

**CHARACTERIZING THE DEVASTATING RICE BLAST FUNGUS,
MAGNAPORTHE ORYZAE AND A POTENTIAL BIOCONTROL
AGENT, *BACILLUS VELEZENSIS* STRAIN S4**

by

Anna Wockenfuss

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Microbiology

Summer 2024

© 2024 Wockenfuss
All Rights Reserved

**CHARACTERIZING THE DEVASTATING RICE BLAST FUNGUS,
MAGNAPORTHE ORYZAE AND A POTENTIAL BIOCONTROL
AGENT, *BACILLUS VELEZENSIS* STRAIN S4**

by

Anna Wockenfuss

Approved: _____
Nicole Donofrio, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Erik Ervin, Ph.D.
Chair of the Department of Plant and Soil Sciences

Approved: _____
Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

ACKNOWLEDGMENTS

Firstly, I would like to thank my advisor, Dr. Nicole Donofrio, for her patience, and dedication to myself and my project. I am very grateful that she decided to take me on as her graduate student. I grew in confidence as a scientist under her guidance. Secondly, I would like to thank my lab mates, Dr. Jessica Cooper-Pancakes, and Kevin Chan, as they answered any questions I had and helped me multiple times with varying aspects of my project. I appreciate the fun times we have had in lab together. Thank you to my committee members, Dr. Kelly Craven, Dr. Jeffry Fuhrmann, and Dr. Julia Maresca, hearing your feedback and suggestions was the backbone of my project. I would also like to thank the Bioimaging Facility and Sequencing Center at the Delaware Biotechnology Institute, and Bioinformatics Data Science Core Facility at the University of Delaware, especially Tim Chaya, Dr. Shawn Polson, and Jaysheel D. Bhavsar, without their help this work would not have been possible. Thank you to the Microbiology Graduate Program and the Graduate College for my funding. Lastly, a big thank you to my family and friends who always support me and remind me to enjoy life outside of science.

TABLE OF CONTENTS

LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	x

Chapter

1 LITERATURE REVIEW OF A FUNGAL PLANT PATHOGEN, <i>MAGNAPORTHE ORYZAE</i> , AND BACTERIAL BIOLOGICAL CONTROL AGENTS.....	1
1.1 Abstract.....	1
1.2 Introduction	2
1.3 <i>Magnaporthe oryzae</i>	3
1.4 Antifungal Bacteria as Biological Control Agents.....	5
1.4.1 <i>Bacillus velezensis</i> strain S4.....	5
1.4.2 <i>Lysobacter enzymogenes</i> strain C3.....	7
1.4.3 <i>Pseudomonas chlororaphis</i> strain EA105.....	8
1.5 Conclusion.....	9
1.6 References	10
2 GENETICS OF THE INTERACTION BETWEEN <i>MAGNAPORTHE</i> <i>ORYZAE</i> AND <i>BACILLUS VELEZENSIS</i> STRAIN S4, A POTENTIAL BIOCONTROL BACTERIUM.....	18
2.1 Abstract.....	18
2.2 Introduction	19
2.3 Methods.....	20
2.3.1 Direct Assays of <i>M. oryzae</i> 4091-wt, HS, and HS mutants	20
2.3.2 Microscopy of fungal hyphal legions after direct contact with antifungal bacterium.....	22
2.3.3 Recovery assay of <i>M. oryzae</i> after contact with antifungal bacteria	22
2.3.4 Diffusible molecule assay.....	23
2.3.5 Spore germination assay.....	24

2.3.6	Statistical analysis	25
2.3.7	Enzymatic activity assessment in <i>B. velezensis</i> strain S4.....	25
2.4	Results	26
2.4.1	Direct assays showed legions in fungal lawn, but was not reliable for screening and confirmation of mutant library.....	26
2.4.2	Hyphal density and recovery after bacterial exposure	28
2.4.3	<i>B. velezensis</i> strain S4 diffusible molecules affect fungal growth of another <i>M. oryzae</i> strain - 7015 and mutants.....	31
2.4.4	<i>B. velezensis</i> S4 disrupts spore germination in <i>M. oryzae</i> 4091..	34
2.4.5	Enzymatic plate assays	35
2.5	Discussion.....	37
2.6	References	45
3	RNASEQ OF <i>MAGNAPORTHE ORYZAE</i> IN THE PRESENCE OF THE BACTERIA, <i>BACILLUS VELEZENSIS</i> STRAIN S4	49
3.1	Abstract.....	49
3.2	Introduction	50
3.3	Methods	52
3.3.1	Indirect assay	52
3.3.2	RNA Extraction and Isolation	53
3.3.3	RNaseq Analysis	54
3.4	Results and Discussion	55
3.5	References	64
4	CONCLUSIONS AND FUTURE DIRECTIONS	70
4.1	References	76
	Appendix	
A	TROUBLESHOOTING	79
4.2	Exudate Assay	79
4.3	Image J.....	79
B	PERMISSIONS	81

LIST OF TABLES

Table 2.1 <i>M. oryzae</i> fungal mutants, strain, and functions.....	21
Table 2.2 Percent (%) growth inhibition of <i>M. oryzae</i> 7015 wt and 7015 mutants on day 7 after inoculation of <i>B. velezensis</i> strain S4. Growth % inhibition was calculated from $[(Rc-Rexp)/Rc] \times 100$. Each value is the average with its respective standard deviation (R=3). p-value was determined by a 2 tailed t-test assuming unequal variance and was significant if < 0.05	33
Table 2.3 Brief overview of the bacterial genome analysis. There are 7 putative cellulase-degrading enzymes, 7 putative lipases and 42 proteases annotated in <i>B. velezensis</i> , S4 genome.	36
Table 3.1 RNA concentrations (ng/ μ L), A260/280, and A260/230 values from RNA isolates. M represents <i>M. oryzae</i> 4091 grown alone (control) and M:S represents <i>M. oryzae</i> 4091 in the presence of <i>B. velezensis</i> strain S4. Desired concentration was $> 50\text{ng}/\mu\text{L}$ with A260 ratios ~ 2.0	54
Table 3.2 logFC (fold change) and FDR (false discovery rate) values of the 69 DEGs (differentially expressed genes) in <i>M. oryzae</i> 4091. Gene ID and descriptions/functions of each DEG. Gray rows represent biologically relevant genes that will be discussed further. Total Gene IDs = 69	57

LIST OF FIGURES

- Figure 2.1** *B. velezensis* strain S4 shows a zone of “inhibition” on fungal hyphae of *M. oryzae* 4091 wt. **a.** Representative of direct assay set up of the secondary portion of the forward genetic screen on the *M. oryzae* HyPerSensor mutant collection. **b.** Example plate of *M. oryzae* 4091 wt on oatmeal agar after one day in the incubator at 25°C inoculated with LB, *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105. 27
- Figure 2.2** Potential mutant of interest, J-67B, was ruled out, due to control (LB) not beading up on the surface after inoculation of LB, *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105 grown on oatmeal agar and imaged after 1 day of incubation at 25°C. ... 28
- Figure 2.3** Microscopy images of inoculation spots of LB (control), *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105 on *M. oryzae* 4091-wt that was grown on OA (100X). Scale bar = 1000µm..... 29
- Figure 2.4** Microscopy images of inoculation spots of LB (control), *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105 on *M. oryzae* 4091-HS that was grown on OA (100X). Scale bar = 1000µm..... 30
- Figure 2.5** Fungal recovery results on PDA of **a.** *M. oryzae* 4091-wt and **b.** *M. oryzae* 4091-HS post-inoculation of (from left to right) LB (control), *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105. *M.oryzae* 4091-wt and -HS were able to grow from the spot treatments of LB and EA105, but not in the case of S4. 31
- Figure 2.6** *B. velezensis* strain S4 shows zone of inhibition on *M. oryzae* 7015 strain and 7015 mutants. There was no significant difference between wt and the mutants in the zone of inhibition. **a.** 7015 wt with LB, **b.** 7015 wt, **c.** *ago1*, **d.** *ago2*, **e.** *dcl1*, **f.** *dcl2*, **g.** *hyr1*, **h.** *rdrp1*, **i.** *yap1*..... 32

- Figure 2.7** *M. oryzae* strain 7015 and mutants percent (%) growth inhibition averages from Table 2.1. Mutants (dark blue) compared to wt (orange) in the presence of *B. velezensis* strain S4 and were determined to be $p > 0.05$ and ruled not significant. “A” represents statistically similar..... 34
- Figure 2.8** Germination of *Magnaporthe oryzae* in the presence of S4 shows reduction of appressoria formation. An overnight culture of *Bacillus velezensis* S4 was added to *M. oryzae* conidia and examined for differences in germination and appressorial formation. The bacterial culture was added to conidia in a 1:1 and 1:100 dilutions, and as a heat-killed culture. * p-value < 0.05 , which was considered statistically significant in a one-tailed student’s t-test (reproduced from Wockenfuss et al., 2024). 35
- Figure 2.9** *B. velezensis* strain S4 shows protease and cellulose activity. **a.** skim milk agar; **b.** tributyrin agar; **c.** carboxymethylcellulose agar with congo red staining (light orange around the bacterial colony indicates halo). For **a.** and **b.**, filter papers were inoculated with LB as the control (left plates) and with S4 (right plates) and incubated for 72 hours. For **c.**, filter papers were dipped in LB control (left plates) or S4 (right plates) and stamped briefly onto the plate, lifted off, and incubated for 48 hours (reproduced Wockenfuss et al., 2024)..... 37
- Figure 3.1** Example plate *M. oryzae* 4091 grown in the presence of *B. velezensis* strain S4. *M. oryzae* whole fungal hyphal colony used for RNA extractions after 7 days of incubation. **a.** Sterile filter paper with LB (left), *M. oryzae* 4091 (right). **b.** Filter paper with 5 μ l of *B. velezensis* strain S4 (left), *M. oryzae* 4091 (right)..... 52
- Figure 3.2** Smear plot showing \log_2 FC vs. \log_2 CPM for each gene. Red dots represent $FDR < 0.05$, meaning statically significant. Blue lines represent \log_2 FC range of -2 - 2. Representing 1 down-regulated gene and 41 upregulated genes (generated by Jaysheel Bhavsar in the Center for Bioinformatics and Computational Biology at UD). 56

Figure 3.3 Differentially expressed genes (DEGs) (69) based on GO Enrichment Terms. FDR < 0.2. Uncharacterized proteins = 29. Oxidoreductase activity = 12. Transferase activity = 9. Transporter activity = 7. Transporter activity = 7. Hydrolase activity = 6. Other molecule function = 4. ATP-dependent activity = 2. Made from UniProt.	61
Figure 4.1 Prediction of <i>M. oryzae</i> gene candidates involved in defense during bacterial-fungal interactions.	74
Figure 4.2 Example plate <i>R. solani</i> grown in the presence of <i>B. velezensis</i> strain S4. <i>M. oryzae</i> whole fungal hyphal colony used for RNA extractions after 7 days of incubation. a. Sterile filter paper with LB (left), <i>R. solani</i> (right). b. Filter paper with 5µl of <i>B. velezensis</i> strain S4 (left), <i>R. solani</i> (right).....	75

ABSTRACT

Plant diseases caused by fungal pathogens cause severe problems for agriculture as they greatly reduce crop yield, with some species causing up to 100% crop loss. Managing these fungal plant pathogens are of the utmost importance as the global economy and crop production relies heavily on cereal crops such as wheat, rice, maize and barley. Traditional methods in mitigating fungal pathogens, typically including fungicides, are ineffective against certain pathogens that have developed resistance, an example of this being the rice blast fungus, or *Magnaporthe oryzae*. *M. oryzae* kills enough rice to feed 60 million people per year, and thus has become a focal point in the field of plant pathology, where it is regarded as a model organism. Since the usage of fungicides in this instance is not effective, scientists must look for alternatives and now favor the use of biological control agents (BCAs), an environmentally friendly way of disease management. Common BCAs are found within the bacterial genus, *Bacillus*, which act as a plant growth promoting bacteria (PGPB). *Bacillus velezensis* strain S4 was isolated and sequenced from biochar-amended agricultural soil in Delaware. Upon genomic analysis and plate assays, *B. velezensis* strain S4 putatively encodes antifungal compounds, and enzymes including cellulases and proteases. *M. oryzae* in the presence of *B. velezensis* strain S4 displays hyphal lesions, reduced radial growth, and decreased appressorium formation, all of which could affect its pathogenicity. RNASeq analysis of this bacterial-fungal interaction revealed 69 differentially expressed genes (DEG) in *M. oryzae*, including toxin biosynthesis. All together *B. velezensis* strain S4 shows potential as a BCA for

M. oryzae; observing specific aspects of this interaction could yield more insight into how fungi defend themselves against bacteria.

Chapter 1

LITERATURE REVIEW OF A FUNGAL PLANT PATHOGEN, *MAGNAPORTHE ORYZAE*, AND BACTERIAL BIOLOGICAL CONTROL AGENTS

1.1 Abstract

The rice blast fungus, *Magnaporthe oryzae*, causes devastating disease in rice crops and other cereals, which leads to severe yield losses and resistance to chemical agents. The bacterial genus *Bacillus* has been proven to contain species that are successful, environmentally friendly biological control agents (BCAs) against plant diseases. A strain isolated from biochar-amended soil - *B. velezensis* strain S4 (S4) - has been shown to disrupt the growth of *M. oryzae*, likely via secretion of secondary metabolites and enzymes. These secondary metabolites and enzymes possibly do this by inhibiting development of *M. oryzae*'s pre-penetration structure, the appressorium. By furthering our understanding of this fungal-bacterial interaction we can hopefully glean more information about S4's potential as a BCA and fungal genetic defense mechanisms.

Keywords: Fungal pathogens, Biological control, Plant diseases, PGPR, Bacillus, Bacteria, *Magnaporthe oryzae*

1.2 Introduction

Managing plant diseases and pathogens is imperative to the success of farming crops. Plant diseases result in approximately \$220 billion per year to the global economy (Nature Publishing Group, 2021). Global crop production, about 15%, is lost to biological threats as well as a warming climate (Delgado-Baquerizo et al., 2020). Temperature changes can affect the microbial communities found in soils (Tedersoo et al., 2014), which are reservoirs for fungal pathogens. Among plant pathogens, soil-borne fungi tend to be the most aggressive. Plant diseases are largely managed by host-plant resistance, fungicides, and biological control agents (BCAs). Fungal pathogens can mutate making them resistant to fungicides, rendering fungicides ineffective (Hahn, 2014). Fungicides can also be dangerous to other organisms in the environment, and aquatic systems from runoff (Bereswill et al., 2012). This has shifted the focus to the more environmentally friendly method of BCAs, such as bacteria in the genus, *Bacillus*. BCAs have many ways of disrupting pathogens, like competition for nutrients and space in the environment, they can even disrupt pathogenicity through the release of different substances, like biosurfactants, which have antimicrobial activity (Babalola, 2010). A strain of *Bacillus*, *B. velezensis* strain S4 (S4) was isolated in Delaware and its genome has since been sequenced and analyzed (Hempel et al., 2020; Wockenfuss et al., 2024). S4 has shown antifungal activity

against *M. oryzae* strain 4091, thus having potential as a BCA, and can be used to better our understanding of fungal defense mechanisms by observing this bacterial-fungal interaction (Wockenfuss et al., 2024).

1.3 *Magnaporthe oryzae*

Magnaporthe oryzae is a hemibiotrophic, filamentous fungal pathogen that causes rice blast disease (Fernandez & Orth, 2018). Hemibiotrophic fungus start by making associations with the host as it is alive without causing symptoms while suppressing their immune system, and later switches and causes plant cell death (Fernandez & Orth, 2018). Three and a half million people rely on rice as a food staple (Rutledge et al., 2024). Rice was originally cultivated in Asia and its largest producers presently are China, India, and Indonesia (Rutledge et al., 2024). Rice provides ~ 19% of daily calories consumed globally, however with an increasing population rice demands will increase as well (Fernandez & Orth, 2018). *M. oryzae* destroys 10-30% of rice globally (Fernandez & Orth, 2018) and kills enough rice to feed 60 million people per year (*Science*, 2010, DOI:10.1126/science.327.5967.804). Different strains of this pathogen can infect other crops, like wheat, barley, and millet (Skamnioti & Gurr, 2009). Due to *M. oryzae*'s prevalence in agriculture, it is well-studied and is a model organism in fungal research (Dean et al., 2012).

Magnaporthe oryzae can infect all parts of the rice plant - stems, leaves, and roots. The infection cycle starts with the conidium (asexual spore) landing onto a rice leaf and adhering itself to the surface, where germination of the spore takes place.

From the spore differentiates a germination tube that grows across the leaf's surface. Then from the germ tube develops an appressorium, which matures causing the conidium to die as osmolytes flow from the conidium into the appressorium. A mature appressorium becomes melanized, and creates turgor, allowing force to be applied to form a penetration peg to enter the leaf cuticle. The appressorium formation process requires cAMP-dependent protein kinase A (PKA) activation and Pmk1 MAPK signaling pathway in order to form and then infect the host (Wilson & Talbot, 2009). Fungal hyphae can now grow into the leaf and cause diamond-shaped “blast” lesions, indicative of infection. Under humid conditions, the lesions will produce spores causing the continuation of the disease to spread (Wilson & Talbot, 2009). *M. oryzae* infection is difficult to control as it can adapt to the plants resistance (R) genes (Zhou et al., 2007; Huang et al., 2014).

M. oryzae genes involved in reactive oxygen species mitigation have been shown to have roles in plant infection, and we questioned whether they could also be involved in fungal defense against bacteria (Huang et al., 2011; Guo et al., 2021). These genes include ROS scavenging (*HYR1*) and a transcriptional activator protein (*YAPI*). Furthermore, former members of the Donofrio lab have worked on the role of fungal small RNAs in virulence, and as such, we have a collection of mutants that include argonaute proteins (*ago1*, *ago2*), dicer-like (*dcl1*, *dcl2*), and an RNA-dependent RNA polymerase (*rdrp1*) (Raman et al., 2017). The AGO protein family is a part of the RNA-induced silencing complex (RISC), which, during and after transcription, regulate gene expression (Campo et al., 2016). AGO proteins are

responsible for modulating target RNAs' abundance and activity serving as effectors (Campo et al., 2016). In the fungal pathogen, *Colletotrichum higginsianum*, knockout mutants - *ago1*, *dcl1* and *dcl1 dcl2* double mutants, had defective conidia morphology and conidiation (Campo et al., 2016). Additionally, RNA silencing is processed by DCL by producing small RNAs (DCL2) and regulating development of mycelia (DCL1) (Guo et al., 2021). *dcl2* and *ago2* mutants were shown to be more susceptible to mycovirus infection in *Cryphonectria parasitica*, a model organism for virus-fungus interactions (Lax et al., 2020). In *Fusarium oxysporum* f. sp. *lycopersici*, a fungal pathogen that infects tomato, displayed decreased virulence when *ago2* was knocked out (Huang et al., 2011). The *RdRP1* gene in *Mucor lusitanicus* functions in the production of antisense RNA from transgene transcripts (Lax et al., 2020). *Hyr1* gene senses and detoxifies ROS in yeast and is a homolog in *M. oryzae* (Huang et al., 2011). When in the presence of ROS, *Hyr1* activates the protein, yAP1, via formation of an intermolecular disulfide bond (Huang et al., 2011). The mutant homolog, *hyr1*, has demonstrated in roles in detoxification and acting as a plant basal immune response (Huang et al., 2011). We have both the *hyr1* and the *yap1* mutants in our lab.

1.4 Antifungal Bacteria as Biological Control Agents

1.4.1 *Bacillus velezensis* strain S4

The *Bacillus* genus is well-studied and has potential usage in agriculture, specifically as a means of biological control, as it acts as a plant growth promoting bacterium (PGPB) for certain crops (Etesami et al., 2023), like rice and wheat

(Dobrzyński et al., 2022). Combining multiple PGPBs have demonstrated efficacy to combat disease in crop plants and against a broad range of plant pathogens (Babalola, 2010). Compounds within the genus that contribute to its antimicrobial activity include bacteriocins, and non-ribosomally synthesized peptides and lipopeptides (Chen et al., 2009).

Bacillus velezensis has clusters of genes that are involved in the production of secondary metabolites, which can promote resistance in plants (Rabbee et al., 2019). *B. velezensis* is characterized by motility and biofilm formation (Xu et al., 2020). *B. velezensis* strain S4 was first isolated from biochar-amended agricultural soil in Delaware in 2011 and later its genome was sequenced in 2020 (Hempel et al., 2020). However, it was first classified as *B. amyloliquefaciens* subsp. *plantarum*, and was then reclassified as *B. velezensis* subsp. *plantarum* upon sequence analysis. Following sequencing, its genome was shown to encode for 3,918 predicted proteins, with a 46.4% GC content, and its size is 4.07 Mbp (Hempel et al., 2020). It has 12 known antibiotic gene clusters, which putatively encode compounds that are antimicrobial or antifungal. The gene cluster products with 100% identity to known gene clusters in the genus, include bacillaene, bacillibactin, bacilysin, difficifin, macrolactin, and mersacidin (Wockenfuss et al., 2024). Products with 91% identity to *Bacillus sp.* genus include plantazolicin, and surfactin. These synthesized compounds have antifungal activity; bacillibactin, bacilysin, fengycin, and surfactin. The *B. velezensis* strain S4 genome has putatively annotated enzymes, including 7 cellulose-degrading

enzymes (1 endoglucanase, 5 β -glucosidases, and 1 β -glucanases), 7 lipases, and 42 proteases (Wockenfuss et al., 2024).

1.4.2 *Lysobacter enzymogenes* strain C3

Lysobacter are gram-negative species can be found in soil and are characterized by having lytic activity against microbes (Christensen & Cook, 1978). *L. enzymogenes* can reduce the viability or kill cells from the pathogenic fungus, *Cryphonectria parasitica* (Patel et al., 2013). *L. enzymogenes* strain C3 is a BCA against plant pathogens (Sullivan et al., 2003) due to its ability to produce chitinases (Zhang & Yuen, 2000), β -1, 3-glucanases, proteases and secondary metabolites (Patel et al., 2013). *L. enzymogenes* strain C3 was first recognized as *Stenotrophomonas maltophilia* (Zhang & Yuen, 2000). One of the first studies to look for candidate fungal defense genes in a plant pathogenic fungus involving challenging *M. oryzae* with *L. enzymogenes* strain C3 and a non-antifungal strain of *L. enzymogenes* DCA, mutated in its ability to secrete enzymes. RNA transcriptomics was performed on both fungal interactions and revealed 100 candidate fungal defense genes that were upregulated when in the presence of DCA. (Mathioni et al., 2013). An example of this is the *PTH11* gene, which when deleted was found to increase lesion development in *M. oryzae* by *L. enzymogenes* strain C3.

1.4.3 *Pseudomonas chlororaphis* strain EA105

Pseudomonads are widespread in the environment. *P. chlororaphis* is characterized as a plant growth promoting rhizobacteria (PGPR) and can be beneficial in increasing resistance to environmental stresses faced by plants (Bertani et al., 2021). *P. chlororaphis* can form biofilms (Calderón et al., 2013) and can be used as BCAs, which have since been commercialized (Nam et al., 2018). *P. chlororaphis* strain EA105 are able to produce molecules that inhibit the growth of fungal pathogens including *M. oryzae* (Spence et al., 2013), and *Rhizoctonia* (Liu et al., 2007). When rice leaves were pretreated with *P. chlororaphis* strain EA105, before infection by *M. oryzae* strain 7015, there was a decrease in lesion size (Spence et al., 2013). *P. chlororaphis* strain EA105 was originally isolated from rice soil, which could mean it has an advantage that allows them to grow better in rice soil conditions versus non-native bacteria (Spence et al., 2013).

Plant pathogenic fungi likely have methods of protection against other microorganisms in the environment that are not well characterized, evidenced by the fact that they persist in soils and on plants. It is hypothesized that by observing these bacterial-fungal interactions utilizing these three known antifungal bacteria in the presence of *M. oryzae* it will reveal candidate fungal defense genes. Our goal was originally to accomplish this through performing forward genetic screens on a randomly mutagenized HS mutant library, however some setbacks presented the opportunity to perform transcriptomics on the interaction between *M. oryzae* and *B.*

velezensis strain S4, and finally on genetic assays of specific small RNA and ROS mutants.

1.5 Conclusion

Bacillus velezensis strain S4 was recently sequenced (Hempel et al., 2020) and has shown potential as biological control agent as it is able to inhibit the growth of several fungal pathogens, as well as an oomycete (Wockenfuss et al., 2024). *M. oryzae* in the presence of S4 caused spore germination to appear deformed and bubble-like (Wockenfuss et al., 2024), which could be due to their secondary metabolites. Moving away from traditional methods of plant pathological control to biocontrol, would mean the potential for a decrease in fungal pathogen genetic resistance as well as increased sustainability. Other possible bacterial biocontrols against *M. oryzae* include *Lysobacter enzymogenes* strain C3 (Mathioni et al., 2013) and *Pseudomonas chlororaphis* strain EA105 (Spence et al., 2013).

1.6 References

Armed and dangerous. (2010). *Science*, 327(5967), 804–805.

<https://doi.org/10.1126/science.327.5967.804>

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology Letters*, 32(11), 1559–1570. <https://doi.org/10.1007/s10529-010-0347-0>

Bereswill, R., Golla, B., Streloke, M., & Schulz, R. (2012). Entry and toxicity of

organic pesticides and copper in vineyard streams: Erosion rills jeopardise the

efficiency of riparian buffer strips. *Agriculture, Ecosystems &*

Environment, 146(1), 81–92. <https://doi.org/10.1016/j.agee.2011.10.010>

Bertani, I., Zampieri, E., Bez, C., Volante, A., Venturi, V., & Monaco, S. (2021).

Isolation and characterization of *Pseudomonas chlororaphis* strain ST9;

Rhizomicrobiota and in Planta Studies. *Plants*, 10(7), 1466.

<https://doi.org/10.3390/plants10071466>

Calderón, C. E., Pérez-García, A., de Vicente, A., & Cazorla, F. M. (2013). The dar

genes of *Pseudomonas chlororaphis* PCL1606 are crucial for biocontrol

activity via production of the antifungal compound 2-Hexyl, 5-propyl

resorcinol. *Molecular Plant-Microbe Interactions*®, 26(5), 554–565.

<https://doi.org/10.1094/mpmi-01-13-0012-r>

Campo, S., Gilbert, K. B., & Carrington, J. C. (2016). Small RNA-based antiviral defense in the phytopathogenic fungus *Colletotrichum higginsianum*. *PLOS Pathogens*, 12(6). <https://doi.org/10.1371/journal.ppat.1005640>

Chen, X. H., Koumoutsis, A., Scholz, R., Schneider, K., Vater, J., Süßmuth, R., Piel, J., & Borriss, R. (2009). Genome analysis of *Bacillus amyloliquefaciens* fzb42 reveals its potential for biocontrol of plant pathogens. *Journal of Biotechnology*, 140(1–2), 27–37. <https://doi.org/10.1016/j.jbiotec.2008.10.011>

Christensen, P., & Cook, F. D. (1978). Lysobacter, a new genus of nonfruiting, gliding bacteria with a high base ratio. *International Journal of Systematic Bacteriology*, 28(3), 367–393. <https://doi.org/10.1099/00207713-28-3-367>

Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., & Foster, G. D. (2012). The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13(4), 414–430. <https://doi.org/10.1111/j.1364-3703.2011.00783.x>

Delgado-Baquerizo, M., Guerra, C. A., Cano-Díaz, C., Egidi, E., Wang, J.-T., Eisenhauer, N., Singh, B. K., & Maestre, F. T. (2020). The proportion of soil-

borne pathogens increases with warming at the global scale. *Nature Climate Change*, 10(6), 550–554. <https://doi.org/10.1038/s41558-020-0759-3>

Dobrzyński, J., Jakubowska, Z., & Dybek, B. (2022). Potential of *Bacillus pumilus* to directly promote plant growth. *Frontiers in Microbiology*, 13. <https://doi.org/10.3389/fmicb.2022.1069053>

Etesami, H., Jeong, B. R., & Glick, B. R. (2023). Biocontrol of plant diseases by bacillus spp.. *Physiological and Molecular Plant Pathology*, 126, 102048. <https://doi.org/10.1016/j.pmpp.2023.102048>

Fernandez, J., & Orth, K. (2018). Rise of a cereal killer: The biology of *Magnaporthe oryzae* biotrophic growth. *Trends in Microbiology*, 26(7), 582–597. <https://doi.org/10.1016/j.tim.2017.12.007>

Guo, F., Liang, J., Xu, M., Zhang, G., Huang, L., & Feng, H. (2021). A novel DCL2-dependent micro-like RNA VM-PC-3P-92107_6 affects pathogenicity by regulating the expression of VM-vps10 in *Valsa mali*. *Frontiers in Microbiology*, 12. <https://doi.org/10.3389/fmicb.2021.721399>

Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *Journal of Chemical Biology*, 7(4), 133–141. <https://doi.org/10.1007/s12154-014-0113-1>

- Hempel, P. P., Yao, M., Yannarell, S., Shevchenko, O., Vogt, F., Donofrio, N., & Maresca, J. A. (2020). Complete genome sequence of *Bacillus velezensis* strain S4, isolated from biochar-treated soil. *Microbiology Resource Announcements*, 9(20). <https://doi.org/10.1128/mra.00352-20>
- Huang, J., Si, W., Deng, Q., Li, P., & Yang, S. (2014). Rapid evolution of avirulence genes in rice blast fungus *Magnaporthe oryzae*. *BMC Genetics*, 15(1). <https://doi.org/10.1186/1471-2156-15-45>
- Huang, K., Czymmek, K. J., Caplan, J. L., Sweigard, J. A., & Donofrio, N. M. (2011). Hyr1-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus. *PLoS Pathogens*, 7(4). <https://doi.org/10.1371/journal.ppat.1001335>
- Lax, C., Tahiri, G., Patiño-Medina, J. A., Cánovas-Márquez, J. T., Pérez-Ruiz, J. A., Osorio-Concepción, M., Navarro, E., & Calo, S. (2020). The evolutionary significance of rnaI in the fungal kingdom. *International Journal of Molecular Sciences*, 21(24), 9348. <https://doi.org/10.3390/ijms21249348>
- Liu, H., He, Y., Jiang, H., Peng, H., Huang, X., Zhang, X., Thomashow, L. S., & Xu, Y. (2007). Characterization of a phenazine-producing strain *Pseudomonas chlororaphis* GP72 with broad-spectrum antifungal activity from green pepper rhizosphere. *Current Microbiology*, 54(4), 302–306. <https://doi.org/10.1007/s00284-006-0444-4>

Mathioni, S. M., Patel, N., Riddick, B., Sweigard, J. A., Czymmek, K. J., Caplan, J. L., Kunjeti, S. G., Kunjeti, S., Raman, V., Hillman, B. I., Kobayashi, D. Y., & Donofrio, N. M. (2013). Transcriptomics of the rice blast fungus *Magnaporthe oryzae* in response to the bacterial antagonist *Lysobacter enzymogenes* reveals candidate fungal defense response genes. *PLoS ONE*, 8(10).

<https://doi.org/10.1371/journal.pone.0076487>

Nam, H. S., Anderson, A. J., & Kim, Y. C. (2018). Biocontrol efficacy of formulated *Pseudomonas chlororaphis* 06 against plant diseases and root-knot nematodes. *The Plant Pathology Journal*, 34(3), 241–249.

<https://doi.org/10.5423/ppj.nt.12.2017.0264>

Nature Publishing Group. (2021, August 5). *Pathogens, precipitation and produce prices*. Nature News. <https://www.nature.com/articles/s41558-021-01124-4#ref-CR3>

Patel, N., Oudemans, P. V., Hillman, B. I., & Kobayashi, D. Y. (2013). Use of the tetrazolium salt MTT to measure cell viability effects of the bacterial antagonist *Lysobacter enzymogenes* on the filamentous fungus *Cryphonectria parasitica*. *Antonie van Leeuwenhoek*, 103(6), 1271–1280.

<https://doi.org/10.1007/s10482-013-9907-3>

Rabbee, M., Ali, Md., Choi, J., Hwang, B., Jeong, S., & Baek, K. (2019). *Bacillus velezensis*: A valuable member of bioactive molecules within plant

microbiomes. *Molecules*, 24(6), 1046.

<https://doi.org/10.3390/molecules24061046>

Raman, V., Simon, S. A., Demirci, F., Nakano, M., Meyers, B. C., & Donofrio, N. M. (2017). Small RNA functions are required for growth and development of *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions*[®], 30(7), 517–530. <https://doi.org/10.1094/mpmi-11-16-0236-r>

Rutledge, K., McDaniel, M., & Teng, S. (2024, January 3). *Food staple*. Education. <https://education.nationalgeographic.org/resource/food-staple/>

Skamnioti, P., & Gurr, S. J. (2009). Against the grain: Safeguarding rice from Rice Blast Disease. *Trends in Biotechnology*, 27(3), 141–150. <https://doi.org/10.1016/j.tibtech.2008.12.002>

Spence, C. A., Raman, V., Donofrio, N. M., & Bais, H. P. (2013). Global gene expression in rice blast pathogen *Magnaporthe oryzae* treated with a natural rice soil isolate. *Planta*, 239(1), 171–185. <https://doi.org/10.1007/s00425-013-1974-1>

Sullivan, R. F., Holtman, M. A., Zylstra, G. J., White, J. F., & Kobayashi, D. Y. (2003). Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. *Journal of Applied*

Microbiology, 94(6), 1079–1086. <https://doi.org/10.1046/j.1365-2672.2003.01932.x>

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., Ruiz, L. V., Vasco-Palacios, A. M., Thu, P. Q., Suija, A., Smith, M. E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., ... Abarenkov, K. (2014). Global diversity and geography of soil fungi. *Science*, 346(6213). <https://doi.org/10.1126/science.1256688>

Wilson, R. A., & Talbot, N. J. (2009). Under pressure: Investigating the biology of plant infection by *Magnaporthe oryzae*. *Nature Reviews Microbiology*, 7(3), 185–195. <https://doi.org/10.1038/nrmicro2032>

Wockenfuss, A., Chan, K., Cooper, J. G., Chaya, T., Mauriello, M. A., Yannarell, S. M., Maresca, J. A., & Donofrio, N. M. (2024). A *Bacillus velezensis* strain shows antimicrobial activity against Soilborne and foliar fungi and oomycetes. *Frontiers in Fungal Biology*, 5. <https://doi.org/10.3389/ffunb.2024.1332755>

Xu, S., Xie, X., Zhao, Y., Shi, Y., Chai, A., Li, L., & Li, B. (2020). Whole-genome analysis of *Bacillus velezensis* ZF2, a biocontrol agent that protects cucumis sativus against *Corynespora* leaf spot diseases. *3 Biotech*, 10(4). <https://doi.org/10.1007/s13205-020-2165-y>

Zhang, Z., & Yuen, G. Y. (2000). Effects of culture fluids and preinduction of chitinase production on biocontrol of *Bipolaris* leaf spot by *Stenotrophomonas*

maltophilia C3. *Biological Control*, 18(3), 277–286.

<https://doi.org/10.1006/bcon.2000.0834>

Zhou, E., Jia, Y., Singh, P., Correll, J. C., & Lee, F. N. (2007). Instability of the *Magnaporthe oryzae* avirulence gene *avr-pita* alters virulence. *Fungal Genetics and Biology*, 44(10), 1024–1034. <https://doi.org/10.1016/j.fgb.2007.02.003>

Chapter 2

GENETICS OF THE INTERACTION BETWEEN *MAGNAPORTHE ORYZAE* AND *BACILLUS VELEZENSIS* STRAIN S4, A POTENTIAL BIOCONTROL BACTERIUM

2.1 Abstract

The rice blast fungus or *Magnaporthe oryzae*, causes devastating disease in rice crops, which leads to severe yield losses and resistance to chemical agents. The bacterial genus *Bacillus* includes species that are successful, and environmentally friendly biological control agents (BCA) against plant diseases. A strain isolated from biochar-amended soil, *B. velezensis* strain S4, has shown to disrupt the growth of *M. oryzae* via secretion of secondary metabolites and enzymes. In this chapter we observed the interaction between *M. oryzae* 4091 wildtype (wt), *M. oryzae* 4091 HyPerSensor (HS), and *M. oryzae* 4091 HS mutants in the presence of *B. velezensis* strain S4, and 3 other antifungal bacteria to assess whether they could be candidate fungal defense mutants of interest that would later be sequenced, and their genomes analyzed. After direct contact, we were interested in seeing whether the fungus could recover. Additionally, genetic assays of small RNA and ROS mutants were performed in a different parental strain of *M. oryzae*, 7015. Next, we were interested in how the

appressoria, the pre-penetrative structure important to the infection by *M. oryzae*, was affected by whole cell cultures of *B. velezensis* strain S4. Lastly, enzymatic activity by S4 was observed phenotypically via plate assays. Demonstrating that the bacterium secretes these secondary metabolites and enzymes may provide evidence that they decrease the number of *M. oryzae*'s pre-penetration structures, the appressorium, as well as disrupt vegetative growth. By furthering our understanding of this fungal-bacterial interaction we can hopefully glean more information about S4's potential as a BCA and fungal genetic defense mechanisms.

Keywords: Forward genetic screen, ROS, small RNA, enzymes

2.2 Introduction

Biological control agents (BCAs), host-plant resistance, and fungicides are the common practices in regulating plant diseases. However, the rice blast fungus, or *Magnaporthe oryzae*, which infects and kills rice, barley and other crops, can mutate in the presence of fungicides and become resistant (Hahn, 2014). BCAs are microorganisms, like bacteria or fungi, that can disrupt the growth of plant and fungal pathogens. *Bacillus velezensis* strain S4 genome contains gene clusters that encode for antimicrobial compounds (Hempel et al., 2020) making it a possible candidate as a BCA against *M. oryzae*. Other bacteria have shown antimicrobial activity against *M. oryzae*, including *Lysobacter enzymogenes* strain C3 (Mathioni et al., 2013) and *Pseudomonas chlororaphis* strain EA105 (Spence et al., 2013).

In response to stress via a pathogen, the plant can employ reactive oxygen species (ROS) as a means of resistance (Mansoor et al., 2022). ROS in plants can include hydrogen peroxide (H_2O_2), which is highly stable. A mutant collection of *M. oryzae* strain 4091 (barley infecting strain) was created for the possible identification of reactive oxygen species-related genes (Huang et al., 2016). Each mutant had an ROS sensor that could detect H_2O_2 , HyPer, which is a permutated yellow fluorescent protein (Huang et al., 2016). The collection contains approximately 2,000 mutants (Huang et al., 2016).

In summary, by observing growth of the fungal pathogen, *M. oryzae* and the HyPerSensor (HS) mutant collection in the presence of the antifungal bacterium, *B. velezensis* strain S4, we could reveal a potential new biological control agent and learn more about *M. oryzae* fungal defense mechanisms.

2.3 Methods

2.3.1 Direct Assays of *M. oryzae* 4091-wt, HS, and HS mutants

To identify mutants of interest from the HS mutant collection, a forward genetic screen was performed, with the idea that once a mutant of interest was identified, it could then be confirmed, and its genome sequenced. The mutated gene would represent a strong candidate as a fungal defense gene against S4.

M. oryzae 4091 wildtype (wt), *M.oryzae* 4091-HyPerSensor (HS), and HS mutants (Table 2.1) were grown out from filter stock papers (Fisher Scientific, PA, USA) that had been stored at $-20^{\circ}C$ onto small plates of potato dextrose agar (PDA) (Becton, Dickinson and Company, MD, USA) for 1 week in an incubator at $25^{\circ}C$.

Table 2.1 *M. oryzae* fungal mutants, strain, and functions.

Name	Strain	Abbreviation	Function
<i>M. oryzae</i> 4091 wildtype	4091	wt	Barley infector
<i>M. oryzae</i> HyPerSensor		HS	Tracks ROS
<i>M. oryzae</i> HyPerSensor mutant, J-67B		J-67B	Identified as potential mutant of interest, later ruled out
<i>M. oryzae</i> 7015 mutants	7015	<i>ago1</i>	Small RNA, argonaute protein
		<i>ago2</i>	
		<i>dcl1</i>	Small RNA, dicer-like
		<i>dcl2</i>	
		<i>hyr1</i>	ROS scavenging
		<i>rdrp1</i>	Small RNA, polymerase
		<i>yap1</i>	ROS, activator protein

The fungal cultures were replated into 6 well-plates (in duplicate) of PDA and grown for a week at 25°C. An overnight culture of *B. velezensis* strain S4 was cultured using Luria Broth (LB) (Fisher Scientific, Pittsburgh, PA, USA) at 37°C for 12-18 hours at 80 rpm. The fungal plates were pipetted with 10 µl of LB on the left side of the plate as a negative control. *M. oryzae* is hydrophobic (Eseola et al., 2021), so when LB is plated directly onto the fungus, it beads up and stays in place. Then 10 µl of *B. velezensis* strain S4 overnight culture was pipetted onto the right side of the plate. The 6-well plates were wrapped with parafilm and placed into a 23 - 25°C incubator for 24 hours. Images were taken of each respective plate. Preliminary direct assay experiments with wt and HS showed that *B. velezensis* strain S4 created small holes or legions in the fungal hyphal mat, which could then be used as an indicator of

inhibition by the bacterium (Figure 2.1). The phenotype we were looking for was a larger “zone of inhibition” compared to the wt and HS, which would be classified as a potential mutant of interest, that would then be used in secondary screens (Figure 2.2).

2.3.2 Microscopy of fungal hyphal legions after direct contact with antifungal bacterium

To assess hyphal response to the bacterial droplet/legion, we viewed direct assay plates under microscopy for both *M. oryzae* 4091-wt and HS. *M. oryzae* wt and HS were grown on a regular sized oatmeal agar (OA) plate for 1 week in a 23 - 25°C incubator. Once the fungal hyphae had grown/reached to the edge of the plates, an overnight culture of *B. velezensis* strain S4 was made using LB (see section 2.3.1). Additionally, overnight cultures of *L. enzymogenes* strain C3 and *P. cholororaphis* strain EA105 were made using tryptic soy broth (TSB), and LB, respectively, at 30°C, and 37°C, respectively. Then 45µl of LB, *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. cholororaphis* strain EA105 were plated onto both wt and HS plates and left in an incubator for 24 hours at 25°C. They were then viewed using a Zeiss AxioZoom microscope and imaged with AxioCam 512 in Zen Blue 2.3 Pro.

2.3.3 Recovery assay of *M. oryzae* after contact with antifungal bacteria

We investigated if *M. oryzae* 4091- wt (wt) or *M. oryzae* 4091-HyPerSensor (HS) once in the presence of antifungal bacteria, including *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. cholororaphis* strain EA105 could recover, which in

this case would be assessed phenotypically via growth of fungal hyphae. A direct assay of wt and HS was performed as described above (see section 2.3.2). Following incubation of fungal plates at 23 °C, a recovery assay was performed from the regions of each treatment of the direct assay plates. A sterile pick was used to take from each treatment from the fungal-bacterial direct assay plates after 1 day, and 4 days post-inoculation and replated onto small PDA plates. The PDA plates were grown in an incubator at 23°C for 1 week and observed.

2.3.4 Diffusible molecule assay

We wanted to investigate whether the *M. oryzae* 7015 wt and mutants of the 7015 strain (Table 2.1) would show similarity to the inhibition shown by the 4091 strain by *B. velezensis* strain S4. Instead of placing the bacteria in direct contact with the fungus, they would be grown together on the same petri plate. All fungal mutants tested in this assay (*ago 1*, *ago 2*, *dcl 1*, *dcl 2*, *hyr1*, *rdrp 1*, and *yap1*) were grown on 50% strength PDA plates for 1 week at 25°C (Table 2.1). An overnight culture of *B. velezensis* strain S4 in LB was made at 30°C for 16 hours with shaking at 80 rpm. Fresh 50% strength PDA plates each had a sterile filter paper (0.8 cm) placed onto the left side of the plate and an 0.8 cm fungal plugs (of both wt and 7015 mutants) on the right side of the plate, each 2 cm away from the edge (Wockenfuss et al., 2024). Then 5 µL of LB (control), or *B. velezensis* strain S4 was pipetted onto the sterile filter paper. The plates were wrapped in micropore tape and incubated at 24°C for 7 days. Measurements on day 1, 3, 5, and 7 were taken of the fungal growth in *Rc* (radius

from the fungal plug to the longest hyphal growth), and R_{exp} (shortest radius of the fungal growth to the bacterium) in cm (Chen et al., 2018). Day 7 was used to calculate the % growth inhibition (Table 2.2).

2.3.5 Spore germination assay

The appressorium is an essential pre-penetrative structure in the lifecycle of *M. oryzae*'s and is imperative to its pathogenicity. Similarly, spores, and germination tubes are also important in its lifecycle and development. Since *B. velezensis* strain S4 has displayed inhibitory properties how would these diffusible molecules affect how spores would develop. *B. velezensis* strain S4 was grown overnight as previously described (see section 2.3.4), and diluted to either 2:1, 1:1, 1:50, or 1:100, as well as an undiluted culture that was heat-killed by incubating for 30 minutes at 95°C. Next, 150µL of *M.oryzae* 4091-wt spores were placed into each well of a 96-well plate with a concentration of 5.9×10^5 spores per mL. Then 150µL of sterile water (control), 150µL of LB (control), and 150µL of each bacterial culture were placed into the wells with the spores. Each treatment was in triplicate on the plate. The plate was incubated overnight at room temperature. A CD7 microscope was used for imaging, and Zen 3.2 was used for the manually counting of ungerminated spores, germ tubes (with no appressoria), and appressoria (with germ tubes). The triplicates were used for statistical analysis with a total of over 700 spores included.

2.3.6 Statistical analysis

To assess significance between each treatment, statistical analysis was performed. Each treatment was compared to the water control using Microsoft Excel t-test function. Significance was given with a p-value < 0.05.

2.3.7 Enzymatic activity assessment in *B. velezensis* strain S4

The genome of *B. velezensis* strain S4 has been previously sequenced (Hempel et al., 2020). The NCBI BLAST sequence was searched for genes that encode for specific enzymes, including cellulase-degrading (endonuclease, β -glucosidase, and β -glucanase), lipase, and protease (Accession CP050424).

In order to assess the enzymatic activity of *B. velezensis* strain S4 an enzymatic plate assay was performed on various media types. Carboxymethylene cellulose (CMC) agar (agar 15 g, sodium salt 5 g (Sigma-Alrich, St. Louis MO, USA, potassium chloride 1 g, sodium nitrate 1 g, yeast extract 1 g, dipotassium phosphate 0.5 g, magnesium sulfate heptahydrate 0.5 g, pH 7.0) was made to test for cellulases (Batubara et al., 2021). Tributyrin agar (TA) (agar 12 g, 10 mL glyceryl tributyrate (Sigma-Aldrich), peptone 5 g, yeast extract 3 g, filter-sterilized 1:1 Tween 20: deionized water per liter 6 drops (Sigma-Aldrich)) was made for testing lipase activity (Wockenfuss et al., 2024). Lastly, skim milk agar (SMA) (skim milk powder 100 g (Bectin, Dickson and Co., Franklin Lakes NJ, USA) and agar 16 g) was made to assess the enzymatic activity of protease (Baard et al., 2023). An overnight culture of *B. velezensis* strain S4 was grown at 30°C for 16 hours at 80 rpm ($OD_{600\text{ nm}} = 0.5-0.7$).

Sterile filter papers were dipped into LB (negative control) and placed into the middle of each type of media plate. Sterile filters were also dipped into overnight culture and placed onto the middle of lipase and protease plates, with the cellulases plates being briefly “stamped” with the filter paper of overnight culture and removed from the plate. Protease, lipase and cellulase plates were incubated at 30°C for 3 days for the protease and lipase, and 2 days for the cellulase plates. Then only the cellulase plates were flooded with 0.1% Congo Red (10 mL/plate) and left at room temperature to incubate for 20 minutes. The plates were then washed with 1M NaCl (~ 30 mL). Following incubation of the protease and lipase, and staining/wash of the cellulase plates, they were imaged.

2.4 Results

2.4.1 Direct assays showed legions in fungal lawn, but was not reliable for screening and confirmation of mutant library

To determine possible fungal defense genes, present in *M. oryzae*, we screened a HS mutant collection against bacteria that secrete antifungal materials. There was a total of 455 fungal mutants out of 2,000 that were screened using the direct contact method. After 111 mutants tested, our controls (*M. oryzae* 4091-wt and HS) started showing either a smaller zone of infiltration than previously seen, or no longer developing a legion in the hyphae with *B. velezensis* strain S4. From those tests there were about 50 potential mutants of interest that were retested for reliability and were

then ruled out as potential mutants of interest after additional direct assays that included *L. enzymogenes* strain C3 and *P. chlororaphis* strain EA105 (Figure 2.1, 2.2). The mutants showed bacterial lesions that were no different from wt in these confirmation tests or did not display the hydrophobicity phenotype.

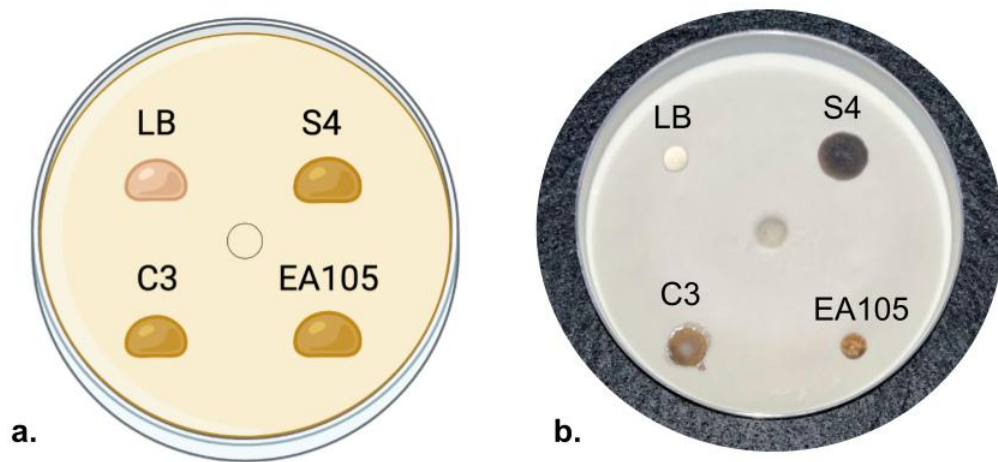


Figure 2.1 *B. velezensis* strain S4 shows a zone of “inhibition” on fungal hyphae of *M. oryzae* 4091 wt. **a.** Representative of direct assay set up of the secondary portion of the forward genetic screen on the *M. oryzae* HyPerSensor mutant collection. **b.** Example plate of *M. oryzae* 4091 wt on oatmeal agar after one day in the incubator at 25°C inoculated with LB, *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105.



Figure 2.2 Potential mutant of interest, J-67B, was ruled out, due to control (LB) not beading up on the surface after inoculation of LB, *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105 grown on oatmeal agar and imaged after 1 day of incubation at 25°C.

2.4.2 Hyphal density and recovery after bacterial exposure

We wanted to know what was occurring in the legions after bacterial inoculation. The images of the holes/spots of different inoculation treatments showed a qualitative decrease in fungal hyphae density in both wt (Figure 2.3) and HS (Figure 2.4). Spores of *M. oryzae* were observed (wt), and their morphology appeared to be normal (data not shown). There were differences, however, in the inoculation spots on the hyphae between wt (Figure 2.3) and HS (Figure 2.4). The spots/zones of inhibition in wt background (Figure 2.1, 2.3) were clearer/better defined than in HS background (Figure 2.2, 2.4). *M. oryzae* 4091-wt, -HS, and HS mutants grow differently on media plates. There is usually a difference in their hyphae with a more flat, even colored appearing hyphae (in wt) (Figure 2.1B) to uneven/blotchy colored hyphae in HS and

HS mutants, which at times would make the results more difficult to interpret (Figure 2.2). *M. oryzae* 4091-HS has multiple copies of the HS gene in its genome (Huang et al., 2016), which could account for the differences in wettability observed (Figure 2.1B, 2.2, 2.3, 2.4). Displaying a wettability phenotype has been shown to affect pathogenicity, in the case of the fungal plant pathogen, *M. grisea* (Kim et al., 2005).

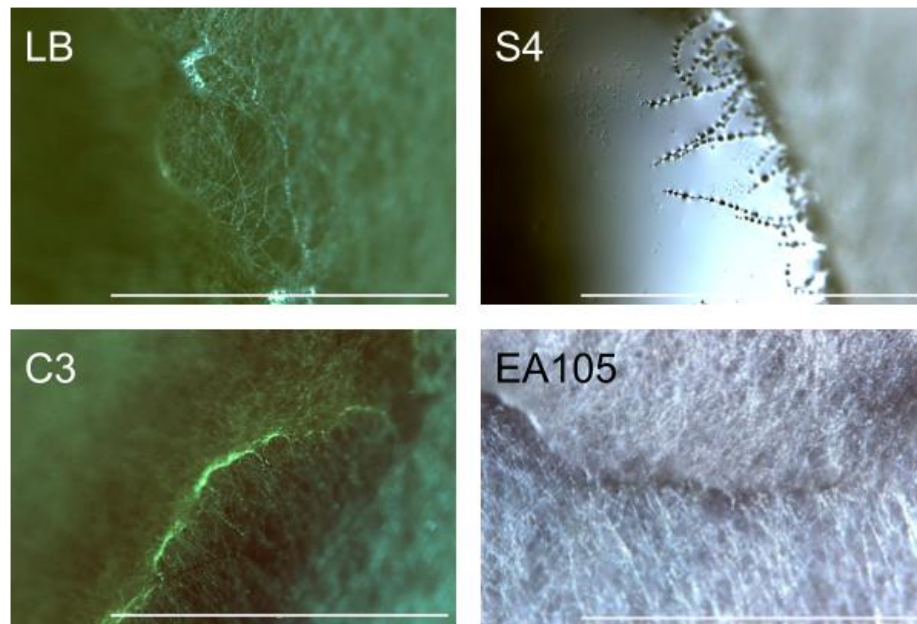


Figure 2.3 Microscopy images of inoculation spots of LB (control), *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105 on *M. oryzae* 4091-wt that was grown on OA (100X). Scale bar = 1000 μ m.

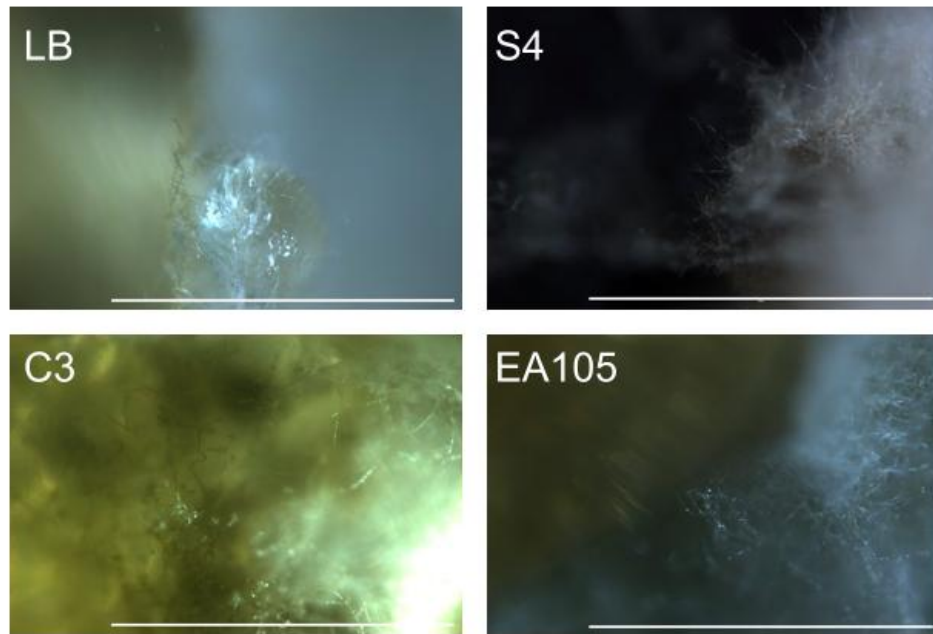


Figure 2.4 Microscopy images of inoculation spots of LB (control), *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105 on *M. oryzae* 4091-HS that was grown on OA (100X). Scale bar = 1000 μ m.

Additionally, we were curious if *M. oryzae* 4091 could grow again/recover after being exposed to antifungal bacterial strains, since *B. velezensis* strain S4 has only been seen as fungistatic, and not fungicidal (Wockenfuss et al., 2024). The recovery control (LB) plates were able to grow *M. oryzae* 4091-wt and HS (Figure 2.5). There was no fungal growth in the *B. velezensis* strain S4, only bacterial growth (Figure 2.5). The *L. enzymogenes* strain C3 recovery plates were contaminated after several attempts of the methodology; however, HS was able to grow some of the time, but since there was contamination, these are not reliable results and would need to be retested (Figure 2.5). *M. oryzae* wt and HS were able to grow out on the *P.*

chlororaphis strain EA105 plates, similar to the control recovery plates, however the hyphae were slightly melanized, indicating stress for the fungus (Figure 2.5). Additionally, there were no significant morphological differences between how long the different bacteria were in contact with the fungus, and their ability to grow (or not grow) (data not shown).

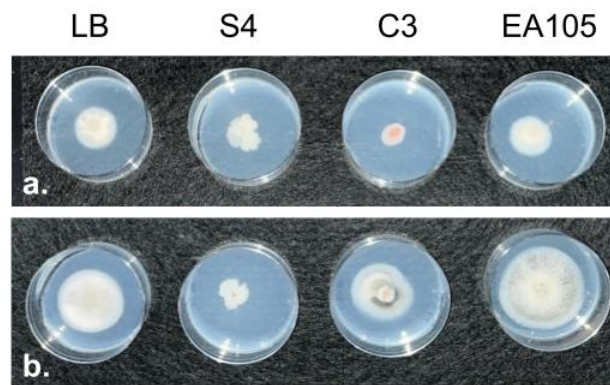


Figure 2.5 Fungal recovery results on PDA of **a.** *M. oryzae* 4091-wt and **b.** *M. oryzae* 4091-HS post-inoculation of (from left to right) LB (control), *B. velezensis* strain S4, *L. enzymogenes* strain C3, and *P. chlororaphis* strain EA105. *M.oryzae* 4091-wt and -HS were able to grow from the spot treatments of LB and EA105, but not in the case of S4.

2.4.3 *B. velezensis* strain S4 diffusible molecules affect fungal growth of another *M. oryzae* strain - 7015 and mutants

M. oryzae strain 7015 mutants are involved in small RNA production, as well as virulence, we were interested in seeing whether they would also be inhibited by *B. velezensis* strain S4. When *M. oryzae* 7015 wt (wt), and 7015 mutants (*ago2*, *dcl1*, *dcl2*, *hyr1*, *rdrp1*, *yap1*) (Table 2.1) were grown in the presence of *B. velezensis* strain

S4 there was a small zone of inhibition compared to when grown with the negative control (LB) (Figure 2.6). Growth inhibition (%) ranged from 28-35% (Table 2.2). There was no significant difference between inhibition between wt and the mutants (Table 2.2). The % growth inhibition was significant (p -value < 0.05) when compared to the LB (negative control). The mutants were compared to the wt % inhibition; they were not significant (p -value > 0.05) (Figure 2.7).

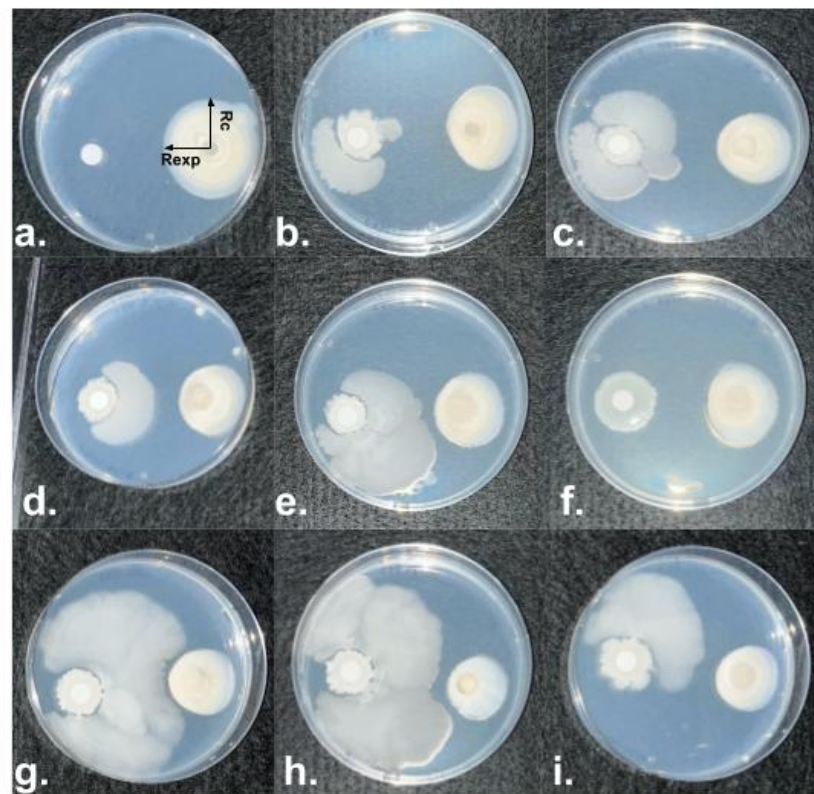


Figure 2.6 *B. velezensis* strain S4 shows zone of inhibition on *M. oryzae* 7015 strain and 7015 mutants. There was no significant difference between wt and the mutants in the zone of inhibition. **a.** 7015 wt with LB, **b.** 7015 wt, **c.** *ago1*, **d.** *ago2*, **e.** *dcl1*, **f.** *dcl2*, **g.** *hyr1*, **h.** *rdrp1*, **i.** *yap1*.

Table 2.2 Percent (%) growth inhibition of *M. oryzae* 7015 wt and 7015 mutants on day 7 after inoculation of *B. velezensis* strain S4. Growth % inhibition was calculated from $[(Rc-Rexp)/Rc] \times 100$. Each value is the average with its respective standard deviation (R=3). p-value was determined by a 2 tailed t-test assuming unequal variance and was significant if < 0.05 .

Mutant	(%) Growth Inhibition
7015 wt	28.00 ± 9.90
<i>ago1</i>	29.59 ± 1.90
<i>ago2</i>	32.16 ± 1.01
<i>dcl1</i>	30.96 ± 4.37
<i>dcl2</i>	22.86 ± 6.67
<i>hyr1</i>	35.22 ± 8.52
<i>rdrp1</i>	31.87 ± 8.08
<i>yap1</i>	29.86 ± 12.66

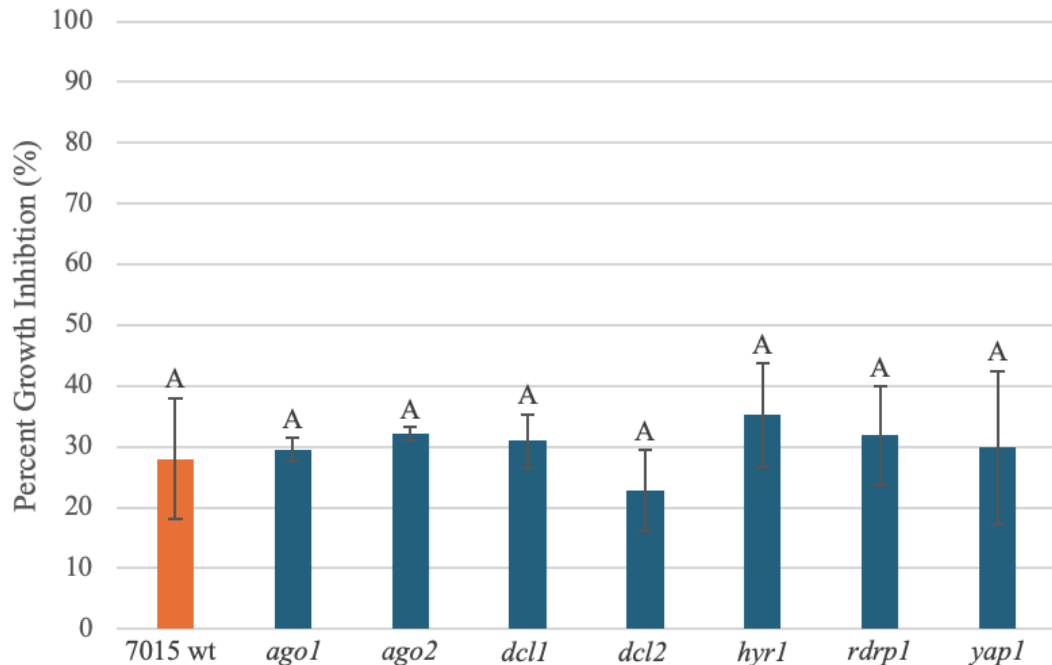


Figure 2.7 *M. oryzae* strain 7015 and mutants percent (%) growth inhibition averages from Table 2.1. Mutants (dark blue) compared to wt (orange) in the presence of *B. velezensis* strain S4 and were determined to be $p > 0.05$ and ruled not significant. “A” represents statistically similar.

2.4.4 *B. velezensis* S4 disrupts spore germination in *M. oryzae* 4091

We hypothesized that there would be some effect on *M. oryzae* strain 4091 spores and their ability to germinate when in the presence of *B. velezensis* strain S4. There were no significant differences between the number of ungerminated spores across treatments (Figure 2.8). The water treatments were able to form an appressoria from their respective germ tubes, thus had a low number of percent of total cells that were germ tube only (Figure 2.8). In the 1:1 dilution of the bacteria there were more germ tubes that formed only, versus appressoria (Figure 2.8). The appressoria formed

more in the lower dilution (1:100) and less germ tube formation only (Figure 2.8). The lower dilution (1:100) was not significantly different from the undiluted heat-killed cultures (Figure 2.8). This illustrates that germination of *M. oryzae* 4091-wt has a decrease in the formation of appressoria.

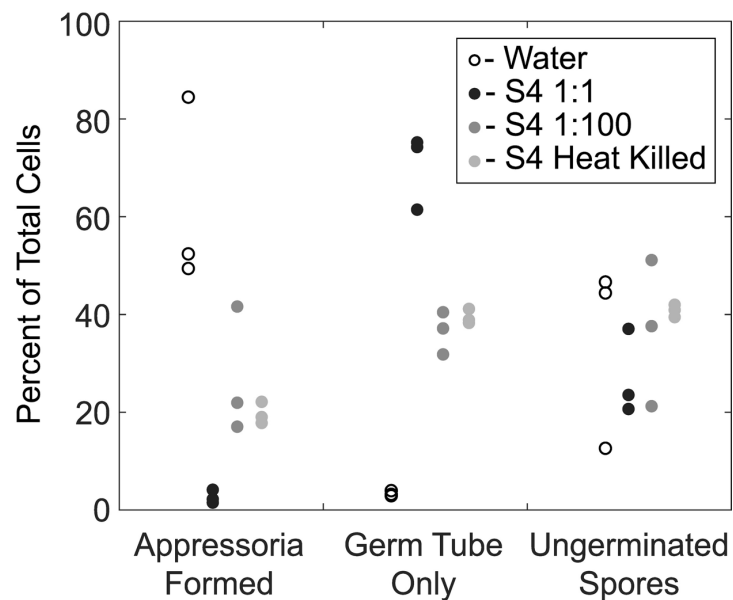


Figure 2.8 Germination of *Magnaporthe oryzae* in the presence of S4 shows reduction of appressoria formation. An overnight culture of *Bacillus velezensis* S4 was added to *M. oryzae* conidia and examined for differences in germination and appressorial formation. The bacterial culture was added to conidia in a 1:1 and 1:100 dilutions, and as a heat-killed culture. * p-value <0.05, which was considered statistically significant in a one-tailed student's t-test (reproduced from Wockenfuss et al., 2024).

2.4.5 Enzymatic plate assays

The *Bacillus* genera possess secondary metabolites and enzymes that contribute to their antifungal activity, so we hypothesized that there would be similar enzymes involved in *B. velezensis* strain S4 ability to inhibit *M. oryzae*. The NCBI

search results revealed 7 total cellulase-degrading enzymes (1 endoglucanase, 5 β -glucosidases, 1 β -glucanase), 7 lipases, and 42 proteases (Table 2.3).

Positive results for a bacterium showing enzymatic activity would be a halo or ring around the colony of growth (Figure 2.9A, C). Negative results would see no halo around the bacterial growth (Figure 2.9B). All the LB (control) plates were negative for enzymatic activity as expected (Figure 2.9). Protease and cellulase plates were positive for both enzymes, with the distinctive halo around the colony (Figure 2.9A, C). There was no halo around the colony for the lipase plates, indicating no lipase activity (Figure 2.9B), despite what was found in its genome (Table 2.3).

Table 2.3 Brief overview of the bacterial genome analysis. There are 7 putative cellulase-degrading enzymes, 7 putative lipases and 42 proteases annotated in *B. velezensis*, S4 genome.

Enzyme	Number in genome
Cellulases	
<i>Endoglucanase</i>	1
<i>Beta-glucosidases</i>	5
<i>Beta-glucanase</i>	1
Protease	42
Lipase	7

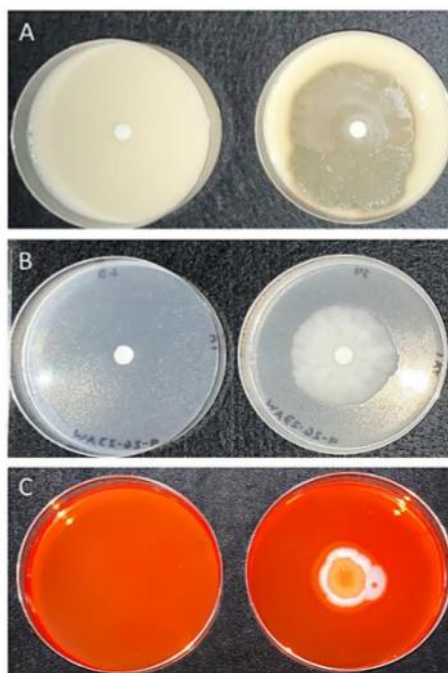


Figure 2.9 *B. velezensis* strain S4 shows protease and cellulose activity. **a.** skim milk agar; **b.** tributyrin agar; **c.** carboxymethylcellulose agar with congo red staining (light orange around the bacterial colony indicates halo). For **a.** and **b.**, filter papers were inoculated with LB as the control (left plates) and with S4 (right plates) and incubated for 72 hours. For **c.**, filter papers were dipped in LB control (left plates) or S4 (right plates) and stamped briefly onto the plate, lifted off, and incubated for 48 hours (reproduced Wockenfuss et al., 2024).

2.5 Discussion

In this chapter we tested roughly 500 *M. oryzae* 4091 HS mutants in the presence of *B. velezensis* strain S4, using a forward genetic screen to identify fungal defense genes. From the initial screen, about 50 candidate mutants were selected for additional testing using 2 other antifungal bacteria. However, after secondary screening the mutants could not be confirmed as the phenotype of the control had changed, with either no lesion in the hyphae being created or no hydrophobicity seen

in the controls, when previously, the LB (negative control) would bead up/stay intact after inoculation on the fungal hyphae. We also investigated how fungi would recover after exposure to *B. velezensis* strain S4 and the 2 other antifungal bacteria, which showed that *B. velezensis* strain S4 outcompetes *M. oryzae* in this instance. Additionally in this chapter we observed how small RNA and ROS mutants from another genetic background strain of *M. oryzae*, 7015 impacted fungal growth inhibition compared to *M. oryzae* strain 7015 wt. Finally, whole cell cultures of *B. velezensis* strain S4 were incubated with *M. oryzae* 4091 spores overnight and displayed a reduction in the number of germinated spores and appressorium formation. This could mean the bacteria can reduce fungal pathogenicity by disrupting the appressoria.

M. oryzae surface hydrophobicity is important for pathogenicity as it can impact its ability to form appressoria (Kim et al., 2005). When *M. oryzae* strain 4091 wt, *M. oryzae* strain 4091 HS, and HS mutants are directly inoculated with *B. velezensis* strain S4 there was a wettable phenotype, whereas on the wild type parent, the droplets beaded up as expected (Figure 2.1). This could indicate that the HyPer sensor background is somehow defective in hydrophobins, which are hydrophobic proteins, found in filamentous fungi, including *M. oryzae* encoded by the gene, *MHP1* (Kim et al., 2005). These aid in attachment to hydrophobic surfaces (Kim et al., 2005). One study showed that when they directly inoculated a detergent solution of commonly used surfactants, 0.2% SDS and 50 mM EDTA, they beaded up on the hyphae of *M. grisea* strain 7015 (Kim et al., 2005). Alternatively, another study using

the same detergent solution on *M. oryzae* strain Guy11 showed the hyphae becoming wettable and the detergent infiltrating the hyphae (Deng et al., 2019). That same study found that a type 2 glycosyltransferase, MoGT2 and the *MPGI* gene, played a role in hydrophobin production (Deng et al., 2019). Members within the *Bacillus* genus can produce amphiphilic compounds (Kakinuma et al., 1969) that can increase solubility of hydrophobic organic compounds, called surfactants (Singh et al., 2007). *B. velezensis* strain S4 has surfactants in its genome (Wockenfuss et al., 2024). Given this, it could be possible that *B. velezensis* strain S4 surfactants could be interfering with the function of *MPGI*, hence the wettable phenotype. A future experiment could involve testing the *mpgI* mutant to see whether the fungus becomes more susceptible to bacterial infiltration.

The change observed in the wettable phenotype during the forward genetic screen could also be attributed to phase variation (PV) in the bacterium. PV is fast acting, reversible gene expression that turns expression either on or off at a greater rate than spontaneous mutants (Gor et al., 2021). Genes that are most susceptible to PV would be those that encode for cell-surface features, like liposaccharide synthesis enzymes and adhesions. PV is thought to be advantageous for bacteria when it is used as a method of defense against infection from bacteriophages and changes in the environment (Gor et al., 2021). We noted that *B. velezensis* strain S4, when growing on plates, started branching and growing rapidly; attempts to triple streak it for single colonies were hampered by its rapid growth at times, however we did sequence it and it was a pure culture of *B. velezensis*, and it retained its fungal-inhibiting capabilities.

PV could be a possible explanation in the changes in the hydrophobicity phenotype, because of the new stress from the environment, in this case the fluctuations (usually an increase) in temperature and changes with the lights, due to a faulty incubator.

The differences in hyphal morphology between *M. oryzae* 4091-wt and HS could be from the multiple random insertions of the HS gene in the HS genome, which has yet to be sequenced (Figure 2.1, 2.2). Additionally, the images of the different spot treatments were difficult to acquire as the liquid droplet makes it so the microscope has to focus through multiple planes at once (Figure 2.3, 2.4).

The particular methodology used for fungal recovery after bacterial inoculation has not been found in literature by the author to this date. However, there is still information to be gained from the results observed (Figure 2.5). *B. velezensis* strain S4 was able to outcompete both *M. oryzae* 4091-wt and -HS after its inoculation, which could result from the bacterium being at a high concentration, so that the spot was saturated with bacterial cells (Figure 2.5). These results are not sufficient to say that *B. velezensis* strain S4 is fungicidal, since it has been shown that *B. velezensis* strain S4 is fungistatic, only inhibiting the growth of several fungal species, including *M. oryzae* 4091, but not killing it in our tests (Wockenfuss et al., 2024). However, it is difficult to plate a single fungal cell that was in contact with diffusible bacterial molecules, which would be more definitive way of testing its ability to recover. The contamination issues seen in *L. enzymogenes* strain C3 treatments multiple times could be attributed to having to inoculate the same plate several times with differing bacteria, which also means opening up the plate additional times as well, even though

this work was performed in a sterilized biological safety cabinet. Despite this experiment being set up in a sterile biological safety cabinet, having to open the plates multiple times to inoculate the other cultures could contribute to the contamination that was sometimes encountered, hampering results. *P. cholororaphis* strain EA105 is a known biological control agent (Spence et al., 2013), however when directly inoculated onto *M. oryzae* 4091-wt and HS, the zone of inhibition/infiltration was smaller than that observed in *B. velezensis* strain S4, and *L. enzymogenes* strain C3 (Figure 2.1, 2.2), which could account for the fungal growth seen (and no bacterial growth) in recovery (Figure 2.5). In this case, we can speculate that *B. velezensis* strain S4 simply might be more aggressive than *P. cholororaphis* EA105 or *L. enzymogenes* C3, but future tests should include recovery assays that were performed in another study (Wockenfuss et al., 2024). In this study, a diffusible molecule assay was performed with *B. velezensis* strain S4 on one side of a petri plate and a fungal pathogen on the other (this plate is what would then be used in the recovery assay) (Wockenfuss et al., 2024). We then set up a 50% PDA plate in quadrants, with two quadrants being from a whole punch of hyphae that was growing closet to the bacteria (zone of inhibition), and the other two quadrants had whole punches from hyphae that was furthest away from the bacteria (control) plated (Wockenfuss et al., 2024). This showed that *B. velezensis* strain S4 was able to grow with the *M. oryzae* strain 4091, and was still able to inhibit its growth, but not completely kill *M. oryzae* strain 4091. Performing this recovery assay in addition to what was done here could potentially

reveal the severity of inhibition by each of the antifungal bacteria, without the stress of other organisms being involved.

We investigated if any strains of *M. oryzae* and their respective mutants would also display similar inhibition as *M. oryzae* strain 4091, in this case the parental 7015 line and associated mutants that are involved in ROS mitigation, virulence, and small RNA production. *M. oryzae* 7015 wt and mutants were inhibited by *B. velezensis* strain S4 (Figure 2.6). Similar results were observed in *M. oryzae* 4091 when grown in the presence of *B. velezensis* strain S4 (Wockenfuss et al., 2024). However, there was no significant difference in inhibition between *M. oryzae* 7015 wt and 7015 mutants (Table 2.1). The greatest % inhibition (35.22) observed was with mutant *hyr1*. The parent strain, *M. oryzae* 7015 wt was no more or less inhibited by the bacterium than the mutants, which could indicate that these processes in this particular strain may not be as influential on genetic defense as originally hypothesized.

M. oryzae strain 4091 spore germination and appressorial formation was affected by *B. velezensis* strain S4 (Figure 2.8). In a related study, we found that *B. velezensis* strain S4 supernatant was able to disrupt germination and appressorial formation (Wockenfuss et al., 2024). We found that at 6-12 hours after *B. velezensis* strain S4 supernatant were placed in the presence of *M. oryzae* 4091 spores, irregular bubbles would form from the germ tube, instead of appressoria (Wockenfuss et al., 2024). Those bubble-like structures and appressorial disturbances were also observed in a separate study using *M. oryzae* and a different biological control bacterium, *B. subtilis* strain BJ-1 (He et al., 2019). Diffusible molecules, and/or genes that produce

bacilysin and fengycin from *B. velezensis* strain S4, could account for the disruption in germination (Wockenfuss et al., 2024), however to our knowledge, there are no studies to date that test this theory.

Previous genomic studies revealed that *B. velezensis* strain S4 has 12 gene clusters that putatively encoded for the production of antifungal compounds (Hempel et al., 2020). The gene clusters included products such as bacilysin, fengycin, surfactin, among other known anti-microbial clusters. Genome sequencing revealed that *B. velezensis* strain S4 was enriched in such clusters (Hempel et al. 2020). From the plate assays we were able to display that *B. velezensis* strain S4 has enzymatic activity, those being protease and cellulases (Figure 2.9A, B), which correlates with its genome (Table 2.3). In endophytic bacteria, both protease and cellulase allows them to enter the plant tissue to infect and fully colonize (Ling et al., 2022). That same study illustrated that *Bacillus licheniformis* strain TG116, which produces serine protease, can inhibit spore germination and mycelial growth (Ling et al., 2022). Cellulase activity was illustrated via the plate assays as well (Figure 2.9C). Cellulase enzymes can degrade fungal oomycetes hyphal walls (comprised of cellulose) and can hydrolyze carboxymethyl cellulose (CMC) (Malik et al., 2023). There was no lipase activity shown phenotypically, as it was able to grow on tributyrin agar (TA), but did not show the halo around the colony, indicating enzymatic activity (Figure 2.9), despite lipases being putatively encoded in *B. velezensis* strain S4 genome (Table 2.3). The difference observed could be due to the media not being conducive for the halo, since several TA recipes were attempted before the one shown here (Figure 2.9) or a

stain, such as 0.1% Congo Red (Figure 2.9A) could have revealed the halo. Future studies would need to be completed to phenotypically show lipase activity as it is reflected in the genome (Table 2.3). Fungal cell walls are made of hard-to-degrade chitin (Ling et al., 2022), so it is hypothesized that *B. velezensis* strain S4 would have multiple “modes of inhibition” against this fungal pathogen.

2.6 References

- Baard, V., Bakare, O. O., Daniel, A. I., Nkomo, M., Gokul, A., Keyster, M., & Klein, A. (2023). Biocontrol potential of *Bacillus subtilis* and *Bacillus tequilensis* against four fusarium species. *Pathogens*, *12*(2), 254.
<https://doi.org/10.3390/pathogens12020254>
- Batubara, U. M., Mardalisa, M., Suparjo, S., Maritsa, H. U., Pujiyanto, E., & Herlini, M. (2021). Isolation and characterization of cellulolytic bacteria diversity in peatland ecosystem and their cellulolytic activities. *IOP Conference Series: Earth and Environmental Science*, *934*(1), 012028.
<https://doi.org/10.1088/1755-1315/934/1/012028>
- Chen, P.-H., Chen, R.-Y., & Chou, J.-Y. (2018). Screening and evaluation of yeast antagonists for biological control of *botrytis cinerea* on Strawberry Fruits. *Mycobiology*, *46*(1), 33–46. <https://doi.org/10.1080/12298093.2018.1454013>
- Deng, S., Sun, W., Dong, L., Cui, G., & Deng, Y. Z. (2019). *mogt2* is essential for morphogenesis and pathogenicity of *Magnaporthe oryzae*. *mSphere*, *4*(5).
<https://doi.org/10.1128/msphere.00309-19>

- Eseola, A. B., Ryder, L. S., Osés-Ruiz, M., Findlay, K., Yan, X., Cruz-Mireles, N., Molinari, C., Garduño-Rosales, M., & Talbot, N. J. (2021). Investigating the cell and developmental biology of plant infection by the rice blast fungus *Magnaporthe oryzae*. *Fungal Genetics and Biology*, 103562.
<https://doi.org/10.1016/j.fgb.2021.103562>
- Gor, V., Ohniwa, R. L., & Morikawa, K. (2021). No change, no life? what we know about phase variation in *Staphylococcus aureus*. *Microorganisms*, 9(2), 244.
<https://doi.org/10.3390/microorganisms9020244>
- Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *Journal of Chemical Biology*, 7(4), 133–141.
<https://doi.org/10.1007/s12154-014-0113-1>
- He, Y., Zhu, M., Huang, J., Hsiang, T., & Zheng, L. (2019). Biocontrol potential of a *Bacillus subtilis* strain BJ-1 against the rice blast fungus *Magnaporthe oryzae*. *Canadian Journal of Plant Pathology*, 41(1), 47–59.
<https://doi.org/10.1080/07060661.2018.1564792>
- Hempel, P. P., Yao, M., Yannarell, S., Shevchenko, O., Vogt, F., Donofrio, N., & Maresca, J. A. (2020). Complete genome sequence of *Bacillus velezensis* strain S4, isolated from biochar-treated soil. *Microbiology Resource Announcements*, 9(20). <https://doi.org/10.1128/mra.00352-20>

- Huang, K., Caplan, J., Sweigard, J. A., Czymmek, K. J., & Donofrio, N. M. (2016). Optimization of the hyper sensor for robust real-time detection of hydrogen peroxide in the rice blast fungus. *Molecular Plant Pathology*, *18*(2), 298–307. <https://doi.org/10.1111/mpp.12392>
- Kakinuma, A., Hori, M., Isono, M., Tamura, G., & Arima, K. (1969). Determination of amino acid sequence in surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*. *Agricultural and Biological Chemistry*, *33*(6), 971–972. <https://doi.org/10.1080/00021369.1969.10859408>
- Kim, S., Ahn, I., Rho, H., & Lee, Y. (2005). *mhp1*, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. *Molecular Microbiology*, *57*(5), 1224–1237. <https://doi.org/10.1111/j.1365-2958.2005.04750.x>
- Ling, L., Cheng, W., Jiang, K., Jiao, Z., Luo, H., Yang, C., Pang, M., & Lu, L. (2022). The antifungal activity of a serine protease and the enzyme production of characteristics of *Bacillus licheniformis* TG116. *Archives of Microbiology*, *204*(10). <https://doi.org/10.1007/s00203-022-03216-x>
- Malik, M. S., Rehman, A., Khan, I. U., Khan, T. A., Jamil, M., Rha, E. S., & Anees, M. (2023). Thermo-neutrophilic cellulases and chitinases characterized from a novel putative antifungal biocontrol agent: *Bacillus subtilis* TD11. *PLOS ONE*, *18*(1). <https://doi.org/10.1371/journal.pone.0281102>

- Mansoor, S., Ali Wani, O., Lone, J. K., Manhas, S., Kour, N., Alam, P., Ahmad, A., & Ahmad, P. (2022). Reactive oxygen species in plants: From source to sink. *Antioxidants*, *11*(2), 225. <https://doi.org/10.3390/antiox11020225>
- Mathioni, S. M., Patel, N., Riddick, B., Sweigard, J. A., Czymbek, K. J., Caplan, J. L., Kunjeti, S. G., Kunjeti, S., Raman, V., Hillman, B. I., Kobayashi, D. Y., & Donofrio, N. M. (2013). Transcriptomics of the rice blast fungus *Magnaporthe oryzae* in response to the bacterial antagonist *Lysobacter enzymogenes* reveals candidate fungal defense response genes. *PLoS ONE*, *8*(10). <https://doi.org/10.1371/journal.pone.0076487>
- Singh, A., Van Hamme, J. D., & Ward, O. P. (2007). Surfactants in microbiology and biotechnology: Part 2. application aspects. *Biotechnology Advances*, *25*(1), 99–121. <https://doi.org/10.1016/j.biotechadv.2006.10.004>
- Spence, C. A., Raman, V., Donofrio, N. M., & Bais, H. P. (2013). Global gene expression in rice blast pathogen *Magnaporthe oryzae* treated with a natural rice soil isolate. *Planta*, *239*(1), 171–185. <https://doi.org/10.1007/s00425-013-1974-1>
- Wockenfuss, A., Chan, K., Cooper, J. G., Chaya, T., Mauriello, M. A., Yannarell, S. M., Maresca, J. A., & Donofrio, N. M. (2024). A *Bacillus velezensis* strain shows antimicrobial activity against Soilborne and foliar fungi and oomycetes. *Frontiers in Fungal Biology*, *5*. <https://doi.org/10.3389/ffunb.2024.133275>

Chapter 3

RNASEQ OF *MAGNAPORTHE ORYZAE* IN THE PRESENCE OF THE BACTERIA, *BACILLUS VELEZENSIS* STRAIN S4

3.1 Abstract

Magnaporthe oryzae is a fungal pathogen that is the causal agent of rice blast disease, which can have severe impacts on crop production. Fungicides are the common method of control for plant pathogenic fungi, however *M. oryzae* has developed resistance to combat the use of fungicides, which is why there is a shift towards biological control agents (BCA). *Bacillus velezensis* strain S4 has been shown to have antifungal properties in its genome and can interrupt the germination of *M. oryzae* spores, hence making it an ideal candidate for a possible BCA. In chapter 2 with the forward genetic screen that is looking at the entire genome, whereas using transcriptomics could give us a preliminary list of potential interesting genes involved in fungal defense. RNAseq was performed on *M. oryzae* 4091 when grown in the absence, and presence of *B. velezensis* strain S4 in the hopes of acquiring a list of genes that could be further tested for defense functions in the fungus. Differential gene expression analysis revealed 69 differentially expressed genes (DEGs) with functions

including toxin biosynthesis, such as cytochrome P450 production, aflatoxin reductases, and trichothecene 3-O-acetyltransferase.

Keywords: RNAseq, differential gene expression, *Magnaporthe oryzae*, *Bacillus*, biological control agents

3.2 Introduction

RNAseq is a Next-Generation Sequencing (NGS) technique that involves transcriptomic reads, or mRNA transcripts, which are aligned to a reference genome (Wang et al., 2016). By comparing entire transcriptomes, researchers can view how RNA expression changes in certain environments and can use the data to speculate on how certain cellular mechanisms and processes function during different treatments (Wang et al., 2016). Differential expression analysis yields output files for each gene in the form of fold change, p-value, and/or adjusted p-value (q-value)/false discovery rate (FDR) (McDermaid et al., 2018). FDR is an adjusted p-value that is intended to solve the multiple testing issues that arise when using the traditional p-value (Jafari and Ansari-Pour, 2018); in our analysis we used the FDR value.

Magnaporthe oryzae is a fungal pathogen that infects and kills cereal crops, including rice and wheat (Hahn, 2014). Biological control agents (BCA) have been used to control plant pathogens, and include bacteria from the genera, *Bacillus* (Babalola, 2010). *B. velezensis* strain S4 has shown potential as a BCA, by presumably

secreting secondary metabolites and enzymes that can disrupt germination in *M. oryzae* 4091 (Wockenfuss et al., 2024).

Most RNAseq studies pertaining to *M. oryzae* to-date have been carried out *in planta* (Shimizu et al., 2019; Yan et al., 2023; Kawahara et al., 2012) as well as with nano fungicides (Ghamari et al., 2022). To date and the knowledge of the author, there have not been publications of RNAseq that examined gene expression changes in *M. oryzae* during interactions with a biocontrol bacterium. Preliminary studies of fungal transcriptomics using RNAseq was performed with *M. oryzae* and the antifungal bacterium, *Lysobacter enzymogenes* strain C3. In this study, they selected time points of 3 and 9 hours post inoculation of *L. enzymogenes* strain C3 from this they were able to reveal 463 fungal genes that were downregulated during those times, that we then used for further analysis (Mathioni et al., 2013). Of the 463 genes, they were able to narrow down to 100 genes that have roles in fungal defense (Mathioni et al., 2013). In the following study, RNAseq was performed with the goal of determining any differences in gene expression between *M. oryzae* grown alone and grown in the presence of *B. velezensis* strain S4 in order to identify a list of candidate genes for further analysis.

3.3 Methods

3.3.1 Indirect assay

Overnight cultures of *B. velezensis* strain S4 was made in Luria Broth (LB) at 30°C for 12-16 hours at RPM = 80. *M. oryzae* 4091 was grown on half-strength potato dextrose agar (PDA). *M. oryzae* 4091 was hole punched (0.8 cm) and plated onto the right side of half-strength PDA plates (2 cm away from the edge of the media plate). On that same plate, sterile filter paper was plated 2 cm away from the left side of the plate with either LB (control), or 5 µL of *B. velezensis* strain S4 overnight culture (R = 3), and the experiment was repeated 3 times (Figure 3.1). The plates were incubated at 24°C for 11 days.

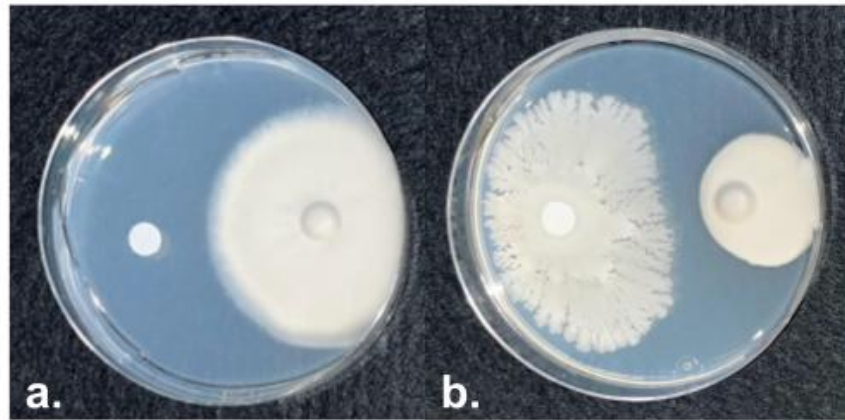


Figure 3.1 Example plate *M. oryzae* 4091 grown in the presence of *B. velezensis* strain S4. *M. oryzae* whole fungal hyphal colony used for RNA extractions after 7 days of incubation. **a.** Sterile filter paper with LB (left), *M. oryzae* 4091 (right). **b.** Filter paper with 5µl of *B. velezensis* strain S4 (left), *M. oryzae* 4091 (right).

3.3.2 RNA Extraction and Isolation

Originally, we only wanted to extract hyphae from the zone of inhibition for plates in the presence of *B. velezensis* strain S4 using a sterilized scalpel. However, the fungal colonies for both the control and experimental plates were small and there were concerns of acquiring sufficient RNA concentrations to submit for sequencing. Instead we used a sterile scalpel to scrap the fungal hyphae off the entire fungal colony for all the plates. RNA extraction was completed using the Nucleospin RNA Plant and Fungi Kit (TaKaRa Bio). Each sample was flash frozen in liquid nitrogen and ground with a mortar and pestle until it was a fine powder.

The RNA isolates were then checked for concentration and quality using a NanoDrop (Table 3.1).

Table 3.1 RNA concentrations (ng/ μ L), A260/280, and A260/230 values from RNA isolates. M represents *M. oryzae* 4091 grown alone (control) and M:S represents *M. oryzae* 4091 in the presence of *B. velezensis* strain S4. Desired concentration was > 50ng/ μ L with A260 ratios ~ 2.0.

Sample Name	ng/ μ L	A260/280	A260/230
M1	693.9	2.18	2.49
M:S1	413.6	2.22	2.52
M2	607.3	2.17	2.49
M:S2	516.7	2.17	2.5
M3	1250.8	2.21	2.48
M:S3	1378.3	2.21	2.41

3.3.3 RNAseq Analysis

RNA samples were delivered to UD's Genotyping and Sequencing Center. The libraries were prepped for Illumina sequencing and were quality control assessed using standard protocols found in the Sequencing Center, which includes Qubit, Fragment Analysis, and digital PCR (ddPCR) (Illumina, San Diego, CA, USA). Transcriptomic sequencing was performed on the Illumina NextSeq2000 at 200 cycles using P2 reagents, which roughly results in 400 million reads/run.

This analysis was performed by Jaysheel D. Bhavsar and Shawn W. Polson at the Bioinformatics Data Science Core Facility (RRID:SCR_017696) at the University of Delaware's Center for Bioinformatics and Computational Biology, using core services and computational resources which were made possible through support from

Delaware INBRE (NIGMS P20GM103446), NIH Shared Instrumentation Grant (NIGMS S10OD028725), the State of Delaware, and the Delaware Biotechnology Institute.

Raw sequence data was analyzed by the Bioinformatics Data Science Core at the University of Delaware using the established RNA-Seq analysis pipeline (adapted from Kalari et al. 2014). Quality of sequencing data was assessed using FastQC (ver. 0.11.6; Babraham Bioinformatics). Reads were trimmed for quality (Q<30) and to remove poly-A and Illumina sequencing adapters using Trim Galore! (ver. 0.6.6; Babraham Bioinformatics) and reads less than 35bp after trimming were discarded. Trimmed reads were aligned to the genome (*M. oryzae* version 8: BioProject PRJNA13840) using HiSat2 (ver. 2.2.1), mapping metrics were assessed using RseQC (ver. 4.0.0), and gene/exon feature counts were calculated using HTseq (ver. 2.0.2). Pairwise differential expression analysis was performed to identify gene-level features which are significantly up or down-regulated between treatments using EdgeR (ver. 3.28.1) analyzing genes with a CPM (count per million reads) of at least one in three or more samples.

3.4 Results and Discussion

The indirect assays resulted in small *M. oryzae* 4091 colonies to be used for RNA extraction (Figure 3.1). There were sufficient RNA concentrations after the extraction protocol, thus if the experiment were to be repeated the sample could be taken solely from the zone of inhibition (hyphae closest to the bacterial colony),

instead of the entire fungal colony, which would amplify gene expression from fungal cells in contact with diffusible bacterial molecules.

Using a FDR value of < 0.05 , there were 41 *M.oryzae* 4091 genes upregulated and 1 gene downregulated in the presence of *B. velezensis* strain S4 (Figure 3.2).

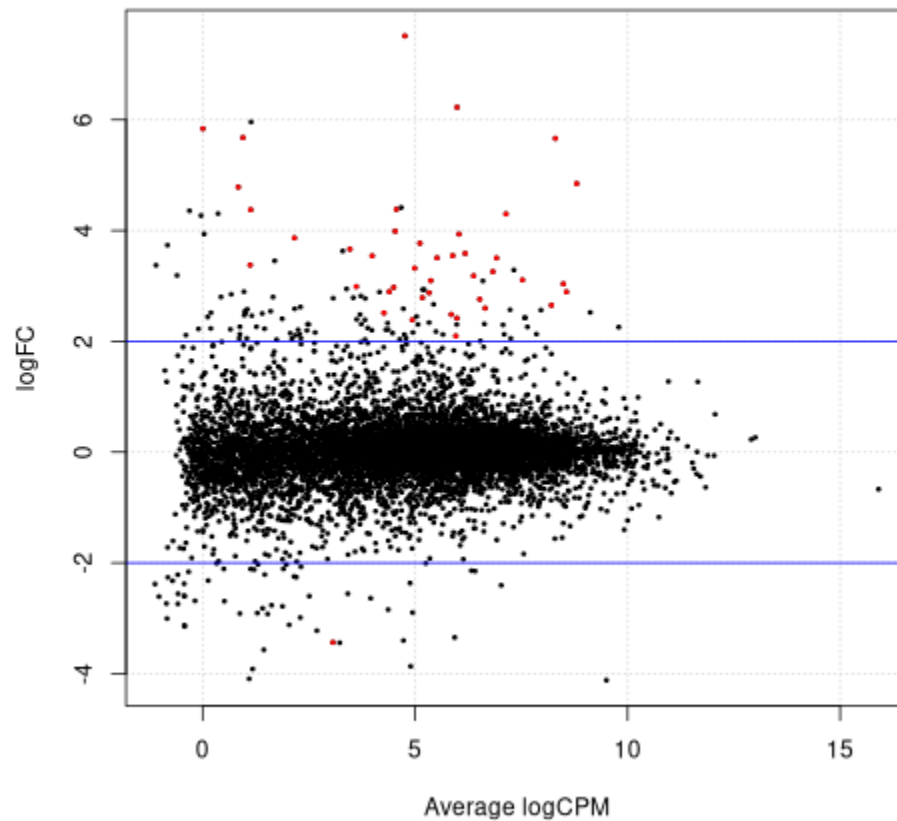


Figure 3.2 Smear plot showing \log_2 FC vs. \log_2 CPM for each gene. Red dots represent $FDR < 0.05$, meaning statically significant. Blue lines represent \log_2 FC range of -2 - 2. Representing 1 down-regulated gene and 41 upregulated genes (generated by Jaysheel Bhavsar in the Center for Bioinformatics and Computational Biology at UD).

Additionally, we then accepted the FDR at a lower threshold of < 0.2 . Given that, we identified 69 genes in *M. oryzae* 4091 that were differentially expressed (DEGs) in the presence of *B. velezensis* strain S4 (Table 3.2). The DEGs were fewer in number than expected and could be attributed to a couple of reasons. Firstly, the samples collected included fungal hyphal that was not in direct contact with the bacterium, and thus the signal could have been diluted out. Secondly, the FDR value threshold could have been too stringent. Having a less stringent FDR would have allowed the window of candidate, differentially expressed genes to be wider. However, given that the signal to noise ratio is likely low because we used the entire colony, a more stringent FDR could mean a reduction in any false-positives and obtaining only the most biologically relevant genes.

Table 3.2 logFC (fold change) and FDR (false discovery rate) values of the 69 DEGs (differentially expressed genes) in *M. oryzae* 4091. Gene ID and descriptions/functions of each DEG. Gray rows represent biologically relevant genes that will be discussed further. Total Gene IDs = 69.

GeneID	logFC	FDR	Gene description
MGG_02247	3.1818	0	SAM-dependent methyltransferase
MGG_03822	7.5131	0	peptidase family T4
MGG_07144	4.8476	0	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MT69]
MGG_08440	5.664	0	Trichothecene 3-O-acetyltransferase [Source:UniProtKB/TrEMBL;Acc:G4NA71]
MGG_08552	3.9355	0	hypothetical protein
MGG_08656	3.5036	0	hypothetical protein
MGG_09777	3.7701	0	trichothecene 3-O-acetyltransferase
MGG_09816	3.5864	0	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NIJ8]
MGG_14883	3.5074	0	Methyltransferase small domain-containing protein [Source:UniProtKB/TrEMBL;Acc:G4N8P0]
MGG_02101	2.7597	4.00E-04	hypothetical protein

MGG_05058	6.2257	5.00E-04	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4N444]
MGG_01989	5.6778	7.00E-04	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MMD2]
MGG_15000	3.5421	0.0017	hypothetical protein
MGG_17102	2.5978	0.0017	hypothetical protein
MGG_09156	4.2995	0.0039	ent-kaurene oxidase
MGG_03301	3.0366	0.0042	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4N9F1]
MGG_04738	4.7856	0.0042	Short chain dehydrogenase/oxidoreductase [Source:UniProtKB/TrEMBL;Acc:G4MQV9]
MGG_03416	3.1077	0.0043	acetyltransferase
MGG_09781	2.4182	0.0049	hypothetical protein
MGG_05994	2.7871	0.0097	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4N4L7]
MGG_08297	4.3759	0.0097	NADH:flavin oxidoreductase/NADH oxidase [Source:UniProtKB/TrEMBL;Acc:G4MX78]
MGG_02139	5.8397	0.0117	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MNT8]
MGG_05880	3.3198	0.0117	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4N3X9]
MGG_08253	4.3821	0.0118	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MXT6]
MGG_11072	-3.4349	0.0118	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NFX3]
MGG_16392	3.2579	0.0118	hypothetical protein
MGG_02907	2.9886	0.0149	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NLL9]
MGG_02907	2.9886	0.0149	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NLL9]
MGG_06062	3.9877	0.0149	Nitrate reductase [Source:UniProtKB/TrEMBL;Acc:G4N550]
MGG_05825	2.8952	0.0154	transferase
MGG_00832	2.4849	0.0183	cytochrome P450
MGG_08074	2.8767	0.0205	NADP-specific glutamate dehydrogenase
MGG_07747	2.386	0.0236	hypothetical protein
MGG_02501	2.0953	0.035	hypothetical protein
MGG_01864	3.5468	0.0387	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MWM3]
MGG_00402	3.6651	0.0401	hypothetical protein
MGG_02100	3.3779	0.0401	hypothetical protein
MGG_06790	3.8661	0.0401	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MM12]
MGG_06799	2.6513	0.0401	hypothetical protein
MGG_00440	2.9745	0.0442	Inactive carboxylesterase 4 [Source:UniProtKB/TrEMBL;Acc:G4NC41]

MGG_00198	3.0961	0.0446	Flavo-hemoglobin [Source:UniProtKB/TrEMBL;Acc:G4NDV3]
MGG_04085	2.8919	0.0446	hypothetical protein
MGG_05716	2.5132	0.0475	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MPG2]
MGG_18030	4.3568	0.0933	hypothetical protein
MGG_09068	2.6685	0.1101	NADPH-dependent 1-acyldihydroxyacetone phosphate reductase [Source:UniProtKB/TrEMBL;Acc:G4N5G2]
MGG_12228	4.3044	0.1137	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NG94]
MGG_14055	-3.2241	0.1137	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NL95]
MGG_14055	-3.2241	0.1137	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NL95]
MGG_14055	-3.2241	0.1137	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NL95]
MGG_14055	-3.2241	0.1137	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NL95]
MGG_15203	1.8779	0.1169	Anion exchange protein 4 [Source:UniProtKB/TrEMBL;Acc:G4N0T8]
MGG_02561	3.9391	0.1318	hypothetical protein
MGG_08405	2.9338	0.1425	retinol dehydrogenase 13
MGG_16792	2.4264	0.1425	hypothetical protein
MGG_02191	2.895	0.1431	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4MP87]
MGG_10704	2.1599	0.1431	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4NIB2]
MGG_05830	5.9605	0.1476	Allantoin transporter [Source:UniProtKB/TrEMBL;Acc:G4N3M6]
MGG_01369	2.3049	0.1537	alpha/beta hydrolase fold-3 domain-containing protein
MGG_11021	2.5632	0.1556	Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G4N280]
MGG_07848	3.3736	0.1633	Multidrug resistance protein CDR1 [Source:UniProtKB/TrEMBL;Acc:G4N1K8]
MGG_09063	4.4164	0.1633	urea active transporter
MGG_09400	2.0065	0.1633	hypothetical protein
MGG_10318	2.5888	0.1633	hypothetical protein
MGG_13793	3.0897	0.1633	nitrate transporter
MGG_03446	2.8897	0.1638	hypothetical protein
MGG_09351	-2.5559	0.1638	aspergillopepsin-F
MGG_08519	2.7738	0.1674	aflatoxin B1 aldehyde reductase member 3
MGG_07571	2.4839	0.1767	LysM domain-containing protein [Source:UniProtKB/TrEMBL;Acc:G4N263]
MGG_14904	1.7751	0.182	histidinol-phosphate aminotransferase

Based on the gene description for each gene the majority (29 genes) were uncharacterized/hypothetical proteins, which could be attributed to inadequate sequence homologs (Rauthan et al., 2023). Gene descriptions with hypothetical proteins could still be useful as they play a role in uncovering pathways, functions, etc. in microbes (Malik et al., 2020).

The 69 DEGs (based on the FDR) were examined for any similar gene functions and then grouped together (Figure 3.3). The top enrichment GO terms were involved in oxidoreductase activity, transferase activity, and transporter activity (Figure 3.3).

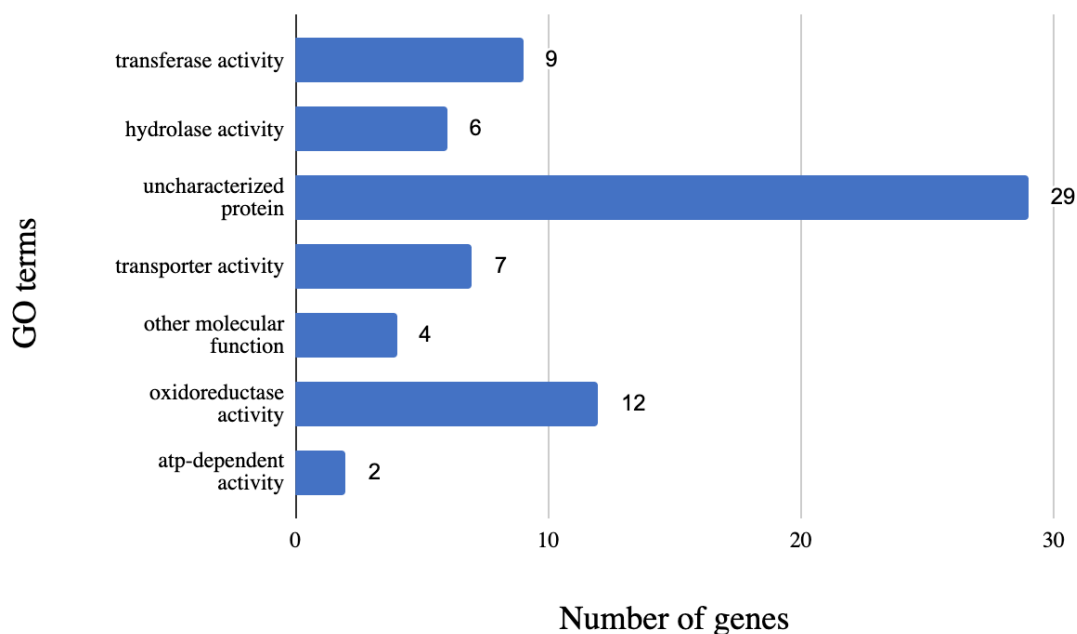


Figure 3.3 Differentially expressed genes (DEGs) (69) based on GO Enrichment Terms. FDR < 0.2. Uncharacterized proteins = 29. Oxidoreductase activity = 12. Transferase activity = 9. Transporter activity = 7. Transporter activity = 7. Hydrolase activity = 6. Other molecule function = 4. ATP-dependent activity = 2. Made from UniProt.

There were two genes (MGG_08440 and MGG_09777) that encoded for trichothecene 3-O-acetyltransferase (Table 3.2). Trichothecenes are secondary metabolites produced by *Fusarium* and other fungi (Kimura et al., 1998). *Fusarium graminearum* gene, *Tri101* encodes trichothecene 3-O-acetyltransferase. *Tri101* has been shown to reduce toxicity in trichothecene mycotoxin deoxynivalenol (DON), a toxin found in grains infected by this fungus (Khatibi et al., 2011), and can protect mycelia (Miguel-Rojas et al., 2023). DON was acetylated by TRI101/TRI102 enzymes

(Khatibi et al., 2011). *M. oryzae* could be pumping out this gene to protect its mycelia when in the presence of *B. velezensis* strain S4.

MGG_09156 (ent-kaurene oxidase) (KO) and MGG_00832 (cytochrome P450) were similar in their involvement in cytochrome P450 enzyme production, which has been shown to allow for fungal detoxification from environmental stressors (Table 3.2) (Črešnar & Petrič, 2011). When placed under a strong promoter, MoMCP1, a cytochrome P450 gene in *M. oryzae*, was determined to be required for pathogenicity (Wang et al., 2019). In that same experiment, they observed no appressorium formation (Wang et al., 2019).

MGG_07848 gene description showed a putative function as a multidrug resistance protein, CDR1 (Table 3.2). The CDR1 protein is mainly studied in *Candida albicans*, a fungal pathogen that can infect humans, especially those who are immunocompromised (Prasad et al., 2015). CDR1 is a transporter belonging to the family of ATP binding cassettes (ABC), which enables the pathogen to combat antifungals (Prasad et al., 2015).

MGG_08519 is an aflatoxin B1 (AFB1) aldehyde reductase member 3 (Table 3.2). Aflatoxin are a group of mycotoxins or metabolites produced by fungi, including *Aspergillus flavus*, with AFB1 being the more dangerous aflatoxin to humans when consumed from infected agricultural products (Shabeer et al., 2022). Field studies with *A. flavus* revealed that environmental stress are factors involved in the production of AFB1 (Shabeer et al., 2022). *Bacillus spp.* can prevent the production of aflatoxins (Shabeer et al., 2022). After co-cultivation with *B. amyloliquefaciens* there was a reduction in *A. parasiticus* (another fungus that produces AFB1) growth as well as degradation of aflatoxins B1, B2, G1, and G2 after several days (Ren et al., 2020). *M.*

oryzae does not have known toxins, so it is a mystery as to why it appears here. A future study could be deleting AFB1 deleted from the genome of *M. oryzae*, to assess function.

MGG_07571 is a LysM domain containing protein (Table 3.2). LysM proteins function in various host-pathogen interactions (Hu et al., 2021). For example, when fungal species are faced with hydrolysis the LysM proteins can protect chitin in fungal cell walls from plant chitinases (Hu et al., 2021). LysM effector Slp1 is secreted from *M. oryzae* to suppress chitin-triggered immune responses (Tian et al., 2022). A similar form of protection could be occurring when *M. oryzae* is in the presence of *B. velezensis* strain S4 diffusible molecules.

B. velezensis strain S4 has been shown to encode for surfactin, fengycin, bacillaene, bacillibactin, bacilysin, which have antifungal activity (Hempel et al., 2020; Wockenfuss et al. 2024). In the presence of this bacterium, *M. oryzae* could be causing the upregulation of the previously discussed DEGs that are involved in toxin biosynthesis, in order to protect its mycelium.

3.5 References

- Anders, S., McCarthy, D. J., Chen, Y., Okoniewski, M., Smyth, G. K., Huber, W., & Robinson, M. D. (2013). Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. *Nature protocols*, 8(9), 1765–1786. <https://doi.org/10.1038/nprot.2013.099>
- Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)*, 31(2), 166–169. <https://doi.org/10.1093/bioinformatics/btu638>
- Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology Letters*, 32(11), 1559–1570. <https://doi.org/10.1007/s10529-010-0347-0>
- Babraham Bioinformatics - FastQC a quality control tool for high throughput sequence data. (n.d.). <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
- Babraham bioinformatics - trim galore! (n.d.). https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

- Črešnar, B., & Petrič. (2011). Cytochrome P450 enzymes in the fungal kingdom. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1814(1), 29–35. <https://doi.org/10.1016/j.bbapap.2010.06.020>
- Ghamari, R., Ahmadikhah, A., Tohidfar, M., & Bakhtiarizadeh, M. R. (2022). RNA-seq analysis of *Magnaporthe grisea* transcriptome reveals the high potential of zno nanoparticles as a nanofungicide. *Frontiers in Plant Science*, 13. <https://doi.org/10.3389/fpls.2022.896283>
- Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *Journal of Chemical Biology*, 7(4), 133–141. <https://doi.org/10.1007/s12154-014-0113-1>
- Hu, S.-P., Li, J.-J., Dhar, N., Li, J.-P., Chen, J.-Y., Jian, W., Dai, X.-F., & Yang, X.-Y. (2021). Lysin motif (LYSM) proteins: Interlinking manipulation of plant immunity and fungi. *International Journal of Molecular Sciences*, 22(6), 3114. <https://doi.org/10.3390/ijms22063114>
- Jafari, M., & Ansari-Pour, N. (2018). Why, When and How to Adjust Your P Values?. *Cell Journal (Yakhteh)*, 20(4), 604-607. doi: 10.22074/cellj.2019.5992
- Kalari, K. R., Nair, A. A., Bhavsar, J. D., O'Brien, D. R., Davila, J. I., Bockol, M. A., Nie, J., Tang, X., Baheti, S., Doughty, J. B., Middha, S., Sicotte, H., Thompson, A. E., Asmann, Y. W., & Kocher, J.-P. A. (2014). Map-RSEQ:

Mayo Analysis Pipeline for RNA sequencing. *BMC Bioinformatics*, 15(1).

<https://doi.org/10.1186/1471-2105-15-224>

Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., & Minami, E. (2012).

Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS ONE*, 7(11).

<https://doi.org/10.1371/journal.pone.0049423>

Khatibi, P. A., Newmister, S. A., Rayment, I., McCormick, S. P., Alexander, N. J., &

Schmale, D. G. (2011). Bioprospecting for trichothecene 3-*o*-acetyltransferases in the fungal genus *Fusarium* yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. *Applied and Environmental Microbiology*, 77(4), 1162–1170. <https://doi.org/10.1128/aem.01738-10>

Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: a fast spliced aligner with

low memory requirements. *Nature methods*, 12(4), 357–360.

<https://doi.org/10.1038/nmeth.3317>

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K.,

& Yamaguchi, I. (1998). Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. *Journal of Biological Chemistry*, 273(3), 1654–1661.

<https://doi.org/10.1074/jbc.273.3.1654>

- Malik, G., Agarwal, T., Raj, U., Sundararajan, V. S., Bandapalli, O. R., & Suravajhala, P. (2020). Hypothetical proteins as predecessors of long non-coding RNAs. *Current Genomics*, *21*(7), 531–535.
<https://doi.org/10.2174/1389202921999200611155418>
- McDermaid, A., Monier, B., Zhao, J., Liu, B., & Ma, Q. (2018). Interpretation of differential gene expression results of RNA-Seq Data: Review and Integration. *Briefings in Bioinformatics*, *20*(6), 2044–2054.
<https://doi.org/10.1093/bib/bby067>
- Miguel-Rojas, C., Cavinder, B., Townsend, J. P., & Trail, F. (2023). Comparative transcriptomics of *Fusarium graminearum* and *Magnaporthe oryzae* spore germination leading up to infection. *mBio*, *14*(1).
<https://doi.org/10.1128/mbio.02442-22>
- Prasad, R., Banerjee, A., Khandelwal, N. K., & Dhamgaye, S. (2015). The abcs of *Candida albicans* multidrug transporter Cdr1. *Eukaryotic Cell*, *14*(12), 1154–1164. <https://doi.org/10.1128/ec.00137-15>
- Rauthan, K., Joshi, S., Kumar, L., Goel, D., & Kumar, S. (2023). Functional annotation of uncharacterized proteins from *Fusobacterium nucleatum*: Identification of virulence factors. *Genomics & Informatics*, *21*(2).
<https://doi.org/10.5808/gi.22065>

- Ren, X., Zhang, Q., Zhang, W., Mao, J., & Li, P. (2020). Control of aflatoxigenic molds by antagonistic microorganisms: Inhibitory behaviors, bioactive compounds, related mechanisms, and influencing factors. *Toxins*, *12*(1), 24. <https://doi.org/10.3390/toxins12010024>
- Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2011). Integrative genomics viewer. *Nature biotechnology*, *29*(1), 24–26. <https://doi.org/10.1038/nbt.1754>
- Shabeer, S., Asad, S., Jamal, A., & Ali, A. (2022). Aflatoxin contamination, its impact and management strategies: An updated review. *Toxins*, *14*(5), 307. <https://doi.org/10.3390/toxins14050307>
- Shimizu, M., Nakano, Y., Hirabuchi, A., Yoshino, K., Kobayashi, M., Yamamoto, K., Terauchi, R., & Saitoh, H. (2019). RNA-Seq of *in planta*-expressed *Magnaporthe oryzae* genes identifies *mosvp* as a highly expressed gene required for pathogenicity at the initial stage of infection. *Molecular Plant Pathology*, *20*(12), 1682–1695. <https://doi.org/10.1111/mpp.12869>
- Tian, H., Fiorin, G. L., Kombrink, A., Mesters, J. R., & Thomma, B. P. (2022). Fungal dual-domain lysm effectors undergo chitin-induced intermolecular, and not intramolecular, dimerization. *Plant Physiology*, *190*(3), 2033–2044. <https://doi.org/10.1093/plphys/kiac391>

- Wang, C., Schröder, M. S., Hammel, S., & Butler, G. (2016). Using RNA-seq for analysis of differential gene expression in fungal species. *Methods in Molecular Biology*, 1–40. https://doi.org/10.1007/978-1-4939-3079-1_1
- Wang, L., Wang, S., & Li, W. (2012). RSeQC: quality control of RNA-seq experiments. *Bioinformatics (Oxford, England)*, 28(16), 2184–2185. <https://doi.org/10.1093/bioinformatics/bts356>
- Wang, Y., Wu, Q., Liu, L., Li, X., Lin, A., & Li, C. (2019). MOMCP1, a cytochrome P450 gene, is required for alleviating manganese toxin revealed by transcriptomics analysis in *Magnaporthe oryzae*. *International Journal of Molecular Sciences*, 20(7), 1590. <https://doi.org/10.3390/ijms20071590>
- Wockenfuss, A., Chan, K., Cooper, J. G., Chaya, T., Mauriello, M. A., Yannarell, S. M., Maresca, J. A., & Donofrio, N. M. (2024). A *Bacillus velezensis* strain shows antimicrobial activity against Soilborne and foliar fungi and oomycetes. *Frontiers in Fungal Biology*, 5. <https://doi.org/10.3389/ffunb.2024.1332755>
- Yan, X., Tang, B., Ryder, L. S., MacLean, D., Were, V. M., Eseola, A. B., Cruz-Mireles, N., Ma, W., Foster, A. J., Osés-Ruiz, M., & Talbot, N. J. (2023). The transcriptional landscape of plant infection by the rice blast fungus *Magnaporthe oryzae* reveals distinct families of temporally co-regulated and structurally conserved effectors. *The Plant Cell*, 35(5), 1360–1385. <https://doi.org/10.1093/plcell/koad036>

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

As fungal plant pathogens continue to increase in their resistance against fungicides, biological control agents (BCAs) research grows in importance. Especially in the case of *M. oryzae*, as it not only is resistance to fungicides (Hahn, 2014), but can also infect several different kinds of crops, which are important to both the global economy and food production (Fernandez & Orth, 2018). The *Bacillus* genus has already proven to contain BCAs (Babalola, 2010), such as *B. subtilis*. *B. subtilis* produces cyclic lipopeptides, several polyketide-derived macrolides, and nonribosomal peptides (Kaspar et al., 2019). *Bacillus velezensis* strain S4 has potential as a BCA, or as a model organism for bettering our understanding of bacterial-fungal interactions.

In this study, we performed a forward genetic screen on ~ 500 fungal HS mutants. Unfortunately, about halfway through this set of mutants, strain S4 stopped producing the characteristic lesion in the fungal hyphae, or if it did, our control would also seep into the hyphae, which confounded the results. For future, similar forward genetic screens, there are a couple of different approaches we would take. Firstly, sequencing both the HS parent line and all the 2,000 HS mutants would vastly

improve the process by indicating number and position of inserts in the genome. However, during the project's timeframe, the funds were not available to make this possible. This information would provide precise insertion locations of HS parent line, since all we currently know is that it is inserted randomly in multiple locations. Performing forward genetic screens for phenotypes is not as common as using NextSeq, as mentioned above, but is still a good method to employ as it can be cheaper than sequencing, however more tedious and will most times give you qualitative data rather than quantitative. Forward genetic screens can find mutants that might be categorized as hypothetical proteins in transcriptomic analysis. Additionally, screening for a phenotype automatically ascribes a function to that gene. *B. velezensis* strain S4 in this particular part of the study displayed phase variation (PV), however in the diffusible molecule assays (and some of the direct contact assays) it still showed inhibition of *M. oryzae*. Questions arise as to if this PV would manifest different if used in the field as a BCA, and if it would still be able to retain its antifungal activities when faced with even more environmental changes/stresses. This brings up an additional future study which would include implementing this *in planta* as done in previous RNAseq studies with *M. oryzae* (Shimizu et al., 2019; Yan et al., 2023; Kawahara et al., 2012) and in field studies.

After inoculation of the three antifungal bacteria onto a confluent lawn of *M. oryzae*, each lesion was imaged (Figure 2.3, 2.4). However, as mentioned previously, the images are not as clear as they could be. If this experiment were to be repeated, the author suggests (if possible) simply pipetting away any remaining bacterial culture

from the hyphae after incubation, without disrupting the hyphae, in the hopes of creating a more even plane of view for imaging.

The recovery assays (Figure 2.5), after being inoculated with the antifungal bacteria, could be improved by following the methodology in our related study (Wockenfuss et al., 2024). They used only one bacterium at a time per fungal pathogen and divided the recovery plates into quadrants to assess growth of either the bacteria or fungi, as well as to show if the bacteria would retain its antifungal abilities. Future studies also should take into consideration if the diffusible molecules from the bacteria are killing the fungal cells or if they are deformed, but able to recover.

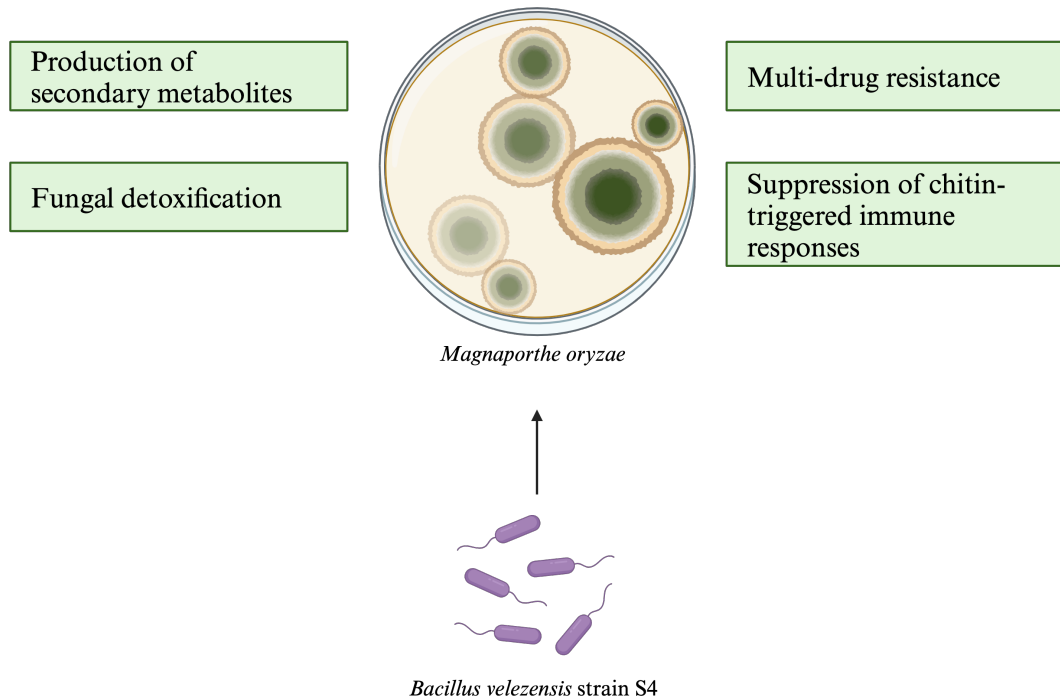
The genetic mutant study took advantage of mutant collections we already had in the lab, so we were able to test ROS mutants, which are important to fungal pathogenicity, as well as small RNA production mutants. We showed that *M. oryzae* strain 7015 and 7015 mutants had no significant difference in percent (%) growth inhibition when challenged with *B. velezensis* strain S4 (Table 2.2, Figure 2.6, 2.7). It was hypothesized that this would not be the case, since most of the mutants are reduced in virulence. It was assumed they would have some difference in inhibition compared to the wt. Future studies could be viewing that interaction microscopically to see if there are any subtle changes in the hyphae.

B. velezensis strain S4 showed enzymatic activity of cellulase and protease, and not lipase, despite its depiction in the genome (Table 2.3, Figure 2.9). Future studies to prove lipase activity (phenotypically) would be the use of a stain, such as Congo Red in the hopes of amplifying any possible halo around the bacterial colony.

Alternatively, co-inoculating the enzymatic plate with *M. oryzae* and the bacteria, as it has been observed that co-inoculation of the fungi produces more antifungal material (data not shown), which may increase the chances of observing a halo, indicating enzymatic activity.

RNAseq analysis revealed 69 DEGs in *M. oryzae* strain 4091 in the presence of *B. velezensis* strain S4 (Table 3.2). A summary of the transcriptomics results and a predicted model of the bacterial-fungal interaction are shown in Figure 4.1. The production of secondary metabolites includes the genes, MGG_08440 (logFC = 5.664) and MGG_09777 (logFC= 3.7701) both of which encode for trichothecenes and for the reduction of toxins (Khatibi et al., 2011). These could be important in protection against *B. velezensis* strain S4 exudates. Fungal detoxification genes also included MGG_09156 (logFC = 4.2995) and MGG_00832 (logFC = 2.4849), which produce cytochrome P450 enzymes (Črešnar & Petrič, 2011). The antifungal materials *M. oryzae* was exposed to in the RNAseq analysis could have been increasing stress for the fungus and hence the production of these enzymes that allow for fungal detoxification (Črešnar & Petrič, 2011). Additionally, MGG_07848 (logFC = 3.3736) is a multidrug resistance protein, CDR1 and in the ATP binding cassettes (ABC), which have been shown in defense against antifungals (Prasad et al., 2015). Finally, the suppression of chitin-triggered immune responses was seen in RNAseq analysis in MGG_07571 (logFC = 2.4839) in which the LysM domain protein as an effector that protects chitin (Hu et al., 2021). *M. oryzae* could be pumping out this gene in an attempt to protect its chitin found in its cell walls.

Figure 4.1 Prediction of *M. oryzae* gene candidates involved in defense during bacterial-fungal interactions.



This was a preliminary study, so additional steps for this would be confirmation of results via real time qRT-PCR. Since we learned that this method does yield DEGs, a future study could use this same method, but at varying time points in the growth of the cultures, to see if further differences in DEGs are revealed with shorter or longer time-courses. The author suggests taking less of the fungal colony, since an adequate amount of RNA was obtained for sequencing. This might decrease the potential for any dilution of signals from hyphae not in contact with the diffusible molecules. To strengthen this study additional fungal pathogens would need to be used to see if there are any similar DEGs that are expressed when in the presence of the

bacteria, which might give us more insight into fungal defense genes. Attempts were made with *Rhizoctonia solani* however issues arose with reference genomes and the server used for RNA analysis (Figure 4.2).



Figure 4.2 Example plate *R. solani* grown in the presence of *B. velezensis* strain S4. *M. oryzae* whole fungal hyphal colony used for RNA extractions after 7 days of incubation. **a.** Sterile filter paper with LB (left), *R. solani* (right). **b.** Filter paper with 5 μ l of *B. velezensis* strain S4 (left), *R. solani* (right).

Overall, this study was able to display that *B. velezensis* strain S4 has potential as a BCA, by excreting diffusible molecules, secondary metabolites, and enzymes that inhibit the growth of the fungal pathogen, *M. oryzae*, as well as create hyphal deformities, and decrease the production of appressoria and spore germination, all of which are critical for virulence on plants.

4.1 References

- Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology Letters*, 32(11), 1559–1570. <https://doi.org/10.1007/s10529-010-0347-0>
- Črešnar, B., & Petrič. (2011). Cytochrome P450 enzymes in the fungal kingdom. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1814(1), 29–35. <https://doi.org/10.1016/j.bbapap.2010.06.020>
- Fernandez, J., & Orth, K. (2018). Rise of a cereal killer: The biology of *Magnaporthe oryzae* biotrophic growth. *Trends in Microbiology*, 26(7), 582–597. <https://doi.org/10.1016/j.tim.2017.12.007>
- Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *Journal of Chemical Biology*, 7(4), 133–141. <https://doi.org/10.1007/s12154-014-0113-1>
- Hu, S.-P., Li, J.-J., Dhar, N., Li, J.-P., Chen, J.-Y., Jian, W., Dai, X.-F., & Yang, X.-Y. (2021). Lysin motif (LYSM) proteins: Interlinking manipulation of plant immunity and fungi. *International Journal of Molecular Sciences*, 22(6), 3114. <https://doi.org/10.3390/ijms22063114>

- Kaspar, F., Neubauer, P., & Gimpel, M. (2019). Bioactive secondary metabolites from *Bacillus subtilis*: A comprehensive review. *Journal of Natural Products*, 82(7), 2038–2053. <https://doi.org/10.1021/acs.jnatprod.9b00110>
- Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., & Minami, E. (2012). Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS ONE*, 7(11). <https://doi.org/10.1371/journal.pone.0049423>
- Khatibi, P. A., Newmister, S. A., Rayment, I., McCormick, S. P., Alexander, N. J., & Schmale, D. G. (2011). Bioprospecting for trichothecene 3-*o*-acetyltransferases in the fungal genus *Fusarium* yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. *Applied and Environmental Microbiology*, 77(4), 1162–1170. <https://doi.org/10.1128/aem.01738-10>
- Prasad, R., Banerjee, A., Khandelwal, N. K., & Dhamgayee, S. (2015). The abcs of *Candida albicans* multidrug transporter Cdr1. *Eukaryotic Cell*, 14(12), 1154–1164. <https://doi.org/10.1128/ec.00137-15>
- Shimizu, M., Nakano, Y., Hirabuchi, A., Yoshino, K., Kobayashi, M., Yamamoto, K., Terauchi, R., & Saitoh, H. (2019). RNA-Seq of *in planta*-expressed *Magnaporthe oryzae* genes identifies *mosvp* as a highly expressed gene required for pathogenicity at the initial stage of infection. *Molecular Plant Pathology*, 20(12), 1682–1695. <https://doi.org/10.1111/mpp.12869>

- Wockenfuss, A., Chan, K., Cooper, J. G., Chaya, T., Mauriello, M. A., Yannarell, S. M., Maresca, J. A., & Donofrio, N. M. (2024). A *Bacillus velezensis* strain shows antimicrobial activity against Soilborne and foliar fungi and oomycetes. *Frontiers in Fungal Biology*, 5. <https://doi.org/10.3389/ffunb.2024.1332755>
- Yan, X., Tang, B., Ryder, L. S., MacLean, D., Were, V. M., Eseola, A. B., Cruz-Mireles, N., Ma, W., Foster, A. J., Osés-Ruiz, M., & Talbot, N. J. (2023). The transcriptional landscape of plant infection by the rice blast fungus *Magnaporthe oryzae* reveals distinct families of temporally co-regulated and structurally conserved effectors. *The Plant Cell*, 35(5), 1360–1385. <https://doi.org/10.1093/plcell/koad036>

Appendix A

TROUBLESHOOTING

4.2 Exudate Assay

Originally, the forward genetic screen of the HS mutant collection was supposed to be performed using the bacterial exudate, mixing it in with the media, plating the different mutants, and measuring radial, fungal growth over the course of a week, with the goal of creating a list of potential mutants of interest if they showed a greater inhibition versus *M. oryzae* 4091 HS. However, multiple attempts to extract the exudate were not successful, as the exudate was not inhibiting the growth of wt or HS, as previously demonstrated. This might be due to the phase variation we observed. However, further experiments would have to determine why the culture filtrate is no longer effective in liquid, yet diffusible molecules are still inhibiting the fungal pathogens on solid media.

4.3 Image J

After the exudate assay, we moved onto what is shown in Chapter 2 using the bacterial whole cell culture for the forward genetic screen. In an attempt to make the results more quantitative, we implemented the use of Image J to measure the lesions in the fungal hyphae, which would then be compared to the parent line, HS. If a larger zone of infiltration/lesion were seen that would be considered a mutant of interest.

However, although we were able to measure the lesions automatically, ImageJ did not always measure the entire lesion, as the software had a hard time distinguishing between the hyphae due to the coloration of the hyphae being similar to each other (even with inoculation of the bacteria). Then we moved on to using Image J to measure manually, however this was extremely time consuming and yielded a significant amount of data, that was not exactly necessary for the scope and time line of this project. This is ultimately why we switched to imaging the 6-well plates and visually comparing the mutants to the parent line, HS.

Appendix B

PERMISSIONS

The paper cited below was used in part in this thesis, the first author is the author of this work. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY), which states that use, distribution, or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in *Frontiers in Fungal Biology* is cited, in accordance with accepted academic practice.

Wockenfuss, A., Chan, K., Cooper, J. G., Chaya, T., Mauriello, M. A., Yannarell, S.

M., Maresca, J. A., & Donofrio, N. M. (2024). A *Bacillus velezensis* strain shows antimicrobial activity against Soilborne and foliar fungi and oomycetes. *Frontiers in Fungal Biology*, 5. <https://doi.org/10.3389/ffunb.2024.1332755>