GENETIC INVESTIGATION OF THE MAIZE PATHOGEN *PHYLLACHORA MAYDIS*

By

Emily Marie Roggenkamp

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Genetics and Genome Sciences – Doctor of Philosophy
Molecular Plant Science – Dual Major

2024

ABSTRACT

Phyllachora maydis is a fungal pathogen of *Zea mays* that causes the disease tar spot of maize. Though *P. maydis* was first identified in Mexico in 1904, the pathogen has only been detected in the United States since 2015. Since this introduction to the US, *P. maydis* has been observed in several states across the Midwest and Canada causing devastating yield losses under conducive conditions. *P. maydis* produces black stromata on maize foliage that resemble spots of tar. A secondary necrotic lesion, termed the fisheye lesion, can often form surrounding the tar spot stroma. Much speculation has arisen surrounding the causal agents of the fisheye lesion. Additionally, *P. maydis* is considered an obligate biotroph that only grows on living host tissue and cannot be cultured axenically. Artificial inoculations of *P. maydis* in controlled environments have previously not been reproducible, and previous understanding of this pathogen was scarce. Also, related species within the classified order have not been extensively studied. Therefore, this dissertation has focused on providing fundamental knowledge of the tar spot of maize pathosystem for future investigation.

In Chapter 2, a detection and quantification assay was developed for specificity to *P. maydis.* A Taq-Man qPCR assay was designed to the internal transcribed spacer (ITS) region of *P. maydis.* Specificity to *P. maydis* was confirmed using herbarium specimens of closely related *Phyllachora* spp. and common maize pathogens and endophytic fungi. The assay was also sensitive, being able to reliably detect 100 femtograms of DNA or 150 *P. maydis* spores. Lastly, reproducibility of the qPCR assay was confirmed for future use in various laboratories.

In Chapter 3, an improved *P. maydis* reference genome is provided. Using long-read sequencing, the contiguity and completeness of the *P. maydis* genome was significantly increased. Gene loss within nitrogen assimilation was found in the *P. maydis* genome indicative of its obligately biotrophic lifestyle. Furthermore, the genome annotation was improved with transcript evidence from RNA extracted *in planta.* A survey of the gene expression at this single timepoint was performed, and prediction of carbohydrate active enzymes and effector proteins was established for future elucidation.

Chapter 4 reports maize differential gene expression in response to *P. maydis* over time. An enrichment of defense response genes was found activated in response to *P. maydis.* Specifically, activation of genes involved in biosynthesis of various compounds was observed. This included terpenoids, phenylpropanoids, flavonoids, and lignin which contain compounds

with anti-fungal properties. Additionally, previously identified candidate genes for tar spot resistance loci were found significantly differentially expressed.

Lastly, in Chapter 5, the fungal and bacterial communities associated with tar spot and fisheye lesions were investigated across the US and Mexico. Bacterial communities did not show significant differences when compared by lesion type but were significantly different by location. Fungal communities showed clear differences between lesion types. Interestingly, different fungi were determined as indicator taxa of fisheye lesions between countries.

In conclusion, the first molecular detection assay was developed for future study on the epidemiology of *P. maydis.* The *P. maydis* genome and annotation was improved. Also, the first differential expression analysis from maize in response to tar spot is reported. Lastly, the microbial communities in fisheye lesions were investigated across countries. Overall, these studies provide tools and initial knowledge on the tar spot of maize pathosystem.

I dedicate this dissertation to my family.
To my dad, whose dedication to agriculture has been an inspiration.
To my mom, whose passion for science has been a guiding light.
To my sister, whose creative spirit has provided new perspectives.
And to my brother-in-law and niece, whose curiosity reminds me of the joy in discovery.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Martin Chilvers, for providing guidance and keeping me focused during my graduate career. I thank you for encouraging me to follow my interests and pursue external professional opportunities. Secondly, I would like to thank the present and previous members of the Chilvers lab for always assisting when I had a question and having my back when I needed it. Additionally, I am grateful to Dr. Chilvers and all the lab members for being patient and understanding during difficult times. To my committee, Dr. Frances Trail, Dr. C. Robin Buell, and Dr. Addie Thompson, I thank you for all your advice throughout my graduate studies.

Next, I would like to thank the Genetics and Genome Sciences and Molecular Plant Science programs for being flexible and accommodating. Specifically, I am grateful to Dr. Cathy Ernst, Dr. Claire Vielle, and Dr. Jianping Hu for their support and Alaina Burghardt for all the administrative support through the years. I also acknowledge the Plant, Soil and Microbial Sciences department for their continued support and the NRT-IMPACTS program for providing funding and training in computational plant sciences. Furthermore, I thank all the friends and colleagues I have met at MSU for their camaraderie and support. I am grateful to the AMPSS student organization for bringing the plant science students together, and SPORE for letting me join their fun plant pathology events. I would specifically like to thank Dr. Hannah Parks for her friendship that helped me make it through grad school. I also appreciate all the new friends I have formed this past year, who provided a source of relief during the last stretch of my Ph.D.

Next, I want to acknowledge all my collaborators from the following institutions: USDA-ARS, CIMMYT, Iowa State University, Ohio State University, Purdue University, University of Illinois, University of Minnesota, and University of Wisconsin-Madison. Your contributions improved the quality and impact of these studies. Furthermore, I am grateful for all the professional connections and friendships these collaborations have formed. Specifically, to Dr. Matt Helm, I thank you for the opportunity to shadow and learn techniques from your lab. To my colleagues at Bayer Crop Science, I thank you for my first experience in industry and for providing new perspectives to my research. I also want to acknowledge all the funding sources for the research in this dissertation including the Corn Marketing Program of Michigan, National Corn Growers Association, National Predictive Tool Modeling Initiative, Project GREEEN, and FFAR. I am also thankful for fellowships from the College of Natural Sciences, the MPS

program, and the NRT-IMPACTS program for funding my stipend during my Ph.D.; and for the travel awards from the College of Agriculture and Natural Resources, the MSU Graduate School, the Council of Graduate Students, the Plant Pathology program, and the NRT-IMPACTS program for funding travel to professional conferences and opportunities.

Lastly, I want to thank all my family and friends back home for their continued support and love, and I thank my dog Charlie for all his cuteness and distractions when I needed a break.

TABLE OF CONTENTS

CHAPTER 1:
LITERATURE REVIEW

Introduction

Tar spot of maize is a fungal disease found in the Americas, caused by *Phyllachora maydis*. Initially observed in Mexico in 1904, the disease has since been confirmed in most of the tropical areas of Central and South America (Maublanc 1904; Valle-Torres et al. 2020). The disease was first confirmed in the continental United States (US) in Indiana and Illinois in 2015 (Ruhl et al. 2016), and the disease quickly spread across the Midwest region of the US, affecting most of the top maize producing states. Tar spot of maize has been shown to cause large reductions in yield through early drying down of the plant (Ceballos and Deutsch 1992; Valle-Torres et al. 2020). In Mexico, Bajet et al. (1994) reported up to a 46% yield loss in fields while Loladze et al. (2019) showed yield losses up to 58% under high disease pressure on susceptible hybrids. In the US, maize hybrids showed yield losses ranging from 1,130 to 2,605 kg per hectare (16.8 to 38.7 bushels per acre) under high disease pressure in 2018 (Telenko et al. 2019). Furthermore, high tar spot disease pressure in 2021 resulted in an estimated disease loss across the US of 5.97 million metric tons (235 million bushels) valued at $1.27 billion USD according to the Field Crop Disease and Invertebrate Loss Calculator Web Tool from the Crop Protection Network (Mueller et al. 2024). Effective management of tar spot disease includes the use of resistant hybrids and fungicide applications (Telenko et al. 2019; Ross et al. 2023; 2024). Further studies have looked at the effects of nitrogen fertilization, planting density, tillage, and irrigation (Check et al. 2023; Ross et al. 2023).

Characteristics of *Phyllachora maydis*, causal agent of tar spot of maize

Phyllachora maydis is characterized by the black spots known as stromata (singular stroma) that form on the maize foliage. Stromata are developed through colonization of the maize epidermal cells and formation of dense hyphal growth and melanization of the epidermal cells (Caldwell et al. 2024). This stromatic structure is defined as a clypeus providing a protective structure around the reproductive fruiting bodies. The oval- to irregularly-shaped stromata range in size from 0.5 to 2.5 mm by 2 to 3 mm and form elongated edges up to 10 mm in some cases (Orton 1944; Rocco Da Silva et al. 2021). Furthermore, stromata are embedded within the leaf tissue having a raised aspect with a convex surface and at later stages are visible on both the abaxial and adaxial sides of the leaf. Common misdiagnoses are observed from insect frass, pesticide damage, and other fungal diseases such as common rust and Physoderma brown spot (Solórzano et al. 2023). Microscopic observations of *P. maydis* fruiting bodies and spores can be

performed to confirm diagnosis. However, the identification of *P. maydis* structures and spores can be challenging depending on the sample quality and accessible resources.

The key morphological characteristics of *P. maydis* include the sexual fruiting bodies known as perithecia where the infective ascospores are produced. *P. maydis* perithecia have a diameter ranging between 170 to 350 µm. Eight ascospores will form uniseriately in an ascus of length ranging 80 to 100 µm. *P. maydis* ascospores are hyaline and aseptate at sizes of 5.5 to 8 µm by 9.5 to 14 µm (Maublanc 1904; McCoy et al. 2018; Rocco Da Silva et al. 2021). The microscopic identification of *P. maydis* ascospores in isolation can be challenging due to their indistinct characteristics. *P. maydis* also reproduces asexually within a single pycnidium centrally located within a stroma. A mature *P. maydis* pycnidium has a multilobed structure where the asexual spores are formed (Caldwell et al. 2024). These asexual spores are hyaline, filiform and aseptate with dimensions of 10 to 15 µm by 0.5 µm (Rocco Da Silva et al. 2021). Microscopic observations of *P. maydis* asexual spores can be difficult as the spores tend to traverse multiple viewing planes of compound microscopes. However, the asexual spores have distinct characteristics compared to the ascospores. Though diagnosis of tar spot of maize through morphological characteristics is relatively simple, molecular diagnostic assays would help in the proper identification of this emerging pathogen as well as provide a baseline for future studies on epidemiology and more.

Infection mechanism and reproductive development of *P. maydis*

P. maydis infection starts with germination of ascospores on maize foliage and formation of appressoria structures for penetration of the leaf surface (Dittrich et al. 1991; Caldwell et al. 2024). Furthermore, within similar fungal species in the *Phyllachora* genus, appressoria began to develop within 2 to 6 hours and reach maturity within 6 to 12 hours after germination (Parbery 1963a). Descriptions of the appressoria from the *Phyllachora* genus indicate that an infection peg will develop for penetration of the leaf epidermis, and the fungus will begin to colonize intracellularly in the epidermal cells (Parbery 1963b). Recently, Caldwell et al. (2024) showed *P. maydis* colonization of the epidermal cells and formation of appressoria-like structures to infect the neighboring plant cell. Dense mycelium will form the clypeus structure to act as protection of the fruiting bodies within the plant mesophyll cells (Parbery 1963b; Caldwell et al 2024). Melanization of the colonized plant epidermis will occur and eventually form the macroscopic black tar spot stroma. Survival of *P. maydis* between growing seasons in the Midwest showed

that viable ascospores were able to germinate from maize residue from the previous season (Groves et al. 2020). The stroma and clypeus may act as protection in overwintering of this fungus.

During clypeus development, the pycnidial fruiting body will begin to form within the mesophyll cells with eventual release of the asexual spores onto the leaf surface (Caldwell et al. 2024). The role of *P. maydis* asexual spores are still under investigation though observations have indicated they act as spermatia and are not infective (Breunig et al. 2023; Caldwell et al. 2024). Spermatia are often pycnidiospores that act as non-motile male gametes for sexual reproduction. Microscopic observations showed that *P. maydis* sexual reproductive development occurs surrounding the plant stomatal chambers (Caldwell et al. 2024). *P. maydis* hyphae were observed coming out of stomata and presumably could interact with the asexual spores or spermatia for perithecia development, though further study is needed for confirmation.

Epidemiology

Tar spot of maize has been historically endemic to tropical regions. However, with the recent introduction into the Midwest region of the US, there has been speculation on the adaptation of this pathogen to more temperate climates (Mottaleb et al. 2019). Studies have shown the overwintering capabilities of *P. maydis* within residue from the US which had not previously been established from tropical climates where maize is grown most of the year (Groves et al. 2020). Mottaleb et al. (2019) predicted the conducive environments in which *P. maydis* could spread and how tar spot disease could impact maize production within the US. Alas, epidemic outbreaks of tar spot have occurred within the US in 2018 and 2021 causing severe economic loss (Mueller et al. 2022; Telenko et al. 2019), and comparisons of disease outbreaks between the US and Honduras showed differences in the progression of tar spot development (Gongora-Canul et al. 2024). Previous environmental factors that lead to disease development in Mexico included moderate temperatures ranging from 17 to 22 ºC, high relative humidity above 75%, and over 7 hours of night-time leaf wetness (Hock et al 1995). Recent investigations identified the most strongly correlated environmental factors for tar spot disease development were the average daily minimum and mean temperatures on a 30-day moving average, noting that relatively moderate temperatures no higher than 23ºC were conducive for disease development (Webster et al. 2024). Furthermore, they showed a negative correlation between disease development and moisture variables including relative humidity, dew point, and leaf wetness.

These findings seem counterintuitive to previous studies focused on initial infection but may indicate weather conditions needed for later stages of disease development. Recent disease risk models have been established to inform effective timing of fungicide applications (Webster et al. 2024; Tar spotter). Relying on weather variables, tar spot disease development is assessed to provide management suggestions based on the risk. The biology and epidemiology of *P. maydis* still remain under investigation.

Taxonomy and Phylogenetics

The taxonomy and phylogenetics of the genus *Phyllachora* are poorly understood due to a lack of research within its entire order. The order Phyllachorales contains ascomycetous plant pathogens that produce mostly black stromata on the foliage of angiosperm hosts (W Silva-Hanlin and Hanlin 1998). The Phyllachorales order contains three families: Phyllachoraceae, Phaeochoraceae, and the newly described Telimenaceae (Mardones et al. 2017, 2022). The Phyllachoraceae are tar spot fungi with mainly Poaceae hosts, while Telimenaceae infect angiosperm hosts apart from the Poaceae family. Phaeochoraceae are tar spot fungi on Aracaceae hosts and morphologically different in that they do not produce a clypeus (Mardones et al. 2017). All species within the Phyllachorales order are considered obligate biotrophs meaning they grow only on living host tissue. Classifications have been historically based on morphological characteristics and classification of the host plant. Furthermore, the host plant has been a main factor in the speciation of these obligate biotrophic pathogens leading to narrow host ranges of the species without proper host range testing. For example, *P. maydis* is thought to only infect *Zea mays* (maize) but this has not been extensively studied. However, *Phyllachora graminis,* a tar spot producing pathogen on grass hosts, is considered to have a wide host range being classified or described on 76 host genera according to the USDA (Farr & Rossman 2024). This may be indicative of the discrepancies in classification within the Phyllachorales order and possible over-splitting of species delimitations (Mardones et al. 2017).

A recent phylogenetic analysis of the *Phyllachora* genus proposed species classifications based on sequences from the ribosomal internal transcribed spacer (ITS) region and large ribosomal subunit (LSU) markers (Broders et al. 2022). Within their collection of *Phyllachora* specimens, five distinct *Phyllachora* species were identified based on the phylogenetic clustering with four species being able to infect maize. Three *Phyllachora* sp. were found in contemporary maize. *Phyllachora* sp. 4 was found to have a wide host range able to infect several grass hosts

including maize from historical herbarium specimens. Previous analyses have also shown variation within the *P. maydis* ITS region (McCoy et al. 2018; 2019). Though these variants could indicate the different species or subspecies of *P. maydis,* the repetitive nature of the ribosomal units does suggest the possibility of intragenomic variation within the ITS region where multiple different copies could be contained within a single nucleus. Variation in the ITS region can be found in ectomycorrhizal and plant pathogenic fungi such as *Phakopsora pachyrhizi,* the causal agent of soybean rust (Rush et al. 2019; Simon and Weiß 2008; Smith et al. 2007). While it is postulated that multiple *Phyllachora* species are able to infect maize, further analysis is needed to confirm the species delineations with the *Phyllachora* genus.

Fisheye Symptomology Background

Tar spot stromata can often be accompanied by a secondary symptom commonly referred to as fisheye. Fisheye lesions are defined by the necrotic ring that surrounds the tar spot stroma. These symptoms were observed in the original tar spot description noting similarity to other diseases on maize (Maublanc 1904). Ovoid in shape, the fisheye lesion measures up to 10 mm by 4 mm with the tar spot stroma centrally located (Müller and Samuels 1984). The fisheye lesion develops approximately 3 to 20 days after tar spot stromata are developed (Hock et al. 1992). Fisheye lesions are ubiquitous in Mexico and South America. However, in the US, observations are relatively random and only detected in some areas of a field and often in the later stage of the growing season. Being necrotic in nature, the fisheye lesion was previously described to be the leading factor in yield reductions (Hock et al. 1992), however early leaf necrosis across the entire leaf can be observed with or without fisheye lesions. Specifically, the infrequent observation of fisheye lesions in the US indicates that tar spot alone can produce reductions in yield under moderate to high disease severity (Valle-Torres et al. 2020; Mueller et al. 2024).

Monographella maydis was first identified in 1984 and found associated with the fisheye lesion (Müller and Samuels 1984). Furthermore, it was hypothesized that *M. maydis* caused the fisheye lesion, however they were unable to confirm causality through Koch's postulates. Currently this fungus has the accepted name of *Microdochium maydis,* however no type specimens exist for confirmations of this fungus (Hernández-Restrepo et al. 2016). Müller and Samuels (1984) found *M. maydis* perithecia immersed in the necrotic host tissue, and ascospores formed within asci. *M. maydis* ascospores were 18 to 22 by 3.5 to 5 µm in size described as hyaline, curved and fusiform with 1 to 3 septa. Additionally, *M. maydis* formed sporodochia

projecting from maize stomata, and asexual conidia were 20 to 46 by 3 to 4 µm in size and hyaline, elongate cylindrical and curved with 3 to 9 septa (Müller and Samuels 1984). Temperatures ranging from 24 to 27 ºC were found optimal for *in vitro* growth of *M. maydis,* aligning with climates where fisheye lesions are observed.

Furthermore, a mycoparasite shown to grow within *P. maydis* reproductive structures was discovered as *Coniothyrium phyllachoraea* (Maublanc 1904). *C. phyllachoraea* was described to form globular structures within the stromata of *P. maydis* where brown elliptical spores of size 6 to 8 by 4 to 4.5 µm would develop (Maublanc 1904). Recent studies have found the mycoparasitic fungus, *Paraphaeosphaeria neglecta,* associated with tar spot and fisheye lesions (McCoy et al. 2019; Caldwell et al. 2024). Interestingly, *Paraphaeosphaeria* spp. are taxonomically classified as *Coniothyrium*-like fungi, and many species within this clade have been re-classfied within the *Paraphaeosphaeria* genus (Verkley et al. 2014). *P. neglecta* has similar morphological characters to *C. phyllachoraea* forming globose conidiomata where ellipsoid hyaline to brown conidia develop and range in size 4–6 × 3–5 µm (Maublanc 1904; Verkley et al. 2014; Caldwell et al. 2024). Therefore, *P. neglecta* is thought to be the *C. phyllachoraea* from the original description.

Depictions of tar spot and fisheye lesions termed the disease as a complex (Hock et al. 1992; Mottaleb et al. 2019). The tar spot disease complex has previously included the three previously described fungi: *Phyllachora maydis, Microdochium maydis,* and *Coniothyrium phyllachoraeae* (*Paraphaeosphaeria neglecta*) (Maublanc 1904; Hock et al. 1992). While *M. maydis* and *P. neglecta* have been associated to the tar spot disease complex, the causal agent(s) of fisheye lesions remain unknown (Muller and Samuels 1984; McCoy et al. 2019; Luis et al. 2024). Over the last 40 years, *M. maydis* has been the supposed causal agent of fisheye lesions (Müller and Samuels 1984; Dittrich et al. 1991; Hock et al. 1992). However, as mentioned, Koch's postulates failed to confirm the causality, and no type specimens exist for *M. maydis.* (Müller and Samuels 1984). Sequence-based fungal communities of tar spot lesions alone and tar spot with fisheye lesions were compared to understand the possible causal agents of fisheye lesions in Michigan (McCoy et al. 2019). As expected, *P. maydis* was found abundantly within both tar spot lesions alone and tar spot with fisheye lesions. Interestingly, *M. maydis* was not found significantly associated with fisheye lesions while *P. neglecta,* a *Fusarium* sp., and a taxon most closely related to *Neottiosporina paspali* were (McCoy et al. 2019). Similarly, two

mycoparasitic fungi were found within *P. maydis* reproductive structures associated with fisheye lesions in Indiana (Caldwell et al. 2024). Sequencing confirmed that one of these fungi was *P. neglecta,* but the other mycoparasitic fungus remains unknown. *Fusarium* spp. have also been extensively isolated from fisheye lesions from the US and Mexico (Luis et al. 2023). Attempting to detect *M. maydis* from fisheye lesions, Luis et al. (2023) found that morphologically identified *M. maydis* isolates were sequence identified within the *Fusarium* genus. Furthermore, isolations from fisheye lesions from Mexico resulted in mostly *Fusarium incarnatum-equiseti* species complex (FIESC) while isolates from the US resulted in a mixture with FIESC, *Fusarium tricinctum* species complex (FTSC), *Fusarium fujikuroi* species complex (FFSC), and mostly from the *Fusarium sambucinum* species complex (FSAMSC) (Luis et al. 2023). While these species have been found associated with fisheye lesions, causality has still not been confirmed.

***Phyllachora maydis* genomics and effector characterization**

Due to the obligate nature of these fungi, sequence data is lacking within the Phyllachorales order. In 2020, the first draft genome within the order was announced from *Phyllachora maydis* (Telenko et al. 2020). Due to the inability to culture this pathogen, the genome was sequenced from excised tar spot lesions and sequenced using Illumina short reads. The benchmarking universal single-copy ortholog (BUSCO) analysis, a common measure for genome completeness, reported 91% of fungal orthologs were present in the genome with 6.9% being fragmented. While this provided the first reference for further genetic analysis and understanding of this pathogen, the genome was highly fragmented having 11,228 scaffolds. Furthermore, the genome was found to be 56.46% repetitive which likely accounted for the high fragmentation that can occur from sequencing repetitive genomes with short reads such as Illumina technologies. Additionally, this can result in reads being misassembled together due to the similarity in the repeats and may result in incomplete genomes (Treangen and Salzberg 2011). Telenko et al. (2020) further showed the *P. maydis* genome as 45 Megabasepairs (Mb) in size with 5,992 protein-coding genes.

Alas, many fungal plant pathogens have increased repetitive genomic content (Duplessis et al. 2011; Sotiropoulos et al. 2022). These repetitive transposable elements can often contain important virulence genes and can help plant pathogens adapt to new environments (Müller et al. 2019; Vossenberg et al. 2019). Often the increase in transposable element content correlates with an increased genome size without an increase in gene content (Raffaele and Kamoun 2012;

Spanu 2012; Aylward et al. 2017). Furthermore, within obligate biotrophic plant pathogens, gene losses within inorganic nitrogen and sulfur metabolism has been prolific (Duplessis et al. 2011). It is theorized that the gene losses within these primary metabolic pathways may be the evolutionary trade-off of advantageous traits involved in the obligate biotrophic plant pathogenic lifestyle (Spanu 2012). Telenko et al. (2020) further determined that the *P. maydis* genome encoded 462 secreted proteins which contained 59 effector proteins. Effector proteins are often secreted into the host to evade defense responses (Stergiopoulos and de Wit 2009). The effector repertoires of various plant pathogens can be highly diverse giving different virulence responses and specificity to certain hosts (Inoue et al. 2023). Additionally, the effector repertoires contribute to the population structure within plant pathogens and can help determine pathotypes within species (Amezrou et al. 2024; Eschenbrenner et al. 2020). A recent study looked to functionally characterize *P. maydis* effector proteins by looking at the localization of effectors in *Nicotiana benthamiana* (Helm et al. 2022; Rogers et al. 2024). Furthermore, microsatellite markers were established in *P. maydis* to determine population structure across geography (Ross et al. 2024).

Maize Disease Resistance

Effective management strategies for tar spot of maize include the planting of resistant hybrids. Currently, no commercial hybrids provide complete resistance to tar spot but partial resistance still provides effective management of tar spot disease (Ross et al. 2023). Tropical variety testing has been successful in identifying lines with increased tar spot resistance (Ceballos and Deutsch 1992; Cao et al. 2017). A major resistance locus to tar spot termed qRtsc8-1 have been identified in tropical maize (Cao et al. 2017, 2021; Mahuku et al. 2016; Ren et al. 2022; Yan et al. 2022). Currently, no candidate genes have been functionally characterized for tar spot resistance development. Several resistance (“R”) genes have been cloned in maize and provide defense to a race-specific pathogen (Johal and Briggs 1992; Collins et al. 1999; Chen et al. 2022). The deployment of R genes within commercial hybrids is highly effective in preventing disease, but often these genes lose efficacy rapidly as the pathogen adapts (Yang et al. 2017). Therefore, small effect quantitative traits in combination provide responses that have a lasting impact and help in slowing the loss of R gene efficacy (Balint-Kurt & Johan 2009; Yang et al. 2017). Genetic mapping of tar spot disease resistance also found loci with minor loci that may be useful in future breeding pipelines (Cao et al. 2017, 2021; Mahuku et al. 2016; Ren et al.

2022; Yan et al. 2022). Furthermore, genetic mapping of resistance loci within temperate maize populations has been ongoing and preliminary results indicate different tar spot resistant loci than tropical lines (Trygestad 2021). Introgression of tropical resistant loci or newly identified temperate resistant loci will be important in future breeding for effective tar spot disease management.

CHAPTER 2:
DEVELOPMENT OF A QPCR ASSAY FOR SPECIES-SPECIFIC DETECTION OF THE TAR SPOT PATHOGEN *PHYLLACHORA MAYDIS*

Source

Roggenkamp, E.M., Check, J.C., Biswal, A.K., Floyd, C.M., Miles, L.A., Camila P. Nicolli, C.P., Shim, S., Salgado-Salazar, C., Alakonya, A., Malvick, D.K., Smith, D.L., Telenko, D.E.P., Chilvers, M.I. (2024) Development of a qPCR for species-specific detection of the tar spot pathogen *Phyllachora maydis*. PhytoFrontiers 4(1): 61-71. Available at: https://doi.org/10.1094/PHYTOFR-04-23-0050-FI

Summary

Molecular detection assays can be used to diagnose plant diseases and confirm indistinguishable samples. Furthermore, the quantification of pathogen can be helpful to understand disease dynamics before observation of disease. Currently, no molecular diagnostic or quantification assay exists for *Phyllachora maydis,* causal agent of tar spot of maize. In this chapter, the development of a TaqMan qPCR assay to *P. maydis* is depicted for future use as a diagnostic tool and quantification of latent infection and airborne spores. The internal transcribed spacer region was used to design primers and probe specific to *P. maydis.* Herbarium specimens of various *Phyllachora* spp. were used to confirm specificity through sequence and performance of the assay. No off-target amplification was reproducible from the various non-target *Phyllachora* spp. and fungal isolates from maize including common endophytes and pathogens. The sensitivity of the assay was tested, and the limit of detection was determined as 100 femtograms of genomic DNA or 150 spores. Additionally, the assay was transferable across various institutions and qPCR thermal cyclers. Overall, this qPCR assay showed exceptional performance and will be used in future epidemiology studies from airborne spore samplers.

CHAPTER 3:
ELUCIDATING THE OBLIGATE NATURE AND BIOLOGICAL CAPACITY OF *PHYLLACHORA MAYDIS*

Source

Roggenkamp, E.M.*, MacCready, J.S.*, MacCready, K.G., Chilvers, M.I. (2023) Elucidating the obligate nature and biological capacity of an invasive fungal corn pathogen. MPMI 36(7): 411-424. Available at: https://doi.org/10.1094/MPMI-10-22-0213-R

Summary

A high-quality genome often advances the molecular understanding of an organism. The fungal order Phyllachorales, containing obligate biotroph plant pathogens that are unable to be cultured axenically, has a scarcity of high-quality genome assemblies making further genetic study difficult. In this chapter, an improved genome assembly and annotation for *Phyllachora maydis,* a pathogen of maize within the Phyllachorales order, was generated and investigated. The genome was assembled into 13 scaffolds totaling 64 Mb in length, being 59.1% repetitive and 98.6% complete according to BUSCO. Genomic elucidation showed that *P. maydis* is deficient in nitrogen assimilation and may be using amino acids as its primary source of nitrogen. The results also predicted that *P. maydis* uses heterothallic sexual reproduction. Furthermore, within the tar spot stroma, *P. maydis* highly expresses genes involved in autophagy and secretion that may play roles in pathogenicity. Lastly, *P. maydis* encodes unique secreted carbohydrate active enzymes and effector proteins that likely contribute to host recognition and subsequent infection. Overall, this study provided fundamental knowledge of the biological capacity of *P. maydis* and the foundation for future genetic research for improved understanding of this pathosystem.

CHAPTER 4:
MAIZE DIFFERENTIAL GENE EXPRESSION UPON EXPOSURE TO THE TAR SPOT PATHOGEN, *PHYLLACHORA MAYDIS*

Abstract

Tar spot of maize, caused by *Phyllachora maydis*, is an emerging threat to crop production across the United States and Canada. Effective management of the disease includes application of fungicides when warranted and planting of partially resistant maize varieties as currently no commercial maize hybrids have complete resistance. Several studies have focused on mapping of tar spot resistant loci from various maize diversity panels resulting in candidate genes of interest. However, no further techniques have been performed for further understanding of the maize host defense response or resistant mechanisms to tar spot. In this study, the first differential expression analysis of maize in response to tar spot disease is presented. Potted B73 maize plants were exposed to infection by placing them into a a tar spot infested maize field. Over the course of disease development, leaves were sampled at two timepoints: 10 days and 24 days post exposure. Differential expression was determined by contrasting the exposed samples to non-exposed controls at the respective timepoints. 3,160 genes were significantly differentially expressed at 10 days post exposure while 3,953 genes were significant at 24 days post exposure. These significantly differentially expressed genes (DEGs) were enriched in gene ontology biological processes involved in defense response, photosynthesis, cell wall biogenesis, signaling cascades, and diterpenoid biosynthesis. At 24 days post exposure, DEGs were enriched in biosynthesis and metabolism of amino acids as well as biosynthesis of flavonoid and phenylpropanoid compounds according to KEGG orthology. Furthermore, several candidate genes from identified tar spot resistant loci were found to be differentially expressed. This study provides a survey of B73 maize response to tar spot and identifies several genes that may be important in host-pathogen interactions and resistance mechanisms.

Introduction

Zea mays (maize) is an important cereal crop produced globally with an estimated 1.1 billion metric tons produced in 2022 (FAOStat, 2024). In the United States (US) alone, 38.3 million hectares were grown that yielded 388 million metric tons of maize in 2023 (USDA-NASS, 2024). However, yield production may be limited by various abiotic and biotic stressors. Biotic stress from pathogens significantly limits maize yield and resulted in an estimated loss of 11.8 million metric tons in the US in 2023 (Mueller et al. 2024). Management of biotic stress usually includes cultural practices, pesticide application, and host genetic resistance. Disease resistance in plants can be either "gene-for-gene," providing major resistance to a race-specific pathogen from a single gene, or quantitative, providing partial resistance additive from several genes (Balint-Kurt & Johal 2009). In maize, single gene resistance has been employed for several fungal pathogens; however, most disease resistance has been quantitative and often protects against multiple pathogens (Yang et al. 2017).

Major and minor resistance genes are often involved in eliciting or regulating an immune response from the plant. Furthermore, the innate immune system of plants had been described to provide defense to pathogens in two tiers: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl 2006). In PTI, plants will recognize pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs). In ETI, plants will recognize effector proteins secreted by the pathogen often using nucleotide-binding, leucine-rich repeat receptors (NLRs) (Zhou & Zhang 2020). Many NLRs are major resistance loci termed R genes involved in the "gene-for-gene" model of resistance. These immune responses will result in signaling cascades involving the mitogen-activated protein kinase (MAPK) cascade, efflux of calcium, and production of reactive oxygen species (ROS), leading to biosynthesis of secondary metabolites and, in ETI, a hypersensitive response (HR).

Phyllachora maydis recently emerged in the US and Canada and causes the destructive disease commonly known as tar spot of maize (Maublanc 1904; Ruhl et al. 2016). This pathogen has remained largely understudied due to the inability to axenically culture and reproduce infection in a controlled environment and its somewhat unpredictable nature in the natural environment (Ceballos and Deutsch 1991). Many ongoing research efforts are dedicated to understanding the management, epidemiology, and genetics of *P. maydis* (Check et al. 2023; Telenko et al. 2019; Webster et al. 2024; MacCready et al. 2023). However, the host-pathogen

interactions of this pathogen have not been extensively studied. Recent work has functionally characterized a subset of *P. maydis* predicted effector proteins (Rogers et al. 2024; Helm et al. 2021) as well as mapped maize resistant loci to *P. maydis* using genome-wide association studies (GWAS) and more techniques (Cao et al. 2017, 2021; Mahuku et al. 2016; Ren et al. 2022; Trygestad 2021). Currently, there are no publications elucidating the maize gene expression response to *P. maydis.* Therefore, this study reports the first differential expression analysis of B73 maize after exposure to *P. maydis* over two time-points.

Materials and Methods

Sample Collection

B73 maize plants were grown in the Michigan State University (MSU) research greenhouse's outdoor courtyard space for 30 days. A subset of plants was then exposed to *Phyllachora maydis* by placing them in a tar spot infested maize field for 10 days at the MSU Plant Pathology Farm. After 10 days of exposure, plants were brought back to the outdoor courtyard and kept there for 14 days to develop tar spot stroma. A respective subset of plants was kept in the outdoor courtyard to serve as non-exposed controls with significantly reduced exposure to tar spot. Single leaves were sampled from three separate replicate plants for both exposed and non-exposed treatments at two timepoints. The first sampling timepoint occurred after the 10-day treatment while the second sampling timepoint occurred after the 14-day incubation period. The resulting sample groups included 10 days post exposure (10 dpe), 10 day non-exposed (10d nonexp.), 24 days post exposure (24 dpe), and 24 day non-exposed (24d nonexp.). From a single leaf, five leaf disks were collected using a 5 mm cork borer near the middle of the leaf lengthwise and spanning the width of the leaf. Leaf disks from the 24 dpe treatment were collected from visible tar spot stroma which varied in length from 0.5 to 2 mm. Leaf disks were flash frozen in liquid nitrogen and kept at -80ºC until ready for extraction. The collection process is illustrated in Figure 4.1.

RNA Sequencing

Samples were prepared for RNA extraction using RNAlater™-ICE Frozen tissue transition solution. Maize tissue was homogenized using lysing Matrix A in a FastPrep® homogenizer (MP Biomedicals, Irvine, CA, USA). Total RNA was extracted using the RNeasy® Plant Minikit with the RLC buffer for lysis (Qiagen, Venlo, Netherlands). Remnant DNA was removed from the RNA samples using the TURBO DNA-free™ kit (Invitrogen, Waltham, MA, USA). RNA was

quantified fluorometrically using Qubit™ RNA Broad Range assay kits (Invitrogen, Waltham, MA, USA), and the RNA quality was assessed using the Agilent 4200 TapeStation high sensitivity RNA ScreenTape® assays. Illumina Stranded mRNA library preparations were performed using the Ligation kit and IDT for Illumina TruSeq RNA Unique Dual Indexes. mRNA libraries were sequenced at 150 bp paired end format using the Illumina NovaSeq v1.5 S4 flow cell with 300 cycle reagent kit. All RNA quality checks, library preparation, and sequencing were performed at the MSU Research Technology Support Facility Genomics Core.

Differential Expression Analysis

Read quality was assessed using FastQC v.0.12.1 (Andrews 2010). Adapters and low-quality reads were removed using cutadapt v.4.6 (Martin 2011). Trimmed reads were aligned to the B73 reference genome version 5 (Zm-B73-REFERENCE-NAM-5.0; Hufford et al. 2021) using hisat2 v.2.2.1 (Kim et al. 2019). Read alignments were counted at gene locations using HTseq v.2.0.4 (Putri et al. 2022), and read counts were supplied to the DEseq2 package v.1.42.0 (Love et al. 2014) for differential expression analysis in R v.4.3.2 (R core team 2023). Significantly differentially expressed genes were defined as having a log2foldchange greater than 1 with an adjusted p-value less than 0.05. Gene set enrichment analysis was performed on all differentially expressed genes with an adjusted p-value above 0.05 using the clusterProfiler R package v. 4.10.1 (Wu et al. 2021). Gene ontology (GO) for biological processes were extracted for *Zea mays* using the biomaRt R package v.2.58.2 (Durinck et al. 2009). KEGG orthology was extracted from the clusterProfiler package. Figures were produced using the ggplot2 package v.3.5.0 (Wickham 2016).

Contaminant Microbiome and Meta-transcriptome Analysis

Raw RNA reads were quality assessed, trimmed, and aligned to the B73 version 5 reference genome as above. Unmapped reads were extracted using samtools v.1.19 and BEDtools v.2.30.0 (Li et al. 2009; Quinlan and Hall 2010) and aligned to the *Phyllachora maydis* genome PM02 (NCBI genome assembly ASM2933922v1; MacCready et al. 2023). The remaining unmapped reads were searched against the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database using the DIAMOND software v.2.1.9 blastx function (Buchfink et al. 2021). Results were analyzed in the MEGAN6 software and results exported for visualization (Huson et al. 2016).

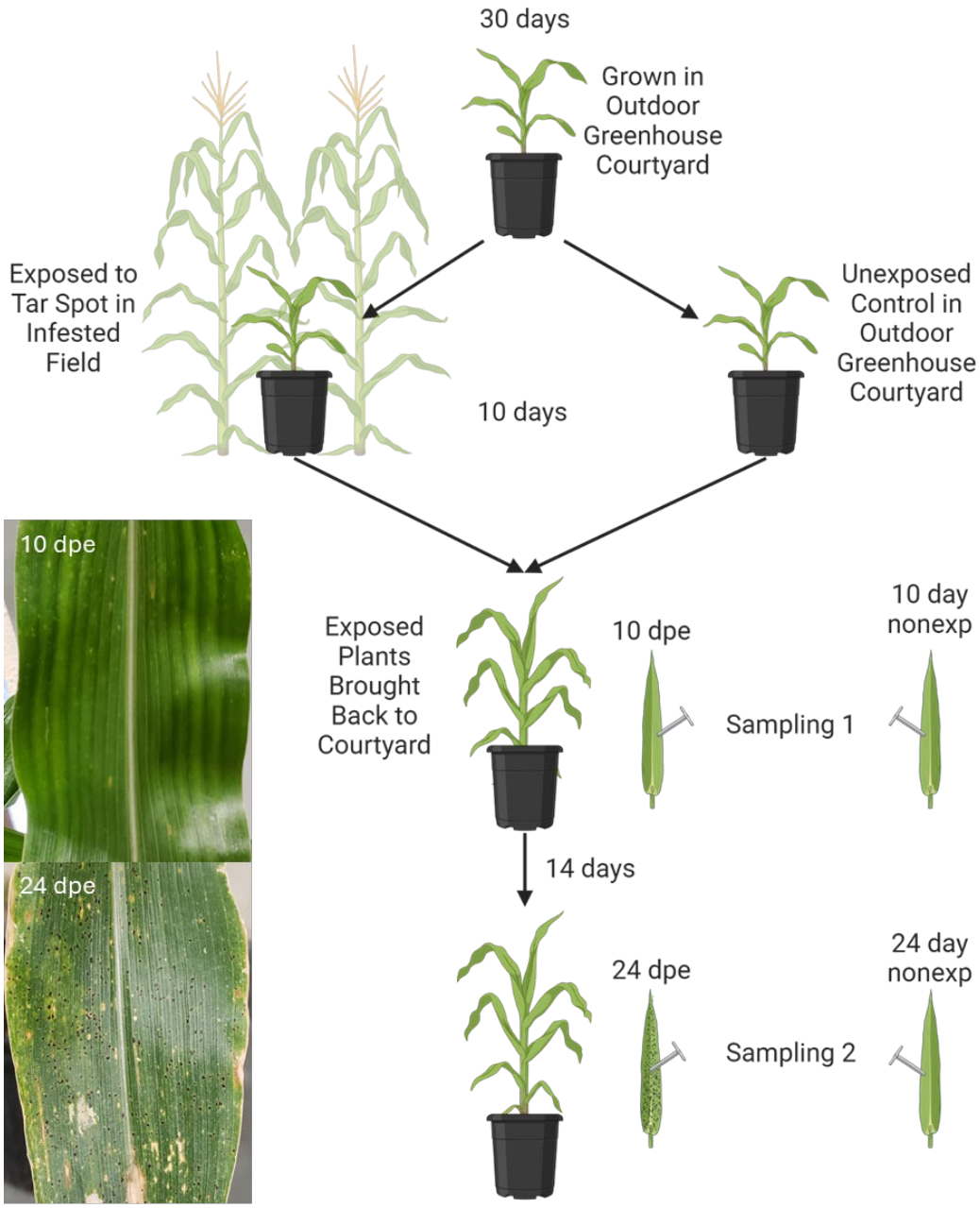

Figure 4.1: RNA sampling schematic. B73 maize plants were grown for 30 days in the greenhouse courtyard. Treated plants were exposed in a *P. maydis* infested field for 10 days and brought back to develop tar spot. Non-treated plants remained in the greenhouse courtyard to serve as non-exposed controls. Exposed plants were sampled at 10 days post exposure (10 dpe) and 24 days post exposure (24 dpe). Non-exposed plants were similarly sampled at 10 days and 24 days. Images showing symptoms sampled on leaves at 10 dpe and 24 dpe. dpe = days post exposure. nonexp = non-exposed control. Illustration created using BioRender.

Results

Alignment and assignment of meta-transcriptome sequences

The alignment rates of the total quality trimmed RNA reads were assessed to determine the amount of *P. maydis* within the samples (Table 4.1; Supplemental Table 4.1). At 10 days non-exposed (10d nonexp), 90.5% of reads aligned on average to the B73 maize genome with an average 209 million reads mapped. The read alignment rates to the *P. maydis* genome at 10 days non-exposed were 0.001% with 2,578 reads mapped on average. Within the 10-day post exposed samples (10 dpe), the average alignment rate to the B73 maize genome was 96.1% with an average of 252 million reads mapped while the alignment rates to the *P. maydis* genome were 0.008% with 21,667 reads mapped on average. At 24 days non-exposed (24d nonexp), 92.0% of reads aligned on average to the B73 maize genome with an average 168 million reads mapped. The average read alignment rate to the *P. maydis* genome at 24 days non-exposed was 0.003% with 5,655 reads mapped. Within the 24-day post exposed samples (24 dpe), the average alignment rate to the B73 maize genome was 50.7% with 119 million reads mapped while the average alignment rate to the *P. maydis* genome was 44.0% with 104 million reads mapped.

Table 4.1: Summary of RNA sequence reads. Read counts of total reads after adapter and quality trimming, mapped reads to the *Phyllachora maydis* and *Zea mays* genomes, and remaining unmapped reads are presented as mean values of three replicates from the respective treatment groups. The average percentage of the total reads for each is listed in parentheses. dpe = days post exposure. nonexp = non-exposed.

	10 day nonexp	10 dpe	24 day nonexp	24 dpe
Unmapped reads (% reads)	21,918,101 (9.48%)	10,202,719 (3.89%)	14,513,235 (7.97%)	12,354,060 (5.25%)
Phyllachora maydis mapped reads (% reads)	2,578 (0.001%)	21,667 (0.008%)	5,655 (0.003%)	103,545,857 (44.0%)
Zea mays mapped reads (% reads)	209,368,062 (90.5%)	252,044,952 (96.1%)	167,535,261 (92.0%)	119,407,961 (50.7%)
Total reads (quality trimmed)	231,288,740	262,269,339	182,054,151	235,307,878

On average, 14.7 million reads did not align to the maize and *P. maydis* genomes

accounting for 6.48% of the total reads. Further analysis was performed on these unaligned transcript reads to determine other environmental exposures. Taxonomically classified reads from all samples were mainly assigned to the Streptophyta phylum within the plant kingdom, and reads from the exposed samples were also assigned to the Ascomycota and Basidiomycota phyla within the fungal kingdom (Supplemental Figure 4.1). The plant genera classified within these unaligned reads included *Zea*, *Vigna*, *Panicum*, *Oryza,* and *Lupinus* while the classified fungal genera included *Puccina*, *Exserohilum*, *Aspergillus*, *Melampsora*, *Coniochaeta*, *Alternaria*, and *Saitoella* (Supplemental Table 4.2). Classified bacterial genera found within these unaligned reads included *Acinetobacter* and *Streptomyces.* Within these remaining unmapped reads, the genera with the highest number of reads were *Zea* with an average of 46,146 reads and *Puccinia* with an average of 25,361 reads (Supplemental Table 4.2).

Differential Expression Analysis

Statistical analyses were performed to confirm that gene expression followed similar patterns between replicates. Principle coordinate analysis showed that each sample replicate separated by treatment and timepoint. Interestingly, the non-exposed control samples grouped separately from the *P. maydis* exposed samples (Supplemental Figure 4.2A). Pearson's correlation between each sample showed that all three replicates were significantly correlated within the treatment and timepoint (Pearson's correlation > 0.9) (Supplemental Figure 4.2B). Similarly, this clustering of sample replicates by timepoint and treatment is shown by the gene expression patterns (Figure 4.2A). Significant differentially expressed genes (DEGs) were determined by the thresholds of a log2foldchange greater than 1 with an adjusted p-value below 0.05. When comparing the 10-day post exposure (10 dpe) to non-exposed samples (10d nonexp), 3,160 genes were significantly differentially expressed with 2,161 genes down-regulated and 999 genes up-regulated within the exposed samples (Figure 4.2B; Supplemental Table 4.3). When comparing the 24-day post exposure (24 dpe) to non-exposed samples (24d nonexp), 3,953 genes were significantly differentially expressed with 2,410 genes down-regulated and 1,543 genes up-regulated (Figure 4.2B; Supplemental Table 4.4). 1,343 DEGs were found to overlap between the timepoints (Figure 4.2C). Several differentially expressed genes within each timepoint had previously been identified as candidate genes for tar spot resistance (Table 4.2).

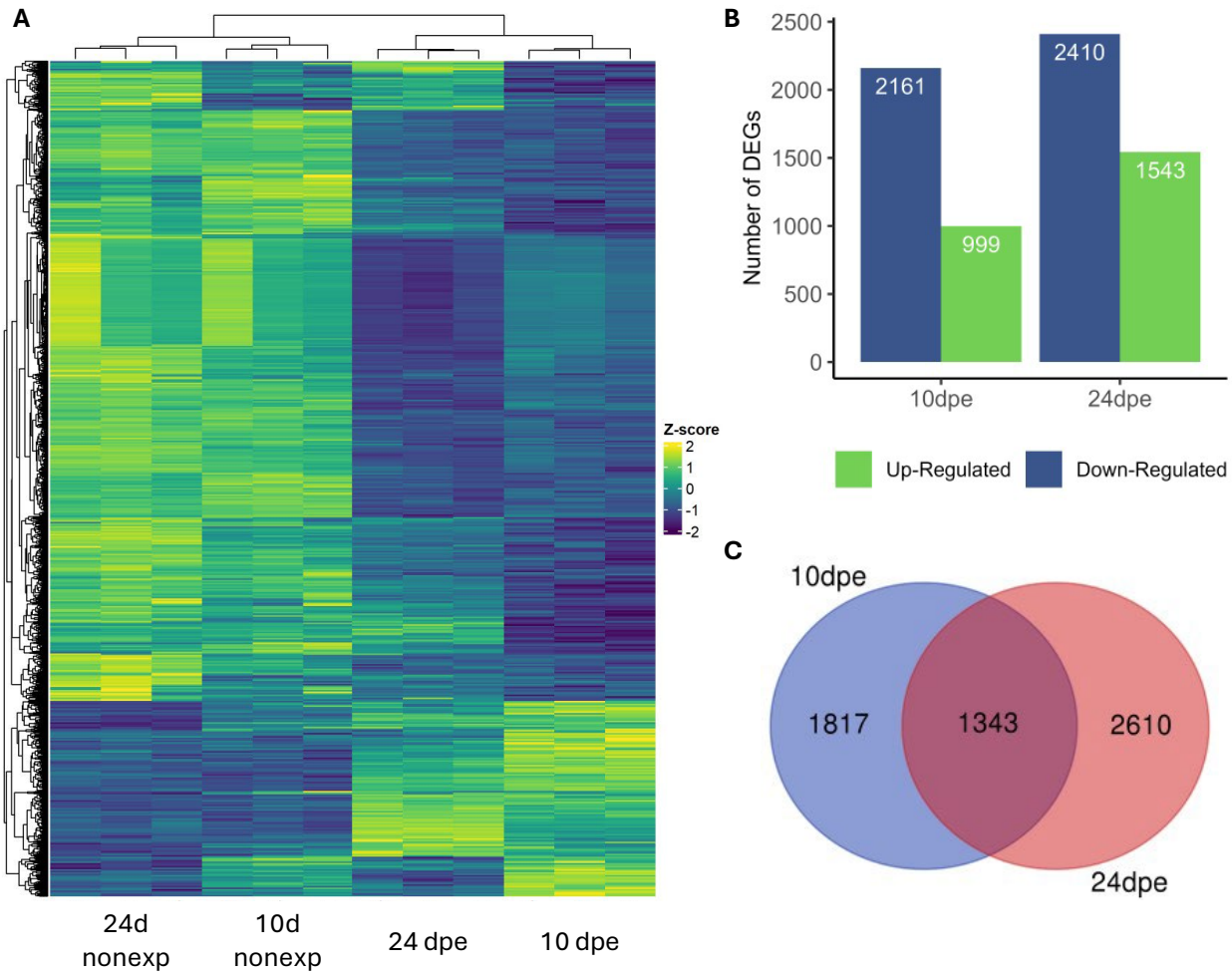

Figure 4.2: Differential expression analysis. (A) Heat map indicating maize gene expression indicated by a Z-score and clustering of genes and sample replicates. Continuous scale with yellow being the highest expressed genes and purple being the lowest expressed genes. (B) Number of significant differentially expressed genes (DEGs) between exposed and non-exposed samples at each timepoint. Up-regulated in green and Down-regulated in purple. (C) Venn diagram showing overlap in DEGs between the 10 dpe and 24 dpe sample groups. dpe = days post exposure. nonexp = non-exposed control.

Table 4.2: Selected significant differentially expressed candidate genes. Significant differentially expressed genes previously identified as tar spot resistant loci are listed with the log2foldchange values between the exposed and unexposed samples at each timepoint separately. dpe = days post exposure.

Gene	Gene product	Log2FoldChange	
		10 dpe	24 dpe
Zm00001eb345910	putative leucine-rich repeat receptor-like protein kinase family protein	2.59	NS

Table 4.2 (cont'd)

Zm00001eb126340	Sarcoplasmic reticulum histidine-rich calcium-binding protein	1.74	NS
Zm00001eb344960	bHLH22 transcription factor FAMA	NS	-1.78
Zm00001eb008920	3-ketoacyl-CoA synthase28 (KCS28)	-2.75	-1.77
Zm00001eb253030	Aspartyl protease (AED1)	-3.39	-2.08
Zm00001eb327450	jasmonate ZIM domain-containing protein, TIFY transcription factor	3.26	2.02
Zm00001eb133200	Terpene synthase (KSL2)	3.61	6.65
Zm00001eb275550	Putative STRUBBELIG family receptor protein kinase	-8.66	NS
Zm00001eb313660	AAA-ATPase	-3.38	NS
Zm00001eb038710	Brachytic2 (br2) ABC transporter	-3.05	NS
Zm00001eb157430	Uncharacterized protein	-1.86	NS
Zm00001eb041410	Putative mannan synthase 7, Glycosyl transferase family group 2	-1.71	NS
Zm00001eb188770	Uncharacterized protein	-1.62	NS
Zm00001eb212520	Photosystem I subunit d1 (psad1)	NS	-3.45
Zm00001eb008910	50S ribosomal protein L15 chloroplastic	NS	-2.12
Zm00001eb036920	NAC transcription factor 48 (nactf48)	NS	-1.84
Zm00001eb279490	DEP domain, electron carrier	NS	2.23

Gene Set Enrichment Analysis

Gene set enrichment analysis based on gene ontology (GO) biological processes was performed on all significant DEGs with an adjusted p-value below 0.05. Genes differentially expressed between the 10-day exposed to unexposed samples showed an enrichment of 22 GO biological processes (Supplemental Table 4.5; Figure 4.3). At 10 dpe, various defense response GO biological processes were activated. This included regulation of defense response (GO:0031347), defense response to other organism (GO:0098542), response to other organism

(GO:0051707), response to wounding (GO:0009611), defense response (GO:0006952), and response to stimulus (GO:0050896). Furthermore, activated DEGs were enriched in the diterpenoid biosynthetic process (GO:0016102), Mitogen-activated protein kinase (MAPK) cascade (GO:0000165), and cell surface receptor signaling pathways (GO:0007166). Suppressed or down-regulated GO biological processes at 10 dpe included photosynthetic processes (GO:0019684; GO:0009772), cell wall biogenesis processes (GO:0009834; GO:0009833), and response to heat (GO:0009408).

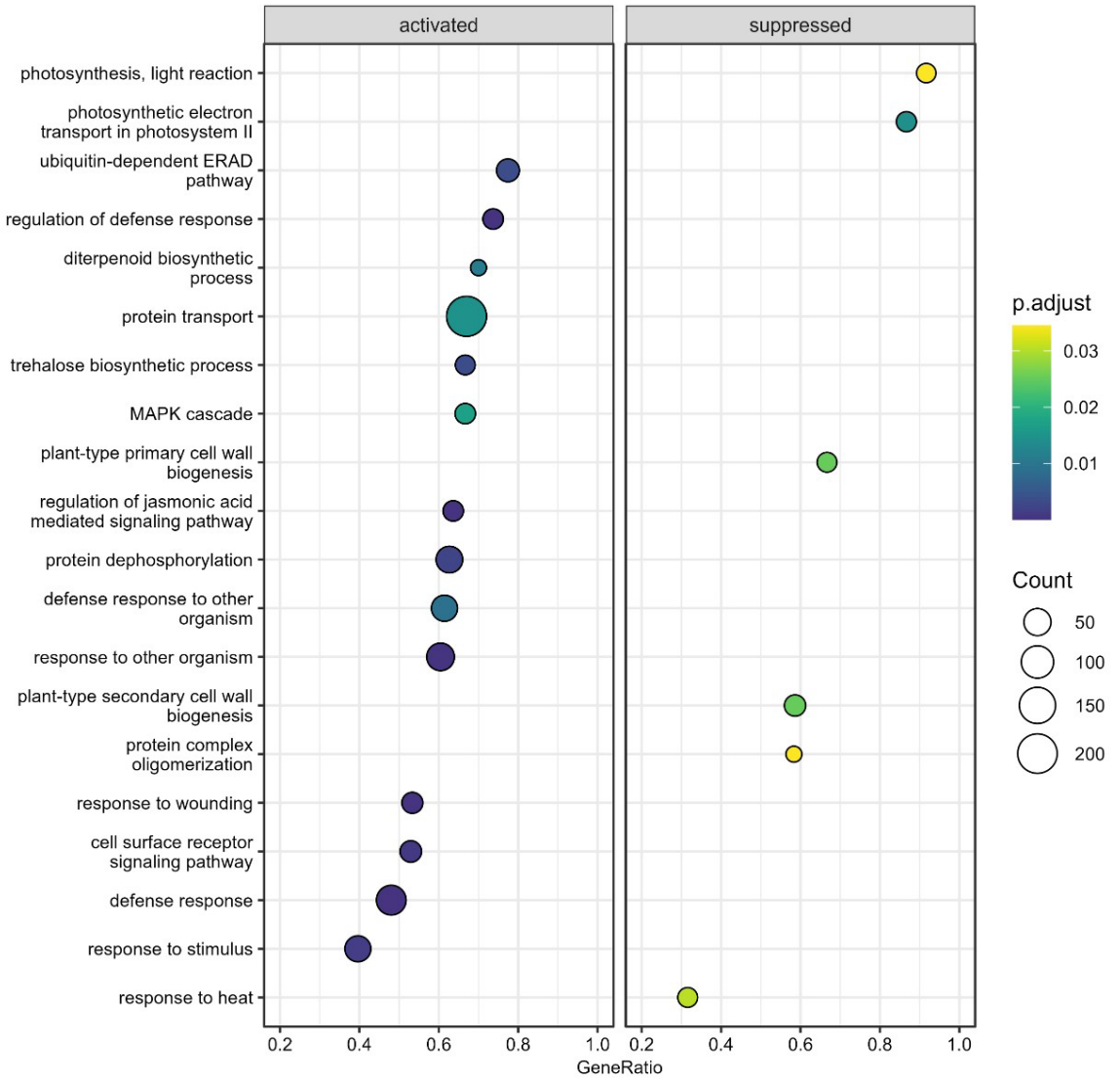

Figure 4.3: Gene set enrichment analysis of differentially expressed genes at 10 days post exposure (10 dpe).

Figure 4.3 (cont'd)

Figure 4.3: Gene set enrichment analysis of differentially expressed genes at 10 days post exposure (10 dpe). Gene ontology biological processes enriched are shown on the y-axis separated by activated (up-regulated) or suppressed (down-regulated). Gene ratio of genes enriched over genes within the pathway are shown on the x-axis. Adjusted p-value indicated by color. Gene count indicated by dot size.

When comparing 24-day exposed to unexposed samples, DEGs were enriched in 68 GO biological processes (Supplemental Table 4.6; Figure 4.4A). Of these, several activated or up-regulated biological processes were involved in defense. This included defense response to other organism (GO:0098542), response to other organism (GO:0051707), defense response (GO:0006952), and defense response to fungus (GO:0050832). Defense-related GO biological processes were also activated including response to oxidative stress (GO:0006979) and transmembrane receptor serine/threonine kinase signaling pathways (GO:0007178). Many biosynthetic and metabolic GO biological processes were enriched and activated at 24 dpe. These biological processes included the metabolism or biosynthesis of amino acids (GO:0009073; GO:0008652; GO:0006520; GO:0006559), aromatic compounds (GO:0019438), phenylpropanoids (GO:0009699), and terpenoids (GO:0016102; GO:0016114). Suppressed or down-regulated GO biological processes included response to herbicide (GO:0009635), response to light stimulus (GO:0009416), RNA modification (GO:0009451), and several photosynthetic processes (GO:0015979; GO:0019684; GO:0009772; GO:0009765; GO:0009768; GO:0009767).

Gene set enrichment analysis was also performed based on KEGG pathways using only the significant DEGs (Supplemental Table 4.7). When comparing 10-day exposed to nonexposed samples, the KEGG pathway enriched only for an activation of the plant-pathogen interaction pathway. When comparing 24-day exposed to unexposed samples, 28 enriched KEGG pathways were identified within the DEGs (Supplemental Table 4.7; Figure 4.4B). Activated or up-regulated KEGG pathways involved the biosynthesis or metabolism of various amino acids including phenylalanine, tyrosine, tryptophan, cysteine, and methionine. Furthermore, the enrichment of KEGG pathways involved in biosynthesis of various compounds were activated within the 24-day exposed samples. These pathways included the biosynthesis of the following compounds: isoquinoline alkaloids, phenylpropanoids, and flavonoids. Suppressed or down-regulated KEGG pathways involved photosynthesis and the ribosome.

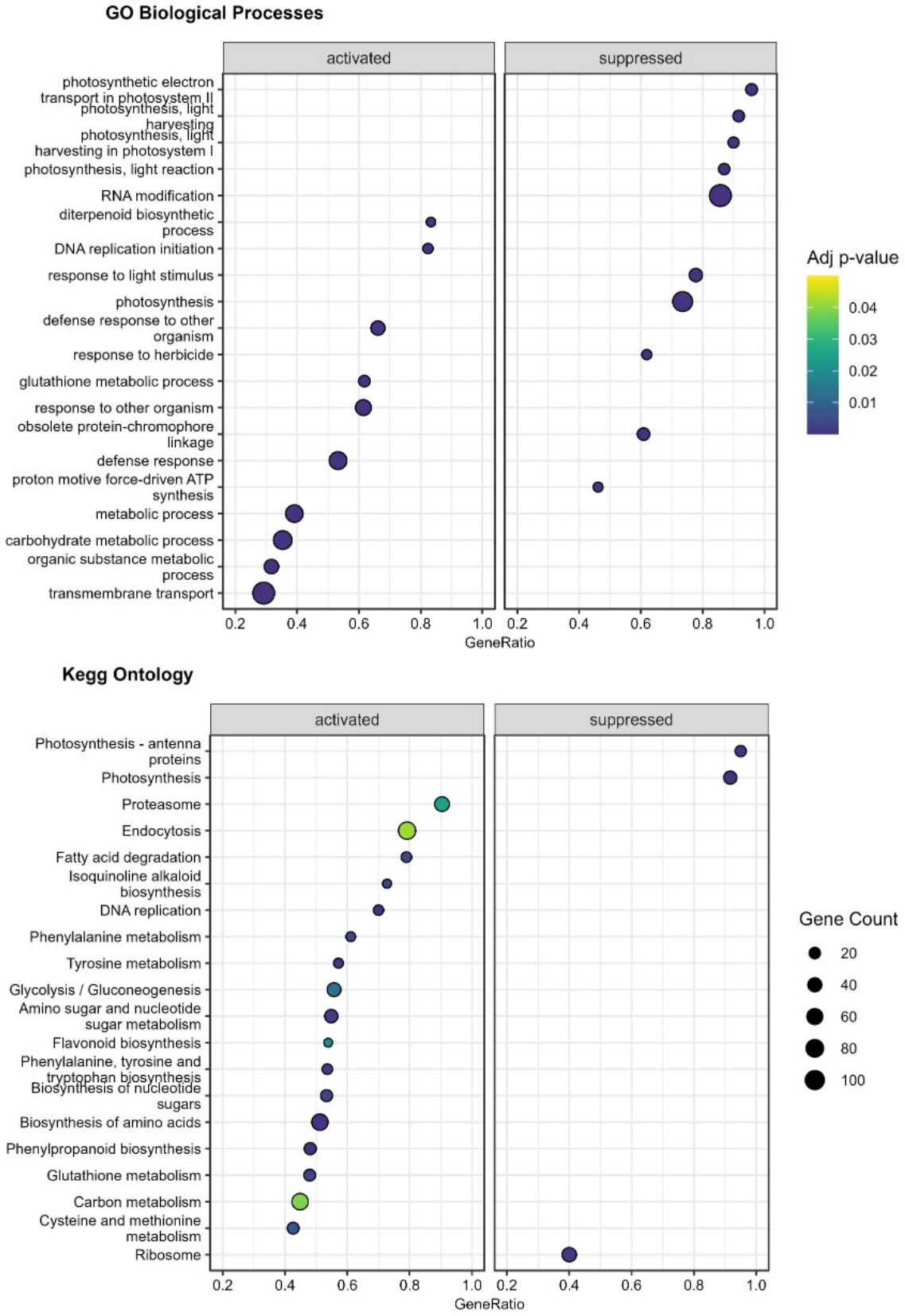

Figure 4.4: Gene set enrichment analysis of differentially expressed genes at 24 days post

Figure 4.4 (cont'd)

exposure (24 dpe). Top graph depicting the gene ontology biological processes enriched. Bottom graph depicting the KEGG orthologous pathways enriched. Enriched processes or pathways listed on the y-axis. Gene ratio of genes enriched over genes within the pathway are shown on the x-axis. Adjusted p-value indicated by color. Gene count indicated by dot size.

Discussion

With recent tar spot epidemics in the US and Canada, improved understanding of the tar spot pathosystem is needed for disease management solutions. Currently, no commercial maize hybrid provides complete resistance to tar spot, but research is being conducted to find tar spot resistant loci. Previous analyses using GWAS, linkage mapping, and genomic prediction methods have identified candidate genes for tar spot resistance (Cao et al. 2017, 2021; Mahuku et al. 2016; Ren et al. 2022; Trygestad 2021). However, no studies have investigated maize gene expression in response to *P. maydis*. This is likely due to the obligate biotrophy of *P. maydis* and inability to culture *P. maydis* axenically. Previously, artificial inoculations in controlled environments were unreliable, and optimization of recently published methods will be necessary (Breunig et al. 2023; Sólorzano et al. 2023). Additionally, much remains unknown about the timing and environmental conditions necessary for infection. Recent studies have shown conducible environmental factors and microscopic structures during tar spot development (Webster et al. 2024; Caldwell et al. 2024). However, initial infection mechanisms and the conditions necessary for spore release for continued infection cycles remain elusive. Thus, in this study, a trap plant method was used to ensure exposure of plants to a high load of natural *P. maydis* inoculum under field conditions (Chilvers et al. 2007). Furthermore, two timepoints during tar spot development were sampled to capture gene expression over time.

Through these methods, maize expression response to the tar spot pathogen was examined, but the plants were also exposed to various environmental stressors. Therefore, the contribution of reads to different organisms was investigated. Within the 10-day timepoint samples, effective alignment of reads to the B73 genome was observed with greater than 88% mapping rates. On average, the number of *P. maydis* reads within the 10-day non-exposed samples accounted for 0.001% while the 10-day exposed samples showed an increase in *P. maydis* reads accounting for 0.008% of the reads on average . Since *P. maydis* is known to have at least a 14-day latency period between infection and tar spot development (da Silva et al. 2020), the 10-day exposed samples were collected from asymptomatic leaf tissue. Therefore, the

increase in *P. maydis* reads confirmed that plants were effectively exposed to this pathogen, and the timepoint was collected during *P. maydis* colonization life stages. Similarly, within the 24-day non-exposed samples, adequate alignment to the B73 genome showed 92% reads mapped on average while alignment to the *P. maydis* genome accounted for 0.003% of the reads on average. Within the 24-day exposed samples, 50.7% of the reads aligned to the B73 genome and 44.0% of the reads aligned to the *P. maydis* genome on average. Since visible tar spots were sampled within the 24-day exposed plants, the number of *P. maydis* reads markedly increased. When investigating the remaining unmapped reads, low numbers of reads were classified to the *Exserohilum* and *Puccinia* genera which are known to be pathogenic on maize (Leonard and Suggs; Schweinitz 1832). Maize is exposed to many pathogens in the environment, and gene expression response to different pathogens can often overlap (Wang et al. 2022; Ding et al. 2019; Yang et al. 2019; Swart et al. 2017; Hoopes et al. 2019). However, within our samples, these pathogens accounted for less than 0.001% of the reads. These results indicated that the main effect within the exposed samples was due to the tar spot pathogen, *P. maydis*, and the gene expression differences could be correlated to response to this pathogen.

When investigating the GO biological processes and KEGG pathways within the maize expression data in response to the tar spot pathogen, significant DEGs at both timepoints were enriched in processes involved in defense. This included the common pathogen associated processes such as cell surface receptor signaling and regulation, MAPK cascade signaling, and response to ROS. Nine genes within the defense response GO biological process were found significantly differentially expressed when exposed to *P. maydis* at both timepoints. These genes encoded two Mildew locus O (Mlo) proteins (Zm00001eb182440 and Zm00001eb182450), two proteins with calcium-dependent lipid binding domains (Zm00001eb330300 and Zm00001eb038880), three terpenoid biosynthetic proteins (Zm00001eb047160, Zm00001eb222660, and Zm00001eb021200), a pathogenesis-related (PR) protein (Zm00001eb410040), and an osmotin-like protein (Zm00001eb047690). Furthermore, these genes were previously found differentially expressed in response to seven other fungal pathogens in maize indicating a conserved defense response (Swart et al. 2017; Hoopes et al. 2018; Ding et al. 2019; Yang et al. 2019; Wang et al. 2022). The significant DEGs encoding the PR protein, Mlo proteins, and terpenoid biosynthetic proteins were up-regulated in response to *P. maydis* at both timepoints. PR proteins can function as chitinases, oxidases, ribonucleases, and more to

defend against pathogens and often provide resistance (Loon and Strien 1999). Additionally, Mlo family proteins have been extensively studied in barley powdery mildew resistance, and the proteins function in response to pathogens as well as abiotic stress (Jørgensen 1992; Nguyen et al. 2016). These findings confirm defense responses to pathogens are induced in maize in response to the *P. maydis*, and future work will be needed to understand the specific plant-pathogen interactions.

Furthermore, as previously stated, three genes encoding biosynthetic proteins of terpenoid compounds were also activated in response to the tar spot pathogen. Defense response to biotic stress in maize often results in biosynthesis of phytoalexin terpenoid compounds such as zealexins and kauralexins (Block et al. 2019; Christensen et al. 2018; Kim et al. 2021) which have anti-fungal activity *in vitro* (Huffaker et al. 2011; Schmelz et al. 2011). When looking further at the zealexin biosynthetic pathway, an up-regulation of the following genes in response to *P. maydis* were found at 24 dpe: Zm00001eb412960 (Zx1), Zm00001eb412970 (Zx2), Zm00001eb412980 (Zx3), Zm00001eb222540 (CYP71Z19/Zx5), Zm00001eb222660 (CYP71Z18/Zx6), Zm00001eb222680 (CYP71Z16/Zx7), Zm00001eb058050 (CYP81A37/Zx8), Zm00001eb058050 (CYP81A38/Zx9), and Zm00001eb058050 (CYP81A39/Zx10). Similarly, within kauralexin biosynthesis, several kaurene synthases and oxidases were found up-regulated: Zm00001eb047160 (KSL4), Zm00001eb133200 (KSL2), Zm00001eb176190 (KSL6), Zm00001eb415430 (KSL5), and Zm00001eb385070 (KO2). These genes were recently implicated in biosynthetic pathways of maize diterpenoid defense compounds, which contribute to defense response to fungal pathogens (Ding et al. 2019; 2020). Furthermore, these compounds were found to accumulate in response to the maize pathogens *Fusarium verticillioides*, *Cercospora zeina*, and *Puccinia sorghi* (Meyer et al. 2017; Veenstra et al. 2018; Kim et al. 2021). The enrichment in these pathways indicates that zealexin and kauralexin compounds may be accumulating at the site of tar spot infection to defend against the pathogen.

Other defense compound biosynthetic genes were also found up-regulated in response to *P. maydis* at 24 dpe. Within the KEGG pathways, phenylpropanoid and flavonoid biosynthesis were enriched. Phenylpropanoids are specialized metabolites in plants that have roles in defense and plant development containing several metabolite classes including monolignols, flavonoids, and anthocyanins (Douglas 1996). Various phenylpropanoid compounds including flavonoids are important to pathogen defense, and many have been shown to have *in vitro* antimicrobial activity

(Dixon et al. 2002). Additionally, production of many phenylpropanoid compounds were observed in maize leaves in response to the pathogens *Cochliobolus heterostrophus* and *Puccinia sorghi* (Obanni et al. 1994; Kim et al. 2021). Biosynthesis of phenylpropanoid compounds arises from the substrate amino acids phenylalanine and tyrosine (Dixon et al. 2002; Gomez-Cano et al. 2020). In the current study, DEGs in response to *P. maydis* were also enriched for KEGG pathways in the biosynthesis and metabolism of amino acids including phenylalanine, tryptophan, tyrosine, cysteine, and methionine. These results indicate these phenylpropanoid defense compounds may also be accumulating at the localized *P. maydis* infection site.

Interestingly, two DEGs (Zm00001eb172420 and Zm00001eb234730) within the phenylpropanoid pathway were up-regulated in response to *P. maydis* at 24 dpe. Mutations within these genes, termed brown midrib mutants, result in a decrease in lignin, which changes the plant cell wall and gives the midrib a brown color (Halpin et al. 2002; Guillaumie et al. 2007; Morrow et al. 1997). Brown midrib maize hybrids are often grown for silage production for their increased digestibility for livestock (Sattler et al. 2010); however, this increase in digestibility also applies to pathogens. Lignin is important in cell wall integrity and often provides a physical barrier to prevent pathogen penetration and colonization (Sattler and Funnel-Harris 2013). These results indicate the plant may be forming a rigid cell wall to prevent the further colonization of *P. maydis.* However, looking further at lignin processes, three genes at 24 dpe (Zm00001eb408530, Zm00001eb254960, and Zm00001eb127360) and two genes at 10 dpe (Zm00001eb012250 and Zm00001eb148270) were highly up-regulated in response to *P. maydis* and involved in the breakdown of lignin. Additionally, plant cell wall biogenesis was an enriched process within the down-regulated DEGs at 10 dpe. The maize cell wall could be a primary target of *P. maydis* for improved infection and colonization. Previous analyses have annotated various secreted cell wall degrading enzymes and carbohydrate active enzymes that would allow for pathogenesis and colonization to occur (MacCready et al. 2023; Rogers et al. 2024). The divergent lignin response in maize shows a complex regulation of the processes at the cellular level, that could result from interaction with *P. maydis.*

While expression of downstream defense responses was shown in response to *P. maydis*, candidate genes for resistance or direct interaction with *P. maydis* needed investigation. Previous studies have shown quantitative trait loci for tar spot resistance in tropical maize varieties (Cao et al. 2017, 2021; Mahuku et al. 2016; Ren et al. 2022). Three candidate genes from the identified

significant resistant loci were also significantly differentially expressed in the current analysis (Zm00001eb345910, Zm00001eb344960, and Zm00001eb126340). All three of these candidate genes were also found differentially expressed in response to another pathogen, *Cochliobolus heterostrophus* (Ding et al. 2019). Zm00001eb345910 is characterized as a putative leucine-rich repeat receptor-like kinase (LRR-RLK). This class of kinases are often found to interact with pathogens at the plasma membrane and are often NLRs involved in ETI response (Jones & Dangl 2009). Furthermore, this specific gene was found significantly differentially expressed in response to *Exserohilum turcicum* and *Colletotrichum graminicola* (Hoopes et al. 2018; Yang et al. 2019). Zm00001eb344960 is characterized as a basic helix-loop-helix Myc transcription factor involved in stomata formation. Interestingly, *P. maydis* growth has been shown to protrude from the maize stomata near the site of infection (Caldwell et al. 2024), and several pathogens use sensing and regulation of plant stomata to invade the host tissue (Melotto et al. 2008; Ye et al. 2020). Lastly, Zm00001eb126340 is characterized as a sarcoplasmic reticulum histidine-rich calcium-binding protein. Calcium ion signaling is an important plant response to different stressors. In wheat, mutations to a histidine-rich calcium binding protein provided resistance to Fusarium head blight (Kushalappa et al. 2022).

Additionally, preliminary studies of tar spot resistant loci in temperate maize varieties have identified candidate genes across the chromosomes with no overlap with the published tropical variety studies (Trygestad 2021). When comparing to the current study, four tar spot resistance candidate genes were also significantly differentially expressed in response to *P. maydis* at both timepoints (Zm00001eb008920, Zm00001eb133200, Zm00001eb253030, and Zm00001eb327450). Zm00001eb008920 encodes 3-ketoacyl-CoA synthase28 (KCS28) functioning in biosynthesis of fatty acids for the leaf cuticle (Campbell et al. 2019). KCS28 was found down-regulated in response to *P. maydis* which may relate to increased permeability of the leaf cuticle. Zm00001eb253030 was also down-regulated in response to *P. maydis* and encodes an aspartyl protease. These proteins have been shown to be involved in plant-pathogen interactions often cleaving a signaling peptide to elicit a defense response (Figueiredo et al. 2021). Zm00001eb327450 encodes a TIFY transcription factor considered a jasmonate ZIM domain-containing protein (JAZ). JAZ proteins have been extensively studied in *Arabidopsis thaliana* and function as transcriptional regulators of jasmonate signaling involved in stress responses (Thireault et al. 2015; Wang et al. 2024). Zm00001eb133200 encodes the kauralexin

synthase (KSL2) as previously mentioned that is involved in synthesizing antifungal kauralexin compounds (Ding et al. 2019). All these gene products are good candidates for future studies on tar spot resistance mechanisms.

In this study, the first expression analysis of maize in response to the tar spot pathogen, *Phyllachora maydis,* is presented and provides an initial molecular understanding of the host defense response. Differentially expressed genes of interest were identified that could be important in host-pathogen interactions or resistance mechanisms. Future work will rely on inoculation methods in controlled environments, which may show a clear effect due to *P. maydis*. With further understanding of the infection mechanisms and timing of this pathogen, detailed experiments on gene expression, translation, and defense compound accumulation will be possible. This study along with recent functional characterization of candidate *P. maydis* effectors proteins have provided a baseline for study of plant-pathogen interactions (Helm et al. 2022; Rogers et al. 2024).

CHAPTER 5:
FUNGAL AND BACTERIAL COMMUNITIES ASSOCIATED WITH TAR SPOT AND FISHEYE LESIONS IN THE UNITED STATES AND MEXICO

Abstract

Tar spot of maize is an emerging disease within the United States and Canada. Originally described in Mexico in 1904, the disease was observed as a complex involving the formation of tar spot stroma followed by necrotic rings termed fisheye lesions developing around the tar spots. The tar spot disease complex consisted of *Phyllachora maydis,* causal agent of tar spot stroma, *Microdochium maydis,* supposed causal agent of fisheye lesions, and *Coniothyrium phyllachoraea,* supposed mycoparasite of *P. maydis.* Previous identification of isolates from fisheye lesions did not find *M. maydis* which has led to speculation over this species. Within Michigan, fungal communities also showed that *M. maydis* was not significantly associated with fisheye lesions. In this study, the fungal and bacterial libraries of tar spot and fisheye lesions were investigated across the United States and Mexico. The significant indicator taxa for fisheye lesions differed by country. A related *Microdochium* sp. was found abundant in fisheye lesions in Mexico, while *Parapherosphaeria* sp., *Fusarium* sp., and a taxon closely related to *Neottiosporina paspali* were found in the US. This study provides confirmation of *Microdochium* sp. associated with fisheye lesions, but points to differences in location that indicate more complex interactions resulting in fisheye lesion development.

Introduction

Tar spot of maize is a devastating fungal disease distributed across the Americas (da Silva et al. 2020; Mueller et al. 2022). The disease is defined macroscopically by the black, raised stromata resembling tar on the maize foliage. The stroma can often be surrounded by a necrotic ring termed fisheye. Tar spot and fisheye lesions were first described in Mexico in the early 1900s with *Phyllachora maydis* identified to cause the tar spot lesions and *Coniothyrium phyllachoraea* identified as a mycoparasite of *P. maydis* associated with fisheye lesions (Maublanc 1904). In 1984, Müller and Samuels reported another fungal species associated with fisheye lesions, *Monographella maydis* synonymous with *Microdochium maydis* (Müller and Samuels 1984; Hernández-Restrepo et al. 2016). This disease was then described as the tar spot disease complex with the three fungal species: *Phyllachora maydis,* causing tar spot lesions*; Microdochium maydis,* supposedly causing fisheye lesions; and *Coniothyrium phyllachoraea,* a reported mycoparasite of *P. maydis.* It is reported that the tar spot disease complex starts by the infection of maize foliar tissue with *P. maydis* and development of tar spot stromata. This is followed by the development of the fisheye lesion supposedly caused by colonization of another pathogen (Hock et al. 1992). Previous descriptions of the tar spot disease complex indicated the fisheye lesion was the leading cause of yield losses (Hock et al. 1992; Bajet et al. 1994). However, while the fisheye is commonly observed in Central and South America, it is only occasionally observed in the US, and severe tar spot outbreaks have caused significant yield losses without an abundance of fisheye lesions (Kleczewski et al. 2020; Valle-Torres et al. 2020; Mueller et al. 2024).

Though *M. maydis* was identified to cause fisheye lesions, there has been much speculation and concerns over the claims. *M. maydis* has not been isolated from fisheye lesions in the US, and morphologically identified *M. maydis* isolates from central America were sequence identified to the *Fusarium incarnatum-equiseti* species complex (FIESC) (Luis et al. 2023). Furthermore, molecular identification of *M. maydis* has not been confirmed as no sequences exist for this species in available databases nor are any type specimens available in culture or herbarium collections (Hernández-Restrepo et al. 2016). Recent isolations of *Microdochium*-like morphologies from fisheye lesions in the US and Mexico resulted in a high abundance of *Fusarium* spp. and no *Microdochium* spp. (Luis et al. 2023). It has been theorized that *M. maydis* could have been misclassified from a *Fusarium* sp. due to the overlap in

morphological characteristics and the lack of evidence in the original species description. Additionally, sequence-based analysis of fungal communities from fisheye lesions in Michigan did not find an abundance of *M. maydis* but showed a high abundance and significant association of a *Fusarium* sp., *Paraphaeosphaeria* sp., and a *Neottiosporina paspali*-related species when compared to tar spot lesions alone (McCoy et al. 2019).

While this initial study has been the baseline for comparing fisheye and tar spot microbial communities, it only looked at the fungal communities from a single field in Michigan (McCoy et al. 2019). To further understand how the microbial communities from the various lesions of the tar spot disease complex differ by location, fungal and bacterial communities were analyzed from field sites in several states across the Midwest United States and Mexico. The lesion type comparisons made included: tar spot lesions alone (tar spot), tar spot with fisheye lesions (fisheye), and healthy leaf controls (non-symptomatic). Trends in average relative abundance of genera were observed on the whole dataset and comparisons were made between lesion types. Diversity measurements and pairwise comparisons were made to confirm the effect of variation from lesion type and location. Indicator taxa for each lesion type by location was determined, and co-occurrence networks were constructed to understand the differences in fisheye lesions between the US and Mexico.

Materials and Methods

Sample Collection and Processing

Leaves were collected containing lesion types including tar spot only (tar spot), tar spot with fisheye lesions (fisheye), and symptom-free/healthy (non-symptomatic). Even sampling was attempted with ten leaves of each lesion type collected from fields where possible. Not all fields had fisheye lesions nor healthy/non-symptomatic leaves due to the prolific nature of this disease. Tar spot infested fields were sampled in August and September of 2019 from the United States (US), and the leaves were kept cool until samples could be processed. Fresh leaf samples from the US were sampled within two days of receiving to prevent contamination. Additionally, leaves from Mexico were collected and dried in March and April 2021. To capture the endophytic microbial communities, leaves were surface sterilized with 70% ethanol, and a single leaf disk surrounding the symptomatic lesion was sampled using a 7 mm cork borer. Freshly sampled leaf disks were stored in lysis buffer and kept at 4ºC until ready for DNA extraction and purification. Leaf disks were homogenized in a FastPrep® homogenizer using lysing matrix A

(MP Biomedicals, Irvine, CA, USA), and DNA was extracted using the Mag-Bind® Plant DNA DS 96-well plate kit (Omega Bio-tek, Norcross, GA). Negative control DNA extraction samples were included in each 96-well plate extraction.

Library Preparation and Sequencing

Amplicon sequencing libraries were prepared as previously described (Noel et al. 2022). Briefly, a three step PCR process was used to amplify the target and ligate sequencing adapters with 10 base pair barcodes. To capture the associated fungal communities, the internal transcribed spacer (ITS) region was amplified using the ITS1F and ITS4 primers (White 1990) with DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). For the bacterial microbiome, the 515F and 806R primers (Caporaso et al. 2010) and Platinum™ II Hot-Start Green PCR Master Mix (Invitrogen, Waltham, MA) were used to amplify the 16S ribosomal subunit. Peptide nucleic acid (PNA) clamps were also used to inhibit the amplification of eukaryotic mitochondrial and chloroplast DNA. Negative control DNA extractions and no DNA negative controls were added to each 96-well plate in the analysis. Amplification was verified using gel electrophoresis. Normalization of PCR products was performed using the SequalPrep™ Normalization Plate Kit (Applied Biosystems, Waltham, MA). The barcoded and normalized amplicons were combined and concentrated using Amicon® Ultra Centrifugal Filters (Millipore Sigma, Burlington, MA). AMPure XP Bead-Based Reagent was used to remove unwanted primer-dimers (Beckman-Coulter, Indianapolis, IN). Libraries were quantified fluorometrically using Qubit™ DNA Broad Range assay kits (Invitrogen, Waltham, MA, USA), and quality was assessed using the Agilent 4200 TapeStation HS DNA1000 assays and Colibri Illumina Library Quantification qPCR. Libraries were sequenced at 300 bp paired end format on the Illumina MiSeq v3 flowcell using MiSeq v3 600 cycle reagents at the Michigan State University Research Technology Support Facility Genomics Core.

Data Analysis

Raw sequence data was quality assessed using FastQC v.0.12.1 (Andrews 2010). The forward reads had higher quality and were analyzed further. Sequences were demultiplexed into the respective samples by matching the unique barcodes using USEARCH v.11.0.667 (Edgar 2010). Following demultiplexing, the Cecilia pipeline was used to process the data (https://github.com/Gian77/Cecilia). In brief, phiX was removed using bowtie v.2.5.3 (Langmead et al. 2019). Quality trimming and adapter sequence removal was performed using

cutadapt v.4.5 (Martin 2011). Clustering of operation taxonomic units (OTUs) and amplicon sequence variants (ASVs) was performed using the UPARSE and UNOISE algorithms of USEARCH v.11.0.667 (Edgar 2010). Taxonomic binning was performed using CONSTAX v.2.0.20 (Liber et al. 2021) with the UNITE eukaryotic database for fungi (version 25.07.2023; Nilsson et al. 2019) and the SILVA SSU reference database for bacteria (version 138.1; Quast et al. 2013). Statistical analysis was performed in R v.4.3.2 (R core team 2023). The phyloseq package was used to analyze the OTU clusters (McMurdie and Holmes 2013). Contaminant taxa were determined from negative control samples and removed from leaf samples using the decontam package (Davis et al. 2018). The betadisper and adonis functions from the vegan package were used to determine the PERMANOVA and Tukey pairwise comparisons (Oksanen et al. 2022). To determine indicator taxa, the multipatt function within the indicspecies package was used (De Caceres and Legendre 2009). The spiec-easi package was used to build the co-occurrence networks (Kurtz et al. 2015), and cytoscape was used for visualization (Shannon et al. 2003). Figures from statistical analyses were built using ggplot2 (Wickham, 2016).

To determine what phylogenetic groups the OTUs generated within this study belong to, a phylogenetic analysis was conducted. Representative internal transcribed spacer (ITS) and large subunit (LSU) rDNA sequences of the 5 *Phyllachora* genotypic groups from Broders et al (2023) were obtained through GenBank and used with the unique OTUs and zOTUs (ASVs) sequences generated in this study for phylogenetic analysis. *Exserohilum turcicum* (CBS 690.71) was used as an outgroup. ITS sequences were concatenated to the LSU sequences for *Phyllachora* genotypic groups and the outgroup before alignment. Sequences and OTUs were then aligned in Geneious (version 2024.0.4) and exported with free ends of the OTUs represented as missing data. Modeltest-NG (version 0.1.7) was then used to identify the best nucleotide substitution model for the dataset (Darriba et al 2020; Flouri et al 2014). TIM1+G4 was identified as the best fitting model with the lowest AIC score of 11,273.94 and lowest AICc score of 11,298.94. Maximum likelihood phylogenetic analysis was conducted with the TIM1+G4 model and 1,000 bootstrap replicates using the RaXML-NG program (version 1.2.1; Kozlov et al 2019). The resulting trees were visualized, and consensus bootstrap tree was produced in Geneious.

Results

Sequence Analysis

The fungal Illumina MiSeq libraries produced 47 million raw reads combined. Decontamination analysis using the negative control samples found a single fungal contaminant. After filtering this contaminant, 34,832,908 million reads remained across 509 samples with 63,508 median reads per sample representing 1,589 operational taxonomic units (OTUs). The bacterial Illumina MiSeq libraries produced 44 million raw reads combined. Decontamination analyzed from negative controls found 58 contaminants. After filtering, 25,140,475 million reads remained across 491 samples with 43,694 median reads per sample representing 1023 OTUs. Rarefaction curves showed the sequencing depth was sufficient to capture the fungal and bacterial diversity within our samples (Supplemental Figure 5.1).

Abundant Genera

The overall average relative abundance of classified taxa by lesion type is shown in Figure 5.1. The top abundant fungal genera in non-symptomatic samples were *Alternaria, Hannaella, Neosetophoma, and Tilletiopsis* (Figure 5.1A). The top abundant fungal genera from samples of tar spot lesions alone also show *Alternaria* and *Neosetophoma,* but a decrease in *Hannaella* and *Tilletiopsis* and increase in *Phyllachora* when compared to the non-symptomatic. Interestingly, in the fisheye lesion type samples, decreases in abundance of the *Alternaria, Hannaella, Neosetophoma, and Tilletiopsis* genera were observed with increases in abundance of the *Fusarium, Paraphaeosphaeria,* and *Phyllachora* genera when compared to the non-symptomatic samples. Within the bacterial libraries, *Methylobacterium* and *Sphingomonas* were identified as abundant genera across all lesion types (Figure 5.1B). Interestingly, relative increases in abundance of the *Hymenobacter* and *Pantoea* genera were observed in both diseased sample types. Further analysis of average relative abundance separated by location was performed and showed differences depending on the location (Supplemental Figures 5.2 and 5.3).

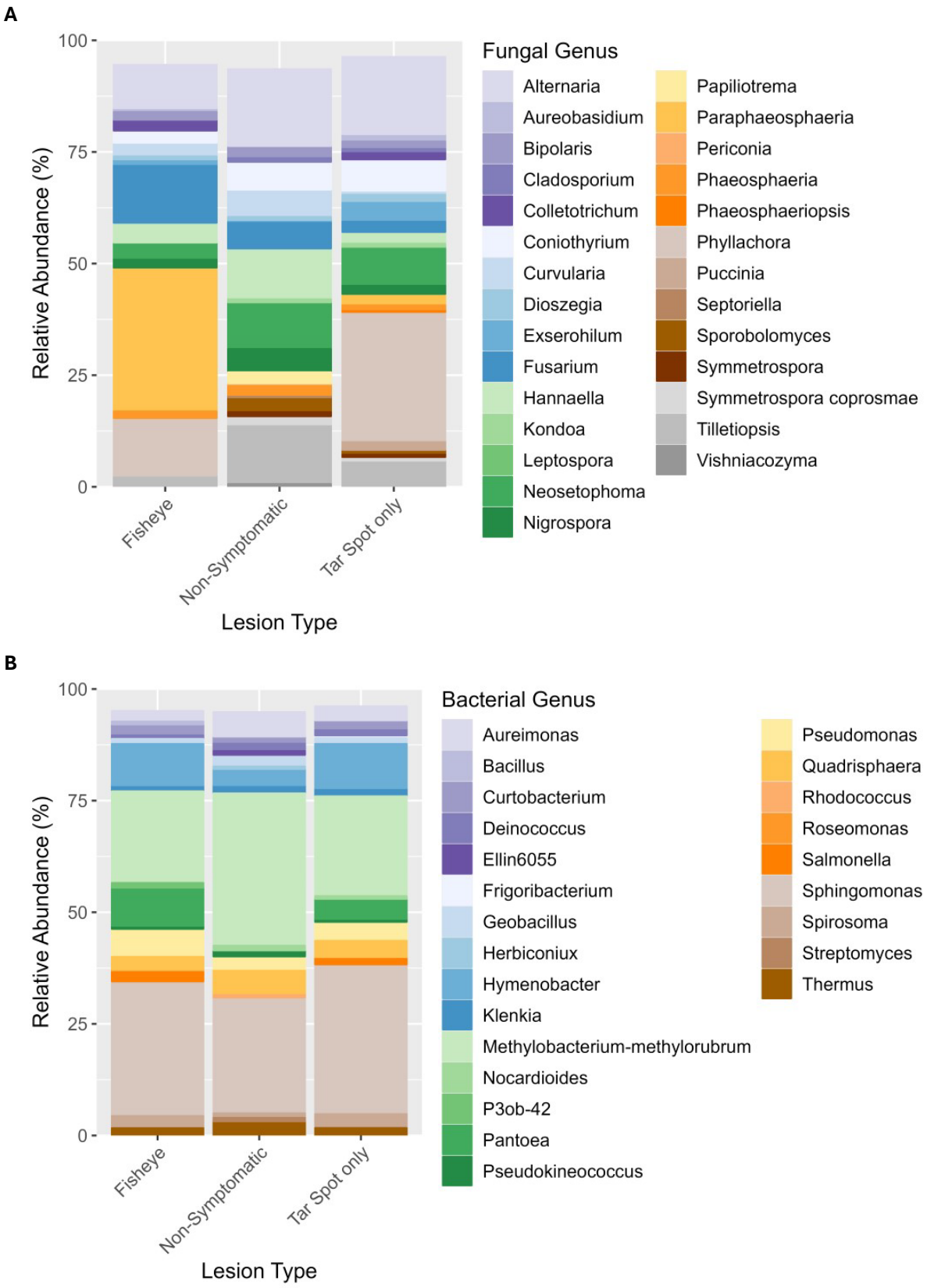

Figure 5.1: Abundant classified genera with microbial communities by lesion type.

Figure 5.1 (cont'd)

Figure 5.1: Abundant classified genera with microbial communities by lesion type. Average relative abundances (%) of classified genera are plotted based on lesion type: fisheye, non-symptomatic, and tar spot only. A) Fungal genera abundance by color B) Bacterial genera abundance by color.

Alpha Diversity

Alpha-diversity was measured to establish within sample diversity by lesion type. The Shannon index showed that fisheye lesions were significantly less diverse in the fungal communities when compared to tar spot lesions alone and non-symptomatic samples for fungal communities (Figure 5.2A). The observed species richness was significantly higher, but species evenness was significantly lower in fisheye lesions when compared to the other lesion types. For the bacterial communities, the Shannon index showed the non-symptomatic sample type is significantly lower when compared to both diseased states (Figure 5.2B). Bacterial species evenness showed no significance between lesion types while the observed species richness shows significance of both diseased states when compared to non-symptomatic samples. There was no significant difference in alpha diversity between fisheye and tar spot only lesion types within the bacterial libraries.

Beta Diversity

Beta-diversity was measured using Bray-Curtis and Jaccard distances to understand how the abundance or presence/absence of taxa impacted the between-sample diversity respectively. Principle coordinate analysis (PCA) of the Bray-Curtis distances show the fungal communities separated by lesion type with 17.38% of the variance explained by axis 1 and 11.08% variance explained by axis 2 (Figure 5.3A). Confidence intervals (95%) indicated clear separations of samples based on lesion type based on the fungal communities. In the bacterial communities, PCA based on Bray-Curtis distances showed 14.07% of variance explained by axis 1 and 9.32% of variance explained by axis 2 (Figure 5.3B). Confidence intervals (95%) between lesion types based on the bacterial communities overlapped and showed no clear separation of samples. However, separation of samples along axis 2 was observed by country for the bacterial communities. PCA based on Jaccard distances showed similar results to Bray-Curtis distances for both fungal and bacterial communities (Supplemental Figure 5.4).

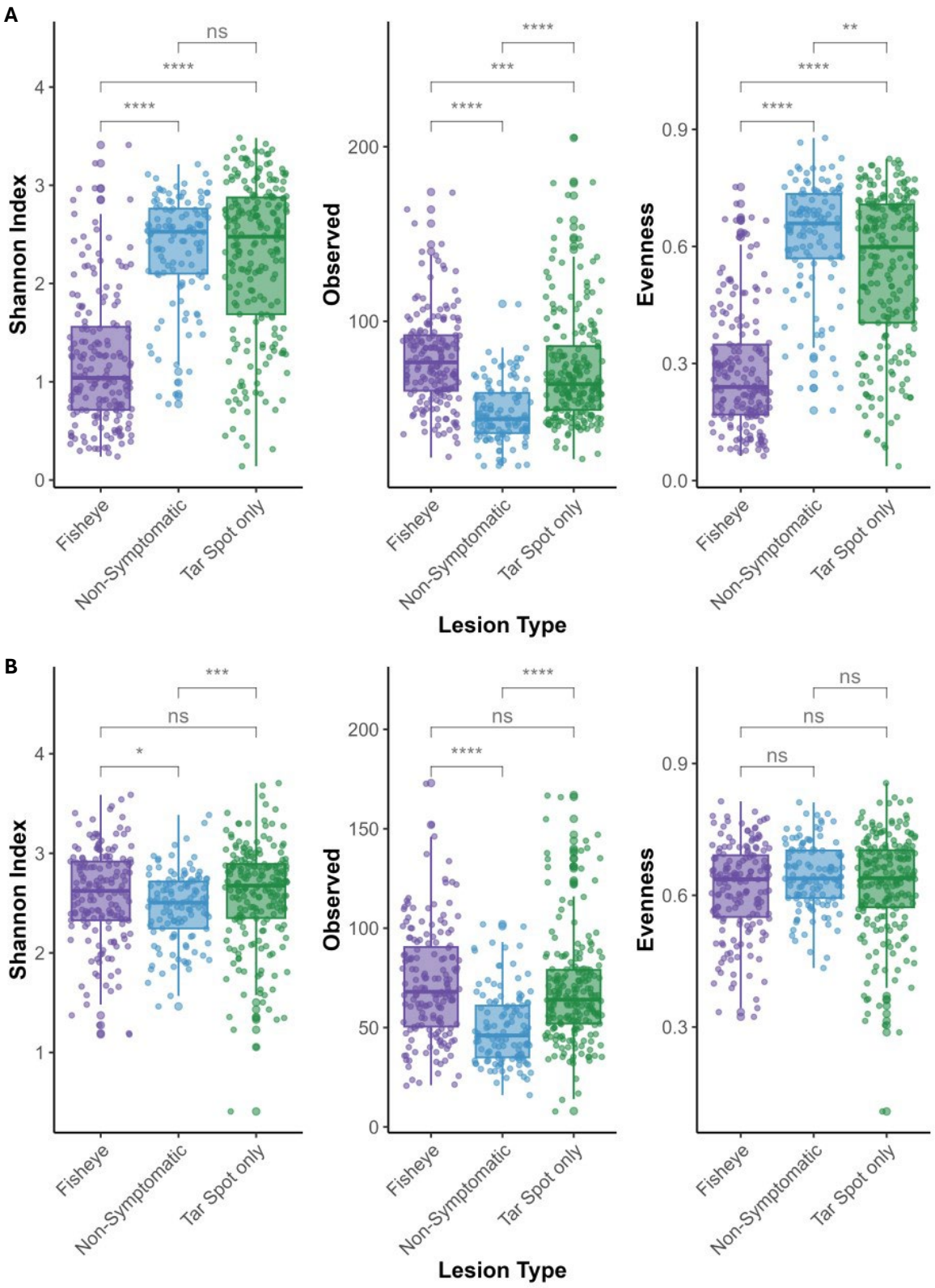

Figure 5.2: Alpha diversity measurements based on lesion type.

Figure 5.2 (cont'd)

Figure 5.2: Alpha diversity measurements based on lesion type. Alpha diversity measurements separated by lesion type are shown as Shannon Index (left), Observed Species Richness (middle), and Species Evenness (right). The Wilcox test was performed to compare means between lesion type. * indicates P <0.1, ** indicates P < 0.01, *** indicates P < 0.001, and **** indicates P < 0.0001. ns = not significant. A) Fungal community alpha diversity. B) Bacterial community alpha diversity.

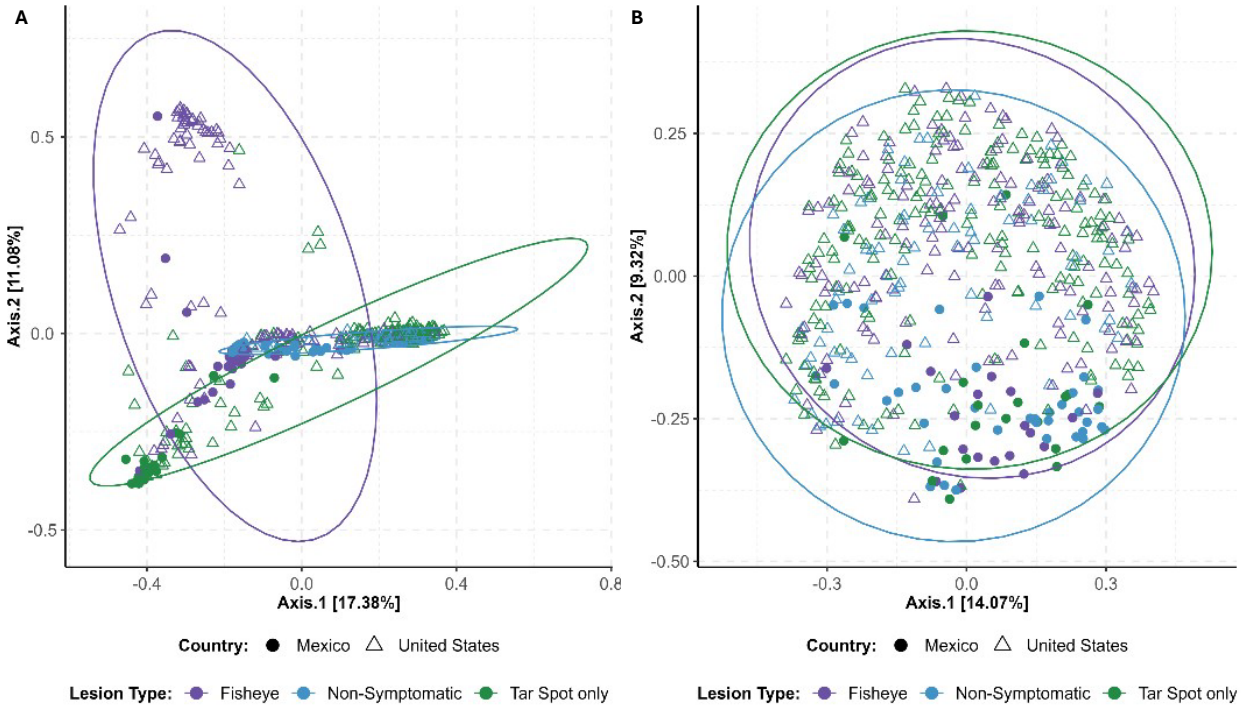

Figure 5.3: Beta-diversity. Principle coordinate analysis based on Bray-Curtis distances. Colors indicate the lesion type. Shape indicates country of origin. 95% confidence interval ellipses based on lesion type. A) Fungal communities. B) Bacterial communities.

To further understand if the differences in communities were due to lesion type or location, PERMANOVA and pairwise comparisons were performed. PERMANOVA results showed a significance by lesion type and country within both fungal and bacterial communities. Lesion type accounted for 9.9% R^2 difference in fungal communities, while bacterial communities only showed 2.3% R^2 difference. Investigations into microbial community differences by country identified 6.4% and 4.6% of the variance explained in fungal and bacteria communities, respectfully (Supplemental Table 5.1). Looking at Tukey pairwise comparisons of lesion types in fungal communities, the most significant comparison was between tar spot and fisheye lesions with a difference of -0.043 and adjusted p-value of 0.003. Furthermore, when

comparing the US and Mexico, a difference of -0.036 was calculated with a 0.001 adjusted p-value in fungal communities. Tukey pairwise comparisons of lesion types in bacterial communities was only significant between tar spot and non-symptomatic samples having a difference of -0.026 with an adjusted p-value of 0.03. However, the pairwise comparison between the US and Mexico showed a difference of -0.04 with an adjusted p-value less than 0.0001 (Supplemental Table 5.2).

Indicator Species

Indicator species analysis was performed to identify taxa that may be significantly associated with each lesion type. Significant taxa (p-value < 0.05) that were highly abundant in samples (stat > 0.75) are reported and further analyzed. Three fungal OTUs were significant in fisheye lesions from all samples: OTU_1, OTU_3, and OTU_7. OTU_1 was identified as *Paraphaeosphaeria neglecta* (98.5% BLAST sequence similarity), OTU_3 was most closely related to *Neottiosporina paspali* (90.5% BLAST sequence similarity), and OTU_7 was identified as a *Fusarium sporotrichioides* (100% BLAST sequence similarity) (Table 5.1). Six fungal OTUs were found significant in tar spot lesions alone from all samples: OTU_2, OTU_80, OTU_13, OTU_18, OTU_21, and OTU_36 (Supplemental Table 5.3). OTU_2 and OTU_80 both classified as *Phyllachora maydis.* OTU_18, OTU_21, and OTU_36 was most closely related to *Colletotrichum* sp. in BLAST searches. OTU_13 was most closely related to a *Thielavia* sp. by BLAST. Two bacterial OTUs were found to be significant indicator species but only within the non-symptomatic samples from all data in this study (Supplemental Table 5.4). These included bacterial OTU_20 and OTU_884 classified within the Hafniaceae family and *Pseudomonas* genus respectively.

Table 5.1: Significant Indicator Taxa in Fisheye lesions. Fungal OTUs identified as significant indicator taxa in fisheye lesions based on all samples or samples from the various countries (Mexico and US). Indicator Taxa Stat shows the output from the indicspecies package and the measure attributing to the prevalence and abundance of the taxon within each sample in a group. N.S. = Not Significant.

OTU_ID	CONSTAX Taxonomy	BLAST results			Indicator Taxa Stat		
		Taxonomy	% Query	% Similarity	All	Mex	US
OTU_1	Genus *Paraphaeosphaeria*	*Paraphaeosphaeria neglecta*	100	98.5	0.96	N.S.	0.99
OTU_3	Order Pleosporales	*Neottiosporina paspali*	100	90.5	0.94	N.S.	0.96
OTU_7	Genus *Fusarium*	*Fusarium sporotrichioides*	100	100	0.83	N.S.	0.84
OTU_8	Class Sordariomycetes	*Microdochium seminicola*	100	100	0.70	0.99	0.47
OTU_215	Species *Moesziomyces aphidis*	*Moesziomyces bullatus*	100	100	N.S.	0.77	0.26

Additional analysis was performed to determine the effect of location on indicator species within fungal communities across each lesion type. Within the US alone, similar to above, fungal OTU_1, OTU_3, and OTU_7 were found to be significant in fisheye lesions (Table 5.1); likewise OTU_2, OTU_80, OTU_13, OTU_18, and OTU_21 were found significant in tar spot lesions alone (Supplemental Table 5.5). Two fungal OTUs were found significant within non-symptomatic samples in the US: OTU_10 and OTU_272, both classified as *Tilletiopsis washingtonensis.* In contrast, two fungal OTUs were significant indicator taxa for fisheye lesions in Mexico: OTU_8, classified as *Microdochium seminicola* (100% BLAST sequence similarity), and OTU_215, classified as *Moesziomyces bullatus* (100% BLAST sequence similarity) (Table 5.1). Nine fungal OTUs were significant in tar spot samples from Mexico: OTU_2, OTU_84, OTU_320, OTU_230, OTU_2095, OTU_25, OTU_31, OTU_1197, and OTU_73 (Supplemental Table 5.6). The *Phyllachora maydis* classified OTUs included: OTU_2, OTU_84, OTU_320,

OTU_230, OTU_1197, and OTU_2095. OTU_25 classified as *Didymella glomerata*, OTU_31 classified as *Epicoccum sorghinum*, and OTU_73 classified as *Papiliotrema* sp. Three fungal OTUs were significant in non-symptomatic samples from Mexico: OTU_12 which classified as *Curvularia lunata*, and OTU_19 and OTU_1966 which both classified as *Nigrospora oryzae* (Supplemental Table 5.6).

Abundance of the fungal indicator taxa within fisheye lesions was further analyzed due to the constraints of the UNITE database classifications (Supplemental Figure 5.5; Supplemental Tables 5.7 and 5.8). OTU_1, classified as *Paraphaeosphaeria neglecta,* was found in fisheye lesions at an average relative abundance of 33.78% in US samples and 7.94% in Mexico samples. OTU_3, classified as *Neottiosporina paspali,* was found at an average relative abundance of 16.79% in the US and only found in 60 samples from Mexico (0% average relative abundance). OTU_7, classified as *Fusarium sporotrichioides,* was found at an average relative 7.9% in US samples and was found in 42 samples from Mexico (0% average relative abundance). OTU_8, classified as *Microdochium seminicola,* was found at an average relative abundance of 37.18% within fisheye lesions from Mexico and was found in 153 samples from the US at low counts (0% average relative abundance).

Co-occurrence network analysis

Networks were constructed to understand the co-occurrence of fungal OTUs in fisheye lesions from each country separately (Figure 5.4). *Phyllachora maydis* OTUs were highly positively associated within both networks. The previously identified indicator taxa showed minor distant interactions with *Phyllachora maydis* OTUs. OTU_1, *Paraphaeosphaeria neglecta,* showed an indirect negative interaction with *Phyllachora maydis* in the US. Furthermore, OTU_3, *Neottiosporina paspali,* showed an indirect but highly positive interaction with a cluster of *Phyllachora maydis* OTUs while OTU_7, *Fusarium sporotrichioides,* had a direct negative interaction with *Phyllachora maydis* in the US. In the co-occurrence network for fisheye lesions from Mexico, OTU_8, *Microdochium seminicola,* is negatively associated with OTU_1, *Paraphaeosphaeria neglecta,* and OTU_1 is indirectly negatively associated with *Phyllachora maydis* OTUs.

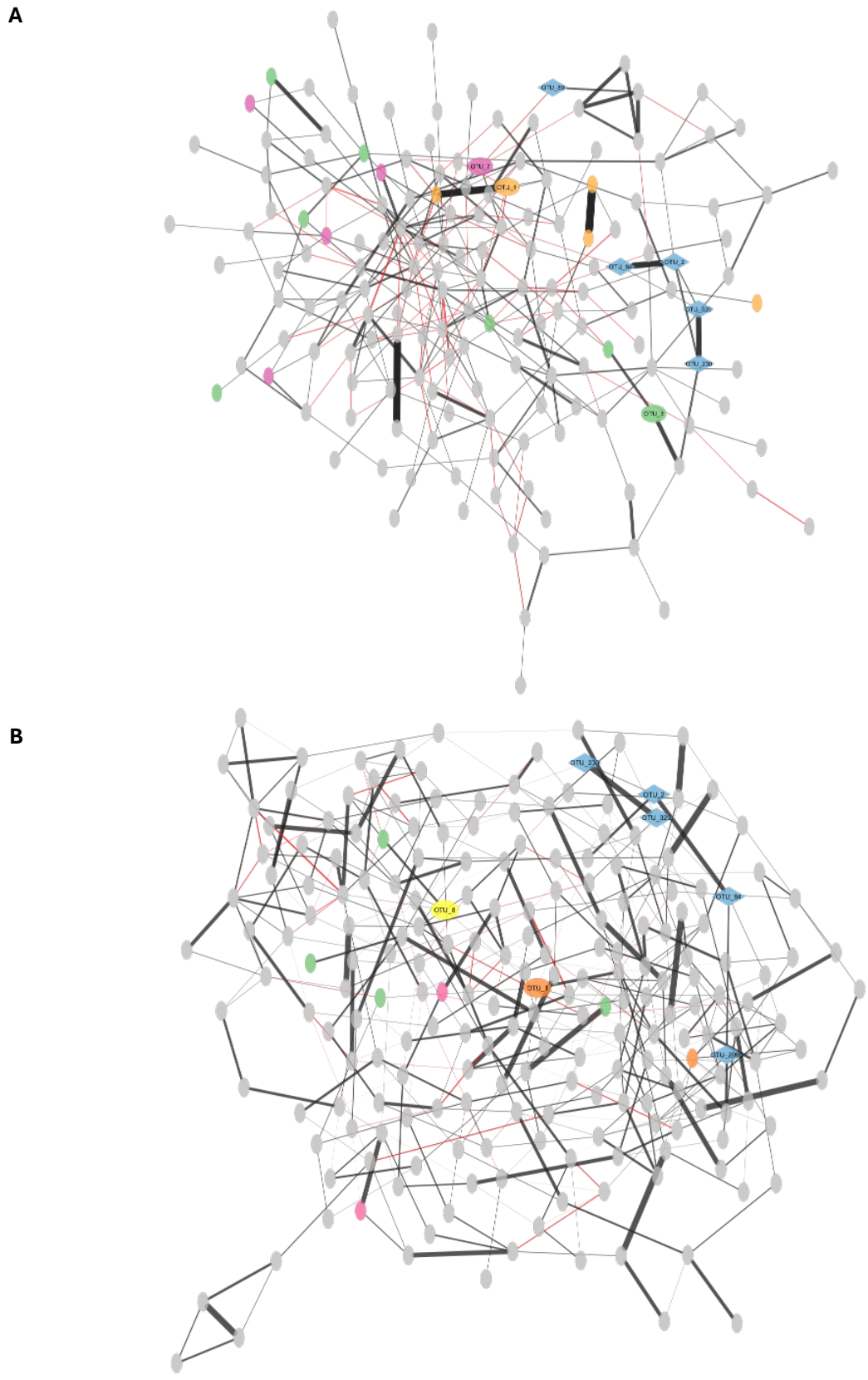

Figure 5.4: Co-occurrence networks.

Figure 5.4 (cont'd)

Figure 5.4: Co-occurrence networks. A) Fisheye within the US B) Fisheye within Mexico. Labeled OTUs indicator taxa and *Phyllachora maydis* OTUs. Color of edges indicate direction of interaction with negative being red. Size of edge indicate the strength of interaction.
Blue diamonds = *Phyllachora maydis* OTUs Orange = *Paraphaeosphaeria* Green = Pleosporales order Pink = *Fusarium* sp. Yellow = *Microdochium seminicola.*

Phylogenetic Analysis of *Phyllachora maydis* OTUs

Multiple *Phyllachora maydis* OTUs were identified in this study. A maximum likelihood phylogenetic analysis was performed to understand the various species groups and how they associated differently between lesion types (Supplemental Figure 5.6). Representative sequences for each of the previously determined *Phyllachora* species groups were aligned with *P. maydis* OTUs and ASVs. With 1,000 bootstraps, most OTUs associated with *Phyllachora* sp. groups 1, 2 and 3 (Broders et al., 2023). Interestingly, no OTUs clustered with groups 4 and 5 with a few OTUs clustered separately from the species groups.

Discussion

Fungal and bacterial communities of the different lesion types associated with the tar spot disease complex from various locations in the US and Mexico were analyzed. Tar spot lesions alone (tar spot), tar spot with fisheye lesions (fisheye), and non-symptomatic controls (non-symptomatic) were compared. As expected, the causal agent of tar spot, *Phyllachora maydis* was found associated abundantly within tar spot and fisheye lesions. Furthermore, multiple OTUs within the fungal communities were classified as *Phyllachora maydis. P. maydis* has been shown to have variation within the internal transcribed spacer (ITS) region (Broders et al. 2022; McCoy et al. 2019; Roggenkamp et al. 2023). Alas, multiple delineations of *P. maydis* were found within a phylogenetic study using the ITS and large ribosomal subunit (LSU) markers (Broders et al. 2023). In the current study, the *P. maydis* classified OTUs and ASVs cluster with the three previously identified species groups found infecting contemporary maize leaves. Whether these delineations confer different species or haplotype separations has yet to be confirmed. Variation of the multicopy ITS region within a single individual does occur in other fungal species (Rush et al. 2019; Simon and Weiß 2008; Smith et al. 2007). The presence of multiple *P. maydis* OTUs within a single sample collected from single lesions from this study may indicate intragenomic variation of the ITS region. However, the abundance of these variations across locations is still not known.

Statistical testing was performed to understand the contributions to variance within the

fungal and bacterial communities. The fungal communities had significant differences when comparing the lesion types. Specifically, differences in abundant fungal genera and lower alpha diversity when comparing fisheye samples to the other lesion types were observed following the trends from the previous study (McCoy et al. 2019). Furthermore, changes in fungal communities by country of origin were observed. Lesion type differences were not as prolific within the bacterial communities. Generally, similar communities were observed between tar spot and fisheye lesions while both were significantly different from non-symptomatic communities. However, location did significantly contribute to the variation in bacterial communities. Several bacterial taxa were identified as indicators for fisheye lesions, but the calculated statistic value was low indicating the taxa were not abundant in all samples. These results show that fisheye lesions are not associated with a specific bacterial species and therefore likely not caused by a bacterium. Previous studies have indicated that the tar spot disease complex is associated with several fungi, and no signs of bacterial infection have been associated with the tar spot disease complex (Valle-Torres et al. 2020).

Within the fungal communities, both lesion type and location contributed to variation. Further analysis was performed to understand the differences of lesion types from the US and Mexico separately. Significant indicator taxa of fisheye lesions from Mexico included an OTU that was binned to the Sordariomycetes class. A BLAST search showed 100% sequence similarity to *Microdochium seminicola.* Upon further analysis, this OTU was found abundantly in fisheye lesions from Mexico, but not abundant in US fisheye communities. Alas, fisheye lesions were previously described as associated and caused by the opportunistic pathogen, *Microdochium maydis* (Müller & Samuels 1984), and *Microdohium seminicola* has been described to cause necrotic lesions on sorghum (de Paula et al. 2022). Our analysis indicated that a *Microdochium* sp. does associate with fisheye lesions in Mexico. However, confirmation of the species with type specimens and sequences has not been performed (Hernández-Restrepo et al. 2016). While *Microdochium* sp. was identified in fisheye lesions, species within this genus can live as saprophytes in the soil and on decaying plant tissue which could mean the fungus only becomes associated after the fisheye necrosis occurs (Hernández-Restrepo et al. 2016). The sampled leaves were collected from Mexico near the end of the spring field season and many of the samples were already senesced. Also, due to limitations in receiving foreign plant material, the samples were dried prior to shipment which may have led to changes within microbial

community makeup (Qui et al. 2020; Singh et al. 2023). Further experiments are needed to understand the association of *Microdochium* sp. within fisheye lesions and to what extent this species is important to fisheye lesions in Mexico.

In contrast, the taxa found in high abundance in fisheye lesions in the US included *Parapheospheria neglecta*, a *Fusarium* sp., and a taxon classified to the Pleosporales order which had BLAST results of 90.5% sequence similarity to *Neottiosporina paspali*. These taxa were found as significant indicator taxa of fisheye lesions within the US, similar to the previous study from Michigan (McCoy et al. 2019). Additionally, both *Fusarium* spp. and *Paraphaeosphaeria* spp. have been isolated in abundance from fisheye lesions in the US (data not shown; Luis et al. 2023). *Paraphaeosphaeria neglecta* has also been shown to be a mycoparasite within *P. maydis* reproductive structures observed in fisheye lesions (Caldwell et al. 2024). Species within the *Paraphaeosphaeria* genus can act as endophytes, plant pathogens, or biocontrol agents due to their antifungal activity (Verkley et al. 2014; de Paula et al. 2022). *Fusarium* spp. are ubiquitous soil microbes with saprophytic lifestyles and are often found as plant pathogens (Blacutt et al. 2018; Chelkowski et al. 1989). Furthermore, colonization of plant tissue by *Fusarium* spp. is not inhibited by prior colonization of other seedborne organisms (Reyes Gaige et al. 2020). Due to these differences in indicator taxa by country, fisheye lesions could be caused by multiple different opportunistic pathogens. While most plant disease outbreaks have been associated with a single pathogen, many diseases can be caused by different similarly related pathogens (Wallace 1978; Fitt et al. 2006). Also, complexes of organisms can work synergistically leading to increased virulence and disease severity (Lamichhane and Venturi 2015), and the species involved in these disease complexes can have different distributions based on location and host genotypes (Fitt et al. 2006). This may indicate the tar spot disease complex may be more complicated than previously thought. In contrast, microbial communities are helpful in the defense response of plants, and associations within communities can also lead to disease outcomes (Gao et al. 2021; Singh et al. 2023). Alas, the microbial community may be a factor leading to disease development in fisheye lesions.

In conclusion, in this study, the abundantly associated microbes in the tar spot disease complex differed by lesion type and location. No bacterial species were found significantly associated with the tar spot disease complex confirming that bacteria are not involved in the disease. Additionally, several differing *Phyllachora maydis* OTUs were observed and clustered

within three previously identified species groups. Future analysis of the geographic distribution of these *Phyllachora maydis* species groups would help in understanding disease dynamic differences between locations. The identification of a *Microdochium* sp. significantly abundant and associated with fisheye lesions in Mexico shows the controversial *Microdochium maydis* may exist, but confirmation of the taxonomy of this species as well as the causation of the fisheye lesion through Koch's postulates will be needed. Furthermore, different associated taxa within the US show that fisheye lesions may be caused by multiple organisms, and the decreased prolific nature of fisheye lesions in the US may be due to the lessened prevalence of *Microdochium* spp. The identification and isolation of the closely related species to *Neottiosporina paspali* will be important to describe this unidentified species. Lastly, the fisheye lesions could be due to the plant response to the parasitization of *P. maydis* caused by *Paraphaeosphaeria neglecta* and other mycoparasitic fungi. Further elucidation of the cause of the fisheye lesions will aid in the understanding of the epidemiology of the tar spot disease complex will help in implications of the future of this disease within the US and future disease management.

CHAPTER 6:
CONCLUSIONS

Tar spot of maize has quickly become one of the top yield reducing diseases in the United States being recently introduced in 2015. Though being of economic concern, this disease has been largely understudied with much still unknown with regards to genetics, biology, and epidemiology. This dissertation has attempted to bridge these gaps by providing foundational studies on detection assay development, genomics and transcriptomics of tar spot pathogen *Phyllachora maydis,* maize differential expression analysis in response to tar spot, and the microbial communities associated with the various lesion types across locations.

Signs of tar spot of maize include the raised, black stromata which are known to be caused by the fungus *Phyllachora maydis.* While diagnostics of tar spot are relatively simple, similar signs and symptoms have been mistaken for tar spot to the untrained eye. Previously no molecular detection assay existed for *P. maydis* therefore the development of a specific and sensitive qPCR assay will be helpful in diagnostics as well as studying latency period and epidemiology of tar spot. The developed qPCR assay was shown to be specific to *P. maydis* and highly sensitive. Further testing showed reproducibility within different laboratories. In the future, this assay can be used *in planta* for detection of *P. maydis* prior to symptom development or for spore quantification from airborne samplers for epidemiology studies.

A quality genome and annotation provide a solid foundation for future research. The first draft genome of *P. maydis* provided the first genetic knowledge of this pathogen besides the common ribosomal identification markers. Even with the challenges with working with this pathogen, they were able to assemble a quality genome with short-reads. However, the genome was highly fragmented due to the prolific transposable element content. In this dissertation, the use of long-read sequencing allowed for the increased contiguity within this second draft genome assembly of *P. maydis*. Additionally, the first transcript evidence is provided for *P. maydis.* This markedly improved gene annotation prediction due to no draft genomes existing within the entire order of fungi. Furthermore, the gene content was surveyed and provided the first description of genes encoded within the *P. maydis* genome. Ongoing research has started functionally characterizing *P. maydis* effectors for improved understanding of plant-pathogen interactions.

Previous studies on the host side of the disease have focused on discovery of resistant varieties and resistance loci for breeding purposes. The response of maize to tar spot at the molecular level has not been previously investigated. In this dissertation, the first differential expression analysis of maize in response to tar spot over time is reported. Once again due to the

previous challenges with inducing disease in controlled environments, plants were exposed to tar spot within an infested field. Fortunately, the samples showed a significant effect from the tar spot pathogen and between sample variation was not significantly different. A survey of the associated defense response genes are provided. Furthermore, candidate genes of previous tar spot resistance loci from GWAS were found significantly differentially expressed. Future detailed studies can investigate earlier infection timepoints and differential expression between resistant and susceptible lines.

Development of tar spot can be followed by development of necrotic halos encompassing the tar spot stroma. The observation of multiple fungi associated with these fisheye lesions initiated the description of the tar spot disease complex. The tar spot disease complex originally consisted of *Phyllachora maydis, Microdochium maydis,* and *Coniothyrium phyllachoraea.* Much contemplation over the existence of *M. maydis* has been of recent concern. Somewhat unexpectedly, a taxon most closely related to *Microdochium semincola* was found significantly abundant and indicative of fisheye lesions within Mexico which confirms the previous described association. However, the causality of fisheye lesions is still questionable given the saprophytic lifestyles of several *Microdochium* spp. Indicator fungal species of fisheye lesions identified within the United States included *Paraphaerosphaeria* sp., *Fusarium* sp., and *Neottiosporina paspali* similarly to the previous study from a single field in Michigan. Questions still remain regarding the cause of fisheye lesions. Can this be caused by multiple different fungi? Is this caused by response to mycoparasites within *P. maydis* structures? Future isolations of the associated fungi and confirmation of Koch's postulates is needed for confirmation of causality.

In conclusion, the tar spot pathosystem has been investigated using various methods within this dissertation. As stated, these initial studies will provide knowledge for future research and elucidation of this difficult, elusive, and interesting pathogen.

List of additional publications from the duration of my PhD

1. Rogers, A., Jaiswal, N., **Roggenkamp, E.**, Kim, H., MacCready, J.S., Chilvers, M.I., Scofield, S.R., Iyer-Pascuzzi, A.S., Helm, M. (2024) Genome-informed trophic classification and functional characterization of virulence proteins from the maize tar spot pathogen *Phyllachora maydis.* Phytopathology First Look.

2. Palande, S., Kaste, J.A.M., Roberts, M.D., Segura Abá, K., Claucherty, C., Dacon, J., Doko, R., Jayakody, T.B., Jeffery, H.R., Kelly, N., Manousidaki, A., Parks, H.M., **Roggenkamp, E.M.**, Schumacher, A.M., Yang, J., Percival, S., Pardo, J., Husbands, A.Y., Krishnan, A., Montgomery, B.L., Munch, E., Thompson, A.M., Rougon-Cardoso, A., Chitwood, D.H., VanBuren, R. (2023) Topological data analysis reveals a core gene expression backbone that defines form and function across flowering plants. PLoS Biol 21(12): e3002397.

3. Solórzano, J.E., Issendorf, S.E., Drott, M.T., Check, J.C., **Roggenkamp, E.M.,** Cruz, C.D., Kleczewski, N.M., Gongóra-Canul, C.C., Malvick, D.K. (2023) A new and effective method to induce infection of *Phyllachora maydis* into corn for tar spot studies in controlled environments. Plant Methods 19, 83.

4. Telenko, D.E.P., Chilvers, M.I., Byrne, A., Check, J., Rocco Da Silva, C., Kleczewski, N., **Roggenkamp E**, Ross, T.J., Smith, D.L. Fungicide efficacy on tar spot and yield of corn in the Midwestern United States. (2022) Plant Health Progress. *Editor's Pick.*

5. Rocco da Silva, C.*, Check, J.*, MacCready, J.S.*, Alakonya, A.E., Beiriger, R., Bissonnette, K.M., Collins, A., Cruz, C.D., Esker, P.D., Goodwin, S.B., Malvick, D., Mueller, D.S., Paul, P., Raid, R., Robertson, A.E., **Roggenkamp, E.M.**, Ross, T.J., Singh, R., Smith, D.L., Tenuta, A.U., Chilvers, M.I., Telenko, D.E.P. Recovery Plan for Tar Spot of Corn, Caused by *Phyllachora maydis*. (2021) Plant Health Progress. *Editor's Pick.*

6. Valle-Torres J, Ross TJ, Plewa D, Avellaneda MC, Check J, Chilvers MI, Cruz AP, Dalla Lana F, Groves C, Gongora-Canul C, Henriquez-Dole L, Jamann T, Kleczewski N, Lipps S, Malvick D, McCoy AG, Mueller DS, Paul, P.A., Puerto, C., Schloemer, C., Raid, R.N., Robertson, A., **Roggenkamp, E.M.**, Smith, D.L., Telenko, D.E.P., Cruz, C.D. (2020) Tar spot: An understudied disease threatening corn production in the Americas. Plant Disease, 104(10):2541-2550.

REFERENCES

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Amezrou, R., Ducasse, A., Compain, J., Lapalu, N., Pitarch, A., Dupont, L., Confais, J., Goyeau, H., Kema, G. H. J., Croll, D., Amselem, J., Sanchez-Vallet, A., & Marcel, T. C. (2024). Quantitative pathogenicity and host adaptation in a fungal plant pathogen revealed by whole-genome sequencing. *Nature Communications*, 15(1), 1–14. https://doi.org/10.1038/s41467-024-46191-1

Aylward, J., Steenkamp, E. T., Dreyer, L. L., Roets, F., Wingfield, B. D., & Wingfield, M. J. (2017). A plant pathology perspective of fungal genome sequencing. *IMA Fungus*, 8(1), 1–15. https://doi.org/10.5598/imafungus.2017.08.01.01

Bajet, N. B., Renfro, B. L., & Carrasco, J. M. V. (1994). Control of tar spot of maize and its effect on yield. *International Journal of Pest Management,* 40(2), 121–125. https://doi.org/10.1080/09670879409371868

Balint-Kurti, P. J., & Johal, G. S. (2009). Maize Disease Resistance. *Handbook of Maize: Its Biology*, 229–250. https://doi.org/10.1007/978-0-387-79418-1_12

Blacutt, A. A., Gold, S. E., Voss, K. A., Gao, M., & Glenn, A. E. (2018). *Fusarium verticillioides*: Advancements in understanding the toxicity, virulence, and niche adaptations of a model mycotoxigenic pathogen of maize. *Phytopathology*, 108(3), 312–326. https://doi.org/10.1094/PHYTO-06-17-0203-RVW

Block, A. K., Vaughan, M. M., Schmelz, E. A., & Christensen, S. A. (2019). Biosynthesis and function of terpenoid defense compounds in maize (*Zea mays*). *Planta*, 249(1), 21–30. https://doi.org/10.1007/S00425-018-2999-2

Breunig, M., Bittner, R., Dolezal, A., Ramcharan, A., & Bunkers, G. (2023). An assay to reliably achieve Tar Spot symptoms on corn in a controlled environment. *BioRxiv*, 2023.01.12.523803. https://doi.org/10.1101/2023.01.12.523803

Broders, K., Iriarte-Broders, G., Bergstrom, G. C., Byamukama, E., Chilvers, M., Cruz, C., Dalla-Lana, F., Duray, Z., Malvick, D., Mueller, D., Paul, P., Plewa, D., Raid, R., Robertson, A. E., Salgado-Salazar, C., Smith, D., Telenko, D., VanEtten, K., & Kleczewski, N. M. (2022). *Phyllachora* species infecting maize and other grass species in the Americas represents a complex of closely related species. *Ecology and Evolution*, 12(4), e8832. https://doi.org/10.1002/ECE3.8832

Buchfink, B., Reuter, K., & Drost, H-G. (2021). Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nature Methods,* 18, 366–368. https://doi.org/10.1038/s41592-021-01101-x

Caldwell, D. L., Da Silva, C., McCoy, A., Avila, H., Bonkowski, J. C., Chilvers, M. I., Helm, M., Telenko, D. E. P., & Iyer-Pascuzzi, A. S. (2024). Uncovering the infection strategy of *Phyllachora maydis* during maize colonization: A comprehensive analysis. *Phytopathology*, 114(5), 1075–1087. https://doi.org/10.1094/PHYTO-08-23-0298-KC

Campbell, A. A., Stenback, K. E., Flyckt, K., Hoang, T., Perera, A. M. D. N., & Nikolau, B. J. (2019). A single-cell platform for reconstituting and characterizing fatty acid elongase component enzymes. *PLoS ONE,* 14(3). https://doi.org/10.1371/journal.pone.0213620

Cao, S., Loladze, A., Yuan, Y., Wu, Y., Zhang, A., Chen, J., Huestis, G., Cao, J., Chaikam, V., Olsen, M., Prasanna, B. M., San Vicente, F., & Zhang, X. (2017). Genome-Wide Analysis of Tar Spot Complex Resistance in Maize Using Genotyping-by-Sequencing SNPs and Whole-Genome Prediction. *The Plant Genome*, 10(2), 0. https://doi.org/10.3835/plantgenome2016.10.0099

Cao, S., Song, J., Yuan, Y., Zhang, A., Ren, J., Liu, Y., Qu, J., Hu, G., Zhang, J., Wang, C., Cao, J., Olsen, M., Prasanna, B. M., San Vicente, F., & Zhang, X. (2021). Genomic Prediction of Resistance to Tar Spot Complex of Maize in Multiple Populations Using Genotyping-by-Sequencing SNPs. *Frontiers in Plant Science*, 12, 672525.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences,* 108, 4516–4522. https://doi.org/10.1073/PNAS.1000080107

Ceballos, H., & Deutsch, J. A. (1992). Inheritance of resistance to tar spot complex in maize. In *Phytopathology*, 82(5), 505–512. https://doi.org/10.1094/Phyto-82-505

Check, J. C., Byrne, A. M., Singh, M. P., Steinke, K., Widdicombe, W. D., & Chilvers, M. I. (2023). Effects of Nitrogen Application Rate and Plant Density on Severity of Tar Spot of Corn. *Plant Health Progress,* 24(4), 416–423. https://doi.org/10.1094/PHP-12-22-0125-RS

Chelkowski, J., Manka, M., Kwasna, H., Visconti, A., & Golinski, P. (1989). *Fusarium sporotrichioides* Sherb., *Fusarium tricinctum* (Corda) Sacc. and *Fusarium poae* (Peck) Wollenw. — Cultural characteristics, toxinogenicity and pathogenicity towards cereals. *Journal of Phytopathology*, 124(2), 155–161. https://doi.org/10.1111/J.1439-0434.1989.TB04910.X

Chen, S., Ren, C., Zhai, J., Yu, J., Zhao, X., Li, Z., Zhang, T., Ma, W., Han, Z., & Ma, C. (2020). CAFU: a Galaxy framework for exploring unmapped RNA-Seq data. *Briefings in Bioinformatics*, 21(2), 676–686. https://doi.org/10.1093/BIB/BBZ018

Chen, G., Zhang, B., Ding, J., Wang, H., Deng, C., Wang, J., Yang, Q., Pi, Q., Zhang, R., Zhai, H., Dong, J., Huang, J., Hou, J., Wu, J., Que, J., Zhang, F., Li, W., Min, H., Tabor, G., Li, B., Liu, X., Zhao, J., Yan, J., & Lai, Z. (2022). Cloning southern corn rust resistant gene RppK and its cognate gene AvrRppK from *Puccinia polysora*. *Nature Communications,* 13(1), 1–11. https://doi.org/10.1038/s41467-022-32026-4

Christensen, S. A., Sims, J., Vaughan, M. M., Hunter, C., Block, A., Willett, D., Alborn, H. T., Huffaker, A., & Schmelz, E. A. (2018). Commercial hybrids and mutant genotypes reveal complex protective roles for inducible terpenoid defenses in maize. *Journal of Experimental Botany*, 69(7), 1693–1705. https://doi.org/10.1093/JXB/ERX495

Collins, A. A., Bandara, A. Y., May, S. R., Weerasooriya, D. K., & Esker, P. D. (2021). First report of tar spot of maize (*Zea mays*) caused by *Phyllachora maydis* in Pennsylvania. *Plant Disease*, 105(8). https://doi.org/10.1094/PDIS-11-20-2456-PDN

Darriba, D., Posada, D., Kozlov, A. M., Stamatakis, A., Morel, B., & Flouri, T. (2020). ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Molecular Biology and Evolution*, 37(1), 291-294. https://doi.org/10.1093/molbev/msz189

da Silva, C. R., Check, J., MacCready, J. S., Alakonya, A. E., Beiriger, R., Bissonnette, K. M., Collins, A., Cruz, C. D., Esker, P. D., Goodwin, S. B., Malvick, D., Mueller, D. S., Paul, P., Raid, R., Robertson, A. E., Roggenkamp, E., Ross, T. J., Singh, R., Smith, D. L., Tenuta, A. U., Chilvers, M. I., Telenko, D. E. P. (2021). Recovery Plan for Tar Spot of Corn, Caused by *Phyllachora maydis*. *Plant Health Progress*, 22(4), 596–616. https://doi.org/10.1094/PHP-04-21-0074-RP

Davis, N. M., Proctor, Di. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, 6(1), 1–14. https://doi.org/10.1186/S40168-018-0605-2

De Cáceres, M., & Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. *Ecology*, 90(12), 3566–3574. https://doi.org/10.1890/08-1823.1

de Paula, L. R. V., Barreto, R. W., & da Nóbrega, T. F. (2022). Sooty leaf spot—a new fungal disease of sorghum caused by *Paraphaeosphaeria sorghi* sp. nov. *Tropical Plant Pathology*, *47*(5), 618–625. https://doi.org/10.1007/S40858-022-00522-Z

Ding, Y., Murphy, K.M., Poretsky, E., Mafu, S., Yang, B., Char, S.N., Christensen, S.A., Saldivar, E., Wu, M., Wang, Q., Ji, L., Schmitz, R.J., Kremling, K.A., Buckler, E.S., Shen, Z., Briggs, S.P., Bohlmann, J., Sher, A., Castro-Falcon, G., Hughes, C.C., Huffaker, A., Zerba, P., & Schmelz, E.A. (2019). Multiple genes recruited from hormone pathways partition maize diterpenoid defences. *Nature Plants*, 5(10), 1043–1056. https://doi.org/10.1038/s41477-019-0509-6

Ding, Y., Weckwerth, P. R., Poretsky, E., Murphy, K. M., Sims, J., Saldivar, E., Christensen, S. A., Char, S. N., Yang, B., Tong, A.-D., Shen, Z., Kremling, K. A., Buckler, E. S., Kono, T., Nelson, D. R., Bohlmann, J., Bakker, M. G., Vaughan, M. M., Khalil, A. S., Betsiashvili, M., Dressano, K., Köllner, T.G., Briggs, S.P., Zerbe, P., Schmelz, E.A., & Huffaker, A. (2020). Genetic elucidation of interconnected antibiotic pathways mediating maize innate immunity. *Nature Plants,* 6, 1375–1388. https://doi.org/10.1038/s41477-020-00787-9

Dittrich, U., Hock, J., Kranz, J., & Renfro, B. L. (1991). Germination of *Phyllachora maydis* ascospores and conidia of *Monographella maydis*. *Cryptogamic Botany*, 2(2–3), 214–218.

Dixon, R. A., Achnine, L., Kota, P., Liu, C. J., Reddy, M. S. S., & Wang, L. (2002). The phenylpropanoid pathway and plant defence - a genomics perspective. *Molecular Plant Pathology*, 3(5), 371–390. https://doi.org/10.1046/J.1364-3703.2002.00131.X

Douglas, C. J. (1996). Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends in Plant Science*, *1*(6), 171–178. https://doi.org/10.1016/1360-1385(96)10019-4

Duplessis, S., Cuomo, C. A., Lin, Y. C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly, D. L., Hacquard, S., Amselem, J., Cantarel, B. L., Chiu, R., Coutinho, P. M., Feaue, N., Field, M., Frey, P., Gelhaye, E., Goldberg, J., Grabherr, M. G., Kodira, C. D., Kohler, A., Kües, U.,

Lindquist, E. A., Lucas, S. M., Mago, R., Mauceli, E., Morin, E., Murat, C., Pangilinan, J. L., Park, R., Pearson, M., Quesneville, H., Rouhier, N., Sakthikumar, S., Salamov, A. A., Schmutz, J., Selles, B., Shapiro, H., Tanguay, P., Tuskan, G. A., Henrissat, B., Van de Peer, Y., Rouzé, P., Ellis, J. G., Dodds, P. N., Schein, J. E., Zhong, S., Hamelin, R. C., Grigoriev, I. V., Szabo, L. J., & Martin, F. (2011). Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proceedings of the National Academy of Sciences*, 108(22), 9166–9171. https://doi.org/10.1073/PNAS.1019315108

Durinck, S., Spellman, P. T., Birney, E., & Huber, W. (2009). Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nature Protocols,* 4, 1184–1191. *h*ttps://doi.org/10.1038/nprot.2009.97

Edgar, R. C., & Bateman, A. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26(19), 2460–2461. https://doi.org/10.1093/BIOINFORMATICS/BTQ461

Eschenbrenner, C. J., Feurtey, A., & Stukenbrock, E. H. (2020). Population genomics of fungal plant pathogens and the analyses of rapidly evolving genome compartments. *Methods in Molecular Biology*, 2090, 337–355. https://doi.org/10.1007/978-1-0716-0199-0_14

Farr, D. F., & Rossman, A. Y. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved June 14, 2024 from https://nt.ars-grin.gov/fungaldatabases/

FAO.STAT. License: CC BY-NC-SA 3.0 IGO. Extracted from: https://www.fao.org/faostat/en/#data/QCL/visualize. Date of access: 04-06-2024.

Figueiredo, L., Santos, R. B., & Figueiredo, A. (2021). Defense and offense strategies: The role of aspartic proteases in plant–pathogen interactions. *Biology,* 10(2), 75. https://doi.org/10.3390/BIOLOGY10020075

Fitt, B. D. L., Huang, Y., van den Bosch, F., & West, J. S. (2006) Coexistence of related pathogen species on arable crops in space and time. *Annual Review of Phytopathology,* 44, 163–182. https://doi.org/10.1146/annurev.phyto.44.070505.143417

Flouri, T., Izquierdo-Carrasco, F., Darriba, D., Aberer, A. J., Nguyen, L. T., Minh, B. Q., von Haeseler, A., & Stamatakis, A. (2014) The phylogenetic likelihood library. *Systematic Biology,* 64(2), 356–362. https://doi.org/10.1093/sysbio/syu084

Gaige, A. R., Todd, T., & Stack, J. P. (2020). Interspecific competition for colonization of maize plants between *Fusarium proliferatum* and *Fusarium verticillioides*. *Plant Disease*, 104(8), 2102–2110. https://doi.org/10.1094/PDIS-09-19-1964-RE

Gao, M., Xiong, C., Gao, C. et al. (2021) Disease-induced changes in plant microbiome assembly and functional adaptation. *Microbiome 9*, 187 https://doi.org/10.1186/s40168-021-01138-2

Geneious version 2024.0 created by Biomatters. Available from https://www.geneious.com

Gomez-Cano, L., Gomez-Cano, F., Dillon, F. M., Alers-Velazquez, R., Doseff, A. I., Grotewold, E., & Gray, J. (2020). Discovery of modules involved in the biosynthesis and regulation of maize phenolic compounds. *Plant Science*, 291, 110364. https://doi.org/10.1016/J.PLANTSCI.2019.110364

Gongora-Canul, C. C., Puerto, C., Jiménez-Beitia, F. E., Telenko, D. E. P., Kleczewski, N., Rosas, J. C., Avellaneda, C., Sanders, A., Rodriguez, I. Y., Goodwin, S. B., Henriquez-Dole, L. E., Fernandez-Campos, M., Lee, D. Y., Cruz, A. P., & Cruz, C. D. (2024). Comparing tar spot epidemics in high-risk areas in the United States and Honduras. *Canadian Journal of Plant Pathology*, 46(3), 288–305. https://doi.org/10.1080/07060661.2023.2300077

Groves, C. L., Kleczewski, N. M., Telenko, D. E. P., Chilvers, M. I., & Smith, D. L. (2020). Phyllachora maydis ascospore release and germination from overwintered corn residue. *Plant Health Progress*, 21(1), 26–30. https://doi.org/10.1094/PHP-10-19-0077-RS

Guillaumie, S., Pichon, M., Martinant, J. P., Bosio, M., Goffner, D., & Barrière, Y. (2007). Differential expression of phenylpropanoid and related genes in brown-midrib bm1, bm2, bm3, and bm4 young near-isogenic maize plants. *Planta*, 226(1), 235–250. https://doi.org/10.1007/S00425-006-0468-9

Halpin, C., Holt, K., Chojecki, J., Oliver, D., Chabbert, B., Monties, B., Edwards, K., Barakate, A., & Foxon, G. A. (1998). Brown-midrib maize (bm1) – a mutation affecting the cinnamyl alcohol dehydrogenase gene. *The Plant Journal*, 14(5), 545–553. https://doi.org/10.1046/J.1365-313X.1998.00153.X

Helm, M., Singh, R., Hiles, R., Jaiswal, N., Myers, A., Iyer-Pascuzzi, A. S., & Goodwin, S. B. (2022). Candidate effector proteins from the maize tar spot pathogen *Phyllachora maydis* localize to diverse plant cell compartments. *Phytopathology*, 112(12), 2538–2548. https://doi.org/10.1094/PHYTO-05-22-0181-R

Hernández-Restrepo, M., Groenewald, J. Z., & Crous, P. W. (2016). Taxonomic and phylogenetic re-evaluation of *Microdochium*, *Monographella* and *Idriella*. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, 36, 57–82. https://doi.org/10.3767/003158516X688676

Hock, J., Dittrich, U., Renfro, B. L., & Kranz, J. (1992). Sequential development of pathogens in the maize tarspot disease complex. *Mycopathologia*, 117(3), 157–161. https://doi.org/10.1007/BF00442777

Hock, J., Kranz, J., Renfro, B. L. (1989). El complejo mancha de asfalto de maiz, su distribucion geografica, requisitos ambientales e importancia economica en Mexico. *Revista Mexicana de Fitopatologia*, 7(2), 129–135.

Hock, J., Kranz, J., Renfro, B. L. (1995). Studies on the epidemiology of the tar spot disease complex of maize in Mexico. *Plant Pathology*, 44(3), 490–502. https://doi.org/10.1111/j.1365-3059.1995.tb01671.x

Hock, J., Renfro, B. L., & Kranz, J. (1992). Tests of standard diagrams for field use in assessing the tarspot disease complex of maize (*Zea mays*). *Tropical Pest Management*, 38(3), 314–318. https://doi.org/10.1080/09670879209371716

Hoopes, G. M., Hamilton, J. P., Wood, J. C., Esteban, E., Pasha, A., Vaillancourt, B., Provart, N. J., & Buell, C. R. (2019). An updated gene atlas for maize reveals organ-specific and stress-induced genes. *The Plant Journal,* 97(6), 1154–1167. https://doi.org/10.1111/TPJ.14184

Huffaker, A., Kaplan, F., Vaughan, M. M., Dafoe, N. J., Ni, X., Rocca, J. R., Alborn, H. T., Teal, P. E. A., & Schmelz, E. A. (2011). Novel acidic sesquiterpenoids constitute a dominant class

of pathogen-induced phytoalexins in Maize. *Plant Physiology*, 156(4), 2082–2097. https://doi.org/10.1104/PP.111.179457

Hufford, M. B., Seetharam, A. S., Woodhouse, M. R., Chougule, K. M., Ou, S., Liu, J., Ricci, W. A., Guo, T., Olson, A., Qiu, Y., Coletta, R. Della, Tittes, S., Hudson, A. I., Marand, A. P., Wei, S., Lu, Z., Wang, B., Tello-Ruiz, M. K., Piri, R. D., Wang, N., won Kim, D., Zeng, Y., O'Connor, C. H., Li, X., Gilbert, A. M., Baggs, E., Krasileva, K. V., Portwood, J. L., Cannon, E. K. S., Andorf, C. M., Manchanda, N., Snodgrass, S. J., Hufnagel, D. E., Jiang, Q., Pedersen, S., Syring, M. L., Kudrna, D. A., Llaca, V., Fengler, K., Schmitz, R. J., Ross-Ibarra, J., Yu, J., Gent, J. I., Hirsch, C. N., Ware, D., & Dawe, R. K. (2021). De novo assembly, annotation, and comparative analysis of 26 diverse maize genomes. *Science*, *373*(6555), 655–662. https://doi.org/10.1126/SCIENCE.ABG5289

Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., & Mitra, S. (2016). MEGAN community edition-interactive exploration and analysis of large-scale microbiome. *PLoS Comput Biol,* 12, *1004957*. https://doi.org/10.1371/journal.pcbi.1004957

Johal, G. S., & Briggs, S. P. (1992). Reductase activity encoded by the HM1 disease resistance gene in maize. *Science*, 258(5084), 985–987. https://doi.org/10.1126/SCIENCE.1359642

Johnson, E. T., Dowd, P. F., Ramirez, J. L., & Behle, R. W. (2023). Potential biocontrol agents of corn tar spot disease isolated from overwintered *Phyllachora maydis* stromata. *Microorganisms*, 11(6), 1550. https://doi.org/10.3390/MICROORGANISMS11061550

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, 444(7117), 323–329. https://doi.org/10.1038/nature05286

Jørgensen, I. H. (1992). Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. *Euphytica*, 63(1–2), 141–152. https://doi.org/10.1007/BF00023919

Kim, D., Paggi, J. M., Park, C., Bennett, C., & Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology*, 37(8), 907–915. https://doi.org/10.1038/s41587-019-0201-4

Kim, S. B., Van den Broeck, L., Karre, S., Choi, H., Christensen, S. A., Wang, G. F., Jo, Y., Cho, W. K., & Balint-Kurti, P. (2021). Analysis of the transcriptomic, metabolomic, and gene regulatory responses to *Puccinia sorghi* in maize. *Molecular Plant Pathology*, 22(4), 465. https://doi.org/10.1111/MPP.13040

Kleczewski, N. M., Plewa, D. E., Bissonnette, K. M., Bowman, N. D., Byrne, J. M., Laforest, J., Dalla-Lana, F., Malvick, D. K., Mueller, D. S., Chilvers, M. I., Paul, P. A., Raid, R. N., Robertson, A. E., Ruhl, G. E., Smith, D. L., & Telenko, D. E. P. (2020). Documenting the establishment, spread, and severity of *Phyllachora maydis* on corn, in the United States. *Journal of Integrated Pest Management*, 11(1), 14–15. https://doi.org/10.1093/JIPM/PMAA012

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B., & Stamatakis, A. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics*, 35(21), 4453–4455. https://doi.org/10.1093/BIOINFORMATICS/BTZ305

Kurtz, Z. D., Müller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., & Bonneau, R. A. (2015). Sparse and compositionally robust inference of microbial ecological networks. *PLOS Computational Biology*, 11(5), e1004226. https://doi.org/10.1371/JOURNAL.PCBI.1004226

Kushalappa, A. C., Hegde, N. G., Gunnaiah, R., Sathe, A., Yogendra, K. N., & Ajjamada, L. (2022). Apoptotic-like PCD inducing HRC gene when silenced enhances multiple disease resistance in plants. *Scientific Reports,* 12(1), 1–13. https://doi.org/10.1038/s41598-022-24831-0

Lamichhane, J. R., & Venturi, V. (2015). Synergisms between microbial pathogens in plant disease complexes: a growing trend. *Frontiers in Plant Science*, 6, 1–12. https://doi.org/10.3389/FPLS.2015.00385

Langmead, B., Wilks, C., Antonescu, V., & Charles, R. (2019). Scaling read aligners to hundreds of threads on general-purpose processors. *Bioinformatics*, 35(3), 421–432. https://doi.org/10.1093/BIOINFORMATICS/BTY648

Leonard, K. J., & Suggs, E. G. (1974). *Setosphaeria prolata*, The ascigerous state of *Exserohilum prolatum*. *Mycologia*, 66(2), 281–297. https://doi.org/10.1080/00275514.1974.12019603

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25(16), 2078-2079. https://doi.org/10.1093/bioinformatics/btp352

Liber, J. A., Bonito, G., & Benucci, G. M. N. (2021). CONSTAX2: improved taxonomic classification of environmental DNA markers. *Bioinformatics*, 37(21), 3941–3943. https://doi.org/10.1093/BIOINFORMATICS/BTAB347

Lipps, S., Smith, D., Telenko, D., Paul, P., Kleczewski, N., & Jamann, T. (2022). Identification of resistance for *Phyllachora maydis* of maize in exotic-derived germplasm. *Crop Science*, 62(2), 859-866. https://doi.org/10.1002/csc2.20709

Loladze, A., Rodrigues, F. A., Toledo, F., San Vicente, F., Gérard, B., & Boddupalli, M. P. (2019). Application of remote sensing for phenotyping tar spot complex resistance in maize. *Frontiers in Plant Science*, 10. https://doi.org/10.3389/fpls.2019.00552

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12), 1–21. https://doi.org/10.1186/S13059-014-0550-8

Luis, J. M., Mehl, H. L., Plewa, D., & Kleczewski, N. M. (2023). Is *Microdochium maydis* associated with necrotic lesions in the tar spot disease complex? A culture-based survey of maize in Mexico and the Midwestern United States. *Phytopathology*, 113(10), 1890–1897. https://doi.org/10.1094/PHYTO-04-23-0109-R/

MacCready, J. S., Roggenkamp, E. M., Gdanetz, K., & Chilvers, M. I. (2023). Elucidating the obligate nature and biological capacity of an invasive fungal corn Pathogen. *Molecular Plant-Microbe Interactions*, 36(7), 411–424. https://doi.org/10.1094/MPMI-10-22-0213-R

Mahuku, G., Chen, J., Shrestha, R., Narro, L. A., Guerrero, K. V. O., Arcos, A. L., & Xu, Y. (2016). Combined linkage and association mapping identifies a major QTL (qRtsc8-1),

conferring tar spot complex resistance in maize. *Theoretical and Applied Genetics*, 129(6), 1217–1229. https://doi.org/10.1007/s00122-016-2698-y

Mardones, M., Trampe-Jaschik, T., Hofmann, T. A., Mangelsdorff, R. D., & Piepenbring, M. (2018). New neotropical species of Phyllachorales based on molecular, morphological, and ecological data. *Mycologia*, 110(5), 835–859. https://doi.org/10.1080/00275514.2018.1481720

Mardones, M., Trampe-Jaschik, T., Hofmann, T. A., & Piepenbring, M. (2018). Contribution to the phylogeny and a new species of *Coccodiella* (Phyllachorales). *Mycological Progress*, 17(1–2), 205–213. https://doi.org/10.1007/S11557-017-1353-6

Mardones, M., Trampe-Jaschik, T., Oster, S., Elliott, M., Urbina, H., Schmitt, I., & Piepenbring, M. (2017). Phylogeny of the order Phyllachorales (Ascomycota, Sordariomycetes): Among and within order relationships based on five molecular loci. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, 39, 74–90. https://doi.org/10.3767/persoonia.2017.39.04

Mardones, M., Trampe-Jaschik, T., Piepenbring, M., Romberg, M. K., & Urbina, H. (2022). *Telimena* (Telimenaceae, Phyllachorales) species on neotropical host plants: New species, combinations and revision of species on Polygonaceae. *Nova Hedwigia*, 114(3–4), 435–460. https://doi.org/10.1127/NOVA_HEDWIGIA/2022/0688

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, 17(1), 10–12. https://doi.org/10.14806/EJ.17.1.200

Maublanc, A. (1904). Especes nouvelles de champignans inferieurs. *Bulletin Trimestriel de La Societe Mycologique de France*, 20, 72. http://www.biodiversitylibrary.org/item/106463

McCoy, A. G., Roth, M. G., Shay, R., Noel, Z. A., Jayawardana, M., Longley, R. W., Bonito, G., & Chilvers, M. I. (2019). Identification of fungal communities within the tar spot complex of corn in michigan via next generation sequencing. *Phytobiomes Journal*, 3(3), 235–243. https://doi.org/10.1094/pbiomes-03-19-0017-r

McCoy, A.G., Romberg, M.K., Zaworski, E.R., Robertson, A.E., Phibbs, A., Hudelson, B.D., Smith, D.L., Beiriger, R.L., Raid, R.N., Byrne, J.M., Chilvers, M. I. (2018). First report of Tar spot on corn (*Zea mays*) caused by *Phyllachora maydis* in Florida, Iowa, Michigan, and Wisconsin. *Plant Disease*, 102(9). https://doi.org/10.1094/PDIS-02-18-0271-PDN

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, 8(4), e61217. http://dx.plos.org/10.1371/journal.pone.0061217

Melotto, M., Underwood, W., & Sheng, Y. H. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annual Review of Phytopathology,* 46, 101–122. https://doi.org/10.1146/ANNUREV.PHYTO.121107.104959/

Meyer, J., Berger, D. K., Christensen, S. A., & Murray, S. L. (2017). RNA-Seq analysis of resistant and susceptible sub-tropical maize lines reveals a role for kauralexins in resistance to grey leaf spot disease, caused by *Cercospora zeina*. *BMC Plant Biology*, 17(1), 1–20. https://doi.org/10.1186/S12870-017-1137-9

Morrow, S. L., Mascia, P., Self, K. A., & Altschuler, M. (1997). Molecular characterization of a brown midrib3 deletion mutation in maize. *Molecular Breeding*, 3(5), 351–357. https://doi.org/10.1023/A:1009606422975

Mottaleb, K. A., Loladze, A., Sonder, K., Kruseman, G., & San Vicente, F. (2019). Threats of tar spot complex disease of maize in the United States of America and its global consequences. *Mitigation and Adaptation Strategies for Global Change*, 24(2), 281–300. https://doi.org/10.1007/s11027-018-9812-1

Mueller, D. S., Wise, K. A., Sisson, A. J. (2022). Corn disease loss estimates from the United States and Ontario, Canada — 2021. *Crop Protection Network.* https://doi.org/10.31274/cpn-20220328-0

Mueller, D. S., Sisson, A. J., Eide, B., Allen, T. W., Bradley, C. A., Faske, T. R., Friskop, A., Lawrence, K., Musser, F., Reisig, D., Tenuta, A. U., & Wise, K. A. (2024). Field crop yield loss calculator for disease and invertebrate pests: An online tool from the Crop Protection Network. *PhytoFrontiers,* 4(2), 255–258. https://doi.org/10.1094/PHYTOFR-08-23-0109-A

Müller, E., & Samuels, G. J. (1984). *Monographella maydis* sp. nov. and its connection to the tar-spot disease of *Zea mays*. *Nova Hedwigia*, 40, 113–121.

Müller, M. C., Praz, C. R., Sotiropoulos, A. G., Menardo, F., Kunz, L., Schudel, S., Oberhänsli, S., Poretti, M., Wehrli, A., Bourras, S., Keller, B., & Wicker, T. (2019). A chromosome-scale genome assembly reveals a highly dynamic effector repertoire of wheat powdery mildew. *New Phytologist*, 221(4), 2176–2189. https://doi.org/10.1111/NPH.15529

Nguyen, V. N. T., Vo, K. T. X., Park, H., Jeon, J. S., & Jung, K. H. (2016). A systematic view of the MLO family in rice suggests their novel roles in morphological development, diurnal responses, the light-signaling pathway, and various stress responses. *Frontiers in Plant Science*, 7, 215993. https://doi.org/10.3389/fpls.2016.01413

Nilsson, R. H., Larsson, K. H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F. O., Tedersoo, L., Saar, I., Kõljalg, U., & Abarenkov, K. (2019). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research*, 47(D1), D259–D264. https://doi.org/10.1093/NAR/GKY1022

Noel, Z. A., Longley, R., Benucci, G. M. N., Trail, F., Chilvers, M. I., & Bonito, G. (2022). Non-target impacts of fungicide disturbance on phyllosphere yeasts in conventional and no-till management. *ISME Communications*, 2(1). https://doi.org/10.1038/S43705-022-00103-W

Obanni, M., Hipskind, J., Tsai, C. Y., Nicholson, R. L., & Dunkle, L. D. (1994). Phenylpropanoid accumulation and symptom expression in the lethal leaf spot mutant of maize. *Physiological and Molecular Plant Pathology*, 44(5), 379–388. https://doi.org/10.1016/S0885-5765(05)80050-5

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., McGlinn, D., Ouellette, M.,

Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C., Weedon, J. (2022). vegan: Community Ecology Package. R package version 2.6-4. https://CRAN.R-project.org/package=vegan

Orton, C. R. (1944). Graminicolous species of *Phyllachora* in North America. *Mycologia*, 36(1), 18–53. https://doi.org/10.2307/3754878

Parbery, D. G. (1963a). Studies on graminicolous species of *Phyllachora* Fckl I. Ascospores—their liberation and germination. *Australian Journal of Botany*, 11(2), 117–130. https://doi.org/10.1071/BT9630117

Parbery, D. G. (1963b). Studies on graminicolous species of *Phyllachora* Fckl. II. The invasion of the host & development of the fungus. *Australian Journal of Botany*, 11(2), 131–140. https://doi.org/10.1071/BT9630131

Parbery, D.G., Langdon, R. F. N. (1964). Studies on graminicolous species of *Phyllachora* Fckl. IV. Evaluation of the criteria of species. *Australian Journal of Botany*, 12(2), 265–281. https://doi.org/10.1071/BT9640265

Parbery, D. G. (1967). Studies on graminicolous species of *Phyllachora* Fckl. V. A taxonomic monograph. *Australian Journal of Botany*, 15(2), 271–375. https://doi.org/10.1071/BT9670271

Peng, X., Wang, J., Zhang, Z., Xiao, Q., Li, M., & Pan, Y. (2015). Re-alignment of the unmapped reads with base quality score. *BMC Bioinformatics*, 16(5), 1–10. https://doi.org/10.1186/1471-2105-16-S5-S8

Putri, G. H., Anders, S., Pyl, P. T., Pimanda, J. E., & Zanini, F. (2022). Analysing high-throughput sequencing data in Python with HTSeq 2.0. *Bioinformatics*, 38(10), 2943–2945. https://doi.org/10.1093/BIOINFORMATICS/BTAC166

Qiu, Z., Wang, J., Delgado-Baquerizo, M., Trivedi, P., Egidi, E., Chen, Y. M., Zhang, H., & Singh, B. K. (2020). Plant microbiomes: do different preservation approaches and primer sets alter our capacity to assess microbial diversity and community composition? *Frontiers in Plant Science*, 11, 554781. https://doi.org/10.3389/FPLS.2020.00993

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 41. https://doi.org/10.1093/NAR/GKS1219

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics Applications Note*, 26(6), 841–842. https://doi.org/10.1093/bioinformatics/btq033

Raffaele, S., & Kamoun, S. (2012). Genome evolution in filamentous plant pathogens: why bigger can be better. *Nature Reviews Microbiology*, 10(6), 417–430. https://doi.org/10.1038/nrmicro2790

R Core Team (2023). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ren, J., Wu, P., Huestis, G. M., Zhang, A., Qu, J., Liu, Y., Zheng, H., Alakonya, A. E., Dhliwayo, T., Olsen, M., San Vicente, F., Prasanna, B. M., Chen, J., & Zhang, X. (2022). Identification

and fine mapping of a major QTL (qRtsc8-1) conferring resistance to maize tar spot complex and validation of production markers in breeding lines. *Theoretical and Applied Genetics*, 135(5), 1551–1563. https://doi.org/10.1007/S00122-022-04053-8

Ríos Herrera, E. N., Ochoa Fuentes, Y. M., Cerna Chávez, E., Landeros Flores, J., Cepeda Siller, M., Rodríguez Guerra, R., Ríos Herrera, E. N., Ochoa Fuentes, Y. M., Cerna Chávez, E., Landeros Flores, J., Cepeda Siller, M., & Rodríguez Guerra, R. (2017). Fungi associated with the tar spot in maize cultivation in Mexico. *Revista Mexicana de Ciencias Agrícolas*, 8(2), 457–462. https://doi.org/10.29312/REMEXCA.V8I2.65

Rogers, A., Jaiswal, N., Roggenkamp, E., Kim, H.-S., MacCready, J. S., Chilvers, M. I., Scofield, S. R., Iyer-Pascuzzi, A. S., & Helm, M. (2024). Genome-informed trophic classification and functional characterization of virulence proteins from the maize tar spot pathogen *Phyllachora maydis*. *Phytopathology First Look* https://doi.org/10.1094/PHYTO-01-24-0037-R

Ross, T. J., Ross, T. J., Jumbam, B., Bonkowski, J., Chaky, J. L., Chilvers, M. I., Goodwin, S. B., Kleczewski, N. M., Mueller, D. S., Robertson, A. E., Smith, D. L., & Telenko, D. E. P. (2023). Small but significant genetic differentiation among populations of Phyllachora maydis in the midwestern United States revealed by microsatellite (SSR) markers. *BioRxiv*, 2023.10.31.563566. https://doi.org/10.1101/2023.10.31.563566

Ross, T. J., Allen, T. W., Shim, S., Thompson, N. M., & Telenko, D. E. P. (2024). Investigations into economic returns resulting from foliar fungicides and application timing on management of tar spot in Indiana hybrid corn. *Phytopathology*, 108(2), 461–472. https://doi.org/10.1094/PDIS-05-23-0932-RE

Ruhl, G., Romberg, M. K., Bissonnette, S., Plewa, D., Creswell, T., & Wise, K. A. (2016). First report of tar spot on corn caused by *Phyllachora maydis* in the United States. *Plant Disease*, 100(7), 1496–1497. https://doi.org/10.1094/PDIS-12-15-1506-PDN

Rush, T. A., Golan, J., McTaggart, A., Kane, C., Schneider, R. W., & Aime, C. M. (2019). Variation in the internal transcribed spacer region of *Phakopsora pachyrhizi* and implications for molecular diagnostic assays. *Plant Disease*. 103:2237–2245. https://doi.org/10.1094/PDIS-08-18-1426-RE

Sattler, S. E., & Funnell-Harris, D. L. (2013). Modifying lignin to improve bioenergy feedstocks: Strengthening the barrier against pathogens? *Frontiers in Plant Science*, 4, 44349. https://doi.org/10.3389/FPLS.2013.00070

Sattler, S. E., Funnell-Harris, D. L., & Pedersen, J. F. (2010). Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. *Plant Science*, 178(3), 229–238. https://doi.org/10.1016/J.PLANTSCI.2010.01.001

Schmelz, E. A., Kaplan, F., Huffaker, A., Dafoe, N. J., Vaughan, M. M., Ni, X., Rocca, J. R., Alborn, H. T., & Teal, P. E. (2011). Identity, regulation, and activity of inducible diterpenoid phytoalexins in maize. *Proceedings of the National Academy of Sciences*, 108(13), 5455–5460. https://doi.org/10.1073/PNAS.1014714108

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., & Ideker, T. (2003). Cytoscape: A software environment for integrated

models of biomolecular interaction networks. *Genome Research*, 13(11), 2498. https://doi.org/10.1101/GR.1239303

Silva-Hanlin, D. M. W., & Hanlin, R. T. (1998). The order Phyllachorales: Taxonomic review. *Mycoscience*, 39, 97–104. https://doi.org/10.1007/BF02461586

Simon, U. K., & Weiß, M. (2008). Intragenomic variation of fungal ribosomal genes is higher than previously thought. *Molecular Biology and Evolution*, 25(11), 2251–2254. https://doi.org/10.1093/molbev/msn188

Singh, B. K., Delgado-Baquerizo, M., Egidi, E., Guirado, E., Leach, J. E., Liu, H., & Trivedi, P. (2023). Climate change impacts on plant pathogens, food security and paths forward. *Nature Reviews Microbiology,* 21(10), 640–656. https://doi.org/10.1038/s41579-023-00900-7

Singh, R., Goodwin, S. B., Singh, R., & Goodwin, S. B. (2022). Exploring the corn microbiome: A detailed review on current knowledge, techniques, and future directions. *PhytoFrontiers*, 2(3), 158–175. https://doi.org/10.1094/PHYTOFR-04-21-0026-RVW

Smith, M. E., Douhan, G. W., & Rizzo, D. M. (2007). Intra-specific and intra-sporocarp ITS variation of ectomycorrhizal fungi as assessed by rDNA sequencing of sporocarps and pooled ectomycorrhizal roots from a Quercus woodland. *Mycorrhiza.* 18, 15–22. https://doi.org/10.1007/s00572-007-0148-z

Solórzano, J. E., Cruz, C., Arenz, B. E., Malvick, D., & Kleczewski, N. (2022). Tar spot of corn: A diagnostic and methods guide. *Plant Health Progress*, 24, 117–122. https://doi.org/10.1094/PHP-04-22-0033-DG

Solórzano, J. E., Issendorf, S. E., Drott, M. T., Check, J. C., Roggenkamp, E. M., Cruz, C. D., Kleczewski, N. M., Gongóra-Canul, C. C., & Malvick, D. K. (2023). A new and effective method to induce infection of *Phyllachora maydis* into corn for tar spot studies in controlled environments. *Plant Methods*, 19(1), 1–14. https://doi.org/10.1186/S13007-023-01052-8/

Sotiropoulos, A. G., Arango-Isaza, E., Ban, T., Barbieri, C., Bourras, S., Cowger, C., Czembor, P. C., Ben-David, R., Dinoor, A., Ellwood, S. R., Graf, J., Hatta, K., Helguera, M., Sánchez-Martín, J., McDonald, B. A., Morgounov, A. I., Müller, M. C., Shamanin, V., Shimizu, K. K., Yoshihira, T., Zbinden, H., Keller, B., & Wicker, T. (2022). Global genomic analyses of wheat powdery mildew reveal association of pathogen spread with historical human migration and trade. *Nature Communications*, 13(1), 1–14. https://doi.org/10.1038/s41467-022-31975-0

Spanu, P. D. (2012). The genomics of obligate (and nonobligate) biotrophs. *Annual Review of Phytopathology*, 50, 91–109. https://doi.org/10.1146/ANNUREV-PHYTO-081211-173024

Stergiopoulos, I., & De Wit, P. J. G. M. (2009). Fungal effector proteins. *Annual Review of Phytopathology*, 47, 233–263. https://doi.org/10.1146/ANNUREV.PHYTO.112408.132637

Swart, V., Crampton, B. G., Ridenour, J. B., Bluhm, B. H., Olivier, N. A., Meyer, J. J. M., & Berger, D. K. (2017). Complementation of ctb7 in the maize pathogen *Cercospora zeina* overcomes the lack of in vitro cercosporin production. *Molecular Plant-Microbe Interactions*, 30(9), 710–724. https://doi.org/10.1094/MPMI-03-17-0054-R

Telenko, D. E. P., Chilvers, M. I., Kleczewski, N., Smith, D. L., Byrne, A. M., DeVillez, P., Diallo, T., Higgins, R., Joos, D., Kohn, K., Lauer, J., Mueller, B., Singh, M., Widdicombe, W. D., & Williams, L. 2019. How tar spot of corn impacted hybrid yields during the 2018 Midwest epidemic. *Crop Protection Network*. https://doi.org/10.31274/cpn-20190729-002

Telenko, D. E. P., Ross, T. J., Shim, S., Wang, Q., & Singh, R. (2020). Draft genome sequence resource for *Phyllachora maydis* —An obligate pathogen that causes tar spot of corn with recent economic impacts in the United States. *Molecular Plant-Microbe Interactions*, 33(7), 884–887. https://doi.org/10.1094/MPMI-03-20-0075-A

Thireault, C., Shyu, C., Yoshida, Y., St. Aubin, B., Campos, M. L., & Howe, G. A. (2015). Repression of jasmonate signaling by a non-TIFY JAZ protein in *Arabidopsis*. *The Plant Journal,* 82(4), 669–679. https://doi.org/10.1111/TPJ.12841

Treangen, T. J., & Salzberg, S. L. (2012). Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nature Reviews. Genetics*, 13(1), 36. https://doi.org/10.1038/NRG3117

Trygestad, B. (2021). *Genetic and Genetic by Environment Effects on Tar Spot Resistance and Hybrid Yield in Maize* [Michigan State University – United States, Michigan]. ProQuest. https://ezproxy.msu.edu/login?url=https://www.proquest.com/dissertations-theses/genetic-environment-effects-on-tar-spot/docview/2572546484/se-2

United States Department of Agriculture National Agricultural Statistics Service. Quick Stats Database. Accessed 3 June 2024.

Valle-Torres, J., Ross, T. J., Plewa, D., Avellaneda, M. C., Check, J., Chilvers, M. I., Cruz, A. P., Dalla Lana, F., Groves, C., Gongora-Canul, C., Henriquez-Dole, L., Jamann, T., Kleczewski, N., Lipps, S., Malvick, D., McCoy, A. G., Mueller, D. S., Paul, P. A., Puerto, C., Schloemer, C., Raid, R. N., Robertson, A., Roggenkamp, E. M., Smith, D. L., Telenko, D. E. P., & Cruz, C. D. (2020). Tar spot: An understudied disease threatening corn production in the Americas. *Plant Disease*, 104(10), 2541–2550. https://doi.org/10.1094/PDIS-02-20-0449-FE

van de Vossenberg, B. T. L. H., Warris, S., Nguyen, H. D. T., van Gent-Pelzer, M. P. E., Joly, D. L., van de Geest, H. C., Bonants, P. J. M., Smith, D. S., Lévesque, C. A., & van der Lee, T. A. J. (2019). Comparative genomics of chytrid fungi reveal insights into the obligate biotrophic and pathogenic lifestyle of *Synchytrium endobioticum*. *Scientific Reports*, 9(1), 1–14. https://doi.org/10.1038/s41598-019-45128-9

Van Loon, L. C., & Van Strien, E. A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology,* 55(2), 85–97. https://doi.org/10.1006/PMPP.1999.0213

Veenstra, A., Moola, N., Wighard, S., Korsman, J., Christensen, S. A., Rafudeen, M. S., & Murray, S. L. (2019). Kauralexins and zealexins accumulate in sub-tropical maize lines and play a role in seedling resistance to Fusarium verticillioides. *European Journal of Plant Pathology*, 153(1), 223–237. https://doi.org/10.1007/S10658-018-1557-X

Verkley, G. J. M., Dukik, K., Renfurm, R., Göker, M., & Stielow, J. B. (2014). Novel genera and species of *Coniothyrium*-like fungi in Montagnulaceae (Ascomycota). *Persoonia:*

Molecular Phylogeny and Evolution of Fungi, 32, 25–51. https://doi.org/10.3767/003158514X679191

Wallace, H. R. (1978). The diagnosis of plant diseases of complex etiology. *Annual Review of Phytopathology*, 16, 379–402. https://doi.org/10.1146/ANNUREV.PY.16.090178.002115

Wanderlei-Silva, D., Neto, E. R., & Hanlin, R. (2003). Molecular systematics of the Phyllachorales (ascomycota, fungi) based on 18S ribosomal DNA sequences. *Brazilian Archives of Biology and Technology*, 46(3), 315–322. https://doi.org/10.1590/S1516-89132003000300002

Wang, Y., Li, T., Sun, Z., Huang, X., Yu, N., Tai, H., & Yang, Q. (2022). Comparative transcriptome meta-analysis reveals a set of genes involved in the responses to multiple pathogens in maize. *Frontiers in Plant Science*, 13. https://doi.org/10.3389/FPLS.2022.971371

Wang, J., Yan, D., Lu, Z., Liu, R., Hong, Y., Wang, Y., Peng, Z., Yu, C., Gao, Y., Liu, Z., Duan, L., & Li, R. (2024). Integration of the metabolome and transcriptome reveals diurnal variability in the effects of melatonin on salt tolerance in maize seedlings. *Journal of Plant Growth Regulation,* 43(5), 1672–1688. https://doi.org/10.1007/S00344-023-11213-7

Webster, R. W., Nicolli, C., Allen, T. W., Bish, M. D., Bissonnette, K., Check, J. C., Chilvers, M. I., Duffeck, M. R., Kleczewski, N., Luis, J. M., Mueller, B. D., Paul, P. A., Price, P. P., Robertson, A. E., Ross, T. J., Schmidt, C., Schmidt, R., Schmidt, T., Shim, S., Telenko, D. E. P., Wise, K. A., & Smith, D. L. (2023). Uncovering the environmental conditions required for *Phyllachora maydis* infection and tar spot development on corn in the United States for use as predictive models for future epidemics. *Scientific Reports*, 13, 17064. https://doi.org/10.1038/s41598-023-44338-6

White, T.J. (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protocols, a Guide to Methods and Applications, 315-322.

Wickham, H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., Fu, X., Liu, S., Bo, X., & Yu, G. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The innovation,* 2(3). https://doi.org/10.1016/j.xinn.2021.100141

Yan, S., Loladze, A., Wang, N., Sun, S., Chilvers, M. I., Olsen, M., Burgueño, J., Petroli, C. D., Molnar, T., San Vicente, F., Zhang, X., & Prasanna Boddupalli, M. (2022). Association mapping of resistance to tar spot complex in maize. *Plant Breeding*, 141(6), 745–755. https://doi.org/10.1111/PBR.13056

Yang, Q., Balint-Kurti, P., & Xu, M. (2017). Quantitative Disease Resistance: Dissection and Adoption in Maize. *Molecular Plant,* 10(3), 402–413. https://doi.org/10.1016/j.molp.2017.02.004

Yang, P., Praz, C., Li, B., Singla, J., Robert, C. A. M., Kessel, B., Scheuermann, D., Lüthi, L., Ouzunova, M., Erb, M., Krattinger, S. G., & Keller, B. (2019). Fungal resistance mediated by maize wall-associated kinase ZmWAK-RLK1 correlates with reduced benzoxazinoid content. *New Phytologist*, 221(2), 976–987. https://doi.org/10.1111/NPH.15419

Ye, W., Munemasa, S., Shinya, T., Wu, W., Ma, T., Lu, J., Kinoshita, T., Kaku, H., Shibuya, N., & Murata, Y. (2020). Stomatal immunity against fungal invasion comprises not only chitin-induced stomatal closure but also chitosan-induced guard cell death. *Proceedings of the National Academy of Sciences*, 117(34), 20932–20942. https://doi.org/10.1073/PNAS.1922319117

Zhou, J. M., & Zhang, Y. (2020). Plant Immunity: Danger Perception and Signaling. *Cell*, 181(5), 978–989. https://doi.org/10.1016/J.CELL.2020.04.028