were emptied, rinsed gently 3 times with sterile water. Crystal violet was solubilized with modified biofilm dissolving solution (MBDS) (Tram et al., 2013). OD_{595} was then measured using Wallac 1420 Plate Reader (PerkinElmer Life and Analytical Science, Wallac Oy, P.O. Box 10, FIN-20101 Tuku, Finland).

2.5 Gene Expression Reporter Assays

2.5.1 Preparation of Treatments for Gene Expression Reporter Assays

Reporter lines for Rm8530 were provided by Dr. Max Teplitski of the University of Florida. All cultures grown in liquid broth shaking at 225 RPM at 30° C. Bacteria primary cultures were grown in TYC with appropriate antibiotics 48 hours to OD_{600} = 2.0-3.0. Cells were further prepared as described in Biofilm Assays section.

2.5.2 Fluorescence Measurement of Gene Expression Reporter Assays

Plates were measured, and data reported as described in (Gao et al., 2012).

2.5.3 Biofilm Measurement of Gene Expression Reporter Assays

After 72 hour fluorescence measurement, 96 well plates were processed as described in 'Biofilm Assays' above to assess qualitative biofilm formation.

2.6 AHL Biosensor Assays: Quorum Interference Analysis

Preparation of the AHL biosensor *Agrobacterium tumefaciens* KYC55 was as described in (Joelsson and Zhu, 2005) with modifications. KYC55 pre-induced cells were inoculated 1:1,000 into MGM medium for X-Gal soft agar plates. Pre-induced KYC55 cells were made as described in (Joelsson and Zhu, 2005). Soft agar plates were treated the same day they were poured.

UD1022 primary culture was inoculated from a fresh plate streaked from glycerol stock into TYC and grown shaking 30° C for 5 hours to OD_{600} = 1.5. Bacteria were sub-cultured 1:100 to MGM media and grown shaking 30° C for 20 hours to OD_{600} = 0.5. Treatments were made using these cultures mixed into sterile micro-centrifuge tubes with standard C8-AHL and oxo-C16-AHL to a final concentration of 10 µM in volume of 200 µL. Controls contained standard AHL only.

Treatments were incubated shaking 30° C for 24 hours. Samples were then centrifuged at 16.1 *x 10,000 g* for 10 minutes at 4° C. Supernatants were transferred to new sterile tubes and sterilized open in biosafety cabinet under UV light for 30 minutes. 2 µL of treatments were applied to KYC55 X-Gal soft agar plates and allowed to dry. Plates were sealed with Parafilm® and incubated right side up for 24 hours at 30° C. Two treatment replicates were included on 2 separate plates. AHL biosensor assay was repeated twice.

2.7 Surfactin Distruption of Rm8530 Biofilm Assay

Bacillus spp. are documented to produce significant quantities of cyclic lipopeptides (cLPs), the most common of which is surfactin (Ongena and Jacques, 2008). To further investigate the possible mechanisms by which UD1022 may be inhibiting Rm8530 biofilm production, biofilm assays were performed using multiple biologically relevant concentrations of pure surfactin derived from *Bacillus subtilis* (Sigma-Aldrich Co., LLC). Assays and conditions were performed exactly as described in section 2.4.2 Preparation of Biofilm Treatments, with the following treatments of final surfactin concentration of: 100 µM, 50µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, 1.56 µM, 0.78 µM, and 0.39 µM. Biofilm assay plates were cultured, harvested and evaluated as described above.

2.8 Surfactin Minimal Inhibitory Concentration Assay

Sterile filter disks were prepared by pipetting 5 µL treatments consisting of 2.5 µg mL^{-1} tetracycline positive control, 100 µM surfactin, 0.4 µM surfactin and ethanol negative control and allowed to dry.

Rm8530 primary TYC cultures were grown to OD_{600} = 1.0 and diluted to 0.02. 200µL diluted culture was spread on TYC plates and allowed to dry. Prepared filter disks were placed onto plates, which were sealed and grown 28° C for 48 hours. Three replicate plates were made, and the experiment was repeated twice.

2.9 Gene Expression Analysis Using Semi-Quantitative Reverse Transcription PCR (qRT-PCR)

2.9.1 Primer Design for qRT-PCR

Gene sequences were derived from GenBank; *S. meliloti* 1021 sequences from genome (accession: AL591688.1), and mega-plasmids pSymA (accession: AE006469.1). ExpR genes derived from *S. meliloti* strain 8530 N-acyl homoserine lactone receptor gene sequence (accession: DQ366275.1). The SinI primer pair from (Gurich and González, 2009) and the rpoE1 primer pair from (Trabelsi et al., 2009).

UD1022 genome was BLASTed using gene sequences from *B. subtilis* 168 NCBI Ref Seq: NC_000964.3. Primers were designed by using gene sequence found in UD1022 genome sequence (accession: NZ_CP011534.1). YtnP sequence was derived from protein sequence as published in (Schneider et al., 2012), and the UD1022 *ytnP* primer was designed based on the UD1022 genome (Bishnoi et al., 2015). Primers from this work were designed using GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer). Amplicon size was restricted to 150 bp or less. All primer sequences (listed in Table 2.9) were cross-checked on all strain sequences to ensure species specificity.

Table 2.2 Primer sequences used in this study.

Primer	Sequence 5'→3'	Amplicon Length	Source
UD1022 RecA F	TCCTCGCGGACGGATTATTG	149 bp	This work
UD1022 RecA R	TTTGCGCGTATACCGGATCT		
UD1022 SrfA F	CGTTGCACATGTTCACTGCT	149 bp	This work
UD1022 SrfA R	ACCAATCCCCGCAAGATTTGA		
UD1022 SinI F	AAATATCAGCCCGGAAGAAA	100 bp	This work
UD1022 SinI R	TCAGAAAGGATTTACGGTATGACT		
UD1022 EPS F	TACCGATACGGTGCTGGATA	80 bp	This work
UD1022 EPS R	TGCGGTCTAGCATCTCCATA		
UD1022 YtnP F	CGGTTGCCGATATAGATGTG	143 bp	This work
UD1022 YtnP R	CATTTCGTCCCATTCAACAG		
Rm8530 WggR F	TCCGTTCGCAGACTTTGGAG	107 bp	This work
Rm8530 WggR R	CGAGCGAATCATCTCCGTCA		
Rm8530 SinI F	CCGGAAATCCGTAGTGCGTC	76 bp	(Gurich and González, 2009)
Rm8530 SinI R	ATGCGCGATCCTGGGAGATT		
Rm8530 rpoE1-fw	CGAGGAAGAGGTCCTGGAAT	100 bp	(Trabelsi et al., 2009)
Rm8530 rpoE1-rv	GACGCAGTCCTGCAACAGAT		
Rm8530 NodA F	CCGACCGAGTCGTAAGCAAT	136 bp	This work
Rm8530 NodA R	ACCACCAGGAGCTCTCAGAA		
Rm8530 ExpR F	CCGCAAATCTACATCCGCAAG	144 bp	This work
Rm8530 ExpR R	CATCATGCTTTCCATGCGCT		

2.9.2 Experimental Protocol for qRT-PCR

For qRT-PCR analysis, cells were 'pre-conditioned' on MGM media as described under 'Biofilm assay' section. Cells were pelleted and re-suspended in fresh MGM plus the treatment. Co-inoculations were combined as Rm8530 OD_{600} = 0.8 and UD1022 OD_{600} = 0.2. Luteolin treatments contained a final concentration of 5 µM luteolin. Treatments were grown shaking at 30° C, and 1.5 mL samples were collected at time points of 12 and 24 hours, centrifuged, decanted and flash frozen in liquid nitrogen. RNA was isolated using NucleoSpin® RNA from Macherey-Nagel (Düren, Germany). cDNA was generated with 500 ng RNA using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (www.appliedbiosystems.com)

and qPCR was performed using PerfeCTa® SYBR® Green SuperMix, ROX, Quanta Biosciences (Gaithersburg, MD), and run on Eppendorf Mastercycler® ep *realplex*^{2} (www.eppendorf.com).

2.9.3 Expression Analysis of qRT-PCR

The relative change in gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method as described in (Schmittgen and Livak, 2008), which calculates the expression of the gene of interest relative to the internal control in the treated sample compared with the untreated control. The internal control gene for UD1022 is *RecA*, and the internal control gene for Rm8530 is *rpo*E1. Genes were considered to be differentially expressed if the fold change in expression was ≥ 2 or ≤ -2.

Chapter 3

RESULTS

3.1 Evaluation of Bacterial Compatibility; Cross Streak Assay

Prior to application of the two strains of bacteria to *M. truncatula*, Rm8530 and UD1022 were cross-streaked to evaluate potential negative interactions. When grown together on TYC plates, Rm8530 and UD1022 exhibited no inhibitory effects on the other as can be observed in Figure 3.1. These bacteria were determined to be compatible in co-culture, and co-inoculation on the model plant *M. truncatula* was considered appropriate.

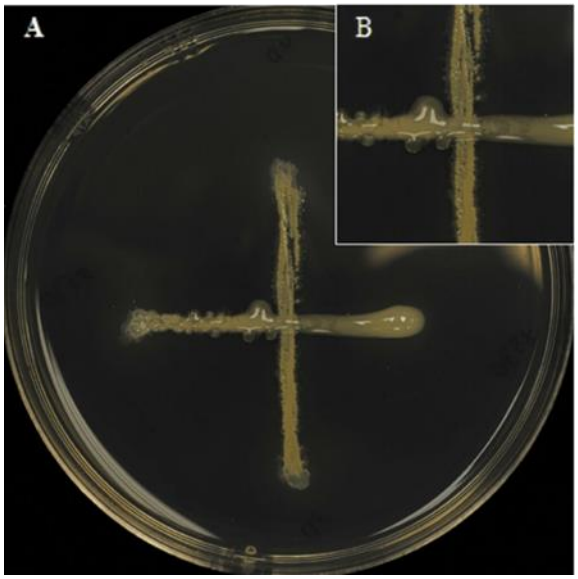

Figure 3.1 Cross Streak Assay. A) Overview of the compatibility between Rm8530 on the horizontal streak, and UD1022 found on the vertical streak. B) The detail of the inset reveals no inhibition of either strain of bacteria on the other.

3.2 Co-Inoculation Plant Growth Assay

Previous published work (Fox et al., 2011; Morel et al., 2015) and observations from a collaborating laboratory (communicated, Dr. Janine Sherrier), found synergistic, or enhanced plant growth promotion upon the co-inoculation of legume nodulating bacteria and other non-mutualistic PGPRs. The original broad goal of this work was to investigate the mechanisms by which rhizobia and PGPRs may be enacting with each other and the plant to increase their plant beneficial activities. The plant model *M. truncatula* A17 and its associated symbiont *S. meliloti* Rm8530 were selected for their sequenced genomes and availability of mutant strains. The PGPR UD1022 was selected as it is highly investigated in this laboratory, and an ideal candidate due to previous observations in other legume-rhizobia systems.

After 7 weeks of growth from the time of inoculation, the plants were harvested, pictures of the shoot and roots were taken to evaluate nodule numbers, and dried prior to weighing. In this tri-trophic model system, the co-inoculation of Rm8530 and UD1022 did not result in any statistical or observable differences in plant growth or nodulation of *M. truncatula*.

Figure 3.2 Co-Inoculated Plant Growth and Nodulation. A) Average plant dry weight of Rm8530 treated control plants and co-inoculated Rm8530 & UD1022 plants did not differ statistically (*P*-value of 0.06). B) There was no statistical difference between average counts of nodules between treatments (*P*-value of 0.59). C) Overall plant growth of both treatments was greater than control (first plant), but no differences were observed between Rm8530 treatment (second plant) and Rm8530 & UD1022 co-inoculation (third plant).

3.3 Rm8530 Biofilm Formation

Based on the inability of the co-inoculations to improve plant growth, focus of this study re-oriented toward how the bacteria may be interacting with each other that may alter their abilities to functionally improve plant growth. Biofilm formation by Rm8530 represents a critical step immediately prior to root invasion and nodulation (Gonzalez et al., 1996). In order to evaluate if UD1022 inhibits Rm8530 biofilm formation, Rm8530 was cultured with 5% culture filtrate supernatant (CFS) of UD1022. UD1022 CFS that was 'heat killed' was included as an additional treatment to determine if the potentially active component in the CFS could be proteinaceous in nature. The experiments were repeated three separate times.

The semi-quantitative analysis of Rm8530 biofilm formation found that treatment with 5% UD1022 CFS significantly inhibited biofilm formation. Treatments with 5% 'heat-killed' UD1022 CFS were no different than the control, indicating that the active component of the UD1022 CFS is not heat-stable, and may be a protein.

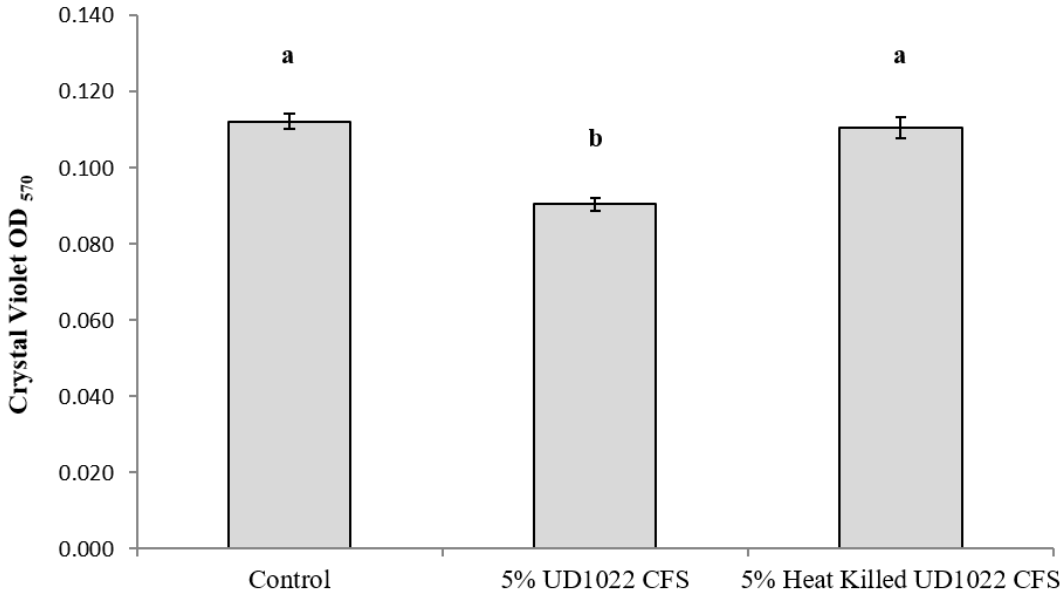

Figure 3.3 Rm8530 Biofilm Formation Assay. Treatment with 5% UD1022 CFS significantly reduced the formation of biofilm by Rm8530 (*P*-value of <0.0001). Treatment with 'heat killed' UD1022 CFS showed no significant difference compared to the control (*P*-value of 0.86).

3.4 Gene Expression Reporter Assays

In order to determine if the inhibition of Rm8530 biofilm by UD1022 CFS involves disruption of elements within the Rm8530 QS system, gene expression reporters for key Rm8530 QS genes were obtained from the lab of Dr. Max Teplitski, University of Florida. These transcriptional reporter lines consist of Rm8530 containing plasmid-borne promoter-*gfp* fusions for the *sin*I and *wgg*R genes. SinI is the AHL synthase responsible for production of Rm8530 long-chain QS molecules such as oxo-C16-AHL. WggR is a transcriptional regulator that activates the *wge* operons responsible for the biosynthesis and polymerization of EPSII. Disruption of *wggR* inhibits the production of EPSII (Gao et al., 2012).

Rm8530 reporter strains were co-cultured with 5% UD1022 CFS in the same manner as for the biofilm assays. Fluorescence of the reporters was measured and assay plates were evaluated for biofilm inhibition as well (results for biofilm formation are reported in Appendix A). Experiments were performed three separate times.

Expression of SinI as measured by the reporter treated with UD1022 CFS was greatly increased after 48 hours of co-culture. The expression of SinI in heat-killed UD1022 CFS treatments was higher than that of the control, but lower than the UD1022 CFS treatment, indicating that the heat-treatment of the CFS may not have thoroughly reduced the action of the active component. SinI expression of the UD1022 CFS treatment tapers off by 72 hours, but is still significantly greater than that of the control.

Expression of the WggR reporter treated with UD1022 CFS was significantly reduced at 48 hours and this reduced difference in expression between treatment and control was greatest at 72 hours. Expression of WggR in the heat killed treatment was not significantly different than the control treatment at 72 hours.

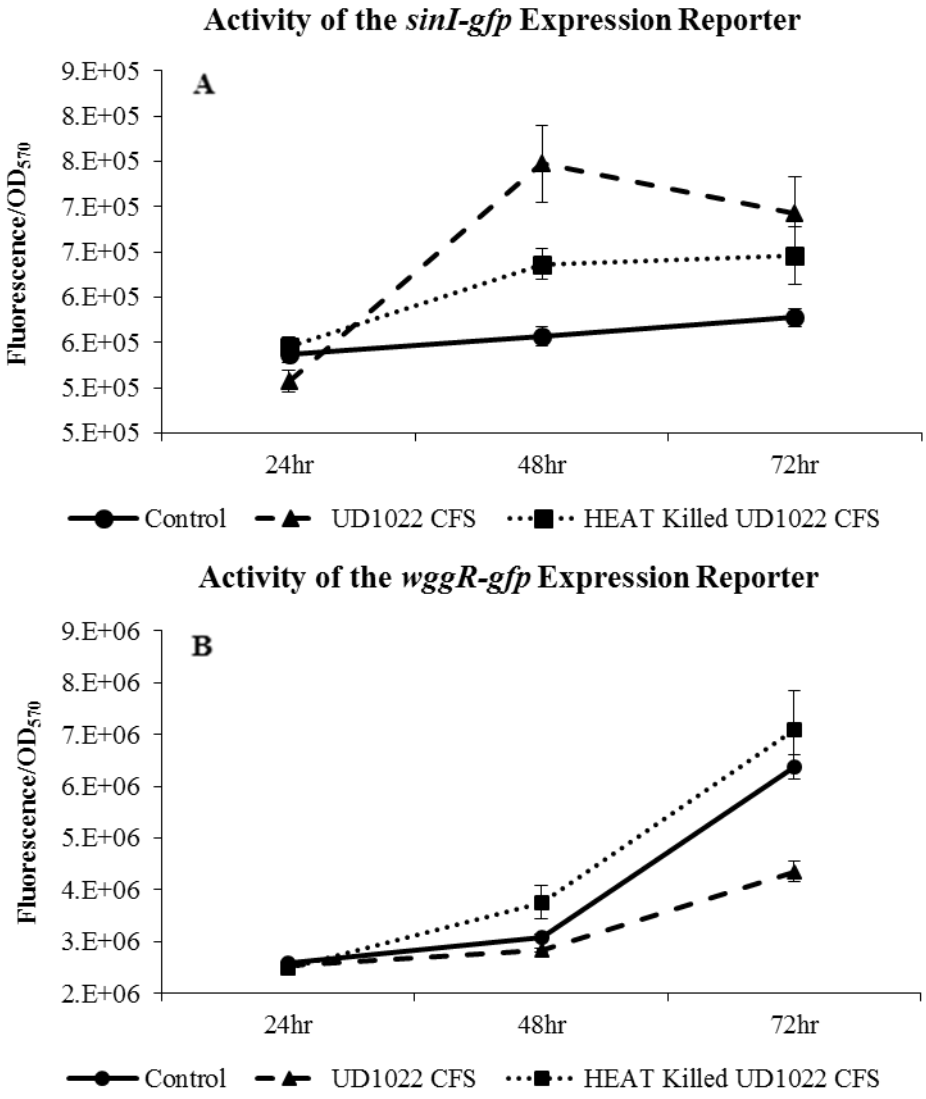
Activity of the *sinI-gfp* Expression Reporter
A
Fluorescence/OD570
9.E+05
8.E+05
8.E+05
7.E+05
7.E+05
6.E+05
6.E+05
5.E+05
5.E+05
24hr
48hr
72hr
Control
UD1022 CFS
HEAT Killed UD1022 CFS
Activity of the *wggR-gfp* Expression Reporter
B
Fluorescence/OD570
9.E+06
8.E+06
7.E+06
6.E+06
5.E+06
4.E+06
3.E+06
2.E+06
24hr
48hr
72hr
Control
UD1022 CFS
HEAT Killed UD1022 CFS

Figure 3.4 Expression of Rm8530 Quorum Sensing Genes. A) Average GFP activity (fluorescence/OD_{570}) of the *sinI-gfp* fusion reporter. The differences between treatments at 48 hours is significant at *P*-value of <0.0001. B) Average GFP activity of *wggR-gfp* fusion reporter. The differences between the treatments and the control at 72 hours is significant with *P*-value of <0.0001. Averages for both assays are from 8 technical replicates and the bars at each time point present standard error.

3.5 Evaluation of Quorum Interference Activity of UD1022: AHL Biosensor Assay

A BLAST search of the UD1022 genome sequence revealed a 98% homology to the putative lactonase gene *ytnP* of *Bacillus subtilis* NCIB 3610 (Schneider et al., 2012). In order to assess the functional capability of UD1022 *ytnP*-like gene (i.e. 'quorum interference' or 'QI'), 10 μM standard AHLs C8 and oxo-C16 were co-cultured with live UD1022 in MGM minimal media. Supernatants of the treatments were applied to soft agar X-gal plates containing the biosensor *Agrobacterium tumefaciens* KYC55. In the presence of both short and long chain AHLs, KYC55 expresses β-galactosidase, which acts on the X-gal substrate to result in a detectable blue coloration in the media.

UD1022 co-cultured with C8-AHL contained detectable levels of AHLs as compared to the controls. Treatments of oxo-C16-AHL co-cultured with UD1022 have no visibly detectable levels of AHL. This result indicates that UD1022 does not affect short-chain AHLs such as C8-AHL, and has the ability to degrade long-chain AHLs such as oxo-C16-AHL.

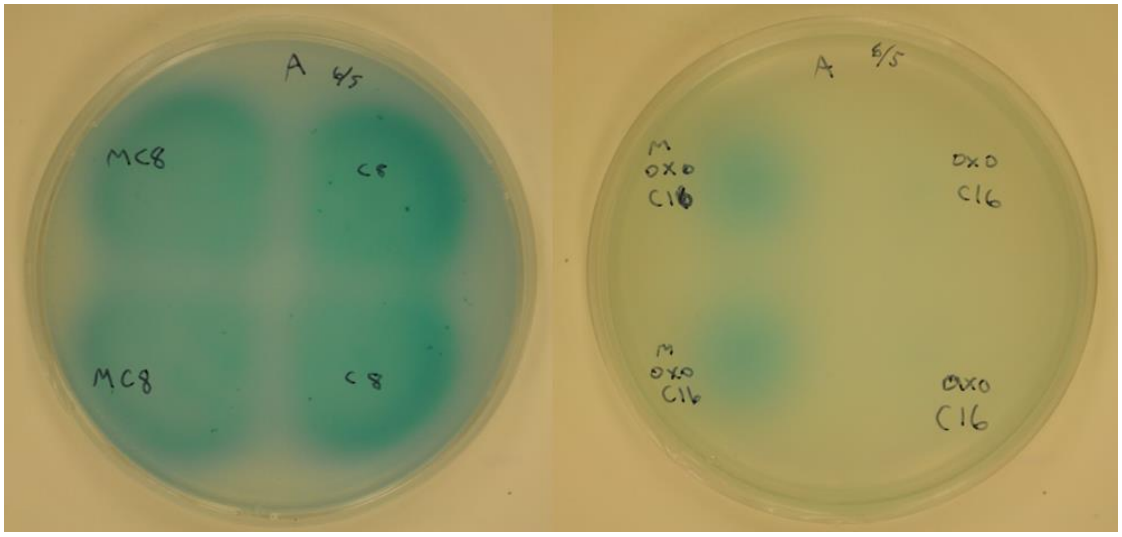

Figure 3.5 Biosensor Assay Plates. The biosensor KYC55-X-gal soft agar plates treated with UD1022-AHL co-cultures. The two spots to the left of each plate are control treatments of standard AHLs with no UD1022. The two spots to the right of each plate are the C-8 and oxo-C16-AHL treated with UD1022 co-cultures, respectively.

3.6 Surfactin Activity on Rm8530 Biofilm Assay

Surfactin production by *Bacillus* spp. is known to be a powerful surfactant and has the potential to influence the growth and behavior of other bacteria in the environment, either as an antagonist or as a signaling molecule (Raaijmakers et al., 2010). To evaluate what role surfactin may play in the interaction between UD1022 and Rm8530, surfactin was applied to cultures of Rm8530 and biofilm formation was assessed. Biofilm formation by Rm8530 in all treatments of surfactin concentrations (graph of all treatments is found in Appendix B), was significantly reduced.

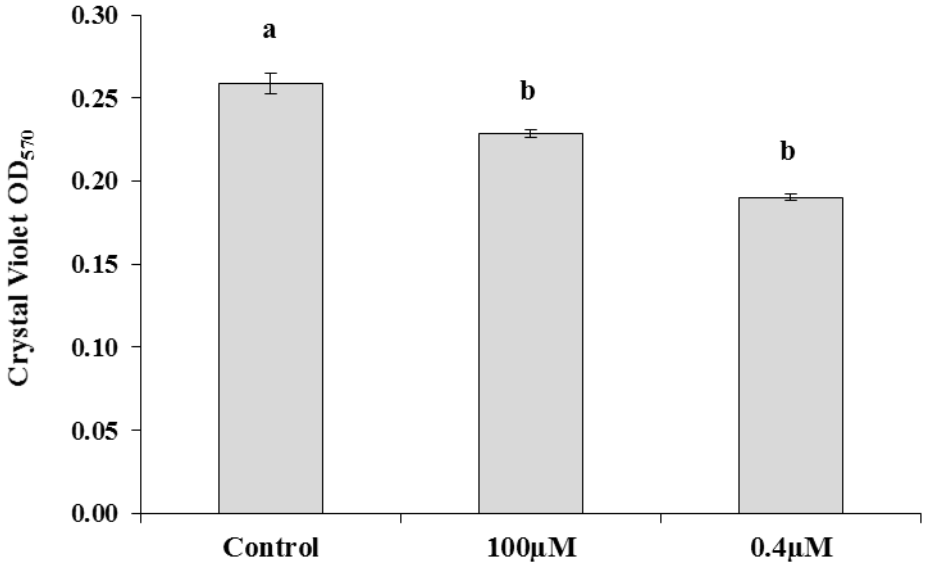

Figure 3.6 Surfactin reduction of Rm8530 Biofilm. The highest and lowest concentrations of surfactin treatments are reported. All concentrations of surfactin reduced the amount of biofilm measured in this semi-quantitative assay (*P*-value of 0.0004).

3.7 Surfactin Minimal Inhibitory Assay

With the finding of Rm8530 biofilm reduction by stock concentrations of surfactin, the ability of surfactin to inhibit the growth of Rm8530 was determined using filter disks treated with 0.4 µM and 100 µM surfactin. Ethanol and tetracycline were used as negative and positive controls, respectively. Three replicate plates were prepared, and the experiment was repeated twice.

On TYC (rich media) agar plates, surfactin does not inhibit the growth of Rm8530. This result coupled with the biofilm reduction results, suggest that the activity of surfactin may be based on its ability to disrupt adhesion of the biofilm itself, rather than inhibiting the planktonic growth of Rm8530.

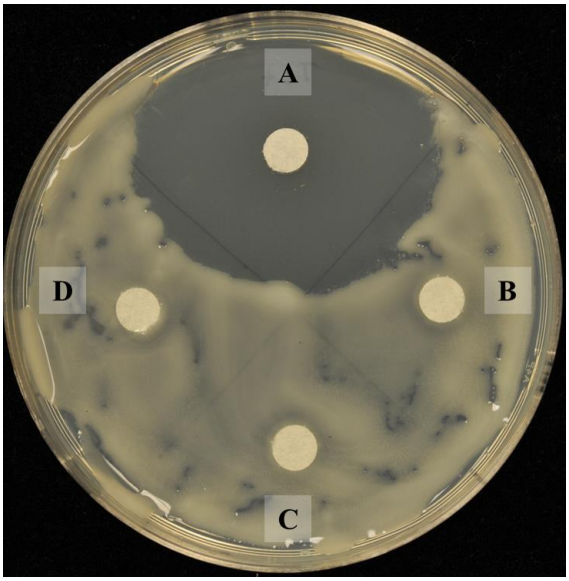

Figure 3.7 Test of Surfactin Inhibition on Rm8530 Growth. A) The positive control of 2.5 µg mL^{-1} tetracycline is the top disk. B) The disk to the right has no treatment. C) The 0.4 µM surfactin disk is positioned at the bottom. D) The 100 µM surfactin is on the left. Planktonic growth of Rm8530 is not inhibited by the concentrations of surfactin assayed.

3.8 Relative Gene Expression Analysis

3.8.1 Evaluation of the Relative Expression of Rm8530 Genes

The QS responsive genes Rm8530 *sinI* and *wggR* were selected for further analysis through semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR). Rm8530 were co-cultured with UD1022; additional treatments include 5 uM luteolin (which is the *M. truncatula* -specific flavonoid that induces Rm8530 Nod genes), and UD1022 co-cultures containing luteolin to induce Rm8530 Nod genes.

The expression of Rm8530 *sinI* (relative to the housekeeping gene *rpo*E1) increased by 4-fold when co-cultured with UD1022. The presence of the plant-root exuded chemical luteolin appears to further increase the expression of *sinI* to 8-fold.

This result is in line with the finding of the GFP expression reporter assays performed earlier.

The relative expression of Rm8530 *wggR* is also in line with that found in the GFP expression reporter assay. Co-culture with UD1022 decreased *wggR* expression by about 3-fold. The presence of luteolin in this case did not greatly alter the extent of the down regulated expression, which is about 3.5-fold.

Co-culture with UD1022 seems to affect the expression of Rm8530 genes responsive to QS and biofilm formation. *SinI* is the QS AHL synthase responsible for production of the long-chain AHL signals. An increase in *sinI* expression could represent an increase in AHL production. *WggR* is required for production of EPSII which is the symbiotically active portion of the biofilm formed by Rm8530. The decrease in *wggR* expression also aligns with the physical decrease in biofilm first detected in the original biofilm assays.

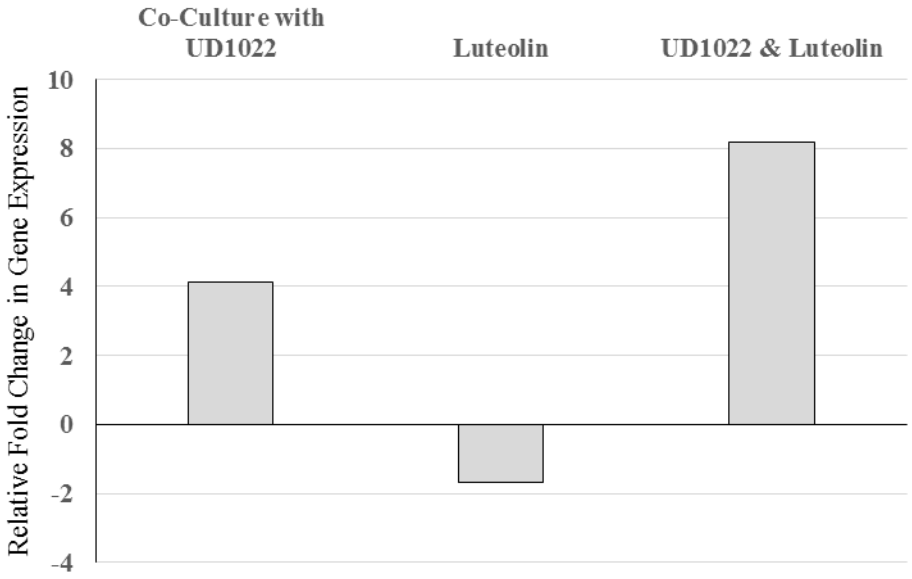

Figure 3.8 Relative Expression of Rm8530 *sinI* in Co-culture with UD1022. Co-culture with UD1022 increased the relative expression of Rm8530 *sinI* by 4-fold. The presence of luteolin (which represents the condition of Rm8530 upregulating Nod genes) doubled the effect of UD1022 on Rm8530 *sinI*, increasing expression 8-fold. Luteolin alone did not significantly change Rm8530 *sinI* expression.

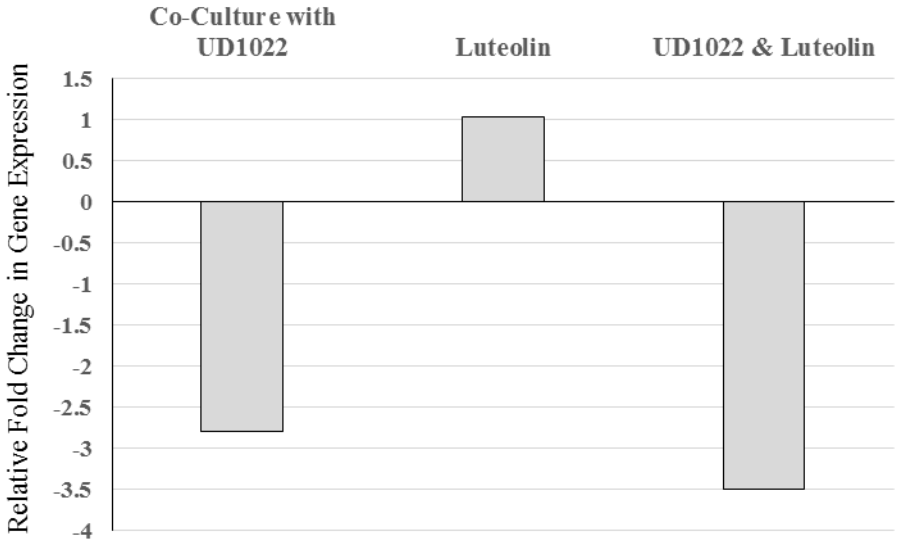

Figure 3.9 Relative Expression of Rm8530 *wggR* in Co-culture with UD1022. Co-culture with UD1022 decreased the relative expression of Rm8530 *wggR* by almost 3-fold. The presence of luteolin slightly enhanced the effect of UD1022 on Rm8530 *wggR*, increasing to 3.5-fold. Luteolin alone did not significantly change Rm8530 *wggR* expression.

The expression of Rm8530 *sinI* and *wggR* were also evaluated for Rm8530 cultured with 0.4 µM surfactin. This concentration of surfactin was shown to reduce biofilm formation as assessed in the biofilm assay performed previously. Both *sinI* and *wggR* did not alter expression to any degree of significance (≥ 2 or ≤ -2).

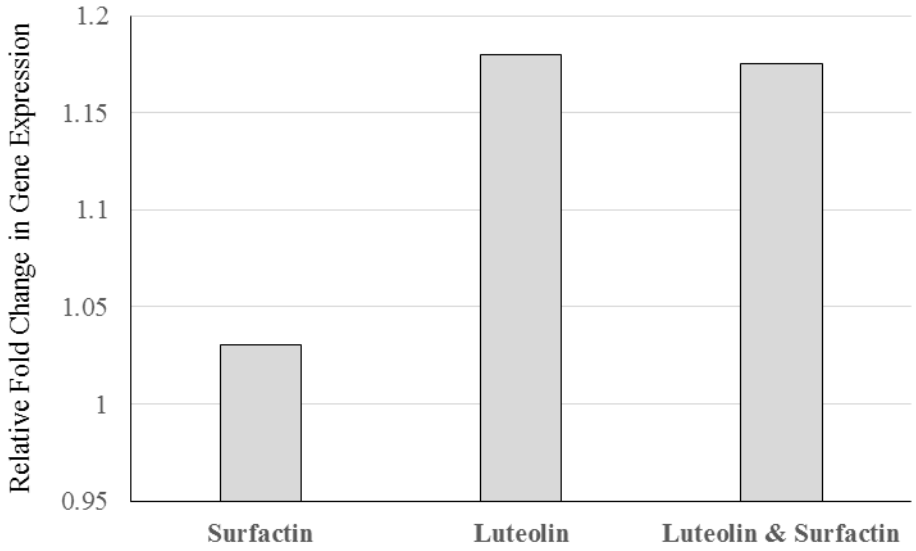

Figure 3.10 Relative Expression of Rm8530 *sinI* Cultured with Surfactin. Co-culture with 0.4 µM surfactin did not change *sinI* expression.

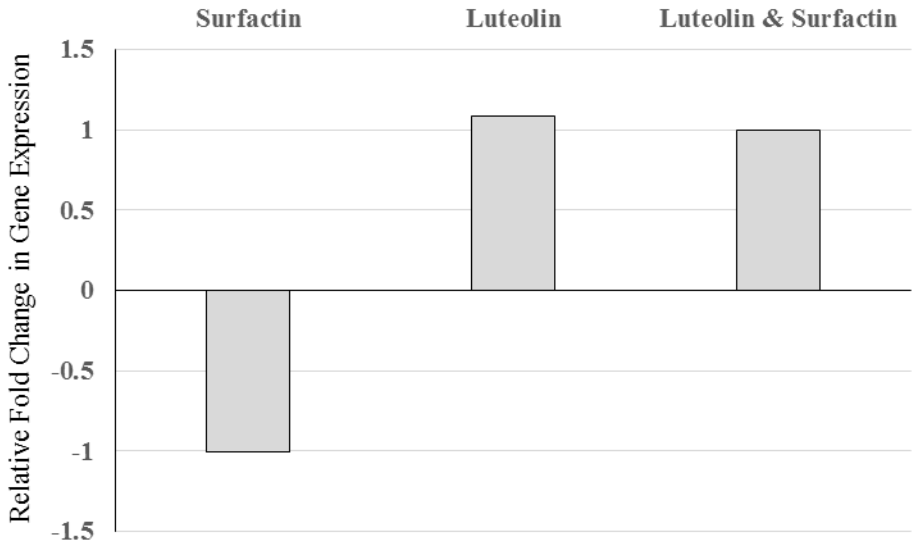

Figure 3.11 Relative Expression of Rm8530 *wggR* Cultured with Surfactin. Co-culture with 0.4 µM surfactin did not change *wggR* expression significantly.

3.8.2 Evaluation of the Relative Expression of UD1022 Genes

The influence of Rm8530 on selected genes of UD1022 was evaluated using qRT-PCR. The following genes were checked; *ytnP, srfA, eps, sinI*. The UD1022 *ytnP*-like lactonase gene was evaluated to assess whether it is responsive to the presence of Rm8530, or the presence of short and long-chain AHLs. Culturing UD1022 with 10 µM C8-AHL, 10 µM oxo-C16-AHL and 200 µM oxo-C16-AHL did not alter the expression of UD1022 *ytnP*, and therefore does not appear to be induced by AHLs (data shown in Appendix C). The relative expression of UD1022 *ytnP* in the Rm8530 co-culture was decreased by over 21-fold. Culturing UD1022 with Rm8530 induced with luteolin appeared to reduce the extremity of the UD1022 *ytnP* downregulation to 7-fold.

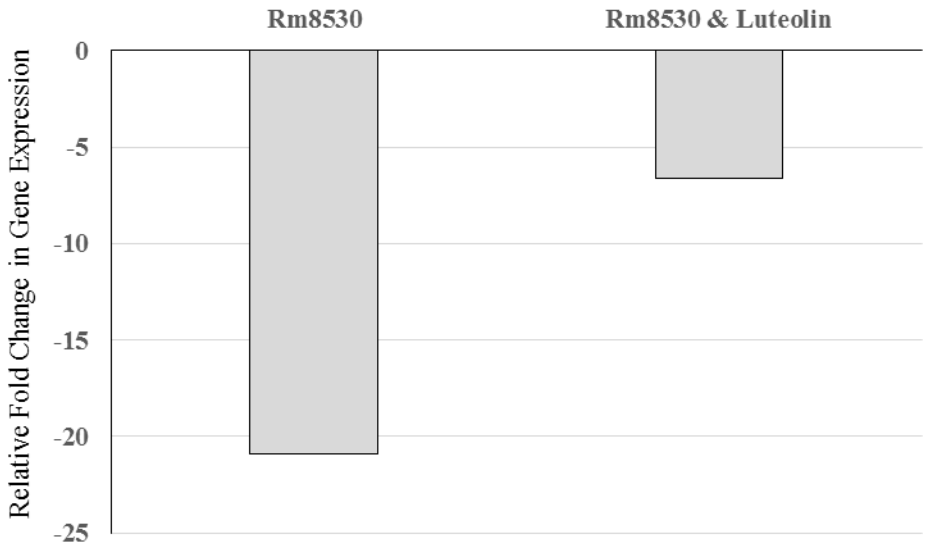

Figure 3.12 Relative Expression of UD1022 *ytnP* Cultured with Rm8530. The relative expression of UD1022 *ytnP* lactonase gene is down regulated by 21-fold in co-culture with Rm8530. This downregulation in expression appears to be attenuated by the induction of Rm8530 Nod genes by the plant flavonoid luteolin.

The UD1022 *srfA* gene is required for the production of surfactin by *B. subtilis* (Nakano et al., 1991). When UD1022 was co-cultured with Rm8530, *srfA* was down regulated by 12-fold. Again, the presence of luteolin appears to nullify this downregulation to result in no significant change in *srfA* expression.

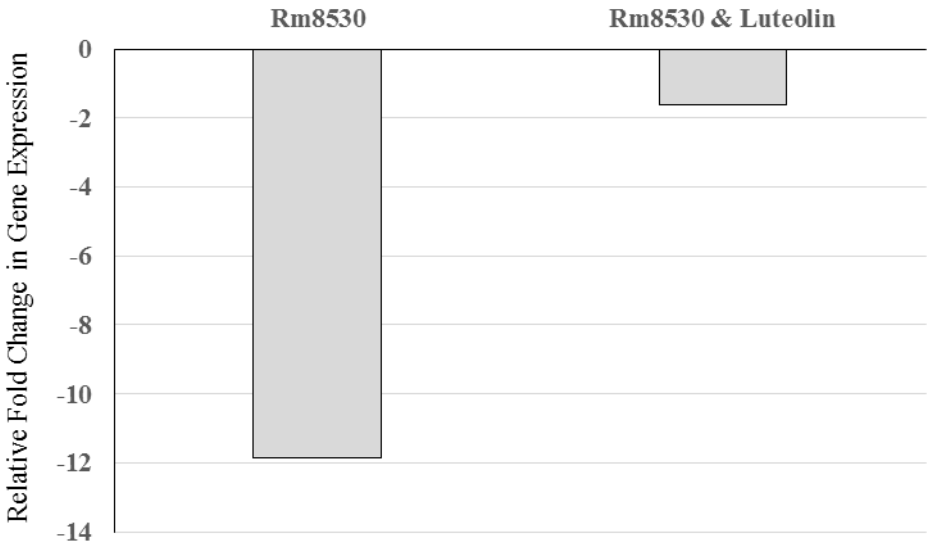

Figure 3.13 Relative Expression of UD1022 *srfA* cultured with Rm8530. Co-culture of UD1022 with Rm8530 downregulates the UD1022 *srfA* gene. The presence of luteolin resulted in the return to relatively normal expression of s*rfA*.

The UD1022 *eps* regulatory region is responsible for the production of *B. subtilis* EPS, which is the critical component biofilm that contributes to adhesion and its complex multicellular structure (Kearns et al., 2005). When UD1022 was co-cultured with Rm8530, UD1022 *eps* gene was down regulated by nearly 150-fold.

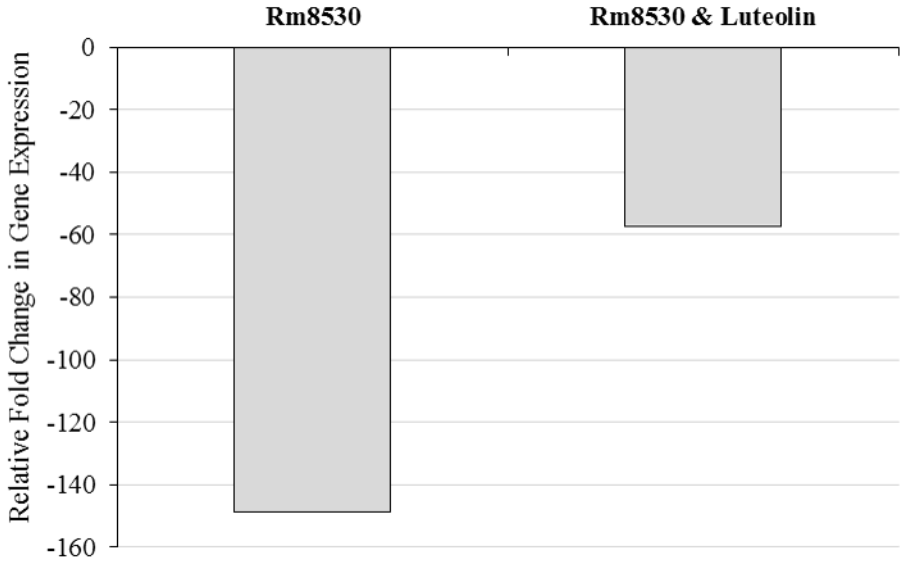

Figure 3.14 Relative Expression of UD1022 *eps* Gene Cultured with Rm8530. The UD1022 *eps* gene is down regulated by an extreme degree when UD1022 is co-cultured with Rm8530; nearly by 150-fold. Similar to other genes analyzed, the influence of luteolin mitigates the extent of the downregulation of *eps* to 57-fold.

In all instances of co-culture with Rm8530, UD1022 gene expression is altered with the inclusion of luteolin. When UD1022 is grown alone with luteolin, the gene expression of *ytnP* and *SrfA* are not altered (results reported in Appendix D). The UD1022 *eps* gene was not tested. The effect of luteolin on UD1022 gene expression in co-culture is therefore unlikely to be directly acting on UD1022.

Chapter 4

DISCUSSION

4.1 Introduction

It is clear that bacteria in the environment interact with each other in ways that are not explicitly expected. Common methods of screening for beneficial microbe compatibility include the use of a simple cross-streak assay or other means of observing growth competition on plates (Maymon et al., 2015; Kang et al., 2014). While antibiosis is a clear indication of incompatibility, simple competition assays, especially on artificially rich media, may not be a suitable standard for evaluating the interactions of plant beneficial bacteria that could influence their growth promotional and plant protective functions.

In this work, the two PGPRs Rm8530 and UD1022 were shown to have no explicit growth interference upon one another. When co-inoculated on the legume *M. truncatula*, however, plant growth promotional activities were not improved, and were slightly, but not statistically significantly, diminished. Co-inoculation did not enhance nodulation as has been reported elsewhere (Fox et al., 2011). Utilizing the tri-trophic model, it was hypothesized that the two bacteria may be interacting in a manner that disrupts the growth promotional mechanisms of the other.

4.2 Rm8530 Biofilm Inhibition

Work by Kang et al. (2014) implicated disruption of biofilm by one PGPR on the other in co-inoculation that was unsuccessful in increasing plant growth. It is well-

established that biofilm production by Rm8530 is necessary for efficient nodulation of its host (Pellock et al., 2000; Rinaudi and Gonzalez, 2009). Biofilm production by Rm8530 is solely manifested through QS systems that rely on long-chain AHLs. In other work, *B. subtilis* NCIB 3610 was shown to disrupt the QS systems and disrupted QS dependent biofilm formation of *Pseudomonas aeruginosa* through activity of the lactonase enzyme YtnP (Schneider et al., 2011). When the lactonase enzyme AiiA of *Bacillus* spp. was heterologously expressed in Rm1021 (the parental strain of Rm8530), its nodulation efficiency was reduced by 12 hours (Gao et al., 2007), similar to the *sinI* mutant strains of Rm1021 and Rm8530 that do not produce significant amounts of symbiotically active biofilm (Gao et al., 2005).

UD1022 was shown to have the ability to inhibit the biofilm production of Rm8530 in semi-quantitative assays. However, these assays were performed using CFS derived from UD1022 grown by itself, and does not account for how Rm8530 may be altering UD1022 functions such as lactonase production during co-culture. The expression of the UD1022 putative lactonase gene *ytnP* was shown to be downregulated when cultured with Rm8530. Thus, functional biofilm inhibition may not be occurring in the environment. This is yet to be shown.

4.3 UD1022 Lactonase Activity

Members of diverse species of bacteria engage in QS signal ‘quenching’ activities termed ‘quorum interference’ (QI) (Crépin et al., 2012; González and Keshavan, 2006; Uroz et al., 2009). QI between bacteria can be manifested in several different ways, but the most common of which is to physically alter the signal molecule structure through enzymatic activity (Helman and Chernin, 2015), rendering it incapable of complexing with its requisite transcriptional activator. The two major

QI enzymes found in rhizosphere bacteria are lactonases and acylases; diverse rhizosphere bacteria such as *A. tumefaciens, Ralstonia* spp., *Bacillus* spp., and *P. aeruginosa* have been found to express one or the other QI enzyme (Helman and Chernin, 2015). These enzymes can have a significant impact on the activities of QS bacteria in the rhizosphere, with an estimated 2-10% of culturable soil and rhizosphere bacteria participate in QI (Riaz et al., 2008).

Here, UD1022 was shown to have the capacity to degrade standard long chain AHLs. However, it has yet to be shown that UD1022 functionally degrades long-chain AHLs when cultured with Rm8530. AHL production by Rm8530 when cultured with UD1022 needs to be determined, as expression of Rm8530 *sinI* was shown to increase in co-culture. UD1022 may have the ability to degrade long-chain AHLs, but Rm8530 may also enact some mechanism of defense/offence to inhibit UD1022 lactonase expression.

4.4 The Role of Surfactin in the UD1022 Interaction with Rm8530

Neither high nor low levels of surfactin inhibited the growth of Rm8530. The presence of surfactin did decrease Rm8530 biofilms as measured through semi-quantitative colorimetric assays. However, the biofilm gene *wggR* was not differentially expressed in treatments of surfactin. Surfactin also did not change the relative expression of the QS gene *sinI*, indicating that it does not interfere with the Rm8530 QS pathway. The likely contribution of surfactin to the reduced biofilm formation results is due to its characteristic surfactant activity preventing biofilm adhesion. This activity could have implications *in vivo*, as Rm8530 biofilm formation and adhesion to the plant root are essential for rhizobia invasion of the plant.

4.5 How Rm8530 May be Affecting UD1022 PGPR Activities

Interestingly, and somewhat unexpectedly, Rm8530 appears to down regulate the UD1022 genes observed in this work. It was anticipated that UD1022 lactonase *ytnP* would be upregulated in the presence of Rm8530, or induced by AHL substrates. UD1022 *ytnP* was not inducible by AHLs, and more significantly, was downregulated by over 20-fold in co-culture with Rm8530. The UD1022 *SrfA* gene was also downregulated; in this case by almost 12-fold. Whether this is resulting in functional changes in surfactin production has yet to be determined. Most striking, the co-culture with Rm8530 appears to have down regulated UD1022 *eps* gene by almost 150-fold. Again, whether this translates to functional reduction in UD1022 biofilm is not known. In all three instances, the nature of the mechanism by which Rm8530 is acting on UD1022 is not known; its activity could be direct or indirect, and may involve some as yet to be described extracellular component or metabolite.

It is important to note that Rm8530 is shown here to down regulate genes that are involved in known PGPR mechanisms of *B. subtilis*, such as surfactin and biofilm production and lactonase expression. The UD1022 *srfA* gene responsible for surfactin production is down regulated. Surfactin is a known component of *B. subtilis* plant growth promotional activities which acts by triggering plant induced systemic resistance (ISR) (Ongena et al., 2007) and inhibiting the root attachment of the pathogen *Pseudomonas syringae* pv *tomato* DC3000 (Bais et al., 2004). The formation of biofilm by *B. subtilis* is essential for plant root attachment and colonization (Liu et al, 2014; Rudrappa et al., 2008), and therefore its PGPR activities. Here, the UD1022 biofilm gene *eps* was downregulated to an extreme extent. The ectopic expression of lactonase *aiiA* gene from the PGPR *Bacillus* spp. A24 transformed into *P. fluorescens* P3 has been shown to contribute to PGPR activity through interference with the QS

based virulence system of the potato soft rot organism *Erwinia carotovora* (Molina et al., 2003). UD1022 *ytnP* expression was down regulated 20-fold, which could affect the ability of UD1022 to interfere with pathogen QS systems as well as those of Rm8530. While much of the focus at the outset of this work centered on the possible mechanisms by which UD1022 could be interfering with the PGPR activity of Rm8530, it is very likely that Rm8530 may be equally contributing towards constraining the PGPR activities of UD1022.

4.6 The Possible Role of the Plant in the Tri-trophic Model

Plant flavonoids are a large group of low molecular weight phenolic compounds classified as secondary metabolites found in root exudates of all plants (Hassan and Mathesius, 2012). They play a critical role in legume-rhizobia signaling and the initiation of the process of plant nodulation (Jones et al, 2007). Flavonoids released by legumes are important signaling molecules, both serving to attract symbiotic bacteria (Dharmatilake and Bauer, 1992), and more significantly, are required to induce transcription of rhizobia Nod factors (NF) through interaction with the transcriptional regulatory protein NodD, which then binds to the nod box (Kondorosi et al., 1989). Peters et al. (1986) demonstrated that the legume-isolated flavone luteolin positively increased expression of the rhizobia *nodABC* operon.

The legume root exuded flavonoid luteolin appears to influence the interaction between UD1022 and Rm8530. Relative expression of Rm8530 QS genes in co-culture with UD1022 and luteolin was enhanced (Figures 3.8 and 3.9).There is evidence that legume flavonoids increase AHL production and AHL synthase expression in rhizobia (Pérez-Montaño et al., 2011). While luteolin in the work presented here did not by itself alter or increase Rm8530 *sinI* expression, its presence

in the co-inoculation treatments resulted in doubling the fold relative expression of *sinI* (Figure 3.8).

Some flavonoids have been documented to have antimicrobial properties (Cushnie and Lamb, 2011; Weston and Mathesius, 2013). The legume flavonoid naringenin was found to have clear antimicrobial activity against *B. subtilis* ATCC 9372 and ATCC 6633 (Rauha et al., 2000). While luteolin antimicrobial activity was not directly tested on UD1022, relative expression of *ytnP, SrfA* were not significantly altered when treated with luteolin alone. However, when luteolin is included with the co-culture, relative negative expression of these genes is minimized to a great degree. This trend may be due to an indirect mechanism. The luteolin induces the transcription and expression of Rm8530 *nod* genes, which leads to the production of Nod Factors (NF), which are signal molecules received by plant receptors to initiate nodulation (Oldroyd, 2013). This change in expression profile may represent a change in resource allotment of the rhizobia, where it may no longer have the ability to produce or enact defensive/offensive activities toward UD1022. This would, in part, account for the lessening of the negative effect of Rm8530 on UD1022 surfactin and lactonase genes in co-culture with luteolin.

4.7 Conclusion

The work in this study shows that Rm8530 and UD1022 interact with each other in ways that can interfere with their individual plant growth promoting functions. UD1022 can degrade AHLs through putative lactonase activity, and can also decrease Rm8530 biofilm production, as well as decrease the expression of the *wggR* gene required for biofilm synthesis. UD1022 increases the expression of Rm8530 *sinI* AHL synthase, possibly through a negative feedback loop by degrading the AHL signal

molecules of Rm8530. Concurrently, growth with Rm8530 appears to affect UD1022 genes key to its growth promotional activities including surfactin and biofilm production. The hypothesis of the incompatibility of the PGPRs Rm8530 and UD1022 can be supported.

Further clarity into the mechanisms employed by both Rm8530 and UD1022 that could be compromising their respective PGPR activities would be gained through more extensive analysis. Determination of UD1022's ability to enact QI *in vivo* could be conducted through high-performance liquid chromatography (HPLC) purification and gas chromatography-mass spectrometry (GC/MS) analysis of AHLs present in co-cultures, as well as a simple qualitative analysis through the AHL biosensor organism KYC55. Isolation and purification of the UD1022 putative lactonase protein for identification and use in assays performed on standard AHLs and Rm8530 cultures could provide strong mechanistic evidence of QI activity.

To gain a greater understanding of the impact of Rm8530 on UD1022, a survey of its metabolome could reveal novel extracellular factors of *S. meliloti* contributing to inter-species interactions in the rhizosphere. HPLC analysis of surfactin production by UD1022 in co-culture with Rm8530 could also illuminate the nature of its response to Rm8530. Simple UD1022 biofilm assays quantifying its response to Rm8530 CFS, surfactin, luteolin, and *M. truncatula* root exudates may provide insight to how the PGPR functionally interacts with the plant. Moreover, microscopy employing fluorescently marked bacteria applied *in vivo* to *M. truncatula* roots could reveal the outcomes of the PGPR interactions in association with the plant. It will be valuable to further explore the distinct mechanisms of association between

all three members of this tri-trophic system in order to better understand how complex inter-species interactions are taking place in the rhizosphere.

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Appendix A

BIOFILM PRODUCTION OF GFP REPORTER LINES

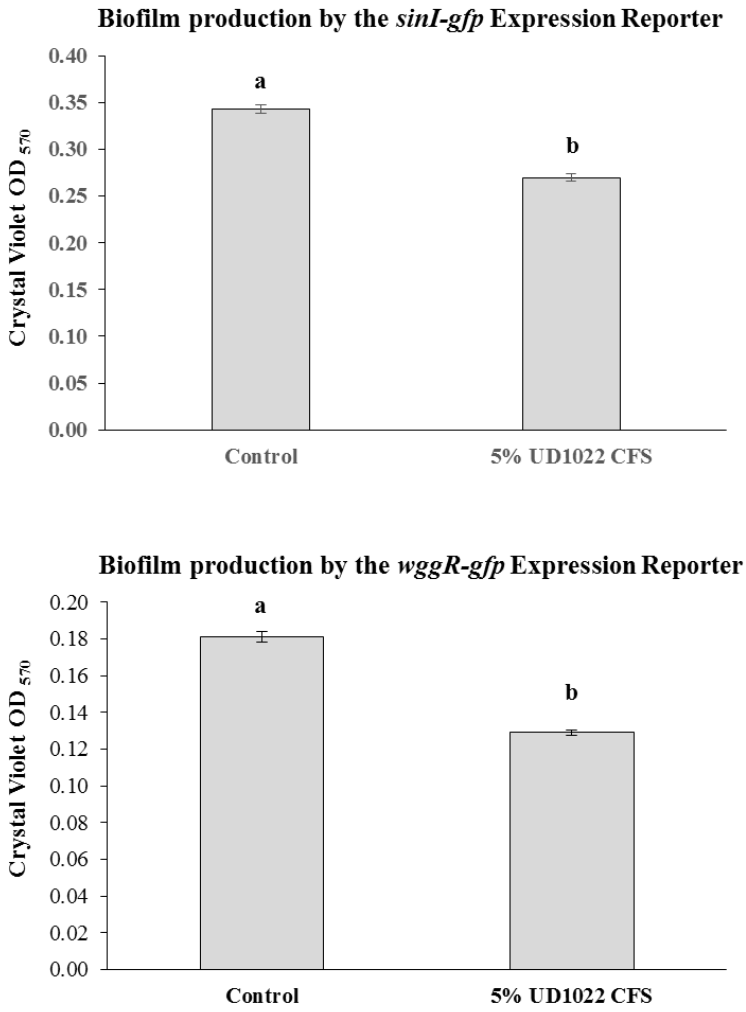

Figure A.1 Biofilm production of Rm8530 *sinI-gfp* & *wggR-gfp* expression reporters. Both expression reporter lines responded to UD1022 CFS similar to WT Rm8530; biofilms were reduced significantly (*P* value of <0.0001).

Appendix B

BIOFILM REDUCTION BY SURFACTIN

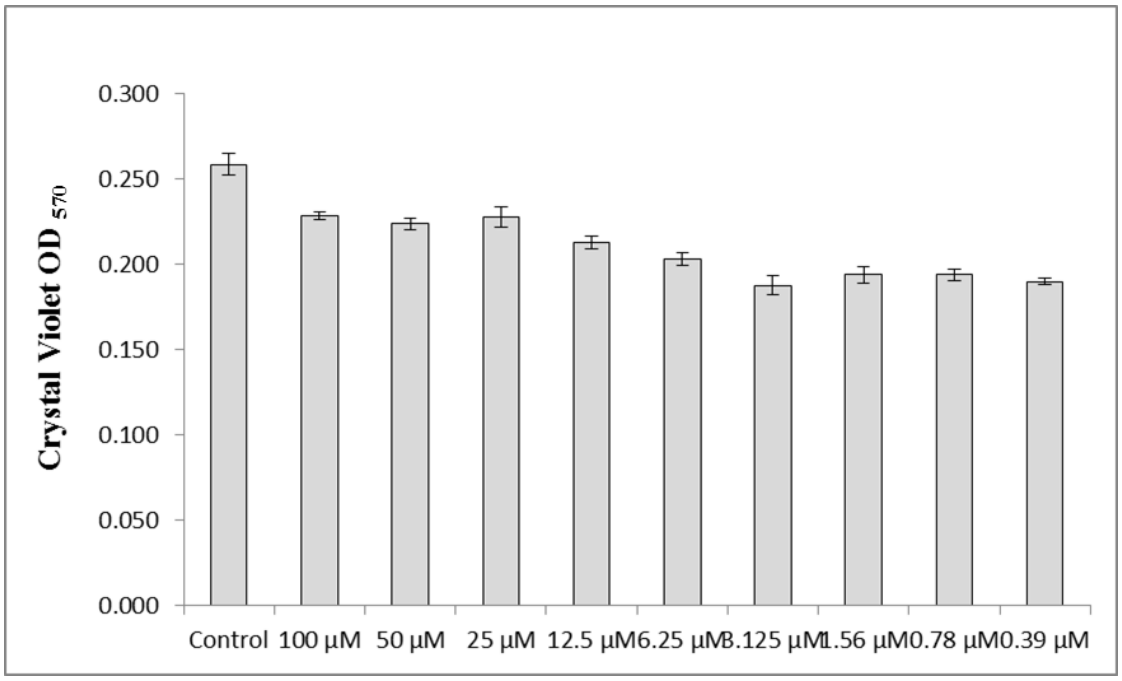

Figure B.1 Rm8530 Biofilm reduction by surfactin. All concentrations of surfactin resulted in reduction of biofilm formed by Rm8530.

Appendix C

RELATIVE EXPRESSION OF UD1022 YTNP

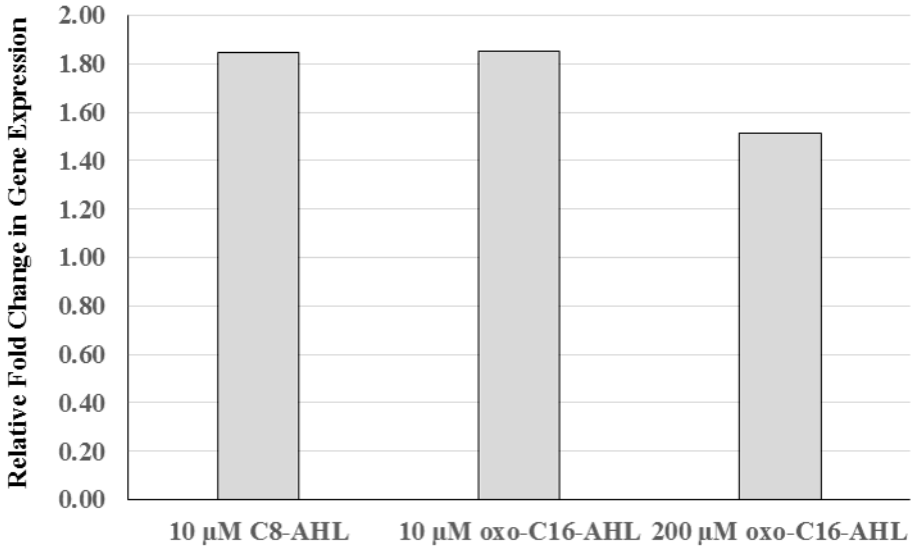

Figure C.1 Relative expression of UD1022 *ytnP* in response to AHLs. Relative expression of UD1022 *ytnP* does not change more than 2-fold.

Appendix D

RELATIVE EXPRESSION OF UD1022 SRFA & YTNP

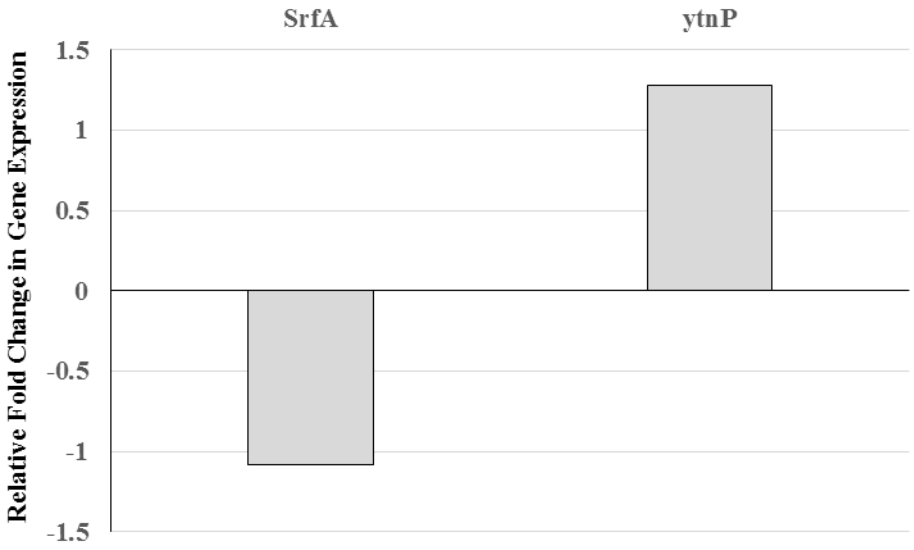

Figure D.1 Relative expression of UD1022 *SrfA* and *ytnP* in response to luteolin. The relative expression of UD1022 genes *SrfA* and *ytnP* are unaltered by the presence of luteolin alone.