DIAGNOSTICS, GENOMICS, AND MANAGEMENT OF *DIAPORTHE HUMULICOLA*, THE CAUSAL AGENT OF HALO BLIGHT OF HOPS

By

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ABSTRACT

This dissertation explores various aspects of the pathogen *D. humulicola*. The research tested multiple fungicide products to determine effective management strategies for the disease. Additionally, a qPCR tool was developed for the detection of *D. humulicola* in different plant materials. Lastly, a genome was produced, and a population genetics study was conducted using *D. humulciola* isolates from the eastern United States and Canada.

The first research objective of this dissertation was to determine if fungicides could be used for the treatment of halo blight. For three years field trials were conducted using 10 fungicides registered for use hop to determine their efficacy against halo blight. To validate field results, the EC_{50} value was determined for some of the active ingredient including flutriafol, tebuconazole + fluopyram, cyflufenamid, and trifloxystrobin + salicylhydroxamic acid (SHAM). A discriminatory dose was used to test the sensitivity of 206 *D. humulicola* isolates in a poison agar assay. Results showed that tebuconazole + fluopyram decreased the incidence and severity of halo blight in the field. Also, this product had EC_{50} values of 2.26×10^{-1} ppm and significantly reduced the growth of most of the isolates tested. Trifloxystrobin + SHAM decreased the presence of halo blight in the field, but some isolates were not inhibited by the discriminatory dose concentration.

The second research objective was to develop a diagnostic tool for *D. humulicola.* A quantitative polymerase chain reaction (qPCR) assay based on the translation elongation factor 1-alpha gene was developed. We assessed this assay for direct detection of *D. humulicola* in plant tissue and investigated aspects of the disease cycle through three distinct experiments: 1) detection of *D. humulicola* in hop rhizomes to determine the colonization range of the pathogen, 2) determining how quickly can *D. humulicola* be detected in hop leaves post inoculation, and 3)

monitoring the presence of *D. humulicola* in cones in a hop yard and comparing isolation methods and the assay. The limit of detection for the assay was 100 fg of DNA. The assay showed no cross-reactivity with other hop pathogens or endophytes, nor with other *Diaporthe* species tested. Detection of *D. humulicola* occurred one day after inoculation. The assay detected *D. humulicola* in both apparently healthy and diseased rhizome tissue. The assay successfully detected the pathogen in individual hop cones and inflorescences throughout the season, surpassing the culture-based method in positive identification rates.

The third objective was to produce and annotate a genome for *D. humulicola*. The draft genome for *D. humulicola*, was assembled with both long and short read sequencing. The draft genome consists of 49.82 MB assembled into 180 contigs, with a GC content of 51.2%. The genome was annotated, and 11,773 genes were predicted, including 2,752 genes with common names.

Finally, the genome was used to conduct a population genetics experiment using 64 different *D. humulicola* isolates from Michigan, New York, Minnesota, and Canada. Single nucleotide polymorphisms (SNPs) were discovered and filtered using GATK. Population Structure was determined using STRUCTURE v.2.3.4 and using the Evanno method the populations cluster into 4 or 6 different populations. Using F_{st} Minnesota isolates appear to have high levels of population differentiation when compared to the different populations. Mating types were determined for each isolate where *Mat-1-2-1* were the larger part of the population with 59 percent of the isolates having this locus. This dissertation also includes an extension fact sheet for halo blight, and disease notes for different occurrences of halo blight in the United States and Canada.

I dedicate this dissertation to the victims of the Triangle Shirtwaist Factory fire

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Thank you to my advisor Timothy Miles for taking a chance on me in undergrad and changing my life for the better! You gave me direction when I had none and will never be able to thank you enough! Your mentorship and kindness have shown me how to be a good scientist while being a kind mentor! I would like to thank the rest of my committee, Dr. Martin Chilvers, Dr. Dan Chitwood, and Dr. Mary Hausbeck for their support and encouragement through this process. Thank you to Dr. Alejo Rojas, Dr. Douglas Higgins, Dr. David Gent, Dr. Jan Byrne and Laura Miles, for being incredible collaborators! Thank you to Dr. Kerri Neugebauer, my lab manager, for being a sounding board for my troubleshooting and for letting me distract you from writing. I would like to thank Shay Szymanski for being my officemate and friend for the past 5 years, you made office work enjoyable. I couldn't have done this without your biting wit and penchant for discourse! I am thankful for the rest of the Miles lab for their support and friendship: Dr. Joel Abbey, Dr. Nancy Sharma, Adam Adaskaveg, Lexi Heger, Sukhdeep Singh, Roger Sysak, and Samantha Thompson. I would like to thank my friends: Dr. Jordan Brock, Dr. Scott Teresi, Erin Kramer, and Mallory St. Clair for being amazing friends and being my support system throughout my PhD! I would also like to thank my parents, siblings, and my chosen family: Micheal Picazo, Bryan Sierra-Rivera, and Alix Rachman for their constant support!

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CHAPTER 1: HALO BLIGHT OF HOP CAUSED BY DIAPORTHE HUMULICOLA: A DIAGNOSTIC GUIDE

Abstract

Diaporthe humulicola, causing halo blight of hops is one of the most impactful pathogens of hop in the Eastern United States and Canada. The pathogen, when left untreated, can cause approximately 50 percent yield loss due to shatter upon harvesting. The disease has a wide variety of symptoms on the cones and leaves, that are not consistent between infections. The pathogen is slow growing on artificial media and can take up to three weeks for the development of pycnidia. This diagnostic guide outlines the current list of symptoms and signs for halo blight, and the current methods for accurate diagnosis of the disease.

Hosts

In North America, *Diaporthe humulicola* can infect common hop (*Humulus lupulus*) causing halo blight of hop on cultivars: Chinook, Cascade, Nugget, AlphAroma, Newport, Saaz, Willamette, Fuggle, Galena, Crystal, and Centennial (Allan-Perkins et al. 2020; Hatlen et al. 2022; Higgins et al. 2021). The pathogen is not known to naturally cause disease on other species of hops or on other species of plant. The pathogen can colonize and produce pycnidia on sterilized alfalfa stems but there is no evidence that the pathogen can cause disease on a living alfalfa plant (Higgins et al. 2021; Sharma et al. 2023). There has been a single report of the pathogen infecting avocado, a genome was produced for this isolate, but the genome sequences do not match other *D. humulicola* genes in the NCBI database (Fostvedt-Austin et al. 2022). Thus, this isolate should not be used for *D. humulicola* diagnostics as it is likely this pathogen is a new species of *Diaporthe*.

Disease

halo blight of hop

Pathogen

Currently the fungus *D. humulicola,* is the only species that causes halo blight of hop.

Taxonomy

Domain *Eukaryota,* Kingdom *Fungi*, Subkingdom *Dikarya* , Phylum *Ascomycota*, Subdivision *Pezizomycotina,* Class *Sordariomycetes,* Family *Diaporhaceae,* Genus *Diaporthe* , Scientific name *Diaporthe humulicola* (accessed from mycobank , Crous et al. 2004)

Symptoms and Signs

D. humulicola can infect and cause symptoms on the leaves and cones (also called strobiles) of the hop plant. The foliar symptom are lesions surrounded by a light green to bright yellow chlorotic halo, (Allan-Perkins et al. 2020; Higgins et al. 2021) but it may not present in all leaf infections (Figure 1.1). Cone symptoms are varied but are generally described as desiccation of the hop cone (Higgins et al. 2021). The desiccation can be on singular bracts of the cone but can also affect whole regions of the hop cone (Figure 1.2). The pathogen can also cause girdling of the hop cone where the middle of the cone is desiccated (Figure 1.2B). The desiccation will then lead to shatter as the cones are harvested and sorted (Figure 1.7) (Higgins et al. 2021). The desiccated lesions on the cones (Figure 1.2A) and the leaves (Figure 1.1A) can develop pycnidia, a sign of the pathogen that appear as small black dots on the surface of desiccated tissue. (Higgins et al. 2021). The mycelia of *D. humulicola* can also colonize the strig (hop cone connective tissue), with white mycelia (Figure 1.3B).

Life Cycle

The pathogen is thought to overwinter on discarded hop materials as pycnidia and then once conditions are favorable the pycnidia will germinate and produce conidia (Figure 1.4) (Hatlen et al. 2023). The conidia will then be able to infect the hop leaves leading to leaf lesions that may be surrounded by a chlorotic halo (Figure 1.1) (Allan-Perkins et al. 2020; Hatlen et al. 2023; Higgins et al. 2021). The leaf lesions can then lead to the production of pycnidia in the desiccated lesions (Figure 1.1A) (Higgins et al. 2021). Finally, the pycnidia can produce conidia to disperse to other hop materials.

The hop burrs can harbor the disease, but little (slight discoloration of the burr) to no symptoms are attributed to the infections of the burr (Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease). In later stages of cone development, the hop cone can become desiccated where individual bracts or whole regions of the cone can display symptoms (Figure 1.2) (Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease); Higgins et al. 2021). Pycnidia can then develop on the desiccated hop cones (Figure 1.2A) (Higgins et al. 2021). *D. humulicola* can colonize the strig (hop cone connective tissue) and can produce a layer of white mycelia on the surface of the strig (Figure 1.3) (Hatlen et al. 2023). When infected with *Diaporthe humulicola* the cone has been reported to give off a “cheese like” aroma (Hatlen et al. 2025).

The pathogen is also able to colonize dried bines that have been left in the field after harvest and produce large amounts of pycnidia (Hatlen et al. 2023) (Figure 1.4). This is thought to be the over wintering stage of the fungi. The pathogen is also able to colonize the rhizomes of the hop plant but there does not to be symptoms attached to the colonization, and the purpose of

the colonization has not been determined (Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease)) (Figure 1.8).

Geographic Distribution

The pathogen has been detected in the eastern United States in New York, Michigan, Minnesota, Connecticut and Indiana (Allan-Perkins et al. 2020; Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease); Havill et al. 2023; Higgins et al. 2021; Sharma et al. 2023). The pathogen has been reported in Quebec, Canada as well (Hatlen et al. 2022). As far as the authors are aware, it has not been reported in major hop production regions such as the Pacific Northwest of the United States or Germany (Agricultural Research Service (USDA) 2025).

Pathogen Isolation

The pathogen can be isolated from symptomatic cone tissue by surface sterilizing the hop cone by submerging the whole cone in a 10% commercial bleach solution for up to 5 minutes while continually agitating the cone, and then two rounds of submerging and vigorously stirring the cone in different containers of deionized water for up to 5 minutes each round (Higgins et al. 2021). The cone should then be dried on sterilized paper towels. Then four small pieces of the leaf lesion margins should be removed from the cone and transferred on to potato dextrose agar (PDA) amended with ampicillin, streptomycin, or penicillin at each cardinal direction (Hatlen et al. 2022; Higgins et al. 2021). Plates should be monitored daily for white stringy mycelial growth. (Figure 1.5) The mycelia can look as if there are two layers of the fungal growth, where at the center there is a dense white to grey mass of mycelia, while at the outer margins of the fungal growth will be less dense and media will be visible between the stands (Figure 1.5) (Allan-Perkins et al. 2020; Higgins et al. 2021). The mycelia can then be transferred to a new

PDA plate to obtain a pure culture. This can also be done with leaf lesions, but the surface sterilization should only be done in 1-minute intervals, otherwise the procedure stays the same. (Allan-Perkins et al. 2020; Higgins et al. 2021).

The pathogen can also be directly isolated from pycnidia growing on hop leaves and cones. Where a sterilized insect needle fixed to a glass pipette tip can be used to remove the pycnidia from the plant materials and place the pycnidia into 75 µl of sterile deionized water. The solution can then be spread onto a fresh PDA plate amended with antibiotics. After 24 hours isolates can then be transferred to obtain a pure culture (Choi et al. 1999; Higgins et al. 2021).

Pathogen Storage

The pathogen can be stored for long term storage as pycnidia and conidia. The pycnidia and conidia can be stored using a glycerol storage solution made up of 200 ml of glycerol, 0.4 g of yeast extract, 1.0 g of malt extract, 0.4 g of Dextrose, 0.2 g of dibasic K_2HPO_4, and 800 ml of Deionized water. (Higgins et al. 2021; Miles et al. 2011). The solution should be mixed thoroughly and then 1.5 ml of the solution should be pipetted into 2 ml cryovials. Cryovials with the solution should then be sterilized in an autoclave. Oozing pycnidia and mycelia should be removed from a pure culture and placed into the cryovials. The cryovials then should be tightly capped and placed into a freezing container, filled with isopropyl alcohol and placed in a -80° C freezer for two days to achieve a freezing rate of –1° C/minute. This step is optional but is recommended. Then the cryovials can be removed from the freezing container and stored at -80° C (Miles et al. 2011). To revive the samples, the cryovials should be thawed on ice and a sterile toothpick should be used to remove fungal tissue from the cryovial. The tissue should be placed onto a fresh PDA plate. Isolates that have not used the freezing container have been revived after

4 years of storage and isolates stored using the freezing container have been revived 6 years post storage.

Pathogen Identification

There are a few characteristics of the pathogen that make it hard to detect on foliar and floral tissue. The halo of chlorotic tissue is not always visible on leaf lesions and thus the lesions can be mistaken for infections caused by other hop pathogens such as *Pseudoperonospora humuli* or *Colletotrichum fioriniae* which both produce small lesions that do not have a chlorotic halo (Hatlen et al. 2023; Johnson et al. 2009). Minor diseases of hop can also be mistaken for halo blight of hop. Phoma wilt (*Phoma herbarum, Phoma herbarum, f. humuli, Phoma aliena, Boeremia exigua*) lesions are almost identical to halo blight leaf lesions as they both have chlorotic halos and a concentric ring appearance, so isolation is required for identification (Mahaffee et al. 2009a). Cercospora leaf spot can be caused by *Cercospora cantuariensis and Cercospora 'sp.* Q' and can cause lesions on the surface of the hop leaf that are dark grey, but unlike *D. humulicola* the conidia of *Cercospora* are produced on conidiophores on the surface of the hop leaf (Pereria et al. 2023, Radisek et al. 2009). The lesions and desiccation on the hop cone can mimic other diseases and disorders of hop such as fusarium tip blight, Alternaria cone disorder, or powdery mildew (Mshaffee et al. 2009; Pethybridge et al. 2001a; Pethybridge et al. 2001b;). Therefore, direct signs of the pathogen are needed for proper identification of the disease. The pycnidia that can develop on the surface of the hop cone and leaf lesions may not develop, so direct isolation of the pathogen may be needed (Figure 1.1A and 1.2A) (Higgins et al. 2021). The pathogen is slow growing and can take up to 30 days to produce pycnidia (Higgins et al. 2021). The time to develop pycnidia can be shortened to ~10 days by growing the culture with sterilized alfalfa stems imbedded in PDA (Higgins et al. 2021; Sharma et al. 2023). The

pycnidia from *D. humulicola* are brown to dark brown (Figure 1.4 and Figure 1.6C), ampulliform, eustoma, and have a singular ostiole (Figure 1.5B-F) (Higgins et al. 2021). The size of the pycnidia is different across nutrient sources, where pycnidia can be up to 267 µm in diameter on leaves and 1,250 µm in diameter on half strength PDA (Allan-Perkins et al. 2020). The alpha conidia produced from the pycnidia are unicellular, hyaline, and cylindrical to ellipsoid and 11.9 ± 2.0 by 4.1 ± 0.5 µm in size, no beta conidia have been described at this time (Figure 1.5a and Figure 1.6E-F) (Higgins et al. 2021).

Molecular Identification

D. humulicola DNA can be extracted from cultures using column-based DNA extraction kits. *D. humulicola* does not produce a profuse amount of mycelia, so multiple plates of the fungus may be required. DNA from leaf tissue can be extracted using an extraction kit such as Zymo Quick-DNA Plant/Seed kit with the recommended amendment of adding beta-mercaptoethanol to the Genomic Lysis Buffer to a final dilution of 0.5% (v/v). Extracting DNA from whole hop cones can be challenging due to the high amounts of secondary metabolites that the hop cone produces, that can become an oily layer on the top of columns. To avoid the oily layer DNA extractions kits using magnets such as the MagMAX plant DNA kit or Omega BIO-TEK Mag-Bind® Plant DNA DS 96 Kit with 40 mM dithiothreitol and 2.5% PVPP40 added to the lysis buffer should be used (Szymanski et al. 2023; Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease)).

The pathogen has been identified using sequence similarity to TEF, ITS, Beta-tub, histone 3, and calmodulin (Higgins et al. 2021; Sharma et al. 2023; Hatlen et al. 2022). Primer sequences and accession numbers for sequences are listed in Table 1.1. A TaqMan assay based on the Tef region that can also be used for the detection of *D. humulicola* from singular hop

cones and leaves. The assay can consistently detect *D. humulicola* DNA down to 0.0001 ng/µl and has been shown to detect *D. humulicola* from singular burrs, cones, and leaves, and small amounts of rhizome tissue. The assay can constantly detect the pathogen in leaves in less than a week after infection. (Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease)). The assay uses an internal plant control, that uses DNA from hop plants. The regents needed for the qPCR assay from both pure culture and from plant tissue are detailed in Tables 1.2 and 1.3. The assay should be run with the following protocol: Lid temp 105° C, 1 cycle of 95° C for 3 minutes, then 40 cycles of 95° C for 10 seconds, 60° C for 30 seconds, and ending each cycle with a plate read step.

Pathogenicity Tests

Pathogenicity of *D. humulicola* isolates can be tested using detached leaves or using whole plants in a greenhouse/misting chamber. Cultivars Cascade, Chinook, or Centennial have been shown to be sensitive to the pathogen and can be used in both detached leaves and whole plant experiments (Hatlen et al. 2022; Higgins et al. 2021;Sharma et al. 2023).

Leaves should be obtained from the 3-5th node of a hop plant grown in a greenhouse. Then a sterile filter paper and cotton ball should be lightly moistened with sterilized deionized water, and then placed in a Petri dishes (100 × 15 mm). Leaves should be placed into the petri dish with the adaxial side up (Higgins et al. 2021). Conidia can be collected from *D. humulicola* cultures in about 21 days if grown on PDA, but this time can be shortened to approximability 10 days by adding autoclaved sterilized alfalfa stems embedded into the media (Higgins et al. 2021, Sharma et al. 2023). In the absence of alfalfa stems 21-day old cultures should be flooded with sterilized deionized water with Tween 20 (polysorbate-20) (1%) until the whole plate is covered. Then a flame sterilized glass rod should be used to agitate the surface of the culture to dislodge

pycnidia and get conidia into the water solution. (Higgins et al. 2021). For cultures embedded with alfalfa stems, 40 ml of sterilized deionized water with Tween 20 (polysorbate-20) (1%) should be added to a 50 ml conical tube. Alfalfa stems covered by *D. humulicola* pycnidia should be carefully removed from the agar using flame sterilized tweezers and placed into the water filled conical tubes. The tubes should then be tightly capped and shaken to get the conidia into the solution (Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease)). For both methods the conidial mixture should then be poured from the petri dish or conical tube into a beaker that is sitting in ice. The solution should then be filtered with two layers of cheese cloth to remove chunks of agar and larger pycnidia that could clog a spray bottle. The solution should have a concentration of 1×10^5 - 2×10^6 conidia/ml (Hatlen et al. 2022; Higgins et al. 2021; Sharma et al. 2023). The mixture should then either be sprayed on to the surface of the hop leave using an aerosol-based spray system (Preval Sprayer; Nakoma, Bridgeview, IL) or a manual spray bottle with a fine mist nozzle. The petri dishes should then be closed and then sealed with parafilm. The leaves should then be incubated at 20° C with a 14-h photoperiod (Higgins et al. 2021). Disease symptoms should be monitored daily, as poor-quality hop leaves will develop symptoms at different rates than healthy leaves. Symptoms may vary and it is likely that the infections on detached leaves will not mimic foliar infections on whole plants. Leaves with low quality seem to become a dark brown necrotic color after 6 days that is not seen in the field. (Hatlen et al. 2025 (unpublished, submitted for review in Plant Disease)).

To test whole plants the conidial solution should be sprayed on to the leaves until run off. The hop plants should then be covered with a plastic bag to achieve roughly 100% relative humidity and then should be stored in a greenhouse set to 20° C with a 14-h photoperiod. (Hatlen et al. 2022; Havill et al. 2023) Plants should be water regularly if in a greenhouse. Hop plants

can also be stored in a misting chamber with alternating 25° C light/18°C dark with a 16 h photoperiod, with an hour of mist daily (Sharma et al. 2023). Plants both in a greenhouse or misting chamber should display symptoms in anywhere from 7 to 28 days post inoculation.

Disease Management

There are currently no fungicides that are labeled in the United States for the treatment of *D. humulicola.* In Canada in 2023, there was an emergency registration for the fungicide flutriafol (Fullback 125 SC, FMC; Philadelphia, PA) (Davidson 2023). Hop products registered for use on powdery mildew of hops belonging to the groups demethylation inhibitors, succinate dehydrogenase inhibitors, and quinone outside inhibitors have been shown to reduce halo blight disease severity (Hatlen et al. 2025). The organic product *Bacillus mycoides* isolate J (FRAC group P6) has also been shown to decrease halo blight severity (Hatlen et al. 2022).

D. humulicola can colonized diseased and damaged hop tissue, so maintaining a clean hopyard is imperative for halo blight control. Crown scratching, a process in which the top 1.9 - 5.1 inches of the hop crown are removed during the start of the hop season can decrease the incidence of powdery and downy mildew and the resulting senescent tissue (Gent et al. 2008, Sirrine et al. 2022).

TABLES

Table 1.1. List of primers and probes used for *D. humulicola* identification.

Primer or probe	Sequence	Reference
Sequencing primers		
EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn 1999
EF1-986R	TACTTGAAGGAACCCTTACC	Carbone and Kohn 1999
ITS1	TCCTAGGTGAACCTGCGG	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik 1997
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson 1995
CYLH3F	AGGTCCACTGGTGGCAAG	Crous et al. 2004
H3-1b	GCGGGCGAGCTGGATGTCCTT	Glass and Donaldson 1995
CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	Carbone and Kohn 1999
CAL-737R	CATCTTTCTGGCCATCATGG	Carbone and Kohn 1999
CAL-235F	TTCAAGGAGGCCTTCTCCCTCTT	Quaedvlieg et al. 2012
Cal2Rd	TG(A/G)TC(A/C/G/T)GCCTC(A/G/T)CGGATCATCTC	Groenewald et al. 2013
LR0R	ACCCGCTGAACTTAAGC	Rehner and Samuels 1994
LR7	TACTACCACCAAGATCT	Vilgalys and Hester 1990
frpb2-5f	GA(T/C)GA(T/C)(A/C)G(A/T)GATCA(T/C)TT(T/C)GG	Liu et al. 1999
RPB2-7cR	CCCAT(A/G)GCTTG(T/C)TT(A/G)CCCAT	Liu et al. 1999
qPCR TEF assay		
DHTEF2F	AATCATCATCGTGCGGGTGT	Hatlen et al 2025 (unpublished)
DHTEF2R	CTCAGCGGCTGTGTAGATGAC	Hatlen et al 2025 (unpublished)
DHTefProbe2	TGTGTTGAACAGGGCAGTGGGATTGGTAGT	Hatlen et al 2025 (unpublished)
Internal plant control		
FMPl2b	GCGTGGACCTGGAATGACTA	Bilodeau et al. 2014
FMPl3b	AGGTTGTATTAAAGTTTCGATCG	Bilodeau et al. 2014
Plant CAL Red probe	CTTTTATTATCACTTCCGGTACTGGCAGG	Bilodeau et al. 2014

[B] Nucleotides in (/) notation denote degenerate oligonucleotide sequences

Table 1.2. Reagent list for master mix for TEF based TaqMan qPCR assay for DNA samples extracted from pure culture.

Reagent	**Starting concentration**	**1x 20 µl RXN**
ToughMix QuantaBio	2X	10 µl
Molecular grade water		5.4
Teffwd2 5'- AATCATCATCGTGCGGGTGT-3'	10 µM	0.5 µl
Tefrev2 5'-CTCAGCGGCTGTGTAGATGAC-3'	10 µM	0.5 µl
Tefprobe2 5'-[HEX]TGTGTTGAACAGGGCAGTGGGATTGGTAGT[BHQ-1]-3'	10 µM	0.4 µl
IPCF 5'-GCGTGGACCTGGAATGACTA-3'	10 µM	0.4 µl
IPCR 5'-AGGTTGTATTAAAGTTTCGATCG-3'	10 µM	0.4 µl
IPCP5'-[CALFluorRed610]CTTTTATTATCACTTCCGGTACTGGCAGG[BHQ2]-3'	10 µM	0.4 µl
Hop DNA free of *D. humulicola*	10 ng/ml	1 µl
Sample DNA	variable	1 µl
Total		20 µl

Table 1.3. Reagent list for master mix for TEF based TaqMan qPCR assay for DNA samples extracted from hop tissue.

Reagent	**Starting concentration**	**1x 20 µl RXN**
ToughMix QuantaBio	2X	10 µl
Molecular grade water		6.4
TEffwd2 5'- AATCATCATCGTGCGGGTGT-3'	10 µM	0.5 µl
Tefrev2 5'-CTCAGCGGCTGTGTAGATGAC-3'	10 µM	0.5 µl
Tefprobe2 5'-[HEX]TGTGTTGAACAGGGCAGTGGGATTGGTAGT[BHQ-1]-3'	10 µM	0.4 µl
IPCF 5'-GCGTGGACCTGGAATGACTA-3'	10 µM	0.4 µl
IPCR 5'-AGGTTGTATTAAAGTTTCGATCG-3'	10 µM	0.4 µl
IPCP5'-[CALFluorRed610]CTTTTATTATCACTTCCGGTACTGGCAGG[BHQ2]-3'	10 µM	0.4 µl
Sample DNA	variable	1 µl
Total		20 µl

FIGURES

Figure 1.1. Images of leaf lesions caused by *Diaporthe humulicola*. A) Photo of leaf with multiple lesions and insect damage. The lesion at the tip of the leaf has pycnidia on the desiccated leaf tissue. B) Leaf with irregular shaped lesion and a bright yellow halo surround the lesions. C) Leaf with chlorotic spots caused by younger infections of *D. humulicola*. D) Leaf with irregularly shaped lesions with a chlorotic halo on the right-hand side of the leaf, next to lesions of unknow origin. E) Young hop leaf with lesions caused by halo blight, while infected tissue can be bleached, some tissue maybe dark brown. F) Large leaf lesion caused by *D. humulicola*, lesion may spread over lag areas of the hop leaf.

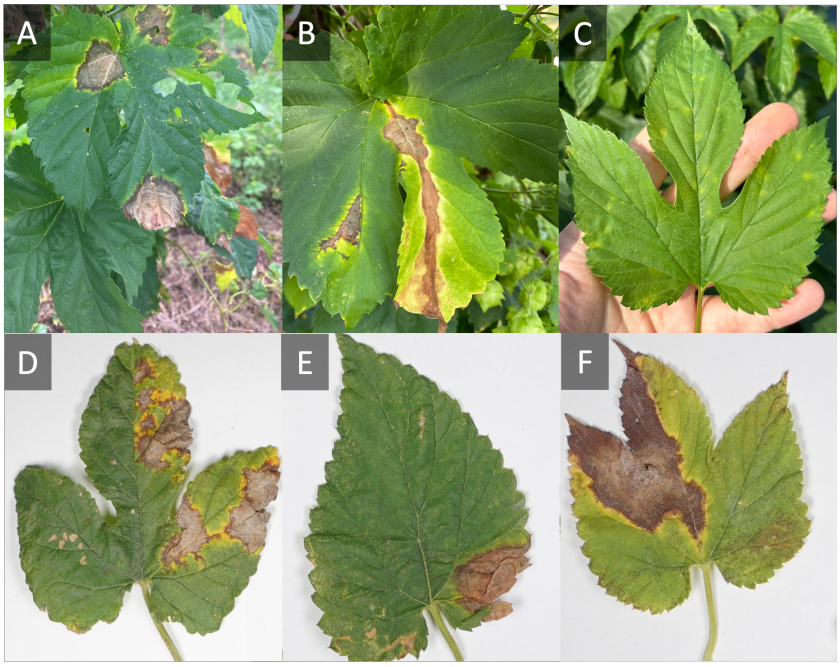


Figure 1.2. Hop cones with desiccation caused by *D. humulicola*. A) Hop cone with a small amounts of infected bracts and pycnidia present on the bracts. B) Hop cone with a infected ring of leaves that cause the hop cone to appear girdled. C) Hop cone where the tip of the cone is blighted, and the upper half of the cone is discolored with small amounts of necrosis. D) Hop cone with a few necrotic bracts. E) Hop cone almost completely desiccated with black pycnidia on the desiccated tissue. F) Hop cone with the upper half of the cone desiccated. G) Hop cone with the left-hand side desiccated, where some bracts still have patches of green tissue. H) Hop cone with girdling and desiccation at the tip, and some necrotic spots on the top of the hop cone.

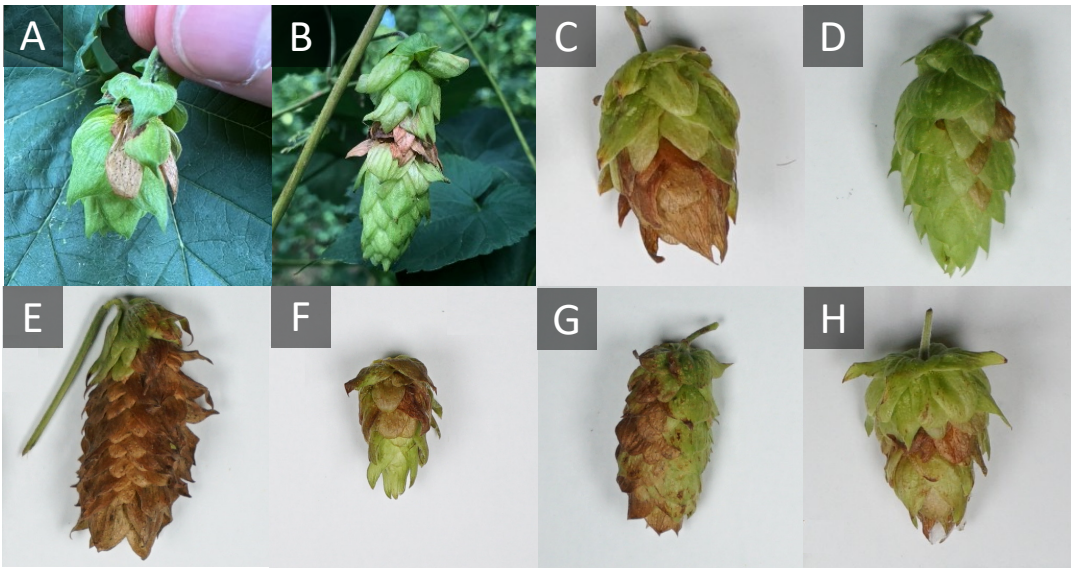


Figure 1.3. Hop cone cross sections. A) Healthy hop cone, B) Hop cone infected with *D. humulicola* with white mycelia in the center of the strig.

Figure 1.4. Images of hop bines that were left in the field during the winter A) pycnidia rupturing through woody hop bines. B) Conidial ooze produced by pycnidia that has become dried. C) Zoomed out image of bine with conidial ooze. D) Ruptured pycnidia with conidial ooze that is dried and a newer ooze that is still wet.

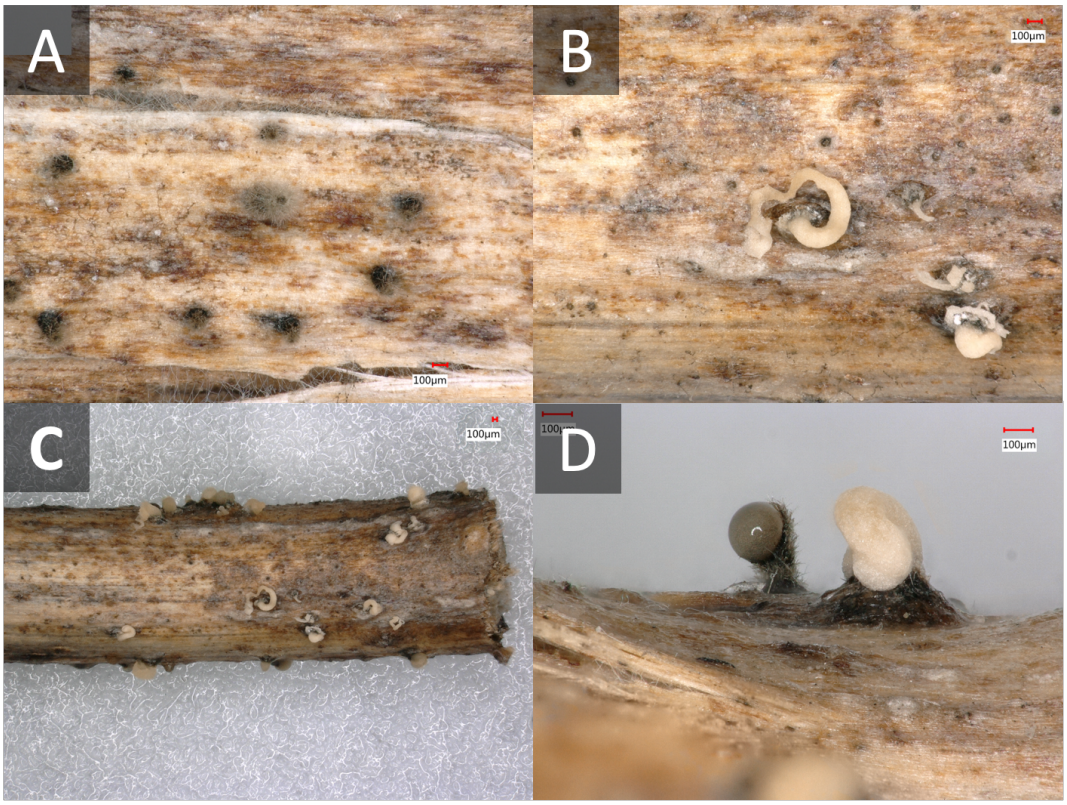


Figure 1.5. Scanning electron microscopy images of *Diaporthe humulicola*. A) Image of conidia. B) Image of a singular pycnidia with fully developed conidia. C) Multiple pycnidia next to each other. D) Pycnidia a mass of mycelia protruding from the hop bine.

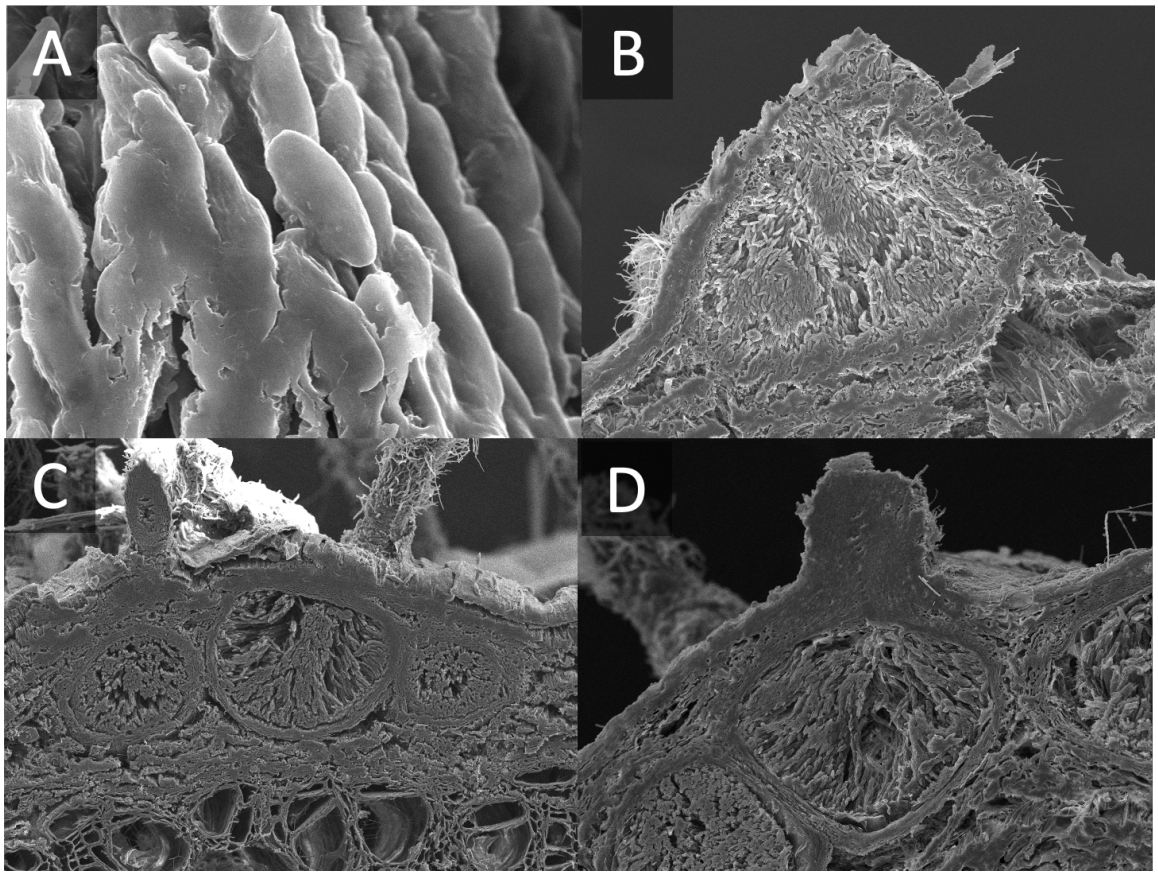


Figure 1.6. Photos of petri dishes with *Diaporthe humulicola* and photos of conidia for Diaporthe humulicola. A-B) *D. humulicola* after 6 days of growth on potato dextrose agar. C) pycnidia in ~30 day old culture. D) Oozing pycnidia of *D. humulicola* on alfalfa stems embedded in PDA. E) conidia from *D. humulicola*. F) Non-germinated and germinated conidia from *D. humulicola*.

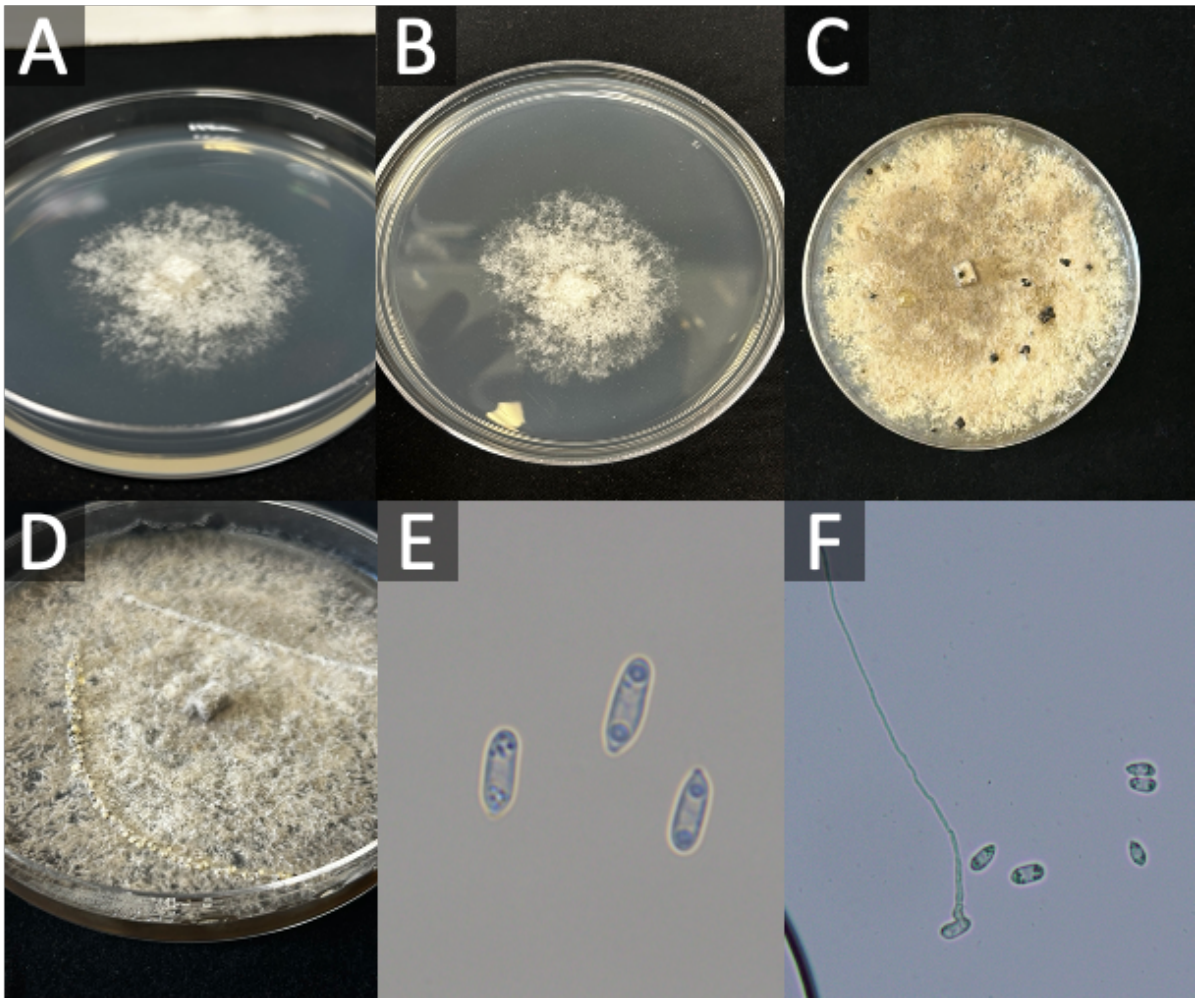
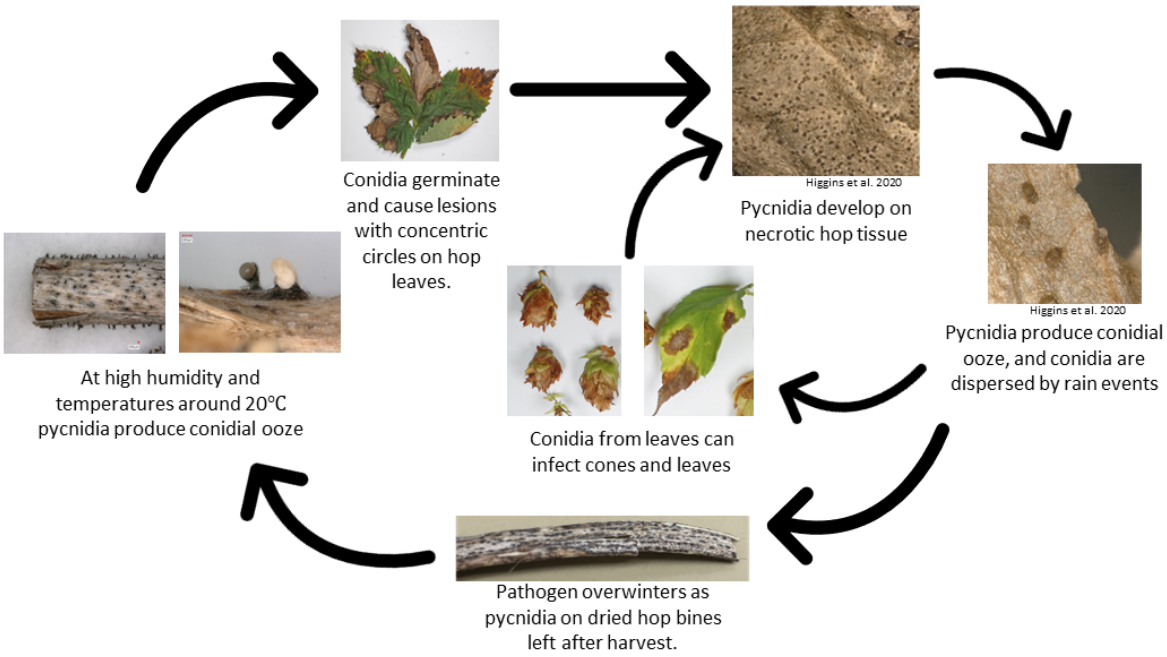


Figure 1.7. Image of hop waste after harvest A) Pile with limited disease B) Waste pile where plants were heavily infected with *Diaporthe humulicola.*

Figure 1.8. Life cycle for *Diaporthe humulicola.*



LITERATURE CITED

Agricultural Research Service (USDA). 2025. Fungal databases: U.S. culture collection. Beltsville (MD): United States Department of Agriculture. [accessed 2025 Jan 9]. https://fungi.ars.usda.gov.

Allan-Perkins, E., Li, D., Schultes, N., Yavuz, S., and LaMondia, J. 2020. The identification of a new species, *Diaporthe humulicola*, a pathogen causing diaporthe leaf spot on common hop. Plant Dis. 104:2377-2390.

Bilodeau, G. J., Martin, F. N., Coffey, M. D., and Blomquist, C. L. 2014. Development of a multiplex assay for genus-and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. Phytopathology 104:733-748.

Carbone, I. and Kohn L. M. 1999. A method for designing primer sets of speciation studies in filamentous ascomycetes. Mycologia 91:553-556.

Choi, Y.-W., Hyde, K. D., and Ho, W. H. 1999. Single spore isolation of fungi. Fungal Divers. 3:29-38.

Crous, P. W., Groenewald, J. Z., Risède, J.-M., Simoneau, P., and Hywel-Jones, N. L. 2004. *Calonectria* species and their Cylindrocladiumanamorphs: Species with sphaeropedunculate vesicles. Stud. Mycol. 50:415-430.

Crous, P. W., Gams, W., Stalpers J. A., Robert, V and Stegehuis, G. 2004. MycoBank: an online initiative to launch mycology into the 21st century. Studies in Mycology 50: 19–22.

Davidson K. 2023. Emergency registration granted for halo blight on hops. The Grower. https://thegrower.org/news/emergency-registration-granted-halo-blight-hops.

Fostvedt Austin, J. E. 2022. Epidemiology and community analysis of stem end rot-associated fungal pathogens and their impact on postharvest disease of commercial avocado (*Persea americana*). PhD thesis. University of Reading.

Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61:1323-1330.

Groenewald, J. Z., Nakashima, C., Nishikawa, J., Shin, H.-D., Park, J.-H., Jama, A. N., Groenewald, M., Braun, U., and Crous, P. W. 2013. Species concepts in *Cercospora*: Spotting the weeds among the roses. Stud. Mycol. 75:115-170.

Hatlen, R.J., Higgins, D.S., Venne, J., Rojas, J.A., Hausbeck M.K., and Miles, T.D. 2022. First report of halo blight of hop (*Humulus lupulus*) caused by *Diaporthe humulicola* in Quebec, Canada. Plant Dis. 106:6: 1750.

Gent, D. G., Nelson M. E., George A. E., Grove, G. G., Mahaffee, W. F., Ocamb, C. M., Barbour J. D., Peetz. A., and Truchek W. W. 2008. A Decade of hop powdery mildew in the Pacific Northwest. PHP, https://apsjournals.apsnet.org/doi/pdf/10.1094/PHP-2008-0314-01-RV.

Hatlen, R. J., Gillett, J. M., Neugebauer, K. A., Sysak, R. W., and Miles, T. D. 2022. Evaluation of fungicides for control of downy mildew and halo blight, 2021. Plant Disease Management Reports. 16.

Hatlen, R.J., Adiar, N., Wismen, M., Sysak, R., Rojas, A., Gent, D., and Miles, T.D. 2023b. Development of Integrated qPCR diagnostic tools to determine the life cycle of *Diaporthe humulicola* in hop (*Humulus lupulus*). Phytopathology 113, S3.1-S3.197.

Hatlen, R. J., Swift, R. M., Miles, L. A., Byrne, J. M., and Miles T. D. 2023a. First report of *Colletotrichum fioriniae* infecting hop (*Humulus lupulus*) in Michigan. Plant Dis. 107:10:3280.

Hatlen, R. J., Szymanski, S. L., Adair, N. L., Fan, Q., Panwar. P., Sysak, R., Miles, L. A., Higgins, D. S., Rojas, J. A., Gent, D. H., and Miles, T.D., 2025 Development of a molecular diagnostic tool for *Diaporthe humulicola*, the causal agent of halo blight of hop. Unpublished, submitted for review in Plant Disease.

Havill, J. S., Hatlen R. J., Muehlbauer G. J., and Miles, T. D. 2023. First report of halo blight on hop (Humulus lupulus) caused by *Diaporthe humulicola* in Minnesota. Plant Dis. 107:8: 2523.

Higgins, D. S., Hatlen R. J., Byrne, J. M., Sakalidis, M. L., Miles, T. D., and Hausbeck, M. K. 2021a. Etiology of halo blight in Michigan hopyards. Plant Dis. 105:859-872.

Liu, Y. J., Wheelen, S., and Hall, B. D. 1999. Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerase II subunit. Mol. Biol. Evol. 16:1799-1808.

Mahaffee, W. F., Engelhard, B., Gent, D. H., and Grove, G. G. 2009. Powdery mildew. Pages 25-30 in: Compendium of Hop Diseases and Pests. W. F. Mahaffee, S. J., Pethybridge, and D. H. Gent, eds. American Phytopathological Society, St. Paul, MN.

Miles, T. D., Day, B., and Schilder, A. C. 2011. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. Mol. Plant Pathol. 12:463-477.

Mahaffee, W. F., Pethybridge, S. J., and Gent, D. H., eds. 2009a. Pages 37-38 in: Compendium of Hop Diseases and Pests. American Phytopathological Society, St. Paul, MN, U.S.A.

O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylo. Evol. 7:103-116.

Pereira, C., Ferreira, B. W., and Barreto, R. W. 2023. First Report of *Cercospora apii* sensu lato 'sp. Q' Leaf Spot on Hop in Brazil. Plant Dis. 107.

Pethybridge, S. J., Hay, F. S., Wilson, C. R., Sherriff, L.J ., and Leggett, G. W. 2001. First report of *Fusarium crookwellense* causing tip blight on cones of hop. Plant Dis 85:1208.

Pethybridge, S. J., Hay, F. S., Wilson, C. R., Sherriff, L. J., and Leggett, G.W. 2001a. First Report of Infection of Hop Cones by *Alternaria alternata* in Australia. Plant Dis. 85:804.

Radisek, S., Leskosek, G., Jakse, J., and Javornik, B., 2009. Occurrence of Cercospora cantuariensis on hop in Austria and Slovenia. Plant Pathol. J. 58:400.

Sharma, S., Strickland, D. A, Hay, F., and Pethybrige S. J. 2023. First report of halo blight on hop caused by *Diaporthe humulicola* in New York. Plant Dis. 107: 216.

Sirrine, R., Miles T., and Lizotte E. 2022. Pruning for disease management and yield benefits in hops. Michigan State University Extension. https://www.canr.msu.edu/news/pruning-for-disease-management-and-yield-benefits-in-hops.

Szymanski, S., Longley, R., Hatlen, R. J., Heger, L., Sharma, N., Bonito, G., Miles, T. 2023. The Blueberry Fruit Mycobiome Varies by Tissue Type and Fungicide Treatment. Phytobiomes 7:2: 208-219.

Vilgalys, R., and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172:4238-4246.

White, T. J., Bruns, T., Lee, J., and Taylor, J.1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR protocols: A guide to methods and applications. Vol. 18. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.

CHAPTER 2: CHEMICAL MANAGEMENT STRATEGIES FOR HALO BLIGHT OF HOP AND IN VITRO SENSITIVITY OF *DIAPORTHE HUMULICOLA* POPULATIONS TO VARIOUS FUNGICIDE CLASSES

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Abstract

Halo blight of hop, caused by *Diaporthe humulicola*, has increased in eastern North America since 2018. When left untreated, the disease can cause yield loss ranging from 17-56%. Currently, there are no fungicides registered for use on halo blight of hop. From 2020 to 2022 field trials were conducted using 10 fungicides registered for use on powdery and downy mildew of hop to determine their efficacy against halo blight. To validate field results, the EC_{50} value was determined for each active ingredient including flutriafol, tebuconazole + fluopyram, cyflufenamid, and trifloxystrobin + salicylhydroxamic acid (SHAM). Each fungicide tested had an EC_{50} value less than 50 ppm. A discriminatory dose was used to test the sensitivity of 206 *D. humulicola* isolates collected from the eastern U.S. and Canada in a poison agar assay. Results showed that tebuconazole + fluopyram decreased the incidence and severity of halo blight in the field. Also, this fungicide combination had EC_{50} values of 2.26×10^{-1} ppm and significantly reduced the growth of most of the isolates tested. Trifloxystrobin + SHAM decreased the presence of halo blight in the field trial, but some isolates were less sensitive in discriminatory dose testing. Our results show that fungicides in FRAC groups 3, 7, and 11 were the most effective to control halo blight. Analyses of field trials showed a positive correlation between the severity of early season downy mildew infections and late season halo blight infections.

Introduction

Hop (*Humulus lupulus* L.) is a dioecious bine that is grown for its hop cones (i.e., female strobili) that provide alpha and beta acids used as bittering and aroma additives in beer production (Lafontaine 2019). The hop industry in Michigan has grown significantly in the past 15 years, peaking in 2018 when 291 ha were harvested (Hop Growers of America 2019 Statistical Report). Michigan is the fourth largest producer of hops in the U.S., following the Pacific Northwest (PNW) states of Washington, Oregon, and Idaho (Hop Growers of America 2020). In contrast to the PNW, Michigan's hop growing season has frequent rainfall and high relative humidity, favoring hop downy mildew (DM) and halo blight, caused by *Pseudoperonospora humuli* and *Diaporthe humulicola*, respectively (Higgins et al. 2021a).

Halo blight of hop is an emerging disease in the eastern regions of the U.S. and Canada (Allan-Perkins et al. 2020; Foster et al. 2024; Hatlen et al. 2021; Havill et al. 2023; Higgins et al. 2021a; Sharma et al. 2023) affecting popular cultivars including 'Chinook' and 'Centennial' (Allan-Perkins et al. 2020; Higgins et al. 2021a; and USDA 2021). Symptoms include irregular necrotic lesions with a chlorotic halo and premature desiccation of cones. Affected hop cones shatter during the sorting processes resulting in yield losses up to 56% (Higgins et al. 2021a). Pycnidia are produced on desiccated leaves and cones leading to repeated infections during the season which may persist on hop stems remaining in the field after harvest (Hatlen et al. 2023; Higgins et al. 2021a). *D. humulicola* may also colonize hop tissue damaged by mechanical pruning (Hatlen et al. 2023). There are no fungicides registered for the control of *D. humulicola* in the U.S. (Lizotte et al. 2023) but in Canada the fungicide flutriafol (Fullback 125 SC, FMC; Philadelphia, PA) has an emergency registration (Davidson 2023).

In Michigan, DM and powdery mildew (PM) caused by *Podosphaera macularis* are managed on hops each season with fungicides. *P. humuli* is endemic, occurs annually in the state and sporulates on young shoots arising from the overwintering hop crown (Higgins et al. 2021b). If these early infections are not controlled, lesions can develop on the leaves and cones leading to desiccation (Lizotte et al. 2023). *P. macularis* infects the leaves and cones causing small necrotic lesions (Gent et al. 2008).

Fungicides registered for PM of hop include systemic broad-spectrum fungicides listed by the Fungicide Resistance Action Committee (FRAC) and belonging in groups 3, 7, and 11. Some of these fungicides are registered for control of *Diaporthe* spp. in other crops (Lizotte et al. 2023; Wise et al. 2023). Trifloxystrobin, pyraclostrobin, and famoxadone are quinone outside inhibitor (Q_oI) fungicides (FRAC group 11) that inhibit fungal respiration by blocking the electron transport chain at the Qo site in the cytochrome *bc1* enzyme complex (Gisi et al. 2000). Trifloxystrobin and pyraclostrobin alone or as a premix (pyraclostrobin + boscalid) decreased *D. humulicola* growth in a poison agar assay but have not been evaluated in a hopyard (Allan-Perkins et al. 2020). Fungicides in FRAC group 11 reduced phomopsis stem canker caused by *Diaporthe helianthi* in sunflower (Dangal et al. 2022) and contributed to an increase in soybean plant stand and health when applied to seeds infected with *Diaporthe* sp. (Haafke 2021). Boscalid, a succinate dehydrogenase inhibitor (SDHI) that targets the complex II electron transport chain and tricarboxylic acid cycle (Gudmestad and Arabiat 2013), is registered to control hop PM but maximum residue level issues associated with its use limits export of the crop to key international markets (Walsh 2018). Fluopyram + tebuconazole (Luna Experience, Bayer CropScience, FRAC groups 3 and 7) is also registered for hop PM. The fungicides flutriafol and tebuconazole (FRAC group 3) are demethylation inhibitors (DMI) of 24-

methylenedihydrolanosterol, a precursor of ergosterol in fungi (Yin et al. 2009). Fungicides in FRAC group 3 reduced *Diaporthe asparagi* (anamorph: *Phomopsis asparagi*) growth in a poison agar study (Shi et al. 2019) and provided a moderate level of control against *Diaporthe ampelina* (anamorph: *Phomopsis viticola*) during dry years (Wilcox 2017).

Bacillus mycoides isolate J (FRAC group P6) decreased halo blight severity (Hatlen et al. 2022) and elicited systemic acquired resistance in sugar beet (Bargabus et al. 2003). Quinoxyfen, a FRAC group 13 fungicide, is used for PM control in hops and appears to inhibit G-proteins in early cell signaling but this has not been confirmed (Wheeler et al. 2003). Metrafenone (FRAC group 50) disrupts normal actin, myosin, and fimbrin function of PM species (Opalski et al. 2006). Cyflufenamid and cymoxanil (FRAC groups U6 and 27) are used to control hop PM but their mode of action is not clear (Haramoto et al. 2006; Ziogas and Davidse 1987). Folpet is a multi-site contact fungicide (FRAC group M04), and its mode of action is thought to be associated with a reaction to thiols (Gordon 2010).

While there are no fungicides currently registered in the U.S. to protect hop from *D. humulicola*, products registered for use on the crop against DM or PM could decrease the incidence and severity of halo blight. Thus, our objectives were to: 1) assess fungicides for their efficacy against halo blight in the field, 2) use a poison agar assay to validate field results and determine an EC_{50} value for each fungicide, and 3) use EC_{50} values to establish a discriminatory dose and screen *D. humulicola* isolates from the eastern U.S. and Canada to determine if there are regional differences in sensitivity. Also, of interest was determining if there is a correlation between DM and halo blight severity.

Methods

D. humulicola isolate collection and storage

Three *D. humulicola* isolates obtained from commercial hop yards in Michigan (3M and 25M), Quebec, Canada (CD6C) and a research hop yard in Connecticut (CT01) (Allan-Perkins et al. 2020; Hatlen et al. 2021; Higgins et al. 2021a) were used to determine the EC_{50} value of each fungicide. All isolates in this study were obtained from tissue by immersing symptomatic cones or leaves in a 10% bleach for 30 s and then rinsing in deionized water for 30 s twice (Higgins et al. 2021a). Tissue from the margin of necrotic and healthy cone or leaf tissue was plated onto potato dextrose agar (PDA) amended with 1% ampicillin and grown for 7 days at 20°C. Cultures that were morphologically similar to *Diaporthe* spp. were hyphal tipped onto PDA. Pure cultures were grown for approximately 30 days; the pycnidia and mycelia were stored in a glycerol-salt solution (Miles et al. 2011) at -80°C.

D. humulicola fungicide field trials

This study was initiated in 2020 in a 4-year-old 'Centennial' hop yard at Michigan State University's Plant Pathology Research Farm located in East Lansing, MI (42.6892037, -84.4850172) and was conducted through 2022. Plants were spaced 1.2 m apart within a row with 2.4 m between rows. Using a randomized complete block (RCB) design, each fungicide treatment (Table 2.1) was assigned to 4 blocks consisting of four adjacent hop plants within a row; treatments were re-randomized each year. Each year, the plants were treated weekly with foliar applications of fluopicolide (Presidio, FRAC 43, Valent, San Ramon, CA) at a rate of 280 mL/ha prior to the start of the experiment to prevent DM foliar infections when the plants were approximately 15.2 cm in height. Treatments were applied using a RYOBI ONE+ 18V Cordless Battery 4 Gal. Backpack Chemical Sprayer with the adjustable nozzle (Ryobi, Australia.) Spray

volume was calculated at ~374 l/ha. In 2020, treatments were applied on 7, 16, 23 May, 3, 12, 21 June, and 3, 11, 18, 25 July. In 2021, treatments were applied on 27 May, 3, 10, 23, 30 June, 8, 15, 22 July, and 5 August. In 2022, treatments were applied on 25 May, 10, 17, 24 June, 1, 8, 15, 22, 29 July, and 11, 17 August. The inoculum for this experiment was collected from D. humulicola isolate 3m. Thirty-day-old PDA plates with oozing pycnidia were flooded with water, and a flame-sterilized glass rod was used to dislodge the embedded pycnidia. The mixture was then pooled into a beaker resting on ice. Subsequently, the mixture was poured through cheesecloth to remove large chunks of agar, and spore concentration was determined using a hemocytometer. Plants were inoculated on 2, 9, 16, 23 June (2020), on 7, 14, 21, 28 June (2021), and 7, 14, 21, 28 June (2022) with a *D. humulicola* conidial suspension ($5.5*10^5$-$7.7*10^5$ conidia/mL water + 1% Tween 20) of isolate 3M that was sprayed until runoff using a 7.6 l hand pump sprayer (2020-2021) or a RYOBI ONE+ 18V Cordless Battery 2 Gal. Chemical Sprayer (2022) (Ryobi Ltd., Australia.). The innermost two plants in each block were evaluated for halo blight weekly from 14 July to 11 August (2020 and 2021) and 6 July to 10 August (2022) by arbitrarily selecting 25 leaves and estimating the leaf tissue (%) with symptoms. Severity was visually assessed in the field as the area (%) of the leaf surface covered by a lesion. Incidence was defined as the number of leaves that exhibited at least one lesion. These ratings were used to produce area under the disease progress curve (AUDPC) values for disease severity and incidence using the trapezoidal method (Madden et al. 2007). For each year's data set in both the final day ratings and AUDPC, normalcy was determined using the Shapiro-Wilk test ($p > 0.5$). If normalcy was not achieved, the data were transformed. For the final day incidence ratings for 2020, the data underwent a squared transformation. For the disease severity assessment for 2020 and 2022 AUDPC values, a square root transformation was required. The disease severity data

for all years were transformed using a λ value obtained from a Box-Cox analysis (λ = 0.10; y = [x^{λ} – 1)/λ]) (Sakia 1992). For data comparing AUDPC values for all years, the Kolmogorov-Smirnov Test was used to test normalcy ($p > 0.5$). When incidence data were combined, the 2020 data were excluded because values were less than 50% of the other years. Variance was assessed using Levene's test ($p > 0.05$). Fisher's protected least significant differences (LSDs) at $p < 0.05$ were used for simple effect comparisons of treatments by year. To compare treatments to the untreated control (UTC), percent reduction was calculated for both incidence and severity in the final day ratings and AUDPC using the equation ((1- (Average treatment value/ Average UTC value)) * 100). Non-transformed data and ANOVA statistics are reported, and statistics were analyzed in Statgraphics Centurion XVI (The Plains, Virginia, USA).

***P. humuli* and *D. humulicola* fungicide field trials**

From 2021 to 2022, a separate field trial was established to determine fungicide efficacy against DM and halo blight. Twenty treatments were included with some being repeated each year (Table 2.5). Treatments consisted of different fungicides applied in alternation or a single fungicide applied through the growing season starting on 27 May (2021) or 25 May (2022) (Table 2.5). Treatments were arranged in an RCB design with four replicates, using four adjacent hop plants within a row as a single replicate. The hop plants were inoculated with *P. humuli* sporangia collected from a commercial hop yard in southwest MI (3.2×10^5-5.0×10^5 sporangia/mL) on 21 May and 2 June (2021) and 23 June (2022). Plants were also inoculated on 21 June (2021, 2022) with a conidial suspension of *D. humulicola* from isolate 3M (4.2×10^5-6.3×10^5 conidia/mL). Twenty-five randomly chosen leaves were visually assessed for DM (Hatlen et al. 2020) on 7 July (2021) and 7 June (2022). On 12 July (2021) and 8 August (2022), 25 randomly chosen leaves were visually assessed for halo blight as previously described in the

D. humulicola fungicide field trials experiment. Twenty-one different correlation models were tested using Statgraphics Centurion XVI.

Fungicide EC_{50} values for *D. humulicola*

The EC_{50} value, the expected concentration in which 50% of mycelial growth is inhibited by a fungicide, was determined for metrafenone, cyflufenamid, flutriafol, quinoxyfen, tebuconazole + fluopyram, folpet, or trifloxystrobin with salicylhydroxamic acid (SHAM), using isolates 3M, 25M, CD6C, and CT01 in a mycelial growth assay (Wang et al. 2017). Prior to autoclaving, the media included 19.50 g of PDA (Difco) and 500 mL of deionized water. Commercial grade fungicides were mixed with sterilized deionized water to produce a concentrated solution of 1,000 ppm. After autoclaving, the medium was allowed to cool to 50°C and then either 50, 5, 0.5, 0, 0.05, 0.005, or 0.0005 mL of liquid medium was removed from the flask and replaced by the same amount of concentrated fungicide solution for a final concentration of 100, 10, 1, 0, 0.1, 0.01, or 0.001 ppm. For the medium amended with trifloxystrobin, SHAM was added at a concentration of 100 µg/mL to suppress the alternative oxidase pathway of the fungus (Floyd and Malvick 2022). The fungicide amended medium was cooled in a laminar flow hood before being stored at 4°C and was used within seven days.

Isolates were grown on PDA amended with 1% ampicillin and streptomycin for 7 days. Mycelial plugs (5 mm) were taken from the edge of the actively growing culture using a flame sterilized corkborer and then plated mycelial side down onto fungicide amended media. Plates were sealed using Parafilm (Amcor, Zurich, Switzerland) prior to incubation. Plates were incubated at 20^{o} C under a 14 hr/10 hr light/dark period. Isolates were measured after 7 days using CD-6”AX Mitutoyo digital calipers (Mitutoyo Kawasaki, Kanagawa, Japan). The diameter of each fungal colony was measured twice on a different axis, and the average diameter growth

and EC_{50} values calculated according to Miles et al. (2012). (Figure 2.1) This experiment was conducted three times.

***D. humulicola* discriminatory dose experiments**

Cyflufenamid, tebuconazole + fluopyram, trifloxystrobin, and flutriafol were used to screen 206 *D. humulicola* isolates from Michigan (n = 118), New York (n = 47), Indiana (n = 2), Arkansas (n = 4), Minnesota (n = 11), and Canada (n = 24). Isolates were identified using morphology and/or Sanger sequencing of the TEF region (data not shown). The dose of each fungicide was determined from the EC_{50} value experiment conducted according to Weber and Hahn (2011). For tebuconazole + fluopyram and trifloxystrobin, 1 ppm of active ingredient was used, while cyflufenamid and flutriafol used 10 ppm. Fungicide amended media were prepared as previously described. A 7-day old 5-mm mycelial plug of *D. humulicola* was placed at the center of a fungicide amended media plate in three replicates for each isolate and fungicide. Plates were incubated, and colony diameter calculated after 7 days as previously described. An ANOVA was used to determine if there were differences in the growth of isolates exposed to each fungicide based on the isolate's geographical origin. Each data set was tested for normalcy using the Kolmogorov-Smirnov Test ($p > 0.05$). The data for tebuconazole + fluopyram were transformed using the natural log. Variance was checked using Levene's test ($p > 0.05$). Fisher's protected least significant differences (LSDs) at $p < 0.05$ were used to make simple effect comparisons of mycelial growth by location. Untransformed data and ANOVA statistics are reported, and all statistics were conducted using Statgraphics Centurion XVI.

Results

Fungicide field trials

Disease incidence for the UTC ranged from 61 to 78% and was highest in 2020 (Table 2.2). At the final assessment, disease severity in the UTC was also highest in 2020 (70.3%) and $\leq$ 38% in subsequent years (Table 2.2). Based on the final disease incidence (2020-2022) and severity (2020, 2021), all treatments were similar to the UTC. For the final disease severity rating in 2022, the UTC was low (19.4%) and all treatments, except pyraclostrobin + boscalid and folpet, were effective in comparison. Trifloxystrobin resulted in the lowest disease severity (6.3%) and was significantly more effective than cyflufenamid, famoxadone + cymoxanil, and folpet (Table 2.2).

Although AUDPC incidence data were similar among the treatments and the UTC in 2020, AUDPC values for severity indicated that all treatments except *Bacillus mycoides* isolate J were significantly lower than the UTC. According to 2021 incidence and severity AUDPC values, all treatments were significantly different from the UTC. Differences among the AUDPC values were noted among the fungicide treatments for incidence but not for severity. Famoxadone + cymoxanil, tebuconazole + fluopyram, and quinoxyfen were significantly more effective than cyflufenamid according to AUDPC values for disease incidence. In 2022, according to AUDPC values for disease incidence, trifloxystrobin, tebuconazole + fluopyram, *Bacillus mycoides* isolate J, and folpet treatments were significantly more effective than the UTC, with reductions of 17.3% to 37.0%. According to the AUDPC for severity, all treatments were significantly more effective than the UTC, with reductions of 38.0% to 63.2% (Table 2.3).

For the combined AUPDC incidence data for 2020 and 2022 all treatments except pyraclostrobin + boscalid and cyflufenamid were significantly more effective than the UTC.

Trifloxystrobin was the most effective treatment with a reduction of 27.9% compared to the UTC. According to the AUDPC data for the 2020-2022 disease severity, all treatments were significantly more effective than the UTC; trifloxystrobin was the most effective treatment. (Table 2.3).

EC_{50} values varied across *D. humulicola* type isolates

Four isolates (3M, 25M, CD6C, CT01) were used to determine the discriminatory doses. A discriminatory dose of 1 ppm was chosen for trifloxystrobin with SHAM (average EC_{50} value = $8.68*10^{-5}$ ppm) and tebuconazole + fluopyram (average EC_{50} value = $2.26*10^{-1}$ ppm). A discriminatory dose of 10 ppm was chosen for flutriafol (average EC_{50} value = 3.39 ppm) and cyflufenamid (average EC_{50} value = 6.93 ppm). Folpet, quinoxyfen, and metrafenone all had average EC_{50} values above 7 ppm and were not used in the discriminatory dose assay (Figure 2.2).

Significant variation was observed using discriminatory dose assays

The mean growth associated with trifloxystrobin was 24.58 mm for the 206 isolates; 50.5% of the isolates exceeded the mean growth while the remainder did not. Mean growth for fluopyram + tebuconazole was 10.23 mm and nearly 40% of the isolates exceeded this. The mean growth associated with flutriafol was 17.25 mm with 48.1% of isolates surpassing the mean growth. The mean growth associated with cyflufenamid was 12.58 mm and 38.3% of isolates grew above the mean. (Figure 2.3).

Differences in fungal growth based on the origin of the isolate were observed (Table 2.4). The isolates from Canada had significantly less growth on media amended with tebuconazole + fluopyram when compared to isolates from the other locations. Isolates from Arkansas had significantly more growth on the medium amended with tebuconazole + fluopyram. The

Arkansas isolates had the least amount of growth on the medium with flutriafol and were significantly different from other locations' isolates. There were no statistical differences based on regionality for the growth of *D. humulicola* when exposed to cyflufenamid. All locations were statistically similar except Arkansas when grown in the presence of trifloxystrobin + SHAM. The Arkansas isolate had a mean growth nearly twice that of isolates from other locations when grown on media amended with trifloxystrobin + SHAM. (Table 2.4).

Correlations were observed between higher levels of *P. humuli* and *D. humulicola*

Of the 26 different regression correlation models, 21 fit the data. Seventeen models had a positive correlation, while four had a negative correlation. The highest correlation values were attributed to the square root Y model that had a correlation value of 0.5676 and a R^2 of 32.22%. The linear model provided a correlation value of 0.5546 with an R^2 of 30.76% (Table 2.6).

Discussion

Hop growers in Michigan, the eastern U.S., and eastern Canada are managing *D. humulicola*, a relatively new pathogen that decreases yield and changes the chemical profile of the hop cone (Higgins et al. 2021a; Hatlen et al. 2023). Hop cultivars preferred by the industry appear to be susceptible to *D. humulicola* (Allen-Perkins et al. 2020; Higgins et al. 2021a). New hop cultivars can take more than 15 years to bring to commercialization (Haunold 1981) and no natural resistance has been found, so fungicides are currently relied on to protect the crop. Since *D. humulicola* was recently described, fungicides registered for use on hop do not include this pathogen. Amending fungicide labels to add a pathogen can be costly so registrants may be reluctant to incur the expense for a relatively small specialty crop. Efficacy data are usually needed to establish application rates and intervals for a new pathogen which could indicate a different use pattern than what is labeled requiring residue data in order to establish a new

tolerance. Thus, we chose to focus on fungicides currently labeled for hop for use against PM to determine their efficacy against halo blight. In our study, fungicides that effectively reduced *D. humulicola*'s growth and the disease incidence and severity in research field plots included trifloxystrobin, tebuconazole + fluopyram, and flutriafol.

While complete control of halo blight was not achieved, trifloxystrobin was the most effective fungicide included in our field trials. A reduction in lesion size should influence the amount of pycnidia produced by the pathogen, resulting in reduced secondary inoculum (Higgins et al. 2021a). Trifloxystrobin is a FRAC 11 fungicide and has a high risk for pathogen resistance (FRAC 2024); pathogen resistance to this group of fungicides has not been reported for hop (FRAC 2020). Pyraclostrobin + boscalid (FRAC 11, 7) was not as effective as trifloxystrobin (FRAC 11) against halo blight. FRAC 3 or DMI fungicides including tebuconazole + fluopyram and flutriafol also reduced halo blight incidence and severity in the field. DMI fungicides have been shown to be effective against other *Diaporthe* species (Shi et al. 2019). While the biological fungicide, *Bacillus mycoides* isolate J, was not consistently effective against halo blight, it could be an option for organic hops production when integrated with cultural disease management strategies or less susceptible germplasm (Shrestha and Hausbeck 2021; Shrestha and Hausbeck 2023). Famoxadone + cymoxanil and folpet appeared to reduce the incidence and severity of halo blight in the field, but the treatments were not tested every year of this study, therefore, more testing is needed to determine their efficacy.

Cyflufenamid, a PM-specific fungicide registered for hops (Claassen et al. 2022; Haramoto et al. 2005), significantly reduced AUPDC disease severity caused by *D. humulicola* compared to the UTC in each year of our study. These results are consistent with those of *Diaporthe citri*, the causal agent of melanose of citrus, which had reduced pathogen growth

when exposed to high rates (6 ppm) of cyflufenamid (Haramoto et al. 2005). Metrafenone, another PM specific fungicide for hops, reduced disease severity compared to the UTC in our trial. In addition to protection against PM, it is effective against *Cladobotryum* sp. and *Trichoderma* sp. in edible mushroom production (Carrasco et al. 2017; Luković et al. 2020). While this fungicide could be used to treat halo blight, residues may persist in the dried hops (Anastassiadou et al. 2021).

Quinoxyfen provided moderate control of halo blight each year even though it is a PM fungicide and not expected to control other fungi (Wheeler et al. 2003). The efficacy of quinoxyfen in our halo blight trials may be a result of good PM control increasing the overall plant health and decreasing its susceptibility for infection by other pathogens including *D. humulicola* (Hampson and Coombes 1985). Cyflufenamid, and metrafenone are also PM fungicides that performed well in our field trials. In the poison agar assay, quinoxyfen and metrafenone had an average EC_{50} value of $1.68*10^{10}$ ppm and $2.64*10^{4}$ ppm and did not limit *D. humulicola* growth. These fungicides limit PM of hop and the resulting senescent tissue potentially limiting *D. humulicola* (Higgins et al. 2021a). Perhaps these fungicides target another aspect of *D. humulicola*'s life cycle that is not represented in a poison agar assay. Cyflufenamid resulted in one isolate with an EC_{50} value above 5ppm, while all other isolates had an EC_{50} under 2 ppm. Cyflufenamid had some activity on *D. humulicola* which is consistent with reports on *Diaporthe citri,* where high amounts of the fungicide can inhibit non-PM causing fungi (Haramoto et al. 2005). The AUDPC incidence and severity for folpet is significantly lower than the UTC, but in the poison agar assay folpet had a higher EC_{50} value than other treatments. The applications of flopet could reduce other diseases such as PM, thus limiting the amount of senescent tissue that *D. humulicola* can colonize.

Differences in fungicide sensitivity among the isolates included in our study were associated with region which may be due to the length of time that hops have been produced in that area. When 206 isolates were evaluated for fungicide sensitivity, isolates from Canada had reduced growth compared to the U.S. isolates when exposed to tebuconazole + fluopyram. Tebuconazole + fluopyram is currently not registered in Canada for hop and its use in the U.S. may select less sensitive *D. humulicola* isolates (Lucas et al. 2015). For flutriafol, Arkansas was the only location that was statistically different from other locations and had the lowest average colony diameter of the 6 regions. Arkansas hop production is relatively new (<10 years) compared to other hop producing states resulting in less time for pathogen selection based on fungicide use (*Personal Communication: Dr. J. Alejandro Rojas*).

Diaporthe isolates have a varied response when grown on trifloxystrobin amended media. Trifloxystrobin (FRAC 11) is at high risk of selecting resistant mutants (FRAC 2024). While the G143A point mutation in cytochrome b is known to cause complete resistance to FRAC 11 chemicals, other mutations exist that cause partial resistance (Gisi et al. 2002). The G137R and the F129L point mutations in cytochrome b can cause partial resistance to Q_oI fungicides in other genera of plant pathogens (Kim et al. 2003; Miao et al. 2020). Currently, none of these mutations have been reported in *D. humulicola*. According to the field trials and the EC_{50} values, trifloxystrobin may be the best choice to control halo blight, but when looking at the results from the discriminatory dose assay it appears that portions of the population may be less sensitive to trifloxystrobin. Differences in sensitivity to trifloxystrobin are not consistent at a single location as isolates from the same hop yard can have different sensitivities (Allen-Perkins et al. 2020). Isolates from Arkansas and Indiana displayed growth that was statistically greater compared to isolates from other regions when exposed to trifloxystrobin, however there was a low number of

samples (n = 2 to 4) tested from each region. Populations of *D. humulicola* should be regularly screened to monitor the prevalence of resistant isolates. Trifloxystrobin is registered for use on hop as a premix (Luna Sensation, fluopryam + trifloxystrobin, 7/11 Bayer; Leverkusen, Germany) and Flint Extra (trifloxystrobin) for PM control in the U.S. Thus, it is possible that *Diaporthe* isolates have been exposed to this chemical and evolved reduced sensitivity. Cytochrome b of *D. humulicola* needs to be explored to search for point mutations or gene structural differences that could be responsible for this loss of sensitivity to Q_oI fungicides (Fernández-Ortuño et al. 2007).

D. humulicola colonizes dead hop tissue and produces pycnidia (Hatlen et al. 2023). In Michigan, hop DM is endemic to the region (Higgins et al. 2021b) and occurs each season. DM symptoms may be first observed in late spring and if not controlled can cause localized leaf lesions (Johnson et al. 2009). A meta-analysis of fungicide trials for DM and halo blight revealed a weak correlation between high DM severity and high halo blight severity. DM leaf infections occur at a range of 15-29^{o}C, while *Diaporthe* grows rapidly from 20-25^{o}C (Higgins et al. 2021a; Johnson et al. 2009). An overlap in growing ranges could be the reason that there is a correlation between the two pathogens. The formation of DM lesions occurs concurrently as *D. humulicola* conidia are released from overwintering pycnidia located on the desiccated bines and leftover hop material from the previous year (Hatlen et al. 2023; Johnson et al. 2009).

Cultural methods that can reduce early season infections include removing diseased tissue that *D. humulicola* can colonize. Chemically removing hop tissue early in the season can reduce PM foliar infections by 30% (Probst et al. 2016; Truechek et al. 2001) and could also potentially limit halo blight. Crown scratching, a process in which the top 4.8 – 12.9 cm of the hop crown are removed during the initiation of shoot development reduces the incidence of PM

and DM and the resulting senescent tissue (Gent et al. 2008; Sirrine et al. 2022). *D. humulicola* overwinters on hop diseased tissue and bines that have been desiccated after harvest (Hatlen et al. 2023). Removing hop tissue postharvest reduces tissue *D. humulicola* can colonize thus removing inoculum sources for the next season (Lizotte et al. 2020).

Trifloxystrobin, tebuconazole + fluopyram, and flutriafol were effective against halo blight in our study and can be used in alternation to limit halo blight disease and mitigate the development of pathogen resistance. Future studies of *Diaporthe* spp., could investigate if variation in the cytochrome b gene explains why *D. humulicola* varies in its sensitivity to FRAC group 11 fungicides. Hop growers may consider limiting early season infections from *P. humuli* and *P. macularis* to prevent desiccated hop tissue and reduce the amount of pycnidia that develop thereby reducing secondary inoculum.

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TABLES

Table 2.1 Active ingredients for fungicide field trials for halo blight of hop from 2020-2022.

Active ingredient	FRAC group[A]	Rate/HA	Trade name	Manufacturer
Trifloxystrobin	11	0.14 g	Flint Extra	Bayer Crop Science
Flutriafol	3	0.51 l	Rhyme	FMC Corporation
Bacillus mycoides isolate J	P6	0.81 g/l	Lifegard WG	Certis Biologicals
Quinoxyfen	13	0.60 l	Quintec	Gowan Company
Metrafenone	U8	1.12 l	Vivando	BASF Corporation
Tebuconazole + fluopyram	7/3	0.73 l	Luna Experience	Bayer Crop Science
Pyraclostrobin + boscalid	7/11	2.60 g/l	Pristine	BASF Corporation
Cyflufenamid	U6	0.58 ml	Torino	Corteva Agriscience
Famoxadone + cymoxanil	11	0.56 kg	Tanos	Corteva Agriscience
Folpet	M04	3.36 kg	Folpan WDG	Adama Agricultural Solutions

[A]FRAC 2024

Table 2.2. Final assessment for halo blight disease incidence and severity caused by *Diaporthe humulicola* of 'Centennial' hop when treated with a foliar fungicide from 2020-2022 in a field study.

	Disease Incidence and Severity (%)					
	2020[A]		2021[B]		2022[C]	
Active ingredient	**Incidence[D]**	**Severity[E]**	**Incidence[D]**	**Severity[E]**	**Incidence[D]**	**Severity[E]**
Untreated control	78	70.3	61	37.6	66	19.4 C
Trifloxystrobin	72	56.8	46	26.0	43	6.3 A
Flutriafol	73	52.3	48	25.9	50	11.7 AB
Bacillus mycoides isolate J	66	52.9	39	27.8	51	11.2 AB
Quinoxyfen	63	56.4	43	22.4	58	11.8 AB
Metrafenone	62	57.8	41	23.5	59	10.4 AB
Tebuconazole + fluopyram	70	64.3	44	22.8	57	11.2 AB
Pyraclostrobin + boscalid	76	56.5	40	28.0	66	14.4 BC
Cyflufenamid	76	56.1	51	25.1	55	12.3 B
Famoxadone + cymoxanil[F]	-	-	46	24.4	62	12.9 B
Folpet[G]	-	-	-	-	53	15.5 BC
Treatment *p* value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	0.01

[A] Data were collected on 25 August 2020 and analyzed with a one-way ANOVA.
[B] Data were collected on 11 August 2021 and analyzed with a one-way ANOVA.
[C] Data were collected on 10 August 2022 and analyzed with a one-way ANOVA. Fisher's LSD was conducted and letters next to severity values denote significance ($p < 0.05$). Values with the same letter are not significantly different.
[D] Disease incidence was estimated by the number of leaves with 1 or more lesion from 25 arbitrarily selected leaves.
[E] Disease severity was estimated by visual rating of 25 arbitrarily selected hop leaves and the percentage of the leaf covered by lesions estimated and averaged.
[F] This fungicide was not tested in 2020.
[G] This fungicide was not tested in 2020 or 2021.

Table 2.3. Area under the disease progress curve (AUDPC) for halo blight of hop incidence and severity ratings for hop cv. 'Centennial' when treated with a foliar fungicide from 2020-2022.

	AUDPC Disease Incidence and Severity (%)[A]							
	2020 + 2022[B]		2020 – 2022[B]		2020[C]		2022[C]	
Active ingredient	**Incidence**	**Severity**	**Incidence**	**Severity**	**Incidence**	**Severity**	**Incidence**	**Severity**
Untreated	2264.5 C	785.8 C	1991.5	1039.0 C	824.5 C	519.4 B	2537.5 C	799.2 C
Trifloxystrobin	1632.0 A	472.1 A	1666.0	719.4 A	546.5 AB	402.5 A	1598.0 A	294.5 A
Flutriafol	1913.8 B	531.5 B	1666.0	686.3 A	593.0 AB	438.5 A	2161.5 BC	469.5 B
Bacillus mycoides isolate J	1948.8 B	562.7 B	1956.5	886.6 BC	574.0 AB	396.0 A	1941.0 AB	405.5 B
Quinoxyfen	1959.0 B	524.8 B	1697.5	708.2 A	478.0 A	404.7 A	2220.5 BC	461.5 B
Metrafenone	1977.3 B	538.1 B	1687.0	765.2 AB	569.5 AB	433.3 A	2267.5 BC	415.7 B
Tebuconazole + fluopyram	1995.5 B	532.8 B	1904.0	783.8 AB	479.5 A	408.5 A	2087.0 B	405.9 B
Pyraclostrobin + boscalid	2041.3 BC	553.0 B	1911.0	751.8 AB	538.5 AB	411.5 A	2171.5 BC	495.8 B
Cyflufenamid	2106.8 BC	532.9 B	2030.0	733.1 A	620.0 B	439.6 A	2183.5 BC	426.0 B
Famoxadone + cymoxanil[D]	-	-	-	-	487.0 A	446.0 A	2261.0 BC	431.9 B
Folpet[E]	-	-	-	-	-	-	2098.0 B	471.6 B
Treatment *p* value	0.0042	<0.001	0.1203	0.0017	0.0001	0.041	0.011	0.0017
Year *p* value	<0.001	<0.001	-	-	-	-	-	-
Year x treatment *p* value	0.1002	0.001	-	-	-	-	-	-

[A] Disease incidence was estimated by how many leaves included a lesion out of 25 randomly selected leaves. Disease severity was estimated by visual rating of hop leaves, where the percentage of the leaf covered by lesions was estimated and then averaged. Values were analyzed using transformed data. Data reported here was back transformed.

[B] Incidence data for 2020 + 2022 and severity data for 2020 - 2022 were analyzed with a multi-factor ANOVA. Fisher's LSD was conducted and letters next to severity values denote significance ($p < 0.05$). Values with the same letter are not significantly different.

[C] Incidence and severity data for 2020 through 2022 was analyzed with a one-way ANOVA. Fisher's LSD was conducted and letters next to severity values denote significance ($p < 0.05$). Values with the same letter are not significantly different.

[D] This fungicide was not tested in 2020.

[E] This fungicide was not tested in 2020 or 2021.

Table 2.4. Mycelial growth of *Diaporthe humulicola* when exposed to different fungicides by region. Letters denote a mean separation test that was conducted on growth diameter.

	Diameter Growth (mm)[B]			
Locations[A]	**Tebuconazole + Fluopyram**	**Flutriafol**	**Cyflufenamid**	**Trifloxystrobin + SHAM**
Canada	07.26 A	14.80 BC	11.48	23.65 A
Michigan	09.86 B	17.48 BC	12.24	25.27 A
New York	10.33 BC	19.11 C	13.46	22.05 A
Minnesota	13.38 CD	15.88 BC	13.56	24.54 A
Indiana	14.65 BCD	11.23 ABC	04.74	24.66 AB
Arkansas	21.96 D	10.37 A	16.81	44.15 B
Location *p* value	<0.01	0.0149	0.3895	0.0268

[A] Number of isolates used from Michigan (n = 118), New York (n = 47), Indiana (n = 2), Arkansas (n = 4) Minnesota (n = 11), and Canada (n = 24).

n [B] Mycelial growth was measured using diameter growth and the relationship between diameter growth of an isolate when exposed to a fungicide and where the isolate was collected was determined using a one-way ANOVA. Fisher's LSD at $P < 0.05$ were used to make comparisons between locations and fungal size. Columns with a letter denote that they are not significantly different.

Table 2.5. Treatments included in a correlation analysis between the percent severity of downy mildew (DM) and halo blight of hop (HB) symptoms in a 6-foot trellis hop yard at Michigan State University in 2021 and 2022.

Treatments, rate/acre	Manufacturer	Years Tested	Active Ingredient	Severity (%)	Severity (%)
				DM	HB
Untreated		2021 2022		21.0[a] 12.4[b]	43.2[b] 8.8[b]
Ridomil Gold Sl (0.5pt drench) Revus (8 fl oz) Rhyme (7 fl oz) Presidio (4 fl oz) Pristine (14 oz/100ga) Ranman (2.75 fl oz) Zampro (14 fl oz) Luna Experience (10 fl oz)	Syngenta Syngenta FMC Valent BASF FMC BASF Bayer	2021, 2022	mefenoxam mandipropamid flutriafol fluopicolide pyraclostrobin + boscalid cyazofamid ametoctradin + dimethomorph fluopyram + tebuconazole	6.95[a] 4.7[b]	25.5[a] 5.0[b]
Ridomil Gold Sl (0.5pt drench) Revus (8 fl oz) Rhyme (7 fl oz) Presidio (4 fl oz) Ranman (2.75 fl oz) Zampro (14 fl oz) Oxidate 5.0 (1%)	Syngenta Syngenta FMC Valent FMC BASF BioSafe Sys.	2022	mefenoxam mandipropamid flutriafol fluopicolide cyazofamid ametoctradin + dimethomorph hydrogen peroxide +peroxyacetic acid	7.2[b]	6.8[b]
LifeGard WG (4.5oz/100gal) Revus (8 fl oz) Ranman (2.75 fl oz) Presidio (4 fl oz) Zampro (14 fl oz) Luna Experience (10 fl oz)	Certis Syngenta FMC Valent BASF Bayer	2021, 2022	*Bacillus mycoides* isolate J mandipropamid cyazofamid fluopicolide ametoctradin + dimethomorph fluopyram + tebuconazole	6.8[a] 7.5[b]	27.3[a] 4.5[b]
Ridomil Gold Sl (0.5pt drench) Revus (8 fl oz) Rhyme (7 fl oz) Presidio (4 fl oz) Ranman (2.75 fl oz) Zampro (14 fl oz) Procure (12 fl oz)	Syngenta Syngenta FMC Valent FMC BASF UPL	2021	mefenoxam mandipropamid flutriafol fluopicolide cyazofamid ametoctradin + dimethomorph triflumizole	19.1[a]	46.8[a]
LifeGard WG (4.5oz/100gal)	Certis	2021, 2022	*Bacillus mycoides* isolate J	9.0[a] 6.2[b]	23.8[a] 7.2[b]

Table 2.5. (cont'd)

Double Nickel (1 qt)	Certis	2021	*Bacillus amyloliquefaciens*	15.2[a]	29.3[a]
Elumin (8 fl oz)	Valent	2022	ethaboxam	6.4[b]	5.8[b]
Elumin (16 fl oz)	Valent	2022	ethaboxam	4.1[b]	5.5[b]
Presidio (4 fl oz)	Valent	2022	fluopicolide	6.6[b]	3.6[b]
Confidential Treatments		2022		4.5[b]	4.3[b]
Confidential Treatments		2021		9.5[a]	36.1[a]
Confidential Treatments		2022		6.1[b]	5.7[b]
Confidential Treatments		2021		13.0[a]	23.2[a]
Confidential Treatments		2021		12.2[a]	25.7[a]

[a] Rating taken in 2021, (DM: July 7th, HB: August 8th)
[b] Rating taken in 2022, (DM: June 7th, HB: July 12th)

Table 2.6. Correlation of lesion severity ratings between downy mildew and halo blight of hop from a single short trellis hop yard ('Centennial') from 2021-2022 using a total of 20 unique treatments with 5 repeated in 2021 and 2022.

Model	Correlation[ab]	R-Squared[b]	ANOVA *p*-value[b]
Square root-Y	0.5676	32.22%	<0.002
Double square root	0.5642	31.83%	<0.002
Linear	0.5546	30.76%	<0.002
Logarithmic-Y square root-X	0.5469	29.92%	<0.002
Square root-X	0.5454	29.75%	<0.002
Exponential	0.5442	29.61%	<0.002
Square root-Y logarithmic-X	0.5394	29.09%	<0.002
Multiplicative	0.5289	27.97%	<0.002
Square root-Y squared-X	0.5281	27.89%	<0.002
Squared-X	0.5277	27.85%	<0.002
Logarithmic-X	0.5161	26.64%	<0.002
Logarithmic-Y squared-X	0.4947	24.47%	<0.002
S-curve model	-0.436	19.01%	<0.002
Square root-Y reciprocal-X	-0.4339	18.83%	<0.002
Squared-Y	0.4299	18.48%	<0.002
Double squared	0.4241	17.99%	<0.002
Squared-Y square root-X	0.4159	17.30%	<0.002
Reciprocal-X	-0.4062	16.50%	<0.002
Squared-Y logarithmic-X	0.3876	15.02%	<0.002
Squared-Y reciprocal-X	-0.296	8.76%	0.0023
Double reciprocal	0.292	8.52%	0.0026
Reciprocal-Y squared-X	-0.2857	8.16%	0.0033
Reciprocal-Y	<no fit>	-	-
Reciprocal-Y square root-X	<no fit>	-	-
Reciprocal-Y logarithmic-X	<no fit>	-	-
Logistic	<no fit>	-	-
Log probit	<no fit>	-	-

[a] Correlation of percent severity ratings using early season downy mildew and late season halo blight of hop leaf infections in a short-trellised hop yard in 2021 and 2022
[b] Data was analyzed using Statgraphics Centurion XVI

FIGURES

Figure 2.1. The average mycelial growth (mm) of *Diaporthe humulicola* when grown on different concentrations (ppm) of different fungicides for 7 days. Isolates originated from Michigan (3M and 25M), Canada (CD6C), and Connecticut (CT01). The vertical line on each graph represents the discriminatory dose (ppm) for each fungicide. A) Trifloxystrobin with SHAM with a discriminatory dose of 1 ppm. B) Tebuconazole + fluopyram with a discriminatory dose of 1 ppm. C) Cyflufenamid with a discriminatory dose of 10 ppm. D) Flutriafol with a discriminatory dose of 10 ppm.

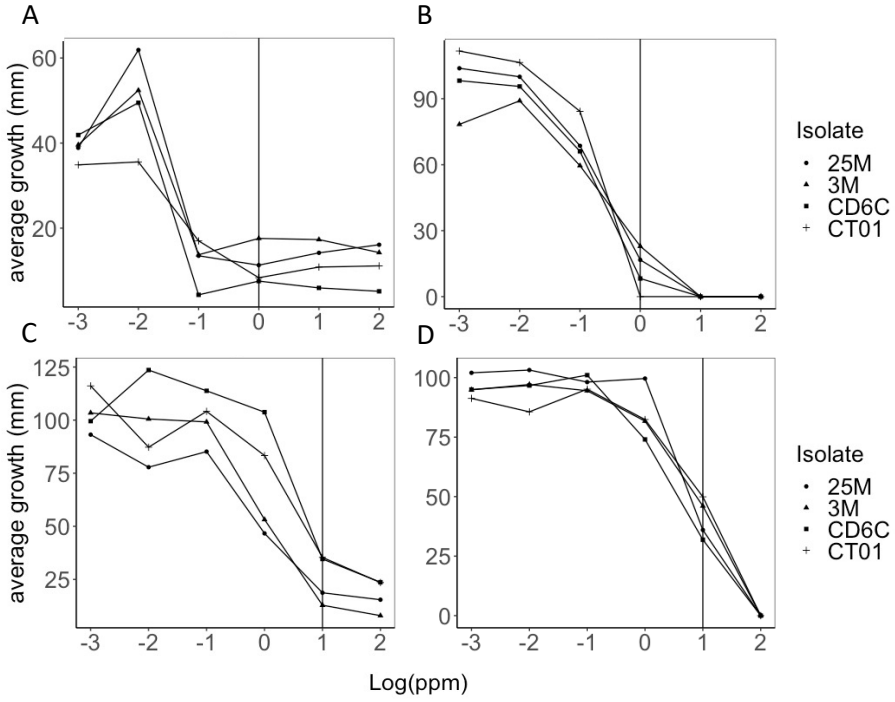


Figure 2.2. Boxplot representing the range of the log (EC_{50}) (ppm) values determined by an inhibition assay of *Diaporthe humulicola* isolates 3M, 25M, CD6C, and CT01. Diameter measurements were taken 7 days post-transfer and used to determine the EC_{50} value.

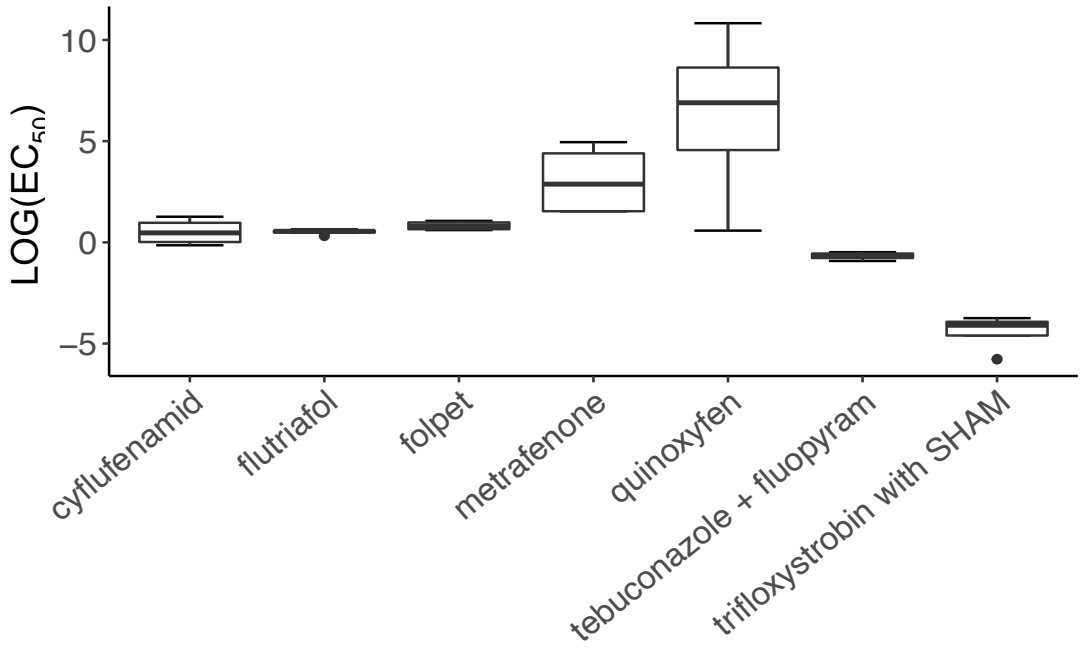
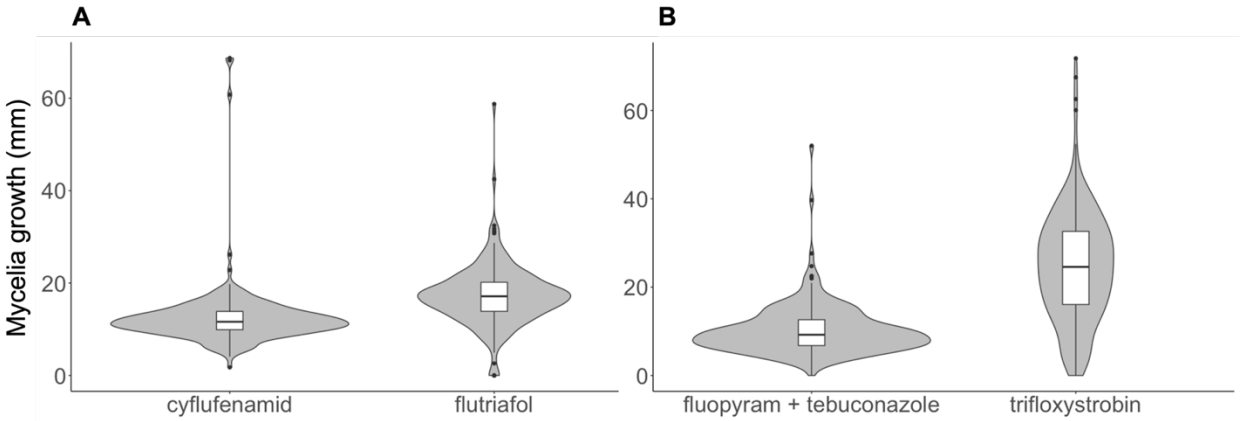


Figure 2.3. Violin plots of average mycelial growth of 206 *Diaporthe humulicola* isolates on fungicide amended media after 7 days. A) Cyflufenamid and flutriafol were tested at 10ppm. B) Fluopyram + tebuconazole and trifloxystrobin with SHAM were tested at a concentration of 1ppm.



LITERATURE CITED

Allan-Perkins, E., Li. D., Schultes, N., Yavuz, S., and LaMondia, J. 2020. The identification of a new species, *Diaporthe humulicola*, a pathogen causing diaporthe leaf spot on common hop. Plant Dis. 104:2377-2390.

Anastassiadou, M., Bellisai, G., Bernasconi, G., Brancato, A., Carrasco, L. C., Ferreira, L., Greco, L., Jarrah, S., Kazocina, A., Leuschner, R., Magrans, J.O., Miron, I., Nave, S., Pedersen, R., Reich, H., Santos, M., Scarlato, A. P., Theobald, A., Vagenende, B., and Verani, A. 2021. Setting of import tolerance for cyflufenamid in hops. EFSA Journal 19:1-25.

Bargabus, R. L., Zidack, N. K., Sherwood, J. E., and Jacobsen, B. J. 2003. Oxidative burst elicited by *Bacillus mycoides* isolate bac J, a biological control agent, occurs independently of hypersensitive cell death in sugar beet. MPMI. 16:1145:1153 https://apsjournals.apsnet.org/doi/epdf/10.1094/MPMI.2003.16.12.1145.

Carrasco, J., Navarro, M. J., Santos, M., and Gea, F. J., 2017. Effect of five fungicides with different modes of action on cobweb disease (*Cladobotryum mycophilum*) and mushroom yield. Ann. Appl. Biol.171:62-69.

Claassen, B. J., Wolfenbarger, S. N., and Gent, D. H., 2022. Fungicide physical mode of action: impacts on suppression of hop powdery mildew. Plant Dis. 106:1244-1252.

Dangal, N. K., Rekabdarkolaee, H. M., Markell, S. G., Harveson, R. M., and Mathew, F. M. 2022. Foliar fungicides containing FRAC 11 mitigates phomopsis stem canker in sunflower (*Helianthus annuus*). Plant Dis. 107:743-749.

Davidson K. 2023. Emergency registration granted for halo blight on hops. The Grower. https://thegrower.org/news/emergency-registration-granted-halo-blight-hops.

Di, Y., Zhu, Z., Lu, X., and Zhu, F. 2016. Baseline sensitivity and efficacy of trifloxystrobin against *Sclerotinia sclerotiorum*. J. Crop Prot. 87:31-36.

Gent, D. G., Nelson M. E., George A. E., Grove, G. G., Mahaffee, W. F., Ocamb, C. M., Barbour J. D., Peetz. A., and Truchek W.W. 2008. A Decade of hop powdery mildew in the Pacific Northwest. PHP, https://apsjournals.apsnet.org/doi/pdf/10.1094/PHP-2008-0314-01-RV.

Gisi, U., Chin. K. M., Knapova, G., Küng Färber, R., Mohr, U., Parisi, S., Sierotzki, H., and Steinfeld, U. 2000. Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. J. Crop Prot. 19:863-872.

Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to QoI inhibitor fungicides. Pest Manag. Sci. 58:859-867.

Gordon, E.B. 2010. Captan and Folpet. Pages 1915-1949 in: Hayes' Handbook of Pesticide Toxicology. R. Krieger, ed. Elsevier, Amsterdam, Netherlands.

Gudmestad, N. C., and Arabiat, S. 2013. Prevalence and impact of SDHI fungicides resistance in *Alternaria solani.* Plant Dis. 97:852-960.

Fernández-Ortuño, D., Torés, J. A., de Vicente, A., and Pérez-García, A. 2007. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology 11:1-9.

Fungicide Resistance Action Committee (FRAC). 2020. List of first confirmed cases of plant pathogenic organisms resistant to disease control agents. Fungicide Resistance Action Committee (FRAC). https://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-first-confirmed-cases-of-plant-pathogenic-organisms-resistant-to-disease-control-agents_05_2020.pdf.

Fungicide Resistance Action Committee (FRAC). 2024. FRAC Code List 2024: Fungal control agents sorted by cross-resistance pattern and mode of action (including coding for FRAC Groups on product labels). Fungicide Resistance Action Committee (FRAC). https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2024.pdf.

Haafke, A.J. 2021. Management of *Diaporthe*-infected soybeans. Iowa State University. https://www.proquest.com/docview/2628260402/fulltextPDF/B9D33711D5664CD8PQ/1?accountid=12598.

Hampson, M. C. and Coombes, J. W. 1985. Stress and stimulus modifications of disease severity of the wart disease of potato. Phytopathology. 75:817-820.

Haramoto, M., Yamanka, H., Sano, H., Sano, S., and Otani, H. 2005. Fungicidal activities of cyflufenamid against various plant-pathogenic fungi. J. Pest Sci. 32:95-101.

Haramoto, M., Hamamura, H., Sano, S., Felsenstine, F.G., and Otani H. 2006. Sensitivity monitoring of powdery mildew pathogens to cyflufenamid and the evaluation of resistance risk. J. Pest Sci 31:397-404.

Hatlen, R .J., Gillett, J. M., Sysak, R. W., Smith, R. L., and Miles, T. D. 2020. Evaluation of fungicides for control of downy mildew in hops, 2019. Plant Disease Management Reports. 15:OT018.

Hatlen, R. J., Higgins, D. S., Venne, J., Rojas, J. A., Hausbeck, M. K., and Miles, T. D. 2021. First report of halo blight of hop (*Humulus lupulus*) cause by *Diaporthe humulicola* in Quebec, Canada. Plant Dis. 106:1750.

Hatlen, R. J., Gillett, J. M., Neugebauer, K. A., Sysak, R. W., and Miles, T. D. 2022. Evaluation of fungicides for control of downy mildew and halo blight, 2021. Plant Disease Management Reports. 16.

Hatlen, R. J., Adiar, N., Wismen, M., Sysak, R., Rohjas, A., Gent, D., and Miles, T. D. 2023. Development of integrated qPCR diagnostic tools to determine the life cycle of *Diaporthe humulicola* in hop (*Humulus lupulus*). Phytopathology 113, S3.1-S3.197.

Haunold, A. 1981. Hop production, Breeding, and Variety development in various countries. J. Am. Soc. Brew. Chem. 39:27-34. https://www.tandfonline.com/doi/pdf/10.1094/ASBCJ-39-0027?needAccess=true.

Higgins, D. S., Hatlen, R. J., Byrne, J. M., Sakalidis, M. L., Miles, T. D., and Hausbeck, M. K. 2021a. Etiology of halo blight in Michigan hopyards. Plant Dis. 105:859-872.

Higgins, D. S., Miles, T. D., and Hausbeck, M. K. 2021b. Fungicide efficacy against *Pseudoperonospora humuli* and point mutations linked to carboxylic acid amide resistance in Michigan. Plant Dis. 105:188-1889.

Johnson, D. A., Engelhard, B., and Gent, D. H. 2009. Downy mildew. Pages 18-22 in: Compendium of Hop Diseases and Pests. W. F. Mahaffee, S. J., Pethybridge, and D. H. Gent, eds. American Phytopathological Society, St. Paul, MN.

Kim, Y., Dixon, E.W., Vincelli, P., and Farman, M. 2003. Field resistance to strobilurin (Q_oI) fungicides in *Pyricularia grisea* caused by mutations in mitochondrial cytochrome b gene. Phytopathology. 93:891-900.

Lafontaine, S. R. and Shellhammer, T. H. 2019. How hoppy beer production has redefined hop quality and a discussion of agricultural and processing strategies to promote it. Tech. Q. Master Brew. Assoc. 59:1-12.

Lizotte, E., Sirrine, R., Hausbeck, M. K., Miles, T., Chaudhari, S., Quintanilla, M., and Issacs, R. 2023. Michigan Hop Management Guide, 2023. Michigan State University Extension. https://www.canr.msu.edu/hops/uploads/files/Michigan+Hop+Management+Guide+2022.pdf.

Lucas, J. A., Hawkins, N. J., and Fraaije, B. A. 2015. Chapter two – the evolution of fungicide resistance. Adv. Appl. Microbiol. 90:29-92.

Luković, J., Milijašević-Marčić, S., Hatvani, L., Kredics, L., Szűcs, A., Vágölgyi, C., Duduk, N., Vico I., and Potočnik, I. 2020. Sensitivity of *Trichoderma* strains from edible mushrooms to the fungicides prochloraz and metrafenone. J. Environ. Sci. Health B Part B 57: 636-643.

Madden, L. V., Hughes, G., and van den Bosch, F. 2007. The Study of Plant Disease Epidemics. American Phytopathological Society, St. Paul, MN.

Miao, J., Zhao, G., Wang, B., Du, Y., Li, Z., Gao, x., Zhang, C., and Lui, X. 2020. Three point-mutations in cytochrome b confer resistance to trifloxystrobin in *Magnaporthe oryzae*. Pest Manag. Sci. 76:4258-4267, https://onlinelibrary.wiley.com/doi/epdf/10.1002/ps.5990.

Miles, L. A., Miles, T. D., Kirk, W. W., and Schilder, A. M. 2012. Strobilurin (Q_oI) resistance in populations of *Erysiphe necator* on grapes in Michigan. Plant Dis. 96: 1621-1628.

Miles, T. D., Day, B., and Schilder, A. C. 2011. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. Mol. Plant Pathol. 12: 463-477.

Opalski K. S., Tresh, S., Nogel., K., Grossmann, K., Köhle, H., and Hückelhoven, R. 2006. Metrafenone: Studies on mode of action of a novel cereal powdery mildew. Pest Manag. Sci. 62:393-401.

Probst C., Nelson M. E., Grove G. G., Towmey C. M., and Gent D. H. 2016. Hop powdery mildew control through alteration of spring pruning practices. Plant Dis. 100:1599-1605.

Sakia, R. M. 1992. The Box- Cox transformation technique: a review. Journal of the Royal Statistical Society: Series D (The Statistician). 41:169-178.

Sharma, S., Strickland, D., Hay, F., and Pethybridge, S.J. 2023. First report of halo blight in hop caused by *Diaporthe humulicola* in New York. Plant Dis. 107:216.

Shi, N., Ruan, H., Gan, L., Dai, Y., Yang, X., Du, Y., and Chen, F. 2019. Evaluating the sensitivities and efficacies of fungicides with different modes of action against *Phomopsis asparagi*. Plant Dis. 104: 448-454.

Shrestha, S. and Hausbeck, M. K. 2021. Evaluation of geranium cultivars and biorational products to control botrytis blight in the greenhouse. PHP. 22: 1535-1025.

Shrestha, S. and Hausbeck, M. K. 2023. Management of *Botrytis cinerea* in petunia using cultivar resistance and biorational products. PHP. 24: 1535-1025.

Sirrine R., Miles T., and Lizotte E. 2022. Pruning for disease management and yield benefits in hops. Michigan State University Extension. https://www.canr.msu.edu/news/pruning-for-disease-management-and-yield-benefits-in-hops.

Truechek W. W., Mahaffee W. F., and Ocamb C.M. 2001. Development of management for hop powdery mildew in Pacific Northwest. Online. https://doi.org/10.1094/PHP-2001-0313-01-RS.

USDA -NASS. 2021 National Hop Report, https://downloads.usda.library.cornell.edu/usda-esmis/files/s7526c41m/08613p220/05742t06k/hopsan21.pdf.

Walsh, D. B., O'Neal, S. D., George, A. E., Groenendale, D. P., Henderson. R. E., Groenendale, G. M., and Hengel, M. J. 2018. Evaluation of pesticide residues from conventional, organic, and nontreated hops on conventionally hopped, late-hopped, and wet-hopped beers. J. Am. Soc. Brew. Chem. 74:53-56.

Wang, J., Bradley, C., Stenzel, O., Pendersen. D., Reuter-Carlson, U., and Chilvers, M. I. 2017 Baseline sensitivity of *Fusarium virguliforme* to fluopyram fungicide. Plant Dis. 101:576:582.

Weber, R. W. S. and Hahn, M. 2011. A rapid and simple method for determining fungicide resistance in *Botrytis*. JPDP 118:17–25.

Wheeler, I. E., Hollomon, D. W., Gustafson, G., Mitchell, J. C., Longhurst, C., Zhang, Z., and Gurr, S. J. 2003. Quinoxyfen perturbs signal transduction in barley powdery mildew (*Blumeria graminis f.sp. hordei*) Mol. Plant Pathol. 4:177-186.

Wilcox, W., 2017. Grape Disease Control: Taking Stock and Looking Forward. Appellation Cornell.

Wise, J. C., Wilson, J., Milbraith M., Mota-Sanchez, D., Quintanilla M., lsaacs, R., Perkins, J., Lindell, C., Mason, K., Miles, T. D., Sundin, G. W., Zandstra B., Beaudry, R., Einhorn, T., Lang G., Shane, W., and Garcia-Salazar, C. 2023. Michigan Fruit Management Guide 2022. Extension Bulletin E-154. Michigan State University, East Lansing.

Yin, Y., Liu, X., Li, B., and Ma, Z. 2009. Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. Phytopathology. 99:438-497.

Ziogas, B. N. and Davidse, L. C. 1987. Studies on the mechanism of action of cymoxanil in *Phytophthora infestans*. Pestic. Biochem. Phys. 29:89-96. https://www.sciencedirect.com/science/article/pii/0048357587900666.

CHAPTER 3: DEVELOPMENT OF A TEF BASED TAQMAN QPCR ASSAY FOR *DIAPORTHE HUMULICOLA*, THE CAUSAL AGENT OF HALO BLIGHT OF HOP

Abstract

Halo blight of hop, caused by the fungus *Diaporthe humulicola*, was first described in 2018 and is a major concern for growers in the eastern United States and Canada. This pathogen can cause quality and yield losses by desiccating hop cones, leading to shatter. However, traditional disease diagnosis is time-consuming, with culture-based morphological features taking up to 30 days to develop, thus reducing the amount of time growers have to make management decisions. To address this issue, a quantitative polymerase chain reaction (qPCR) assay based on the translation elongation factor 1-alpha (TEF) gene was developed. We assessed this assay for direct detection of *D. humulicola* in plant tissue and investigated aspects of the disease cycle through three distinct experiments: 1) detection of *D. humulicola* in hop rhizomes to determine the colonization range of the pathogen, 2) determining how quickly can *D. humulicola* be detected in hop leaves post inoculation, and 3) monitoring the presence of *D. humulicola* in cones in a hop yard and comparing isolation methods and the assay. The limit of detection for the assay was 100 fg/µl of DNA. The assay showed no cross-reactivity with other hop pathogens or endophytes, nor with other *Diaporthe* species tested. Detection of *D. humulicola* occurred one day after inoculation. The assay detected *D. humulicola* in both apparently healthy and diseased rhizome tissue, but further investigation is required to determine the cause of the observed symptoms. The assay successfully detected the pathogen in individual hop cones and inflorescences throughout the season, surpassing the culture-based method in positive identification rates. This assay will provide time-limited diagnosticians a tool for detection of *D. humulicola*.

Introduction

Hop (*Humulus lupulus*) is a diecious perennial plant used to produce beer, nonalcoholic teas, and waters (Macchioni et al. 2022; Schönberger and Kostelecky 2011). The United States (US) is the largest producer of hops in the world, with the Pacific Northwest being the main growing region in the US. The Eastern US makes up a smaller but thriving hop industry. Michigan is the fourth largest hop-producing state with over 380 acres (154 hectares) harvested in 2022 (Hop Growers of America 2022). Establishing a hop yard is an expensive endeavor; in 2014, it could cost approximately $13,688 USD per acre to establish a hop yard and $15,505 USD per acre to maintain it (Sirrine et al. 2014). New hop yards plants are clones derived from softwood cuttings, rhizomes, or micropropagation from a mother plant, but these methods can introduce and spread diseases to the new clones (Higgins et al. 2022; Howard 1965; Neve 1991; Peredo et al. 2009). Pathogens of hop may decrease quality, yield, and the amount of alpha and beta acids in cones; thus, having disease-free plants when establishing and maintaining a hop yard is important (Agehara et al. 2020; Cerenak et al. 2009; Gent et al. 2013; Pethybridge et al. 2008). New pathogens are expected to become problematic as hop production spreads into new growing regions, as is the case with eastern US production (Higgins et al. 2021a; Tomlan 2013).

Diaporthe humulicola, an ascomycete fungus, is the causal agent of halo blight of hop that was formally described in 2018 in Michigan and Connecticut (Allan-Perkins et al. 2020; Higgins et al. 2021a). Since then, the disease has been detected in other growing regions in the eastern US and Canada (Foster et al. 2024; Hatlen et al. 2022; Havill et al. 2023; Sharma et al. 2023). The pathogen can cause up to 60% yield loss in a single hop yard though the desiccation of hop cones that shatter during harvest (Higgins et al. 2021a). It is not known how the disease spreads to other plants or between hop yards. The pathogen can infect and colonize most above-

ground hop tissue (leaves, cones, and bines) and seems to overwinter on dormant hop bines as pycnidia (Hatlen et al. 2022). It is currently unknown if the pathogen can infect below-ground tissue like rhizomes or the hop crown, or if the pathogen can asymptomatically colonize plant tissue.

Proper diagnosis of *D. humulicola* can be a complicated and time-consuming process. The main symptoms of halo blight on hop are lesions on the surface of the hop leaf surrounded by a chlorotic margin and partial to full desiccation of the hop cone (Higgins et al. 2021a). Underdeveloped leaf lesions can mimic foliar infections from other hop pathogens like *Colletotrichum fiorinae*, *Pseudoperonospora humuli*, or *Cercospora 'sp. Q'* (Hatlen et al. 2023a; Johnson et al. 2009; Pereira et al. 2020). The distal end of affected cones can become desiccated similar to the disease Fusarium cone tip blight (Bienapfl et al. 2001; Pethybridge et al. 2001). However, the symptoms on cones are not consistent, as the pathogen can also infect bracts near the center of the cone and cause girdling (Higgins et al. 2021a). This leads to desiccation of the cone, similar to other diseases such as powdery mildew, Alternaria cone disorder, or gray mold (Higgins et al. 2021a; Mahaffee et al. 2009; Mahaffee and Engelhard 2009; Pethybridge et al. 2021a). *Phoma exigua var. exigua*, causing Phoma wilt, can also produce pycnidia and conidia on infected hop tissue that are smaller, but similarly shaped to *D. humulicola*, thus can lead to misdiagnosis of the disease (Higgins et al. 2021a; Radišek et al. 2008). The hop cone strig can also become infected and white mycelia can be present after the desiccation of the hop cone, though this sign is not always observed (Hatlen et al. 2023b). The production of *D. humulicola* pycnidia and conidia on the hop leaves and cones is not consistent, making visual diagnosis difficult (Higgins et al. 2022). Therefore, isolating the pathogen from infected tissues is necessary for proper identification when using morphological features.

Diaporthe humulicola is slow growing and can take over 30 days to produce pycnidia and conidia, the main diagnostic features of the pathogen, making diagnosis a slow process (Higgins et al. 2020). The production of pycnidia is not consistent as some isolates will not produce pycnidia even after 30 days of incubation on potato dextrose agar (PDA). Identification by mycelial characteristics can be misleading because different isolates of *D. humulicola* exhibit varying colors and growth rates when grown on PDA (Higgins et al. 2021a). *Diaporthe humulicola* can produce pycnidia in approximately 10 days when grown on PDA with embedded sterilized alfalfa stems (Higgins et al. 2021a; Sharma et al. 2023). However, this method requires having space and time for the cultivation of alfalfa, making it unlikely to be widely used. Genes for translation elongation factor 1-alpha (TEF), β-tubulin, histone 3, calmodulin, and the internal transcribed spacer (ITS) have been used for the identification of *D. humulicola* though Sanger sequencing, but this process is costly and time consuming (Allan-Perkins et al. 2022; Hatlen et al. 2022; Havill et al. 2023; Sharma et al. 2023).

Quantitative polymerase chain reaction (qPCR) is a diagnostic technique that can detect small amounts of specific DNA sequences using primers and probes with an attached fluorophore and quencher (Kubista et al. 2006). These tools can detect low amounts of their DNA target and are faster than other diagnostic techniques (Guillemette et al. 2004; Wang et al. 2006). Diagnostic qPCR assays based on TEF have been developed for the soybean pathogens *D. helianthi* and *D. gulyae*, in which 100 pg/ml of fungal DNA was detected from infected plant tissue (Elverson et al. 2020). In hop, TaqMan qPCR assays have been developed for the detection of *Pseudoperonospora humuli* (causal agent of downy mildew) and *Podosphaera macularis* (causal agent of powdery mildew) (Crandall et al. 2021; Gent et al. 2024; Higgins et al. 2022). A conventional PCR assay has also been developed for *P. humuli* using the ITS region

(Gent et al. 2009). Thus, developing a TaqMan qPCR assay for *D. humulicola* could be advantageous and provide diagnosticians with a wider array of detection tools for hop pathogens.

The objective of this study was to develop a TEF-based TaqMan qPCR assay for the detection of *D. humulicola* to be used by plant diagnosticians. The assay was validated for its sensitivity and specificity and confirmed through ring testing. Finally, the assay was used in three experiments: (1) testing of hop rhizomes for the presence of *D. humulicola*, *P. humuli*, and *Phytophthora spp*.; (2) determining the time required to detect *D. humulicola* in hop leaves after inoculation; and (3) comparing traditional plating methods with the qPCR assay to monitor *D. humulicola* in a hop yard.

Methods

DNA extractions for developing TEF based TaqMan qPCR assays

Five *D. humulicola* isolates were grown on PDA (BD Franklin, Lakes, NJ) for 21 days prior to initial DNA extraction (PP779614, PP779615, PP779616, PP779617, PP779618) (Table 3.7). DNA was extracted using the Zymo Quick-DNA Fungal/Bacterial Kit (Zymo Research, Irvine, CA). TEF sequences were then amplified using PCR with the following reagents: 15.75 µl of molecular grade water, 5 µl of 5X Green GoTAQ reaction buffer (Promega, Madison, WI), 1 µl of 10 mM dNTP Mix (ThermoFisher Scientific, Waltham, MA) 0.25 µl of GoTaq DNA polymerase (Promega), 1 µl of 10 µm forward primer EF1-728F, and 1 µl of 10 µM reverse primer EF1-986R (Table 1) and 1 µl of the sample DNA. The PCR was conducted on a C1000 Touch Thermal Cycler with a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA) using the following conditions: an initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min (Higgin et al. 2021a). Amplicons were confirmed using gel electrophoresis with 1% agarose gel

(UltraPure Agarose, Invitrogen Carlsbad, CA) stained with GelRed Nucleic Acid Gel Stain (Biotium, Fremont, Ca). The amplicons were purified using the Zymo DNA Clean & Concentrator-5 (Zymo Research) before Sanger sequencing. All sequencing was done by the Research Technology Support Facility Genomics Core at Michigan State University. Sequences were then manually trimmed and identified using NCBI BLAST (Camacho et al. 2009).

Development of TEF based TaqMan qPCR Assay

Thirty-eight translation elongation factor 1 alpha (TEF) sequences from different species of *Diaporthe* were downloaded from NCBI and trimmed to match the length of the TEF region for *D. humulicola* isolate CD6C (OK001342) (Table 3.7) (Hatlen et al. 2022). The NCBI sequences in addition to five *D. humulicola* TEF sequences collected for this study were aligned using Geneious Prime v2023.0.4 (https://www.geneious.com) using the MUSCLE alignment set to default settings (Edgar 2021). The TEF region from *D. humulicola* CD6C was analyzed using Primer-BLAST with the following conditions: 200 to 300 bp amplicon product and a primer melting temperature of 59 to 70°C (Ye et al. 2012). Results from Primer-BLAST were manually checked against the alignment and were shifted by approximately 10-15 bp, so primers pairs spanned regions with high amounts of genetic differences. Two primers DHTEF2F (length = 20, Tm = 60.1°C, GC = 50%) and DHTEF2R (length = 21bp, Tm = 60.8°C, GC = 57.1%) were identified as candidates for the assay with a product size of 260 bp. TaqMan probes were manually designed using Geneious Prime in regions that spanned gaps and base pair differences in the alignment and had the following features: 30 to 40 bp in length, Tm of 65 to 75□C, and the start of the probe was within 50 bp of the end of one of the primers. The final dual labeled hydrolysis probe (TaqMan probe) was denoted as DHTEFprobe2 (length = 30 bp, Tm = 69.2°C, GC = 50.0%). The TaqMan probe was labeled with the fluorophore HEX and quencher black

hole quencher 2 (BHQ2). All probes were purchased from Biosearch Technologies (Hoddesdon, United Kingdom) and all primers were purchased from Invitrogen (Carlsbad, California).

DNA from *D. humulicola* (CD6C, 10 ng/µl), D. amygdali (1 ng/µl), and D. eres (1 ng/µl) was extracted using the Zymo Quick-DNA Fungal/Bacterial Kit (Hatlen et al. 2022; Klein-Gordon et al. 2023). Each single plex reaction of the TEF based TaqMan qPCR assay included: 10 □l of PerfeCTa qPCR ToughMix (Quantabio, Beverly, Massachusetts), 0.4µl of 10µM DHTEFprobe2, 0.5 µl of 10 µM forward primer (DHTEF2F), 0.5 µl of 10 µM reverse primer (DHTEF2R), 1 µl of sample DNA, and 7.6 µl of HyPure molecular biology grade water (Cytiva Marlborough, Massachusetts) for a total of 20 µl per reaction. All qPCR, except those for the ring test, were conducted in either hard-shell 96-well PCR plates, low profile, thin wall, skirted, white/clear with Microseal 'B' Seals or in individual PCR 8-tube strips, clear with optical flat 8-cap strips (Bio-Rad laboratories, Hercules, CA).

Amplicons from CD6C with a 60°C annealing temperature were purified using the Zymo DNA Clean & Concentrator-5 kit and sequenced using Sanger sequencing at the RTSF Genomics Core at Michigan State University. Sequences were verified to be the TEF region of CD6C (OK001342) using NCBI BLAST where each sequenced amplicon showed 100% similarity (Hatlen et al. 2022; Camacho et al. 2009).

The internal plant control (IPC) was developed by Bilodeau et al. (2014). The primers (FMP12b and FMP13B) and probe (Plant CAL Red probe) span a conserved portion of the cox1 gene in plants (Higgins et al. 2022). For the detection of *P. humuli*, a TaqMan qPCR assay developed by Higgins et al. (2022) was used. For the detection of *Phytophthora sp*p. an atp9-nad9 genus-specific-assay was used (Bilodeau et al. 2014). All primers and probes used for qPCR and sequencing are listed in Table 3.1.

Analytical sensitivity

The limit of detection of the TEF based TaqMan qPCR assay were determined using a 10-fold serial dilution of DNA from isolate CD6C. DNA concentration of the stock DNA was determined using the Qubit 1X dsDNA BR Assay Kit (Invitrogen Waltham, MA) on a Qubit 4 fluorometer (ThermoFisher Scientific). DNA was diluted to 10 ng/µl and the following concentrations were created: 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 ng/µl. Three technical replicates were tested at each concentration using the previously mentioned singleplex reaction reagents on a C1000 Touch Thermal Cycler with a CFX96 Real-Time System (Bio-Rad Laboratories) with the following conditions: an initial 3 min at 95°C and 40 cycles of 90°C for 10 s and 60°C for 30 s. A standard curve plot of the TEF based TaqMan qPCR assay was developed using Bio-Rad CFX Maestro software (version 2.3, Hercules, CA).

The internal plant control (IPC, i.e. Plant CAL Red probe) assay was multiplexed with the TEF based TaqMan qPCR assay, and another standard curve was produced using the methods above. Each multiplex reaction for the standard curve of the TEF based TaqMan qPCR assay + IPC assays included: 10 µl of PerfeCTa qPCR ToughMix, 0.4 µl of 10 µM DHTEFprobe2, 0.5 µl of 10 µM forward primer (DHTEF2F), 0.5 µl of 10 µM reverse primer (DHTEF2R), 0.4 µl of 10 µM the Plant CAL Red probe, 0.4 µl of 10 µM forward primer (FMPl2b), 0.4 µl of 10pM reverse primer (FMPl3b), 1 µl of sample DNA, 1 µl of 5 ng/µl hop DNA free of *D. humulicola* and *P. humuli*, and 5.4 µl of HyPure molecular biology grade water. Hop DNA, free of *D. humulicola* and *P. humuli* DNA, was extracted from healthy leaves and bines from a 'Nugget' hop plant that was grown in a Michigan State University greenhouses using the Quick-DNA Plant/Seed Kit (Zymo research). DNA concentration was determined using Qubit 1X dsDNA BR Assay Kit (ThermoFisher Scientific). The DNA from the hop plant

was validated to be free of *D. humulicola* using the TEF based TaqMan qPCR assay and free from *P. humuli* using the orf359 assay (Higgins et al. 2022). The limit of detection (LOD) for the singleplex and multiplex assays were determined using a modified definition described by Bustin et al. (2009) where 100% of the samples needed to react instead of their suggested 95%. Efficiency was calculated as E = 10(–1/slope) – 1.

Repeatability and analytical specificity

The TEF based TaqMan qPCR + IPC assay was run using 10 ng/µl of hop DNA free of *D. humulicola* to test if hop DNA would be amplified by the TEF based TaqMan qPCR assay using the protocol above. To test the repeatability of the assay, isolates of *D. humulicola* from different regions were tested at 1 ng/ul (n = 39) using the TEF based TaqMan qPCR assay + IPC assay (Table 2) (Allan-Perkins et al. 2020; Hatlen et al. 2022; Higgins et al. 2021a). To test specificity, different *Diaporthe* species (n = 8), hop endophytes (n = 6), and different pathogens of hop (n = 3) were tested against the TEF based TaqMan qPCR assay + IPC assay (Table 3.3). DNA from the different *D. humulicola* isolates were extracted using 50 mg of the ground tissue and the Mag-Bind Plant DNA DS 96 Kit (Omega Bio-Tek, Norcross, Georgia) with the addition of 40mM dithiothreitol and 2.5% PVPP40 to lysis buffer A and samples were incubated at 60°C for 1 hour (Szymanski et al. 2023). DNA for other organisms were extracted using the Zymo Quick-DNA Fungal/Bacterial Kit, except for DNA from *P. humuli* and *P. macularis* where DNA was extracted using the DNeasy Plant Pro Kit (Qiagen) and a Chelex extraction procedure, respectively (Brewer and Milgroom 2010). DNA from each isolate was standardized at either 10 or 5 ng/µl of DNA depending on the starting concentration of DNA, except for *P. macularis* that was diluted to 0.1 ng/µl. Each isolate was run with three technical replicates and a negative

(molecular grade water) and positive (1 ng/µl of *D. humulicola* DNA from culture) control. Each reaction was spiked with 1 µl of 10 ng/µl of disease-free hop DNA.

Detection of pathogens in hop rhizomes

Hop plants (cv. Centennial) at the Michigan State University Plant Pathology Farm (42.68925, -84.4847) were selected to determine if D. humulicola can colonize hop rhizome tissue. Plants were selected based on severity of foliar symptoms of halo blight from a fungicide efficacy trial. The plants were separated into three categories: high severity (>12% average severity over 25 rated hop leaves), low severity (<12% average severity of 25 rated hop leaves), and plants that were not inoculated with *D. humulicola* in the previous year (Hatlen et al. unpublished). A power washer, Simpson PowerShot PS60869 Honda 4000 psi Gas 3.5 gpm Pressure Washer (Simpson, Elk Grove Village, Illinois), was used to spray the base of the plant to expose the hop rhizomes. Using ethanol-sterilized pruners, five rhizomes with new shoots that were approximately 0.45 meters away from the crown were removed from the hop plant and stored at 4°C until processing. Two samples were taken from each plant, one from the margin of symptomatic tissue and one from asymptomatic rhizome tissue, the tissue from each of the five rhizomes was pooled together for both the symptomatic and asymptomatic samples. Rhizome samples were subjected to cryodesiccation for 48 hours and ground with a Mixer Mill MM 400 (Retsch, Haan, Germany) at 30 hertz using a 4 mm metal bead for approximately three minutes. DNA was extracted using 50 mg of the ground tissue and the Mag-Bind Plant DNA DS 96 Kit (Omega Bio-Tek, Norcross, Georgia) with the addition of 40mM dithiothreitol and 2.5% PVPP40 to lysis buffer A and samples were incubated at 60°C for 1 hour (Szymanski et al. 2023). The concentration of each sample was determined using the Qubit 1X dsDNA BR Assay Kit. For the TEF based TaqMan qPCR assay + IPC assay each reaction included: 10 µl of

PerfeCTa qPCR ToughMix, 0.4 µl of 10 µM DHTEFprobe2, 0.5 µl of 10 µM forward primer (DHTEF2F), 0.5 µl of 10 µM reverse primer (DHTEF2R), 0.4 µl of 10 µM the Plant CAL Red probe, 0.4 µl of 10 µM forward primer (FMPl2b), 0.4 µl of 10 µM reverse primer (FMPl3b), and 6.4 µl of HyPure molecular biology grade water. For the *P. humuli* orf359 assay each reaction included: 7.5 µl of PerfeCTa qPCR ToughMix, 0.5 µl of 10 µM forward primer (TqFPhum), 0.5 µM of 10 µM of reverse primer (TqRFPhum), 0.3 µl of 10 µM TqProbePhum, 0.3 µl of 10 µM forward primer (FMPl2b), 0.3 µl of 10 µM reverse primer (FMPl3b), 0.3 µl of 10 µM the Plant CAL Red probe, 1.2 µl of 50mM $MgSO_4$ (Invitrogen), 2.5 µl molecular grade water, and 1 µl of the sample (Higgins et al. 2022). The rhizome DNA was also tested for the genus *Phytophthora* using an atp9-nad9 assay with the following reagents: 7.5 µl of PerfeCTa qPCR ToughMix, 0.75 µl of 10 µM forward primer (PhyG_ATP9_2FTail), 0.75 µl of 10 µM reverse primer (PhyG-R6_Tail), 0.15 µl of 5 µM ATP9_PhyG2_probeR, 1.8 ul of 50 mg $MgCl_2$, 2.55 µl molecular grade water, and 1.5 µl of the sample (Bilodeau et al. 2014; Higgins et al. 2022). Each reaction was conducted with three technical replicates. Thermocycling conditions for both the orf359 and atp9-nad9 assay were both taken from Higgins et al. (2022). The DNA concentration for the results from the *D. humulicola* assay was determined using the standard curve for the DHTEF2.

Weekly time course of hop cone infections

To validate if detection of the pathogen using the assay is suitable throughout a hop growing season, and to show that the assay can detect the pathogen more often than morphological methods, a weekly time course was conducted. Ten hop cones or burrs were collected weekly for ten weeks from a ‘Centennial’ hop plant in the Michigan State University Plant Pathology Farm starting on 22 May 2022. Burrs and hop cones had symptomatic tissue removed and were surface sterilized using a wash of 10% bleach for 30 seconds and two washes

of sterile deionized (DI) water for 30 seconds. The tissue was then dried using sterile paper towels. The tissue was transferred to PDA amended with 1% penicillin-streptomycin (10,000 U/mL) (Gilbo Waltham, MA) and grown for 5 days at 22°C with a photoperiod of 12 hours. Diaporthe humulicola isolates were identified using morphology and transferred to a new PDA plate amended with 1% penicillin-streptomycin (10,000 U/mL). Plates were incubated at 22°C with a photoperiod of 12 hours for 21 days to promote the development of pycnidia. Conidia were removed from the culture and visualized to validate that the isolate was *D. humulicola*.

During the initial plating of hop cones, the remaining cone tissue was flash frozen in liquid nitrogen and stored at -80°C until DNA extraction. The hop cones were separated into 10 different samples of a singular cone or burr per week and were cryodesiccated for 24 hours. Samples were ground for three 30 second intervals at 30 Hz with a Mixer Mill MM 400 (Retsch, Haan, Germany) using 4 mm sterile metal beads. DNA was extracted using the same conditions from the rhizome experiment and the concentration was determined using the Qubit 1X dsDNA BR Assay Kit (Invitrogen). The DNA was run with three technical replicates using the TEF based TaqMan qPCR assay + IPC assay under the same reaction setup as the rhizome samples.

Daily inoculation series

PDA was amended with autoclaved alfalfa stems to promote the development of *D. humulicola* conidia for inoculations (Sharma et al. 2023). Isolate 3M was grown on alfalfa amended plates for 10 days at 22°C with a photoperiod of 12 hours. On day 11, the stems were removed from the media with sterile forceps and placed into 15 ml of sterilized deionized water (DI) in a 50 ml conical tube. The tube was capped and shaken to mix the conidia and water. The stems were removed from tube and 35 ml of sterilized DI was added to each of the tubes and then mixed. An aliquot of 10 µl of the conidial suspension was used to determine the

concentration of conidia using a hemocytometer and repeated twice. This suspension of 1.51 × 10^5 conidia/ml was stored on ice until inoculation. Asymptomatic hop leaves from the cultivar 'Nugget' were removed from hop plant grown in a greenhouse and placed into a 100 x 15 mm Petri dish (VWR West Chester, PA) with a DI moistened 70 mm Whatman 1 filter papers (Cytiva). Prior to inoculation, the conidial suspension was shaken to mix the solution and 20 µl of the conidial suspension was pipetted onto the adaxial surface of the leaf in two 10 µl droplets. The leaves were dried for 30 min before plates were sealed with parafilm (Amcor, Zürich, Switzerland). Three leaves were not inoculated but were rinsed with a 10% EtOH and sterile deionized water solution and stored at -20°C for the zero-day time point. Three leaves were collected each day using the same methods as the zero-day time point. DNA was extracted from leaves using the Zymo Quick-DNA Plant/Seed Kit. Each of the three DNA samples per day were ran with three technical replicates using the TEF based TaqMan qPCR assay + IPC assay.

Assay reproducibility, diagnostic sensitivity, and specificity

The TEF based TaqMan qPCR assay + IPC assay was given to three research labs (Labs 2, 3, and 4) and 1 diagnostic clinic (Lab 1) to conduct ring testing. Each lab was given a set of reagents: DHTEF2 probe, DHTEF2F and DHTEF2R primers, Plant CAL Red probe, FMPl2b and FMPl3b primers, 5 ng/µl of hop DNA free of *D. humulicola*, and PerfCTa qPCR ToughMix. The labs were tasked with two experiments to test assay reproducibility and diagnostic sensitivity and specificity: (1) reproduce the standard curve using a tenfold dilution series of 10ng/□l – 0.1 fg/µl; and (2) test a variety of blind DNA samples: three *D. humulicola* (CD6C, hh1ba, and hh7ba) isolates, three *D. vaccinni* (JMK290, JMK291, and JMK292) isolates, and two samples of molecular grade water. *D. humulicola* DNA for the standard curve and *D. vaccinni* DNA for the blind samples were extracted using the Quick-DNA Fungal/Bacterial Kit.

D. humulicola DNA for the blind samples were extracted using the E.Z.N.A. Plant & Fungal DNA Kit (Omega Bio-Tek). Hop DNA for the IPC was extracted using the Quick-DNA Plant/Seed Miniprep Kit (Zymo Research) and tested using the TEF based TaqMan qPCR assay + IPC assay to confirm the DNA did not contain *D. humulicola* DNA.

Results

Analytical sensitivity and specificity of TEF based TaqMan qPCR assay + IPC assay

The TEF based TaqMan qPCR assay + IPC assay was able to amplify DNA (1 ng/µL) of all *D. humulicola* isolates from different geographical locations with an average Cq value of 22.74 with a standard error of 0.15 (Table 3.2). The TEF based TaqMan qPCR assay duplexed with the IPC assay did not amplify off-target DNA (Table 3.3). When the IPC was multiplexed with the TEF based TaqMan qPCR assay, there was a loss of amplification efficacy from 100.1 to 88.1% (Figure 3.1). The regression coefficient of determination experienced a slight drop from 0.994 to 0.991. The slope of the assay decreased in the presence of the IPC assay and hop DNA, from -3.32 to -3.64. The limit of detection was calculated at 0.0001 ng/µl of DNA because amplification was observed in 100% of the positive samples. This did not change when duplexing the TEF based TaqMan qPCR assay and IPC assays. In both the single and duplexed reactions, there were occasional positive amplifications from DNA concentrations lower than the LOD (0.00001 and 0.000001 ng/µl). On average the TEF based TaqMan qPCR assay + IPC assay had lower Cq values than the Cq values of the singleplex assay, with most reactions (except for 0.001 ng/µl) amplifying less than 1 Cq earlier.

Hop rhizomes

Apart from the positive controls, there were no positive reactions for *P. humuli* when using the orf359 qPCR assay, and no positive reactions for *Phytophthora* using the atp9-nad9

assay. Twenty-five out of the 30 sample's DNA passed the threshold set by the Bio-Rad CFX Maestro software. From the 25 samples that passed the threshold, 12 of the samples were above the limit of detection for the assay, enabling the quantification of *D. humulicola* DNA present in the sample. For the symptomatic tissue, there were eight samples that could be quantified with concentrations ranging from 5.25×10^{-4} to 1.96×10^{-1} ng. For the asymptomatic tissue, four samples were quantified with concentrations ranging from 1.38×10^{-4} to 3.04×10^{-3} ng (Table 3.4).

Weekly sampling of cones and burrs

The TEF based TaqMan qPCR assay + IPC assay detected *D. humulicola* more frequently than isolation in all weeks except week 2. In week 1, four out of ten cones tested positive with the TEF based TaqMan qPCR, while traditional methods identified only one. In week 2, both methods detected D. humulicola in one cone. Week 3 recorded six positives with the TEF based TaqMan qPCR assay compared to two with traditional methods. In Week 4 there was one more positive detection when using the assay. In week 5 TEF based TaqMan qPCR assay identified eight positives, while traditional plating found three. Weeks 6 and 7 recorded ten and nine positives, respectively. By week 8, TEF based TaqMan qPCR assay identified four additional positives, totaling nine. In weeks 9 and 10, the qPCR assay identified ten positives each week. (Figure 3.2).

Leaf inoculations

Diaporthe humulicola was not detected in the non-inoculated leaves on day 0, when using the TEF based TaqMan qPCR assay + IPC assay. Every leaf tested thereafter from days 1 to 9 was positive for D. humulicola. For the first three days, the average Cq values were within 1 cycle of each other, ranging from 35.51 to 36.44. Days 4 and 5 had similar average Cq values at

31.73 and 31.74 respectively. From days 6 – 9, the Cq values decreased daily, ranging from 30.03 – 25.19. The DNA concentrations in leaves ranged from 2.71 x 10^{-04} to 8.73 x 10^{-01} ng/µl. (Table 3.5).

Assay reproducibility and diagnostic sensitivity and specificity

Four standard curves were developed by the collaborating labs. The slopes from Labs 2, 3, and 4 were steeper than the original standard curve for the TEF based TaqMan qPCR assay + IPC assay (-3.643) at -4.180, -3.850, and -3.810, respectively. The slope for lab 1 was less steep than the original standard curve at -3.489. Amplification efficiency for each lab decreased to 93.8%, 73.5%, 87.9%, and 82.4% respectively. The limit of detection in the validation testing (0.001 ng/µl) was constantly higher than that of the original standard curve (0.0001 ng/µl). There was sporadic amplification of lower concentrations in runs conducted in the labs 1 and 3 (0.0001 and 0.00001 ng/µl). All blind samples were successfully identified by each lab (Table 3.6).

Discussion

The DHTEF2 assay was developed for use in diagnostic labs, where hop growers can submit samples for halo blight evaluation. This assay can detect *D. humulicola* from individual leaves and cones with greater accuracy than traditional diagnostic methods. This tool can also be used to study the biology of the pathogen, demonstrating that all parts of the hop plant can be colonized by *D. humulicola*.

The DHTEF2 assay has acceptable specificity and sensitivity for accurately detecting the pathogen from individual hop cones and leaves. The TEF region has been previously used for the identification of *Diaporthe* species due to the high amount of polymorphism across species and limited similarity with other genera (Everson et al. 2020; Gomes et al. 2013; Hossini et al. 2021; Udayanga et al. 2014). The assay did not react with endophytes or other common pathogens of

hop or other *Diaporthe* species tested. The assay can detect the *D. humulicola* from less than 50 mg of dried plant materials from cones, burrs, leaves, or rhizome tissue. The assay had a limit of detection of 0.0001 ng/µl (100 fg), but the LOD was higher in the ring testing at 0.001 ng/µl. This loss of sensitivity could be attributed to degradation of target DNA from thawing during shipment or could have been a pipetting error when preparing the dilution series for shipment (Rojas et al. 2017). While the LOD is higher in the ring testing, higher LOD does not mean that the assay cannot detect lower amounts of DNA or that amplifications lower than the LOD are false (Kralik and Ricchi 2017). In ring testing, there was occasional amplification at concentrations lower than 0.001 ng/µl, demonstrating the assay can potentially detect lower concentrations of target DNA.

The TEF based TaqMan qPCR assay was able to provide insight into the biology of the pathogen. *Diaporthe humulicola* was originally thought to colonize only the leaves, cones, and bine tissue (Allan-Perkins et al. 2020; Hatlen et at. 2023b; Higgins et al. 2021a), but the assay was able to detect the pathogen in both asymptomatic and symptomatic rhizome tissue. The assay detected the pathogen in the symptomatic tissue more often than the asymptomatic tissue, thus, when collecting tissue for sampling, the symptomatic margins should be sampled. Detection of the pathogen in the rhizomes does not confirm that the pathogen is responsible for any lesions or rotting of the rhizome tissue, as *D. humulicola* was not detected in some of the symptomatic rhizome tissue (plants E and H) and the pathogen was detected in low concentrations in the asymptomatic rhizome tissue (plants A, B, D, E, G, H, I, K, L, M, and N). *Diaporthe* species can be endophytic, saprophytic, or hemibiotrophic in other plant species, thus a positive identification does not corroborate plant disease (Gomes et al. 2013; Hilário and Gonçalves 2023). This tool will allow researchers to study the relationship between colonization

of hop tissue and the presence of disease symptoms. The assay could help determine the life cycle for *D. humulicola* and determine when and where the pathogen is an endophyte or a pathogen.

Phytophthora citricola sensu lato (causal agent of black root rot) and *P. humuli* can also cause lesions and necrosis on hop rhizomes (Hay et al. 2009; Higgins et al 2021b). While previous papers have noted that *P. humuli* can cause dark brown to black necrosis on the rhizome, *P. humuli* was not detected on any of the rhizome samples when using the ORF359 assay (Higgins et al. 2022). This result is unusual because this yard is regularly used for fungicide field trials and has a history of downy mildew. In the three previous years the plants in that field were used for a *D. humulicola* targeted fungicide trial, where fluopicolide (Presidio, Valent, San Ramon, CA) was applied at the maximum rate specified on the manufacturer's label (280 mL/ha) multiple times in the season to minimize downy mildew associated lesions. Thus, we could hypothesize that the application of the fungicide may have halted the pathogen from colonizing new rhizomes. This product is also labeled for many *Phytophthora* pathogens, so this could also explain the lack of positive detections using the atp9-nad9 assay.

The TEF based TaqMan successfully detected the pathogen on burrs during the early season of hop production, an aspect not previously explored in previous studies (Allan-Perkins et al. 2022; Higgins et al. 2021a). There were little to no symptoms associated with halo blight on the hop burrs. We demonstrate that *D. humulicola* colonization is possible during the burr stage of the hop flower, but it is not known if there is a latent stage where burrs are infected, and disease develops over the season. While partial ontogenetic resistance has been reported in hops for powdery mildew (Twomey et al. 2015), we are currently unable to determine if this holds true for *D. humulicola*. While symptoms seem to be more severe in the later season, and yield

loss is attributed to the cone shattering during harvest, it is not known when the hop cone is infected by the pathogen and how long it takes for severe symptoms to develop (Higgins et al. 2022).

For detached hop leaves, disease symptoms developed in most of the leaves after 6 days post inoculation, while some leaves did not develop symptoms until day 9. This contrasts with work done by Higgins et al. (2022), where hop leaves developed symptoms at day 10 post inoculation. This could be attributed to abiotic factors that could increase the rate in which infection and the development of disease occur (Chojak-Koźniewska et al. 2018). There could also be difference in virulence across different isolates of *D. humulicola*. Nonetheless, the TEF based TaqMan qPCR assay was able to detect the presence of the pathogen every day post inoculation, thus allowing for early detection of the disease.

In summary, the TEF based TaqMan qPCR assay is a quick assay that can detect low concentrations of *D. humulicola* DNA from small amounts of hop tissue. The assay will be viable for its intended purpose of use in a diagnostic clinic. Results can be obtained in less than a day, thus allowing for growers to implement management strategies quickly during the growing season. The assay was able to determine that *D. humulicola* can colonize all above ground tissue and the rhizome of the hop plant. While the pathogen is present in almost all parts of the plant, more testing needs to be done to determine if the presence of the pathogen causes disease in the rhizomes and burrs. The annealing temperature of the TEF based TaqMan qPCR assay makes it a great candidate for multiplexing this tool with the *P. humuli* assay from Higgins et al. (2022), thus saving time and resources for plant diagnosticians.

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TABLES

Table 3.1. List of primers and probes used to sequence the TEF region in *Diaporthe humulicola*, detect *Diaporthe humulicola* and *Pseudoperonospora humuli*, and an internal control using the plant *cox1* gene.

Name[A]	Sequence[B]	Reference
Sequencing primers		
EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn 1999
EF1-986R	TACTTGAAGGAACCCTTACC	Carbone and Kohn 1999
Diaporthe humulicola		
DHTEF2F	AATCATCATCGTGCGGGTGT	This study
DHTEF2R	CTCAGCGGCTGTGTAGATGAC	This study
DHTefProbe2	[HEX}TGTGTTGAACAGGGCAGTGGGATTGGTAGT[BHQ2]	This study
Pseudoperonospora humuli		
Ph359F	TTTTTATCAAAATGAATACCAAGATT	Higgins et al. 2022
Ph359R	AGTATAACTGTTGGTAGTGTAT	Higgins et al. 2022
Phorf359q_probe	[FAM]TAAAATACAGAATAAGAAATTTGATCTTCAAAAAG[BHQ1]	Higgins et al. 2022
Phytophthora		
PhyG_ATP9_2FTail	AATAAATCATAACCTTCTTTACAACAAGAATTAATG	Bilodeau et al. 2014
PhyG-R6_Tail	AATAAATCATAAATACATAATTCATTTTTATA	Bilodeau et al. 2014
ATP9_PhyG2_probeR	[FAM]AAAGCCATCATTAAACARAATAAAGC[BHQ1]	Bilodeau et al. 2014
Internal plant control		
FMPl2b	GCGTGGACCTGGAATGACTA	Bilodeau et al. 2014
FMPl3b	AGGTTGTATTAAAGTTTCGATCG	Bilodeau et al. 2014
Plant CAL Red probe	[CALFluorRed610]CTTTTATTATCACTTCCGGTACTGGCAGG[BHQ2]	Bilodeau et al. 2014

[A] Primers were procured from Integrated DNA Technologies (Coralville, IA) and probes were procured from LGC Biosearch Technologies (Hoddesdon, UK)

[B] Fluorophores and quenchers are denoted by brackets and were synthesized by Biosearch Technologies (Novato, Ca, USA)

Table 3.2. List of *D. humulicola* samples isolated from hop used to test sensitivity and repeatability.

Sample[A]	Location	DNA concentration (ng/µl)	Cycle
CD_1	Canada	Variable	-[B]
CD_2	Canada	5	22.93
CD_3	Canada	5	24.68
CD_4	Canada	5	23.49
CD_5	Canada	5	23.12
CD_6	Canada	5	22.62
MI_1	Michigan	5	23.47
MI_2	Michigan	5	24.31
MI_3	Michigan	5	25.03
MI_4	Michigan	5	23.07
MI_5	Michigan	5	22.39
MI_6	Michigan	5	21.10
MI_7	Michigan	5	22.05
MI_8	Michigan	5	23.33
MI_9	Michigan	5	22.72
MI_10	Michigan	5	21.14
MI_11	Michigan	5	22.44
MI_12	Michigan	5	23.03
MN_1	Minnesota	5	21.54
MN_2	Minnesota	5	23.92
MN_3	Minnesota	5	22.19
MN_4	Minnesota	5	23.02
MN_5	Minnesota	5	23.25
MN_6	Minnesota	5	22.26
MN_7	Minnesota	5	22.13
MN_8	Minnesota	5	21.79
MN_9	Minnesota	5	22.02
MN_10	Minnesota	5	22.81
NY_1	New York	5	22.04
NY_2	New York	5	23.35
NY_3	New York	5	22.06
NY_4	New York	5	24.00
NY_5	New York	5	23.52
NY_6	New York	5	21.81
NY_7	New York	5	22.27
NY_8	New York	5	22.12
NY_9	New York	5	22.14
NY_10	New York	5	21.32
NY_11	New York	5	23.08
NY_12	New York	5	23.45

[A] All samples were tested with three technical replicates and with a positive and negative control. DNA of *D. humulicola* isolates was diluted to 1 ng/µL.
[B] Isolate was used in the creation of the standard curve.

Table 3.3. List of samples that were used to test the specificity of the *Diaporthe humulicola tef* assay.

Sample	Sample host	Location	Concentration (ng/µl)[A]	Cycle[B]
Diaporthe ampelina	*Vitis vinifera*	Michigan	1	-
Diaporthe amygdali	*Vaccinium corymbosum*	Michigan	1	-
Diaporthe eres	*Vaccinium corymbosum*	Michigan	10	-
Diaporthe longicolla	*Glycine max*	Arkansas	10	-
Diaporthe sojae	*Glycine max*	Arkansas	10	-
Diaporthe spinosa	*Glycine max*	Arkansas	1	-
Diaporthe caulivora	*Glycine max*	Arkansas	1	-
Diaporthe vaccinii	*Vaccinium corymbosum*	Michigan	1	-
Alternaria alternata	*Humulus lupulus*	Michigan	10	-
Botrytis cinerea	*Fragaria × ananassa*	Michigan	10	-
Colletotrichum fioriniae	*Humulus lupulus*	Michigan	10	-
Epicoccum nigrum	*Humulus lupulus*	Michigan	5	-
Epicoccum layuense	*Humulus lupulus*	Michigan	5	-
Fusarium sp.	*Humulus lupulus*	Michigan	5	-
Fusarium sp.	*Humulus lupulus*	Michigan	5	-
Podosphaera macularis	*Humulus lupulus*	Oregon	0.1	-
Pseudoperonospora humuli	*Humulus lupulus*	Michigan	5	-
Humulus lupulus		Michigan	10	-

[A] *D. humulicola* DNA from Michigan was used for the standard curve at a concentration of 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 ng/µl. All samples except *P. macularis* and *P. humuli* were extracted using the Zymo research Quick-DNA Fungal/Bacterial Kits (all fungal samples) or the Zymo research Quick-DNA Plant/Seed Kit (plant samples). All samples were tested in triplicate with a positive and negative control.

[B] The cycles for *D. humulicola* from Michigan are shown in Figure 3.1 of the standard curve. The cycle represents when the fluorescence signal is above the threshold of the *D. humulicola* assay.

Table 3.4. Detection of *Diaporthe humulicola* in hop rhizomes from the Michigan State University Plant Pathology Research Farm using the TEF based TaqMan qPCR assay for *D. humulicola*.

Plant	Average Cq	Symptomatic average (ng)[A]	Standard error	Average Cq	Asymptomatic average (ng)[A]	Standard error
Not inoculated						
A	30.76	5.25×10^{-3}	1.94×10^{-3}	37.62	< LOD	-
B	29.42	1.04×10^{-2}	1.22×10^{-3}	35.94[C]	1.66×10^{-4}	6.20×10^{-7}
C	39.03[B]	< LOD	-	-	-	-
D	30.34	$9.77\ 10^{-3}$	4.44×10^{-3}	38.64	< LOD	-
E	-	-	-	37.19[B]	< LOD	
Low severity of halo blight						
F	37.69[B]	< LOD	-	-	-	-
G	34.19	5.25×10^{-4}	9.92×10^{-5}	37.32	< LOD	-
H		-	-	38.58	< LOD	-
I	30.84	4.28×10^{-3}	6.70×10^{-4}	38.89[C]	< LOD	-
J	30.03	7.01×10^{-3}	2.53×10^{-4}	-	-	-
High severity of halo blight						
K	38.06[B]	< LOD	-	37.10[C]	< LOD	-
L	24.86	1.96×10^{-1}	4.54×10^{-2}	36.00	1.91×10^{-4}	1.04×10^{-4}
M	31.10	3.67×10^{-3}	6.75×10^{-4}	36.27	1.38×10^{-4}	1.97×10^{-5}
N	38.80	< LOD	-	32.65	3.04×10^{-3}	1.54×10^{-3}
O	29.10	1.26×10^{-2}	1.13×10^{-3}	-	-	-

[A] DNA concentration was derived from the standard curve equation from this study ($y = -3.643x + 22.175$) where Y equals the cycle that the relative fluorescence units exceeded the threshold for the assay and X equals the concentration present log10 (ng).
[B] Cq value from 1 sample (other two samples did not amplify).
[D] Average Cq value from 2 samples (other sample did not amplify).
< LOD represents samples that did amplify and cross the threshold set by the CFX Maestro Software but were under the limit of detection and not quantified

Table 3.5. qPCR–based quantification of *Diaporthe humulicola* DNA in a time series of hop leaves inoculated with *D. humulicola* using a TEF based TaqMan qPCR.

Day[A]	Cq values[B]	Std. error	Concentration (ng)[C]	Std. error
0	-	-	-	-
1	35.51	2.53×10^{-1}	2.71×10^{-04}	3.55×10^{-05}
2	35.33	1.00	4.74×10^{-04}	3.25×10^{-04}
3	36.44	8.18×10^{-1}	1.62×10^{-04}	7.60×10^{-05}
4	31.73	1.50	3.68×10^{-03}	2.26×10^{-03}
5	31.74	1.84	9.90×10^{-03}	8.36×10^{-03}
6	30.03	2.18	3.02×10^{-02}	2.62×10^{-02}
7	28.95	2.68	4.06×10^{-02}	2.90×10^{-02}
8	27.21	1.91	1.30×10^{-01}	1.09×10^{-01}
9	25.19	2.78	8.73×10^{-01}	7.09×10^{-01}

[A] Days post inoculation of hop leaves with 20 µl of a conidial suspension with a concentration of 1.51 x 10 ^ 5 conidia/ml, three leaves were collected each day. Day zero represents hop leaves that were not inoculated with *D. humulicola.*

[B] Cq values are defined as the average cycle in which the relative fluorescence units exceeded the threshold for the *D. humulicola* assay for three inoculated leaves each tested in triplicate.

[C] Average concentration for three leaves tested in triplicate using the equation $y = -3.643x + 22.175$ where Y equals the Cq value and x = concentration (ng).

Table 3.6. Results from the technology transfer of the *Diaporthe* TEF based TaqMan qPCR assay.

Lab #	Thermocycler model	Equation[A]	R^2	E[B]	Max RFU	Limit of detection (ng/µl)[C]	Agreement with blind samples (%)[D]
1	BioRad CFX	Y = -3.480x + 24.517	0.98	93.8	1279.4	0.001	100%
2	BioRad CFX	Y = -4.180x + -15.471	0.99	73.5	9236.9	0.001	100%
3	BioRad CFX	Y = -3.850x + 48.771	0.99	81.9	4398.6	0.001	100%
4	CFX Opus 96	Y = -3.810x + 52.267	0.98	82.4	10626.0	0.001	100%

[A] Equation is derived from standard curve using the following DNA concentrations 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 ng/µl. The equations were calculated using CFX Maestro Software.
[B] E = Amplification efficiency.
[C] Limit of detection is defined as the lowest concentration with 3 positive replicates from the standard curve concentrations.
[D] Blind DNA targets were 1 to 10 ng/µl of *D.* v*accinii, D. humulicola,* and a water control. In total 3 blind samples were positive for *D. humulicola* and 5 samples were negative.

Table 3.7. List of *Diaporthe* sequences used for TEF-based alignment for the development of the TEF2DH assay.

Identity	Accession number	Host[A]	Location
D. acuta	MK654803.1	*Pyrus pyrifolia*	China
D. biguttulata	KJ490461.1	*Citrus x Limon*	China
D. caryae	MK654896.1	*Pyrus pyrifolia*	China
D. caulivora	HM347689.1	*Dipsacus laciniatus*	Croatia
D. chrysalidocarpi	MT855876.1	N/A	China
D. cinnamomi	MH121546.1	N/A	China
D. citrichinensis	KJ490499.1	*Citrus japonica*	China
D. endocitricola	MT409336.1	*Citrus maxima*	China
D. eres	OM752197.1	*Vaccinium corymbosum*	Poland
D. foeniculacea	KC343830.1	*Foeniculum vulgare*	Portugal
D. fraxinicola	MH121560.1	*Fraxinus chinensis*	China
D. guangxiensis	MK523566.1	*Vitis vinifera*	China
D. helianthi	GQ250308.1	*Helianthus annuus*	Serbia
D. hongkongensis	KC343845	*Dichroa febrifuga*	China
D. huangshanensis	MN224670	*Camellia oleifera*	China
D. hubeiensis	MK523570.1	*Vitis vinifera*	China
D. humulicola	OQ256245	*Humulus lupulus*	USA
D. humulicola	MN180209	*Humulus lupulus*	USA
D. humulicola	MN180208	*Humulus lupulus*	USA
D. humulicola	OK001342	*Humulus lupulus*	Canada
D. humulicola	PP779618	*Humulus lupulus*	USA
D. humulicola	PP779617	*Humulus lupulus*	USA
D. humulicola	PP779614	*Humulus lupulus*	Canada
D. humulicola	PP779615	*Humulus lupulus*	Canada
D. humulicola	PP779616	*Humulus lupulus*	USA
D. infecunda	KC343852.1	*Schinus terebinthifolius*	Brazil
D. kadsurae	MH121566.1	*Acer sp.*	China
D. longicolla	HM347686.1	*Glycine max*	Croatia
D. melonis	GQ250314.1	*Cucumis melo*	USA
D. neotheicola	KC533443.1	*Vaccinium sp.*	Chile
D. novem	HM347696.1	*Glycine max*	Croatia
D. ovoicicola	KF576239.1	*Lithocarpus glaber*	China
D. paranensis	KC343897.1	Maytenus ilicifolia	Brazil
D. sambucusii	KY852507.1	*Sambucus williamsii*	China
D. sinensis	MK660449.1	*Amaranthus* sp.	China
D. sojae	JQ697860.1	*Glycine max*	Serbia
D. theicola	GQ250318	*Foeniculum vulgare*	Portugal
D. ueckeri	MN651265.1	*Glycine max*	Arkansas
D. undulata	KX999190.1	N/A	China
D. vaccinii	GQ250326.1	*Oxycoccus macrocarpos*	Poland
D. viniferae	MK500107.1	*Vitis vinifera*	China
D. viticola	GQ250327.1	*Vitis vinifera*	Portugal
D. zaobaisu	MK654855	*Pyrus x bretschneideri*	China

[A] N/A = not available

FIGURES

Figure. 3.1. Standard curve plot for the TEF-based TaqMan assay using a D. humulicola DNA dilution series (10 to 0.000001ng/µl) in triplicate. Amplification that occurred below the limit of detection (0.0001 ng/µl) was removed from analysis due to sporadic amplification. The linear correlation with a regression coefficient (R2) and the assay efficacy (E) are presented in each graph. A. Standard curve plot without the IPC. B. Standard curve plot with the IPC and hop leaf DNA (10ng/µl). Cq values from the IPC are not shown.

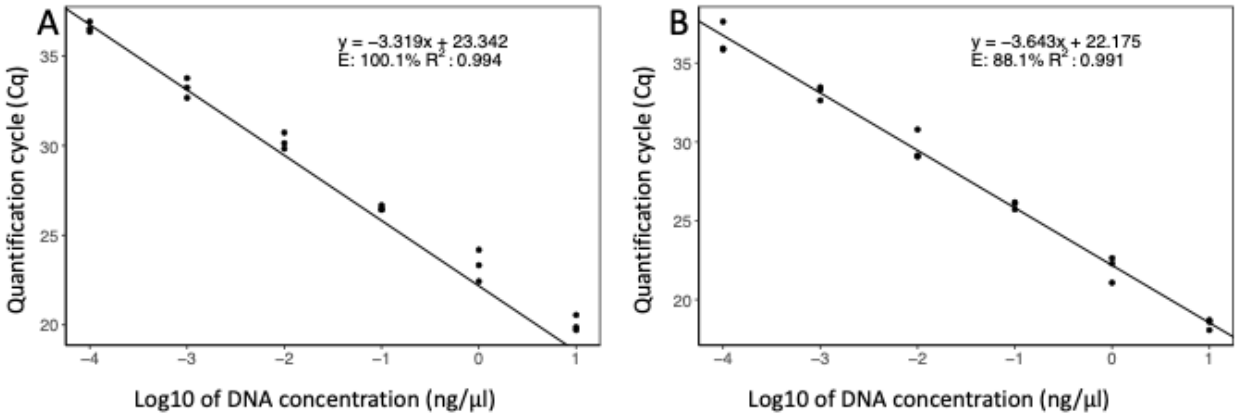
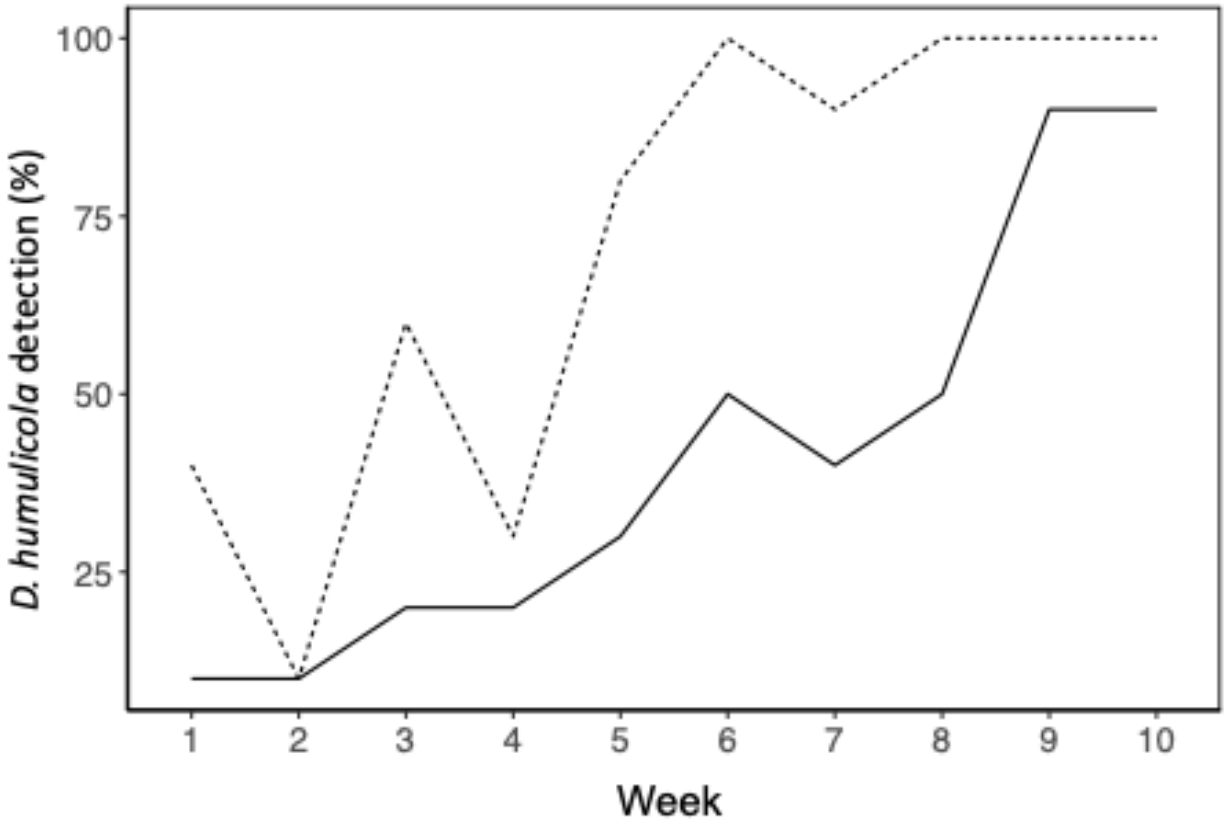


Figure 3.2. Line graph representing the percentage of hop cones (n=10 per week) that had D. humulicola detected using two different methods, plating of cone tissue onto potato dextrose agar (solid line) and a TEF based TaqMan qPCR assay that was run on DNA extracted from the cones used for isolation (dotted line). Cones were collected from the same plant in a research yard in East Lansing, Michigan over 10 weeks (starting 22 May) in 2022.



LITERATURE CITED

Agehara, S., Acosta-Rangel, A., Gallardo, M., and Vallad G. 2020. Selection and preparation of planting material for successful hop production in Florida. UF/IFAS Extension. https://www.researchgate.net/profile/Zhanao-Deng/publication/343656652_Hop_Yard_Establishment_and_Trellis_Construction_in_Florida/links/5f36c152458515b7291f449a/Hop-Yard-Establishment-and-Trellis-Construction-in-Florida.pdf.

Allan-Perkins, E., Li, D., Schultes, N., Yavuz, S., and LaMondia, J. 2020. The identification of a new species, *Diaporthe humulicola*, a pathogen causing diaporthe leaf spot on common hop. Plant Dis. 104:2377-2390.

Beaver-Kanuya, E., Wright, A. A., Szostek, S. A., Khuu, N., and Harper, S. J. 2021. Development of RT-qPCR assays for the detection and quantification of three carlaviruses infecting hop. J. Virol. Methods 292:114124.

Bienapfl, J. C., Ocamb, C. M., Klein, R., and Nelson, M. 2001. Fusarium cone tip blight: A new disease of *Humulus lupulus*. (Abstr.). Phytopathology 91:S8.

Bilodeau, G. J., Martin N. F., Coffey M. D., and Blomquist, C. L. 2014. Development of a multiplex assay for genus- and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. Phytopathology 104:733-748.

Brewer, M. T., and Milgroom, M. G. 2010. Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator,* from diverse *Vitis* species. BMC Evol. Biol.. 10:268.

Bustin, S, A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Muller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. L. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611–622.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421.

Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets of speciation studies in filamentous ascomycetes. Mycologia 91:553-556.

Cerenak, A., Kralj, D., and Javornik, B. 2009. Compounds of essential oils as markers of hop resistance (*Humulus lupulus*) to powdery mildew (*Podosphaera macularis*). Acta agriculturae Slovenica 93:267-273.

Chojak-Koźniewska, J., Kuźniak E., and Zimny, J. 2018. The effects of combined abiotic and pathogen stress in plants: insights from salinity and *Pseudomonas syringae pv lachrymans* interaction in cucumber. Front. Plant Sci. 9:1691.

Crandall, S. G., Ramon, M. L., Burkhardt, A. K., Rodriguez, J. C. B., Adair, N., Gent, D. H., Hausbeck, M. K., Quesada-Ocampo, L. M., and Martin, F. N. 2021. A multiplex TaqMan qPCR assay for detection and quantification of clade 1 and clade 2 isolates of *Pseudoperonospora cubensis* and *Pseudoperonospora humuli.* Plant dis.105: 3154-3161.

Edgar, R.C. 2021. MUSCLE v5 enables improved estimates of phylogenetic tree confidence by ensemble bootstrapping, Nat. Commun. 13.

Elverson, T. R., Kontz, B. J., Markell, S. G., Haverson, R. M., and Mathew, F. M. 2020. Quantitative PCR assays developed for *Diaporthe helianthi* and *Diaporthe gulyae* for phomopsis stem canker diagnosis and germplasm screening in sunflower (*Helianthus annuus*). Plant Dis. 104:793-800.

Foster, A., Mills, A., and Mccallum, J. 2024. First report of *Diaporthe humulicola* causing halo blight of hop (*Humulus lupulus* L.) in Prince Edward Island, Canada Can. J. Plant Pathol. Preprint.

Gent, D. H., Nelson, M. E., Fransworth, J. L., and Grove, G. G. 2009. PCR detection of *Pseudoperonospora humuli* in air samples from hop yards. Plant Pathol. 58:1081-1091.

Gent, D. H., Grove, G.G., Nelson, M. E., Wolfrenbarger, S. N., and Woods, J. L. 2013. Crop damage caused by powdery mildew on hop and its relationship to late season management. Plant Pathol. 63:625-639.

Gent, D. H., Adair, N., Hatlen, R. J., Miles, T. D., Richardson, B., Rivedal, H. M., Ross, C. J., Wiseman, M. S. 2024. Detection of *Podosphaera macularis* in air samples by quantitative PCR. Plant Dis. 108:2820-2829.

Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., and Crous, P. W. 2013. *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia 31:1-41.

Guček, T., Jakše, J., and Radišek, S. 2023. Optimization and validation of singleplex and multiplex RT-qPCR for detection of citrus bark cracking viroid (CBCVd), hop latent viroid (HLVd), and stunt viroid (HSVd) in hops (*Humulus lupulus*). Plant Dis. 107:3592-3601.

Guillemette, T., Iacomi-Vasilescu, B., and Simoneau, P. 2004. Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. Plant Dis. 88:490-496.

Hatlen, R. J., Higgins, D. S., Venne, J., Rojas, J. A., Hausbeck, M. K., and Miles, T. D. 2022. First report of halo blight of hop (*Humulus lupulus*) caused by *Diaporthe humulicola* in Quebec, Canada. Plant Dis. 106:1750.

Hatlen, R. J., Swift, R. M., Miles, L. A., Byrne, J. M., and Miles, T. D. 2023a. First report of *Colletotrichum fioriniae* infecting hop (*Humulus lupulus*) in Michigan. Plant Dis. 107:3280.

Hatlen, R. J., Adair, N., Wisemen, M., Sysak, R., Rojas, A., Gent, D., and Miles, T. D. 2023b. Development of integrated qPCR diagnostic tools to determine the life cycle of *Diaporthe humulicola* in hop (*Humulus lupulus*). Phytopathology 113:S3.1-S3.197.

Havill, J. S, Hatlen, R. J., Muehlbauer, G. J., and Miles, T. D. 2023. First report of halo blight on hop (*Humulus lupulus*) caused by *Diaporthe humulicola* in Minnesota. Plant Dis. 107:2523.

Hay, F. S. 2009. Black root rot. Pages 16-17 in: Compendium of Hop Disease and Pests. Mahaffee, W. F., Pethybridge, S. J. and Gent, D. H., eds. American Phytopathological Society, St. Paul, MN.

Higgins, D. S., Hatlen, R. J., Byrne, J. M., Sakalidis, M. L., Miles, T. D., and Hausbeck, M. K. 2021a. Etiology of halo blight in Michigan hopyards. Plant Dis. 105:859-872.

Higgins, D. S. and Hausbeck, M. K. 2021 Susceptibility of hop cultivars and rootstock to downy mildew caused by *Pseudoperonospora humuli*. HortScience 56:543-550.

Higgins, D. S., Miles, T. D., Byrne J. M., 2022. Optimizing molecular detection for the hop downy mildew pathogen *Pseudoperonospora humuli* in plant tissue. Phytopathology 112:2426-2439.

Hilario, S., and Gonçalves, M. F. M., 2023. Mechanisms underlying the pathogenic and endophytic lifestyles in *Diaporthe*: an omics-based approach. Horticulturae 9:4:423.

Howard, B. H. 1965. Regeneration of the hop plant (*Humulus lupulus* L.) from softwood cuttings. I. the cutting and its rooting environment. J. Hortic. Sci. 40:181-191.

Hop growers of America, 2022. USA Hops 2022 Statistical Report, https://www.usahops.org/img/blog_pdf/435.pdf.

Hosseini, B., Voegele, R. T., and Link, T. I. 2021. Establishment of a quadruplex real-time PCR assay to distinguish the fungal pathogens *Diaporthe longicolla*, *D. caulivora*, *D. eres*, and *D. novem* on soybean. Plos One 16:e0257225.

Johnson, D. A., Engelhard, B., and Gent, D. H. 2009. Downy mildew. Pages 18-22 in: Compendium of Hop Diseases and Pests. W. F. Mahaffee, S. J., Pethybridge, and D. H. Gent, eds. American Phytopathological Society, St. Paul, MN.

Kealik, P. and Ricchi, M. 2017. A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. Front. Microbiol. 8:108.

Klein-Gordon, J. M., Hatlen, R. J., and Miles, T. D., 2023. First report of stem blight caused by *Diaporthe eres* on highbush blueberry (*Vaccinium corymbosum*) in Michigan. Plant Dis. 107:2529.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, J., Sindelka, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., and Zoric, N. 2006. The real-time polymerase chain reaction. Mol. Asp. Med. 27:95-125.

Macchioni, V., Picchi, V., and Carbone, K. 2022. Hop leaves as an alternative source of health-active compounds: effect of genotype and drying conditions. Plants 11:99.

Mahaffee, W. F. and Engelhard, B. 2009. Grey mold, Pages 24-25 in: Compendium of Hop Diseases and Pests. Mahaffee, W. F., Pethybridge, S. J, and Gent, D. H., eds. American Phytopathological Society, St. Paul, MN.

Mahaffee, W. F., Engelhard, B., Gent, D. H., and Grove, G. G. 2009. Powdery mildew. Pages 25-30 in: Compendium of Hop Diseases and Pests. Mahaffee, W. F., Pethybridge, S. J., and Gent, D. H. eds. American Phytopathological Society, St. Paul, MN.

Neve, R. A. 1991. Hops, first edition, Suffolk, Chapman and Hall.

Peredo, L. E., Arroyo-Garcia R., Revilla, M. A. 2009. Epigenetic changes detected in micropropagated hop plants. J. Plant Physiol. 166:1101-1111.

Pereira, C. M., Ferreira, B. W., Barreto, R. W., 2020. First report of *Cercospora apii* sensu lato 'sp. Q' Leaf Spot on hop in Brazil. Plant Dis. 107:947.

Pethybridge, S. J., Hay, F. S., Barbara, D. J., Eastwell, K. C., and Wilson, C. R. 2008 Viruses and viroids infecting hop: significance, epidemiology, and management. Plant Dis. 92:324-338.

Pethybridge, S. J., Hay, F. S., Wilson, C. R., Sherriff, L. J., and Leggett, G. W. 2001. First report of *Fusarium crookwellense* causing tip blight on cones of hop. Plant Dis 85:1208.

Pethybridge, S. J., Hay, F. S., Wilson, C. R., Sherriff, L. J., and Leggett, G. W. 2001a. First report of infection of hop cones by *Alternaria alternata* in Australia. Plant Dis. 85:804.

Radišek, S., Jakse, J., Javornik, B., and Gruyter, J. 2008. First report of *Phoma exigua* as a pathogen of hop in Slovenia. Plant Pathol. 57:381.

Rojas, J. A., Miles, T. D., Coffey, M. D., Martin, F. N., Chilvers, M. I. 2017. Development and application of qPCR and RPA genus- and species-specific detection of *Phytophthora sojae* and *P. sansomeana* root rot pathogens of soybean. Plant Dis. 101:1171-1181.

Schönberger, D. and Kotelecky, T, 2001. 125th anniversary review: the role of hops in brewing., JIB 117:259-267.

Szymanski, S., Longley, R., Hatlen, R. J., Heger, L., Sharma, N., Bonito, G., and Miles, T. D. 2023. The blueberry fruit mycobiome varies by tissue fype and fungicide treatment. Phytobiomes 7:208-219.

Sirrine, R., Lizotte, E., Brown, D., O'Brien, T., and Leach, A. 2014. Michigan State University Extension, https://www.canr.msu.edu/uploads/resources/pdfs/estimated_costs_of_producing_hops_in_michigan_(e3236).pdf.

Sharma, S., Strickland, D. A, Hay, F., and Pethybrige, S. J. 2023. First report of halo blight on hop caused by *Diaporthe humulicola* in New York. Plant Dis. 107:216.

Tomlan, M. A. 2013. Tinged with Gold: Hop Culture in the United States. The University of Georgia Press. Athens, GA.

Twomey, M. C., Wolfenbarger, S. N., Woods, J. L., and Gent, D. H. 2015. Development of partial ontogenic resistance to powdery mildew in hop cones and its management implications. PLoS ONE 10:3.

Udayanga, D., Castlebury, L. A., Rossman, A.Y., and Hyde, K. D. 2014. Species limits in *Diaporthe*: molecular re-assessment of *D. citri*, *D. cytosporella*, *D. foeniculina* and *D. rudis*. Persoonia 32:83-101.

Wang Y., Ahang, W., Wang, Y., and Zheng, X. 2006. Rapid and sensitive detection of *Phytophthora sojae* in soil and infected soybeans by species-specific polymerase chain reaction assays. Phytopathology 96:1315-1321.

Ye, J., Couloutis, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134.

CHAPTER 4: A DRAFT GENOME ASSEMBLY FOR *DIAPORTHE HUMULICOLA* THE CAUSAL AGENT OF HALO BLIGHT OF HOP

Abstract

Diaporthe humulicola is the causal agent of halo blight in hops. Halo blight is a major concern in the northeastern United States and Canada capable of causing significant yield loss in hopyards. Although there is currently a reference genome for this species in GenBank, a four-gene maximum likelihood phylogeny shows that this genome is not the same as previously described *D. humulicola*, but is likely a novel species within the *Diaporthe* genus. In this study, we report a draft genome for *Diaporthe humulicola* that was assembled with both long and short read sequences. The draft genome consists of 49.82 MB assembled into 180 contigs, with a GC content of 51.2%. The genome was annotated, and 11,773 genes were predicted, including 2,752 genes with common names. This paper represents the first genomic resource for *Diaporthe humulicola*.

Diaporthe humulicola is an important pathogen of hop (*Humulus lupulus)* causing halo blight of hop in the northeastern United States and Canada (Allan-Perkins et al. 2020, Hatlen et al. 2021, Higgins et al. 2021, Sharma et al. 2023, Foster et al. 2024, Havill et al. 2023). The pathogen can colonize all tissue in the hop plant and can cause lesions on the leaves and desiccation of the hop cone (Allan-Perkins et al. 2020; Higgins et al. 2021; Hatlen et al. 2025 unpublished). Infections when left untreated can cause up to 56% percent yield loss (Higgins et al. 2021). Currently there is a reference genome for *D. humulicola* (ASM4020912v1) isolate BL38 on GenBank. Based on a maximum likelihood phylogram using the translation elongation factor 1-alpha (TEF), internal transcribed spacer (ITS), beta tubulin (B-TUB) and histone (HIS) genes of 17 different *Diaporthe* species and from isolate BL38, the BL38's genome is not *D.*

humulicola but is likely a novel species within the *Diaporthe* genus (Figure 4.1). This study presents the first verified annotated draft genome for *D. humulicola.*

Diaporthe humulicola isolate MI-0318 was isolated from hop leaf tissue collected from a commercial hopyard in Barry County, Michigan. The pathogen was isolated onto potato dextrose agar (PDA) and conidia were stored in a glycerol solution as previously described (Miles et al. 2011). The isolate was identified using morphological and molecular analysis, and results from the analysis are featured in a publication by Higgins et al. 2021.

For high molecular weight (HMW) DNA extraction, *D. humulicola* isolate MI-0318 was first grown on PDA and then transferred to potato dextrose broth (PDB) for mycelial production. Flasks were inoculated with four mycelial plugs and incubated at room temperature for one week in a MaxQ 4000 Benchtop Orbital Shaker (ThermoFisher Scientific, Waltham, MA). Mycelia were harvested by filtering the media using Büchner funnels (VWR International, LLC, Radnor, PA) and vacuum suction. Mycelia were washed twice with sterile water to remove excess media. The dry mycelia collected on sterile filter paper was then transferred into 50 ml Falcon tubes. HMW DNA was extracted according to the protocol by Schwessinger and Rathjen (2017), with the following modifications: samples were incubated overnight under rotation in a Bambino II hybridization oven (Boekel Scientific, Feasterville, PA) at 30ºC after adding proteinase K, ice-cold isopropanol was added to precipitate the DNA and incubated at -20ºC for 20 minutes, and samples were incubated overnight at 4ºC instead of room temperature until the DNA pellet was completely dissolved. The quality of the HMW DNA was assessed using agarose gel-based electrophoresis and UV-Vis measurements on a ND-1000 Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA), while the total DNA concentration was quantified by Qubit (ThermoFisher Scientific, Waltham, MA) using the dsDNA BR assay. For the Oxford

Nanopore Technology sequencing, the obtained DNA, without size selection or shearing, was used for library preparation using the SQK-LSK109 kit following the manufacturer's specifications. The sequencing was performed on a MinION R9 flow cell on a Nanopore MinION (Oxford Nanopore Technologies, Oxford, United Kingdom) device by the Microbial Genome Sequencing Center (Pittsburgh, PA; currently SeqCoast https://seqcoast.com/). The resulting files were provided as fastq files after base calling was performed using Guppy v4.2.2 by the sequencing center. A total of 2.2 million Nanopore reads with a read length N50 value of 1,874 bp were assembled using the software Flye v2.8.3 (Kolmogorov et al. 2019) with default settings. The initial draft genome assembly consisted of 472 contigs with a total length of 50.66 MB, so that the sequencing coverage was estimated as 31.8X.

After the initial assembly, DNA was extracted from the MI-0318 isolate using the Omega Bio-Tek E.Z.N.A HP fungal DNA Kit (Omega Bio-Tek Norcross, GA), and purified using the Zymo Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA) for the purpose of genome polishing. Libraries were prepared using the IDT xGen DNA EZ kit (Integrated DNA Technologies, Coralville, IA), and then a 2 x 150 bp sequencing run was conducted on the Illumina NovaSeq X Plus (Illumina, San Diego, CA). Sequencing for genome polishing was conducted by the Oklahoma Medical Research Foundation NGS Core.

Reads from the Illumina sequencing were then trimmed and filtered using the BBTools v39.01 (Bushnell et al. 2017) . The assembly was then polished with the 150 paired end sequencing data for 5 rounds using Pilon v1.24 (Walker et al. 2014) with default settings. The assembled genome was then filtered by length and contigs below 3500 bp were removed from the draft assembly. The final assembly included 49.82 MB with 180 contigs. The genome has an N50 of 541.62 KB, a L50 of 25, and 51.2% GC content. The genome completeness was

determined using Benchmarking Universal Single-Copy Orthologs (BUSCO) v5.4.7 (Manni et al. 2021). The BUSCO analysis was done with the sordariomycetes_odb10 library using 3817 BUSCO groups and the assembled genome has 97.2% (3704) of the genes tested, 97% of the genes were complete and single copies, 0.2% of the genes were duplicated, 0.8% of the genes were fragmented, and 2% of the genes were missing.

Repeat sequences were detected by first using RepeatScout v1.0.6 (Price et al. 2005) and then feeding the results into RepeatMasker v4.1.6 (Smit et al. 2013-2015) with default settings, where 15.12% of the genome was masked. The genome was annotated using the Funannotate v1.8.17 (Palmer and Stajich 2020) pipeline using RNA seq data from *Diaporthe vaccinii* (SRR29094103). The pipeline predicted 11,773 genes with 2,752 of the genes having common names. In addition, 134 tRNAs were predicted, as well as 630 carbohydrate-active enzymes. Secreted proteins were predicted using SignalP v6.0 (Teufel et al. 2022) using the fast model, and putative cytoplasmic and apoplastic effectors were identified using EffectorP v3.0 (Sperschneider and Dodds 2021); 1150 signal peptides were predicted, with 314 predicted as effectors, 84 were predicted cytoplasmic effectors and 230 were predicted apoplastic effectors. The program AntiSMASH v7.0 (Blin et al. 2023) was run, and 11 gene clusters were predicted representing 1052 genes. Genome statistics are reported in Table 4.1.

This genome is the first annotated genome for *Diaporthe humulicola.* The annotated genome provides genetic resources for an emergent pathogen in the United States and Canada. This genome provides the basis for comparative genomics studies of *D. humulicola* from different hop growing regions to better understand the ecology and demographics of *D. humulicola*.

Data availability

This Whole Genome project has been deposited at DDBJ/ENA/GenBank under the accession JBLTED000000000. The version described in this paper is version JBLTED010000000.The reads used for assembly and the reads for polishing were both deposited to the SRA under the accession numbers SRR32321459 and SRR32321458, respectively. The BioSample number is SAMN46792095 and BioProject number is PRJNA1222868.

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TABLES

Table 4.1. Genome assembly and annotation statistics for *Diaporthe humulicola* isolate Mi-3.

Feature	***D. humulicola***
Long reads[A]	2,202,858
Short reads[B]	7,925,158
Contigs	180
Assembly size (Mb)	49.82
N50 (bp)	541,620
GC (%)	51.2
BUSCO Completeness (%)	97.2
Genes	11,773
Named genes	2,752
mRNAs	11,644
tRNAs	134
Genome accession	JBLTED000000000

[A] long reads used for assembly of the genome.
[B] short reads used for the polishing of the genome.

FIGURES

Figure 4.1. Maximum likelihood phylogram of concatenated ITS, TEF, B-TUB, and HIS of 17 different Diaporthe species. The 17 sequences were aligned using Geneious prime (Biomatters Ltd.) The alignment was then used to determine the best substitution model using MEGA11 (Stecher et al. 2020).

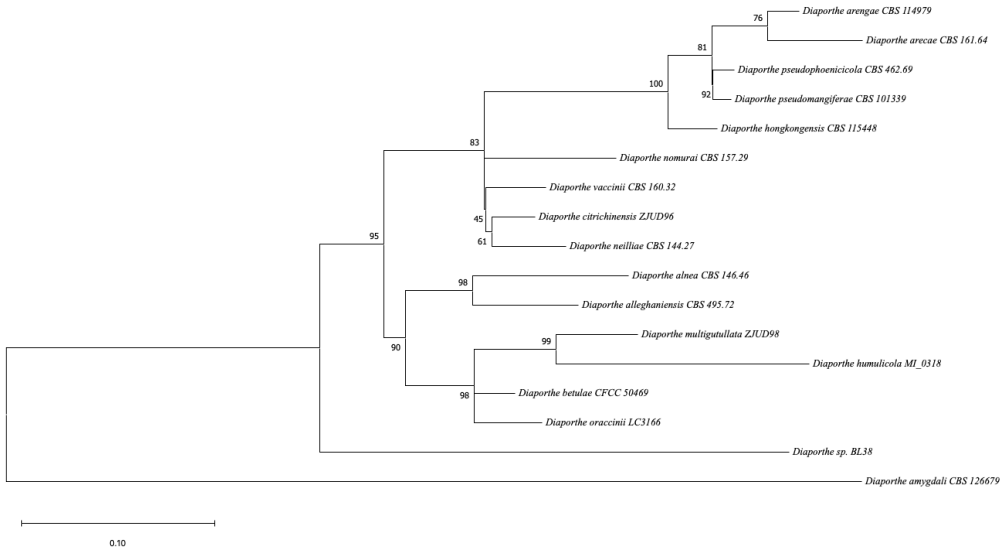

Figure 4.1. (cont'd)

The Hasegawa-Kishino-Yano model was used with a discrete Gamma distribution (5 categories (+G, parameter = 0.2152)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 31.48% sites). 1000 replicates were conducted. The tree with the highest log likelihood (-7996.15) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All named species sequences were obtained from Du et al. (2016), Gao et al. (2016), Gomes et al. (2013), Higgins et al. (2021). Huang et al. (2015), Udayanga et al. (2014). The *Diaporthe* sp. sequences were obtained from the genome assembly ASM4020912v1. CFCC = China Forestry Culture Collection Center, Chinese Academy of Forestry, Beijing, China; CBS = Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; ZJUD = Zhe Jiang University, China; LC = Working collection of Lei Cai, housed at Institute of Microbiology, CAS, China.

LITERATURE CITED

Allan-Perkins, E., Li. D., Schultes, N., Yavuz, S., and LaMondia, J. 2020. The identification of a new species, *Diaporthe humulicola*, a pathogen causing diaporthe leaf spot on common hop. Plant Dis. 104:2377-2390.

Blin, K., Shaw., S., Augustin, H. E., Reitz, Z. L., Biermann, F., Alanjary, A., Fetter, A., Terlouw, B. R. Metcalf, W. W., Helfrich, E. J. N., Van Wezel, G. P., Medema, M., H. M., and Weber. T. 2023. antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. Nucleic Acids Res. 52:W1:W46-W50.

Bushnell, B., Rood, J., Singer, E. 2017. BBMerge – Accurate paired shotgun read merging via overlap. PLoS ONE. 12:10

Du, Z., Fan, X. L., Hyde, K. D., Yang, Q., Liang, Y.-M., and Tian, C.-M. 2016. Phylogeny and morphology reveal two new species of *Diaporthe* from Betula spp. in China. Phytotaxa 269:90-102.

Gao, Y., Liu, F., Duan, W., Crous, P. W., and Cai, L. 2017. *Diaporthe* is paraphyletic [published correction appears in IMA Fungus 2017;8:82]. IMA Fungus 8:153-187.

Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., and Crous, P. W. 2013. *Diaporthe*: A genus of endophytic, saprobic and plant pathogenic fungi. Persoonia 31:1-41.

Foster, A., Mills, A., and Mccallum, J. 2024 First report of *Diaporthe humulicola* causing halo blight of hop (Humulus lupulus L.) in Prince Edward Island, Canada. Can. J. Plant Pathol. 858-873.

Hatlen, R. J., Higgins, D. S., Venne, J., Rojas, J. A., Hausbeck, M. K., and Miles, T. D. 2021. First report of halo blight of hop (Humulus lupulus) cause by *Diaporthe humulicola* in Quebec, Canada. Plant Dis. 106:1750.

Havill J. S, Hatlen R. J., Muehlbauer G. J., and Miles, T. D. 2023. First report of halo blight on hop (*Humulus lupulus*) caused by *Diaporthe humulicola* in Minnesota. Plant Dis. 107:8: 2523.

Higgins, D. S., Hatlen, R. J., Byrne, J. M., Sakalidis, M. L., Miles, T. D., and Hausbeck, M. K. 2021a. Etiology of halo blight in Michigan hopyards. Plant Dis. 105:859-872.

Huang, F., Udayanga, D., Wang, X., Hou, X., Mei, X., Fu, Y., Hyde, K. D., and Li, H. 2015. Endophytic *Diaporthe* associated with Citrus: A phylogenetic reassessment with seven new species from China. Fungal Biol. 119:331-347.

Kolmogorov M., Yuan., J., Lin, Y., and Prevzner, P., 2019. Assembly of long, error-prone reads using repeat graphs. Nat. Biotechnol 37:540–546.

Manni, M., Berkeley, M. R., Seppey, M., Simão, F. A., Zdobnov, E. M. 2021. BUSCO Update: Novel and Streamlined Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of Eukaryotic, Prokaryotic, and Viral Genomes. Mol. Biol. Evol. 38:10: 4647–4654.

Miles, T. D., Day, B., and Schilder, A. C. 2011. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. Mol. Plant Pathol. 12: 463-477.

Palmer, J. M., and Stajich J. 2020. Funannotate v1.8.1: Eukaryotic genome annotation (v1.8.1). Zenodo. https://doi.org/10.5281/zenodo.4054262.

Price, A. L., Jones, N. C. and Pevzner P. A., 2005. De novo identification of repeat families in large genomes. Bioinform. 21: Issue suppl_1: i351–i358.

Schwessinger, B. and Rathjen, J. P. 2017. Extraction of High Molecular Weight DNA from Fungal Rust Spores for Long Read Sequencing. In: Periyannan, S. (eds) Wheat Rust Diseases. Methods in Molecular Biology, vol 1659. Humana Press, New York, NY.

Sharma, S., Strickland, D., Hay, F., and Pethybridge, S. J. 2023. First report of halo blight in hop caused by *Diaporthe humulicola* in New York. Plant Dis. 107:216.

Smit, A. F. A., Hubley, R., and Green, P. 2013-2015. RepeatMasker Open-4.0. http://www.repeatmasker.org.

Sperschmeider, J., and Dodds, P. N. 2022. EffectorP 3.0: Prediction of Apoplastic and Cytoplasmic Effectors in Fungi and Oomycetes. MPMI. 35: 0894-0282.

Stecher G., Tamura, K., and Kumar S. 2020. Molecular Evolutionary Genetics Analysis (MEGA) for macOS. Molecular Biology and Evolution 37:1237-1239.

Teufel, F., Armenteros, J. J. A., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., Heijne, G. V., Nielsen, H., 2022. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nat Biotechnol 40: 1023–1025.

Udayanga, D., Liu, X., Crous, P. W., McKenzie, E. H., Chukeatirote, E., and Hyde, K. D. 2012. A multi-locus phylogenetic evaluation of *Diaporthe* (*Phomopsis*). Fungal Divers. 56:157-171.

Walker B. J., Abeel, T., Shea., T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo C., A., Zeng, Q., Wortman, J., Young, S. K., and Earl, A. M. 2014. Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement. PLoS ONE 9(11): e112963.

CHAPTER 5: DIVERSITY OF FUNGI ISOLATED FROM HOP CONES IN MICHIGAN AND GENETIC DIVERSITY OF *DIAPORTHE HUMULICOLA* POPULATIONS IN THE EASTERN UNITED STATES AND CANADA

Abstract

Diaporthe humulicola isolates from Michigan, New York, Minnesota, and Canada, were sequenced using 150 x 150 bp reads with a desired coverage of 10x. Single nucleotide polymorphisms (SNPs) were discovered and filtered using GATK. After filtering and clone correction 64 isolates were left for analysis. Population Structure was determined using STRUCTURE v.2.3.4 and using the Evanno method the populations cluster into 4 or 6 different populations. SNPs were used to produce PCA plots that show that Michigan isolates are closely clustered with isolates from Canada and New York, and with two isolates from Minnesota. The rest of the Minnesota samples clustered in an independent cluster. F_{st} values for Minnesota isolates appear to have high levels of population differentiation when compared to the different populations. Mating types were determined for each isolate where the *Mat1-2-1* loci were the larger part of the whole population with 59% of the isolates having this locus. We also detected signals of recombination in the fungal populations. Results from these experiments can be used as a baseline to study D. humulicola in future population genetics studies.

Introduction

Hop (Humulus lupulus) is a dioecious perennial plant traditionally used in beer production, but it's also valued for its role in nonalcoholic teas, flavored waters, and for the medicinal properties of its cones (Macchioni et al. 2022; Schönberger and Kostelecky 2011; Rossini et al. 2021). Historically, hops were grown in the eastern United States. In 1895, the majority of the hops grown in the United States were produced in New York. By 1879, hop

production was still predominantly in New York, but it had spread to the western states of California and Washington and to the midwestern states of Michigan and Wisconsin. By 1889, hop production had left the Midwest due to insect and disease pressure and became focused on the western United States (Tomlan et al. 2013).

In 2007, low hop yields in the United States led to global shortages and a resurgence of hop cultivation in the Midwest and eastern United States (Sirrine et al. 2014). As of 2023, the Pacific Northwest remains the leading producer of hops, but Michigan and New York are now the fourth- and fifth-largest hop growers in the United States (Hop Growers of America 2023). With hop production shifting back to the eastern United States, new pathogens are emerging as challenges for hop growers.

Hop production differs in the eastern United States as the summers are more humid and there is a higher chance of rain than the Pacific Northwest (Higgins et al. 2021). This change in climate allows a conducive environment for new and different pathogens to infect hops (Lahlali et al. 2024). Thus, research must be done to determine what new diseases are relevant for growers in the eastern United States and Canada.

Two new fungal pathogens have recently been discovered to infect hop. There have been reports of *Colletotrichum fioriniae* infecting hop cones in the Midwest, but the pathogen does not cause significant yield loss (Hatlen et al. 2023). In 2015 the first report of halo blight of hop was detected in the New York (Sharma et al. 2022). This pathogen was not described until 2020 by two researchers in Michigan and Connecticut (Allen-Perkins et al. 2020; Higgins et al. 2021). The pathogen can cause significant yield loss by desiccating the hop cone, leading to shattering during harvest (Higgins et al. 2021). The pathogen is widespread in the eastern United States and Canada (Hatlen et al. 2022; Havill et al. 2023; Sharma et al. 2023; Foster et al. 2024). The

pathogen has not been detected in the Pacific Northwest of the United States or Germany (Agricultural Research Service (USDA) 2025).

There are a limited options for control for *D. humulicola* as there are no fungicides registered for this disease (Hatlen et al. 2025). Understanding the genetic diversity and structure of the pathogen is important as there seems to be varied response to different modes of action (Allan-Perkins 2021; Hatlen et al. 2025). Understanding the genetic structure may have applications for developing resistant hop plants. (Litrico and Violle 2015).

Diaporthe is a large genus of fungi and can be plant pathogens, non-pathogenic endophytes or saprobes (Gomes et al. 2013). The genera can either be heterothallic or homothallic, and mating is controlled by two homologous loci (Kanematsu et al. 2007). Heterothallic *Diaporthe* species have one of the mating loci per mating type and homothallic *Diaporthe* species contain both of the homologous loci or portions of both loci (Santos et al. 2010). While the sexual phase of *D. humulicola* has not been detected (Allan-Perkins 2021), it is important to understand if the pathogen has the ability to sexually recombine, as sexual recombination in fungi can lead to new combinations of resistance mutations (Taylor and Cunniffe 2023).

In this work we determine what fungal pathogens are present in hop cones across the state of Michigan. We then use the *D. humulicola* samples collected from the survey along with isolates from the eastern United States and Canada in a population genetics study to determine the genetic diversity and structure of the species *D. humulicola.* We also determine the mating strategy of *D. humulicola,* and which mating genes are present in our populations.

Methods

Fungal survey of Michigan hopyards

From August to September from 2019 to 2021 a survey of the diseased hop cones was conducted in Michigan. Surveys were conducted on 15 farms in 2019, 11 in 2020, and 10 in 2021 (Table 5.5). Hop fields were scouted for diseased cones and 10 hop plants in different rows were selected for sampling (Figure 5.1). From each hop plant ten diseased hop cones were removed from the plant and stored in a cooler on ice while being transported to a -20 ° C freezer, where cones were stored until processing. Hop cones were then surface sterilized and fungal isolates were isolated according to methods found in Higgins et al. (2021). Isolates were then transferred to fresh potato dextrose agar (PDA) (Difico, Sparks, Maryland), yeast or bacteria like organism were discarded. Fungi collected were initially identified based on morphology to determine the genera, using a microscope and tape mount slides. All fungal samples were stored as mycelia and/or conidia, in a glycerol stock solution at -80° C degrees (Higgins et al. 2021; Miles et al. 2011)

Other *Diaporthe* samples

Other *D. humulicola* samples were collected from New York, Minnesota, and Canada for the purpose of expanding the range of *D. humulicola* (Minnesota and Canada) (Hatlen et al. 2022; Havel et al. 2023.) or to help growers detect if *D. humulicola* in their yards (New York). Fungi were isolated from leaf or cone tissue following the same isolation techniques used in Higgins et al. (2021). Only *D. humulicola* isolates were collected and stored from these yards. All fungal samples were stored as mycelia and conidia, in a glycerol stock solution at -80 º C (Higgins et al. 2021; Miles et al. 2011).

Molecular Identification of *Diaporthe humulicola* and other fungal isolates

Isolates that were collected and suspected to be *D. humulicola* based on fungal morphology (n = 97) and other fungi form the survey (n = 42), were revived from long term storage onto PDA. *Diaporthe* spp. cultures were grown in an incubator for ~30 days at 21.5 ° C with a 12-hour photoperiod to allow for the production of pycnidia and conidia. Other genera of fungi were grown for a week at room temperature before processing. The mycelia, pycnidia, and conidia of the fungal isolates were collected into 1.1-ml DNA extraction tubes with a metal bead (DOT Scientific, Burton, MI) using a sterilized toothpick. Mycelia, pycnidia and conidia were lyophilized for 24 h and pulverized for 15 s at 30 Hz with a Mixer Mill MM 400 (Retsch, Haan, Germany).

DNA was extracted from the lyophilized fungal tissue using MagMAX plant DNA kit (Applied Biosystems, Foster City, CA) and the Kingfisher Flex (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The extracted DNA was then further processed using the Zymo DNA Clean & Concentrator-25 kit (Zymo, Tustin, California) to remove polymerase chain reaction (PCR) inhibitors from the DNA. The internal transcribed spacer (ITS) region of each sample was then amplified using primers ITS1/ITS4 (White et al. 1990), using reagents and thermocycling conditions for *D. humulicola* from Higgins et al. (2021). This procedure was also used for the all fungi. The amplicons were purified using the Zymo DNA Clean & Concentrator-25 kit before sequencing. Amplicons were then sequenced in both directions by the MSU RTSF genomics core, using Sanger sequencing technology. DNA sequences were then manually trimmed using Geneious Prime (v2024.0.5) (Dotmatics, Bishop's Stortford, United Kingdom). Sequences were run though NCBI BLAST to determine if fugal isolates were *D. humulicola,* or to determine what species the other isolates belong to (Table 5.6)

(Camacho et al. 2009). *D. humulicola* isolates chosen for the population genetics study also had the translocation elongation factor 1-alpha (Tef) amplified using primers EF1-728F/EF1-986R (Carbone and Kohn 1999) using thermocycling conditions from Higgins et al. (2021) (Table 5.1).

DNA extraction and sequencing for population genetics

Diaporthe humulicola isolates (72) from four hop growing regions Michigan, Minnesota, New York, and Canada, were grown for 31 days on PDA in an incubator set at 21.5 °C with a 12-hour photoperiod to allow for the production of pycnidia and conidia (Table 5.1). Isolates were processed in groups of four for each DNA extraction using the E.Z.N.A HP Fungal DNA kit (Omega Bio-Tek, INC., Norcross, GA) using the Fresh/Frozen Samples protocol with an added step of adding 0.01g of sterilized quartz sand (Honeywell, Charlotte, North Carolina) before grinding the sample with a sterilized disposable pestle with liquid nitrogen. DNA quality was then determined using a Denovix Ds-11 Fx+ Spectrophotometer/Fluorometer (DeNovix, Wilmington, DE) and quantified using a Qubit 4 with the 1x dsDNA BR working solution (Invitrogen, Waltham, MA). Sixty-eight of the samples were processed by the RTSF genomics core MSU. DNA libraries were prepared using the Roche Kapa HyperPrep DNA Library Preparation Kit (Kapa Biosystems, Wilmington, MA) with Kapa Unique Dual-Indexed adapters following manufacturer's recommendation. Completed libraries were checked for quality and quantified using a combination of Qubit dsDNA HS and Agilent 4200 TapeStation HS DNA1000 (Agilent, Santa Clara, CA) assays. Sequencing was performed in a 2x150bp paired end format using an Illumina NovaSeq v1.5, 300 cycle reagent kit (Illumina, San Diego, CA. Base calling was done by Illumina Real Time Analysis (RTA) (v3.4.4) and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq (v2.20.0). Additional samples (4) were processed by the Oklahoma Medical Research Foundation NGS Core and

samples had libraries prepared with the IDT xGen DNA EZ kit (IDT, Newark, NJ), and quality checks were done with Kapa qPCR and Agilent's D1000 High Sensitivity Screen Tapes. Sequencing was performed in a 2 x 150bp paired end format on a llumina NovaSeq X Plus - 10B.

Alignments and SNP calling

Reads files were inspected for quality and adaptor content using FastQC (v0.12.1). Reads were then trimmed for adaptors and quality using the BBmap program (v39.01). Read files were then reinspected using FastQC before alignment. Sequences were aligned to the nuclear contigs of the draft genome of *D. humulicola* isolate MI_003 (JBLTED000000000) using the program Burrows-Wheeler Alignment tool (BWA) (v0.7.17) (Li et al. 2009) using default settings.

The alignments files were then sorted and converted to bam format using SAMtools (v1.19.2). The Picard toolkit (v3.0.0) (CollectWgsMetrics) was then used to calculate the average coverage of reads for each genome. Alignments that exceeded 15x coverage were then down sampled using the Picard toolkit (DownsampleSam) to 15x coverage to reduce the effects of the batch effect caused by using two different sequencing runs. (Lou et al. 2021). After down sampling alignments had duplicated reads marked using the Picard toolkit (v2.25.1). The single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) were then called using the Genome Analysis Toolkit (GATK) (v4.5.0.0) haplotype caller producing a VCF file for each sample. The VCF files were then combined using the GATK command CombineGVCFs. The indels were then removed from the combined VCF file, and only SNPs were used for analysis.

The VCF file was then hard filtered using GATK with the following conditions QD < 2.0, QUAL < 30.0, SOR > 3.0, FS > 60.0, and MQ < 50. A modified version of VCFtools (v0.1.17) (https://github.com/vcftools/vcftools/pull/69), that allows for calculations on haploid

organisms, was then used to remove the SNPs that did not pass the filtering steps, SNPs that were not biallelic, and that were monomeric. The modified VCFtools was then used to filter the VCF using a minimum allele frequency of 0.05. VCftools was also used to thin the VCF file to where 3 SNPs cannot be within 10 bp from each other. VCF files were also filtered for completeness using VCFtools and only SNPs that appear in 95% of all the samples and in 30% of each population were kept for analysis. The 95% VCF file was also filtered so that all SNPs were in all the samples, creating a 100% dataset. The 95% data set was also pruned based on linkage disequilibrium using plink with the conditions $r^2 > 0.3$ in 100k windows, creating a third data set (Table 5.6).

Clone correction

SNPs from the 95% data set were converted to a genind format using the R (v4.2.0) program vcfR (v1.15.0). Multilocus genotypes (mgl) were assigned to the different isolates, and the clone correction was conducted using poppr (v2.9.6). BCFtools (v 1.19) (Danecek et al. 2021) was used to removed samples so that only one sample from each mgl would remain in the vcf file, leaving 64 samples with unique mlgs (Table 5.1).

Phylogeny of *Diaporthe humulicola* samples

The clone corrected 95% VCF file was converted to a phylip file format using vcf2phylip (v2.8) (Ortiz 2019). IQ-tree (v2.2.2.7) (Minh et al. 2020) was initially run without replicates to determine the best model for the maximum likelihood phylogeny, and then the TVM + F + ASC + R3 model was selected with 10,000 bootstraps replicates. The consensus maximum likelihood phylogeny was then visualized with bootstrap support using FigTree (v1.4.4) (http://tree.bio.ed.ac.uk) (Figure 5.4).

Analyses of genetic structure

The population structure for the 64 samples was determined using two different methods principal component analysis (PCA) and Bayesian clustering analysis (STRUCTURE). The PCA was conducted in PLINK (v2.00a3.7) (Purcell et al. 2007) using each clone corrected dataset (95%, 100%, and LD pruned). We produced PCA for all populations together and for each individual population. PCA plots were then visualized in R using ggplot2 (Wickham 2016) (Figure 5.3B, Figure 5.10 and 5.7).

For the STRUCTURE (v2.3.4) (Pritchard et al. 2000) analysis the LD pruned dataset was used as STRUCTURE assumes loci are independent of each other. The LD pruned data set included 35,916 SNPs. Prior to the main STRUCTURE runs, STRUCTURE was run using 100,000 Monte Carlo Markov Chain (MCMC) iterations from K = 1 to K = 10 to determine when alpha is stabilized, with a floating lambda, to determine if the values should be changed, and finally with population data to determine if the location data are significantly affecting the population structure. (Hubisz et al. 2009; Porras-Hurtado et al. 2013). STRUCTURE (v2.3.4) was then run for 20 reps for each K value (K = 1-10), each run had 60,000 Monte Carlo Markov Chain (MCMC) iterations, with 30,000 burn in iterations and then 30,000 collected iterations. The STRUCTURE runs were conducted using the admixture model, with correlated allele frequencies, without prior information about the demography, and with a fixed lambda ($\lambda = 1$) (Falush et al. 2023). The results from STRUCTURE were then processed using STRUCTURE HARVESTER (Earl and VonHoldt, 2012), using both the evanno and clumpp options. The optimal K value was determined as the maximized rate of change in log likelihood values (deltaK) using methods developed by Evanno et al. (2005) and the Ln Pr(X|K) selection method. Files from the STRUCTURE HARVESTER clump option were then fed into CLUMPAK

(Kopelman et al. 2015) to determine the optimal ancestry cluster membership proportions for each sample for each K value. The major and minor clusters were then visualized using ggplot2 (Figure 5.3C, Figure 5.10).

Population statistics

Summary statistics for the populations were calculated with VCFools (v1.16), using a patch that allows computation with haploid datasets. Within population diversity was addressed by calculating pi (the average number of differences between individuals) for each individual in 100kb non-overlapping windows and then averages were taken for each population. (Takahata and Nei 1985). Tajima's D was calculated in 100 kb non-overlapping windows (Tajima, 1989), and 95% confidence intervals were conducted for each population for Tajima's D.. Weir and Cockerham F_{st} was calculated using VCFtools using patch that allows computation with haploid datasets (Table 5.3) (Weir and Cockerham 1984). F_{st} was calculated of each pair of the populations and weighted mean F_{st} estimate were produced for each population pair (Table 5.2). One Sample t-test, ANOVA, and Fisher's LSD were conducted in R to confirm if values were significantly different from zero and each other. Linkage decay was calculated using the 95% missing data set using PLINK. Linkage disequilibrium was calculated between pairs of SNPs 2151.76 kb apart and averages were calculated in 100 kb windows. As the genome was highly fragmented and the order of the contigs is not known, average LD for each window was calculated across the whole genome for all populations (Figure 5.9), and the largest contig, contig 1 in all populations (Figure 5.8) and in each population (Figure 5.8). The index of association was calculated using poppr (v2.9.6), using the samp.ia function with 1000 randomly selected loci and 1000 replicates, one sample t-test and an ANOVA were conducted to determine

if the value was statically greater than zero and if the population were statically different from each other (Table 5.3).

Mating type genes

To determine the mating type of *D. humulicola* samples the sequences for *MAT1-1-1*, *MAT1-1-2*, *Mat1-1-3*, and *Mat1-2-1* (AB199324.1, AB199325.1) were searched against a custom blast database using the isolate MI_0318 genome. *MAT1-1-1*, *MAT1-1-2*, and *MAT-1-1-3* had a match in the genome while *MAT1-2-1* was not present in the *D. humulicola* genome. The region for the *MAT1-1-1*, and the TEF gene were then extracted from each of the isolate alignments using SAMtools and then were visualized in Geneious Prime. Alignments with read coverage for this region including all three genes were denoted as containing *MAT1-1-1*. Reads from all of Isolates were then aligned to the *MAT1-2-1* gene using BWA using default settings. Alignments were then visualized using Geneious Prime and isolates that had read coverage for *MAT1-2-1* but not *Mat1-1-1* were then determined to be *MAT1-2-1*.

Results

Michigan fungal cone survey

Over the three years 879 fungal isolates were recovered. In 2019, 400 isolates were collected, and survey data from 2019 has also been published in Higgins et al. (2021), and samples collected from this year were not used in the population genetics study. In 2020, 216 isolates were collected and in 2021, 217 fungal isolates were collected.

Alternaria species were isolated most frequently with 41.6% of the samples (n = 366). A subset of the *Alternaria* isolates had the ITS region sequenced and NCBI BLAST was used to identify the samples and sequences matched to *Alternaria alternata* and *A. longipes*. *Diaporthe* was the second most recovered genera with 23.0 % samples (202), that were identified with

morphology. Of these samples 110 had their ITS sequence amplified and sequenced. Blast results were 100% similar to *D. humulicola* (NR_172855). From these isolates denoted as *D. humulicola,* 72 had the TEF gene amplified and sequenced. BLAST results were 100% similar to *D. humulicola* (OQ256245.1). *Fusarium spp.* represented 11.3% of the samples collected, 10 sample were sequenced and ITS sequences matched to *Fusarium sporotrichioides, F. citri, and F. equiseti*. *Epiccocum spp,* represented 12.3% of the samples and sequences from 4 isolates were identified as *Epicoccum sp*.. *Colletotrichum* spp. represents 0.57% (5) of samples collected in this survey. Of the fungal isolates collected 11.15 % could not be identified using morphology and were denoted as other. A subset of the other samples (n = 4) had their ITS region amplified and sequence were either unculturable fungus or *Periconia byssoides*. Accession for ITS sequences for *Diaporthe* isolates are located in Table 5.1 and Table 5.6, and Accession numbers for other fungi are in Table 5.6.

Over the course of the study incidence of *Alternaria spp.* and *Epicoccum* sp. isolates decreased each year from 48.8% to 32.5% and from 11.3% to 8.7%, respectively. The percent of *Colletotrichum* sp. and fungi denoted as other increased over the three years from 0% to 1.4% and 5.25% to 18.8% of the samples respectively. There was not a consistent trend for *Fusarium* spp. and *D. humulicola* isolates. The amount of *Fusarium* isolates decreased by 9.25% from 2019 (14.3%) to 2020 (5%). In 2021, *Fusarium* isolates increased to 13.3% of the total samples. The amount of *Diaporthe* samples in 2020 (20.5%) increased by 4.8% when compared to 2019 (25.3%) but decreased by 3.6% in 2022 (16.9%). (Figure 5.2)

Other *Diaporthe* samples

Diaporthe samples from Minnesota, New York, and Canada were collected for either the publication of disease notes, or to help growers identify pathogens in their hop yards. From

Minnesota, 12 isolates were recovered from either leaf or cone samples and were used in Havill et al. 2021. Canada had 28 *D. humulicola* samples collected and samples have been in used in Hatlen et al. 2022. There was 45 samples that were collected from leaf and cone tissue from New York. A subset of the samples were identified using ITS sequencing, and accession numbers are located in Table 5.5.

Structure analysis

The ancestry clusters of the 64 *D. humulicola* isolates was determined using 20 runs of the program STRUCTURE. The Evanno method's rate of change of K and the logarithm probability of K agree that there are two possible K values that fit the data, K = 4 and K = 6 (Figure 5.3A and Figure 5.5). The K of 6 STRUCTURE results have higher values in the deltaK plot, but the variance after the K = 6 value is lower than the variance after K = 4 in the log of the probability of K plot. (Figure 5.3C and Figure 5.5). In the delta K plot, K = 2 – K = 4 are all increasing with a peak at K = 4.

The results show some clustering by different geographical populations, but Michigan isolates seem to be a mixture of each population. Isolates from Minnesota, Canada, and New York isolates have high amounts of their ancestry attributed to different clusters, with some mixing of the clusters.

At K = 2 all samples are mixtures of the two proposed clusters, and at K = 3 all isolates expect for two isolates from Michigan and Minnesota derive their ancestry from all three clusters. The other two samples have 100% ancestry to cluster 3. Results from K = 2 and K = 3 are included in the Figure 8.12.

The K = 4 results show that there is a major (n = 10) and minor pattern (n = 9) of clustering across the data. The Canada population clustering stayed the same between the major

and minor clusters at K = 4. While there is variation in the other three populations at K = 4. The Michigan isolates in both the major and minor structure seem to have a high level of similarity to samples from Canada as most of their ancestry is attributed to cluster 1. In the K = 4 major population clusters there are four isolates that do not follow this trend and have the same clustering as isolates from Minnesota (n = 1) and New York (n = 3). The 4th cluster in the K = 4 major population structure seems to be in every population but Canada. In the K = 4 minor population structure the 4th cluster only appears in 8 samples, 7 of which are from Michigan and 1 from Minnesota. There are two Michigan isolates that have 100% ancestry to the 4th population cluster in the minor plot and the rest have under 10% ancestry to this population. In the K = 4 STRUCUTRE analysis results there was one population structure that appeared once out of the 20 runs of STRUCUTRE and can be found in Figure 5.13.

For the K = 6 clustering structure there is one major structure (n = 14) and two minor (n = 4 and n = 2) structures. In the major plot isolates from New York and Minnesota are in agreement with the population structure found in K = 4 major with low amounts of ancestry to another cluster. All Canadian isolates except for one in K6 major belong to cluster 1, and the singular isolate has less then 25% ancestry to cluster 2. In the K = 6 major structure all population are present in the Michigan isolates. In the K = 6, minor clustering patterns, the Canadian isolates no longer have 92 – 100 % ancestry to cluster 1 but now a mixture of clusters 1, 2, and 6. The Minnesota samples are relatively the same as the major population with some samples now have > 10% of their ancestry derived from different populations than that of the major structure at K6. The Michigan isolates are varied when compared to the major structure at K6 where some samples that a 100% ancestry to one of the populations becomes mixtures of other populations.

Phylogenetic tree

Phylogenetic analysis of the isolates produced a tree that has mostly high bootstrap support for most clades (Figure 5.4). In the maximum likelihood phylogeny, there is clustering based on location of the samples as in the STRUCURE analysis, and Michigan samples are represented in every clade. There is 4 clades in the consensus tree. Where clade 1 consists of samples from Michigan. In clade 2 there is a mixture of samples from Minnesota and Michigan. Clade 3 includes samples from Michigan, New York and all of the samples from Canada. In clade 4, there are samples from Michigan, New York, and Minnesota. (Figure 5.4).

PCA derived structure

The PCA results for the LD pruned data set mostly agree with results from the STRUCRE analysis and the phylogenetic tree, where most of the groupings seem to be a combination of all the different populations. There seems to be four different groupings. One of the groupings includes isolates from all populations and in this grouping all isolates from Canada are included. There then is a grouping of samples that have samples from Michigan, New York, and Minnesota. The third grouping has isolates from New York and Michigan. Finaly there is a grouping that is just most of the isolates from Minnesota (Figure 5.4B).

Population statistics between populations

The highest pairwise F_{st} value were consistently high between populations pairs that included Minnesota (0.22 - 0.43), with one pairing Canada and New York that is also high (0.28). Indicating that there is lower levels of gene flow between the population pairs. The lowest F_{st} value was between samples from Canada and Michigan (0.08) and New York and Michigan (0.11) which indicate higher amounts of gene flow between the two population pairs. (Table 5.1**)**

Population statistics within populations

Mean genome wide nucleotide diversity (π) ranged from 1.24×10^{-03} to 7.78×10^{-04}, and all mean values were significantly different from each other (p-value < 2.2×10^{-16}) and significantly different from zero (p-value < 2.2e-16). Michigan had the highest diversity value and Minnesota has the lowest value. The Canada (1.11×10^{-03}) and New York (9.03×10^{-04}) populations were both higher than Minnesota (Table 5.3 and Figure 5.15).

Mean Tajima's D was significantly different from zero for all populations (p-value < 2.2×10^{-16}) but New York (p-value = 0.5297) , and the mean values were all significantly different from each other (p = < 2.2×10^{-16}). For the New York population the mean Tajima's D is 0.03 but is not significantly different from zero, so there is no evidence of selection from the samples collected. Samples from Canada and Michigan both had mean Tajima's D values that were both positive indicating that rare alleles are scarce in these populations. While samples from Minnesota had a mean Tajima's D value that was less than zero indicating an abundance of rare alleles.

The index of association was all significantly different from zero and significantly different from each other ($p < 0.05$). The Minnesota (0.17) and New York (0.056) populations have significantly higher indices of association than the Canadian (0.0021) and Michigan (0.0059) populations (Table 5.3).

Analysis of linkage disequilibrium linkage

On contig 1 linkage decay was low and ($R^2 = 0.16$) and decreased to > 0.08 in approximately 100kb for the combined population data set (Figure 5.8). For the individual populations on contig one all R^2 values were > 0.5. The Michigan and Canada populations decayed quickly and then plateaued in approximately 100kb. The New York and Minnesota

populations both started with a high R^2 and decayed much slower than the other two populations. (Figure 5.9). For the whole genome linkage decay analysis R^2 was higher than the individual populations starting at 1 and then decaying to less than > 0.25 in approximately 100kb. (Figure 5.10).

Mating type genes

For all the populations samples were skewed to *Mat-1-2-1* loci where there was 37.5% of the samples having the *Mat-1-1-1* loci and 62.5% of the samples having Mat-1-2-1 loci. This trend continues for all populations except Minnesota where there was a 50% split between all isolates (Table 5.4, Figure 5.6).

Discussion

Our survey of diseased cones in the state of Michigan indicated that *Alternaria spp.* was the largest proportion of fungi isolated. In hops, *Alternaria alternata* is the causal agent of Alternaria cone disorder in hops. The pathogen can cause complete necrosis on detached hop cones in 10 days, and the pathogen is exacerbated by wounding (Darby 1984; Darby 1988; Pethybridge et al. 2001a). The pathogen does not cause disease on detached hop leaves (Higgins et al. 2021). The pathogen does not cause yield loss in hops and may be a secondary colonizer (Darby 1984, Darby 1988, Higgins et al. 2021). In hops, *Fusarium spp.* can cause two minor diseases of hop fusarium tip blight and fusarium canker, and *Fusarium* spp. can also be isolated from asymptotic hop tissue (Bienaplf 2004; Pethybridge et al. 2001b; Sabo et al. 2021). *Fusarium* is a common endophyte of plants and can be a pathogen of many plant species (Ahmed et al 2023). While the ITS region is the standard barcoding region, it is not able to distinguish closely related species of *Fusarium* (Torres-Cruz et al. 2022). NCBI blast was able to identify *Fusarium* isolates as *Fusarium sporotrichioides*, which is part of the *Fusarium sambucinum*

species complex. Thus it is unknow if the *Fusarium* isolates, we collected are actually *Fusarium sambucinum*, one of the casual agents of fusarium tip blight, or another closely related species within the *Fusarium* genus (Laraba et al. 2021). *Epicoccum nigrum* is considered a ubiquitous fungus for hop and has been recovered in high amounts in other hop disease surveys (Phalip et al. 2006). *Colletotrichum spp*. have recently been shown to cause disease on both common and Japanese Hop (*Humulus scandens*) but yield loss has not been attributed to either pathogen (Frederick et al. 2022; Hatlen et al. 2022).

The second most recovered pathogen of hops from the survey was *D. humulicola*, a newer pathogen of hops, first detected in New York in 2015 (Sharma et al. 2023). In 2018 the pathogen was detected and described in Connecticut and Michigan (Allen-Perkins et al. 2020; Higgins et al. 2021). The pathogen has also been detected in other states such as Minnesota, Indiana, and Massachusetts and it has been detected in 2 different Canadian providences (Foster et al. 2024; Hatlen et al. 2025; Havill et al. 2023; Stamborski et al. 2022;). The pathogen was detected in all major growing region in the lower peninsula of Michigan. Over the three years of sampling the amount of *Diaporthe* isolates detected decreased, this could be due to improved management decisions as fungicide efficacy work was conducted and disseminated during this period (Hatlen et al. 2025). The pathogen is a major problem for hop growers in the eastern United States and Canada and fungicides seem to have varied effects on the growth of the pathogen (Allen-Perkins et al. 2021; Hatlen et al. 2025). As the pathogen continues to spread and cause yield loss, more needs to be known about the genetic differences in populations of the fungi, to better test fungicides and eventually test hop cultivars with different populations to identidy plant resistance for hop breeding programs.

The structure results indicate that there are four or six different clusters of the *D. humulicola* samples that were tested (Figure 5.3A). In both K value clusters, the Michigan samples are mixtures of the other three populations. Michigan samples were the most represented in this study and could lead to bias when using the STRUCTURE program, where the underrepresented populations will cluster together artificially (Meirmans 2019). Using a high number of markers can help deter the bias of oversampling but is not a complete fix to oversampling of populations (Toyama et al. 2020). Thus, the results from structure should not be used as the definite populations for *D. humulicola*, and the results should be combined with other analysis to determine the ancestry of populations. In this project we used PCA and maximum likelihood phylogeny to corroborate our results. The PCA for the 95% data set has most of the same patterns of separation as the K4 major plot. (Figure 5.3B). Where samples from Canada form a distinct cluster that is also present in the Michigan populations with a single sample from both New York and Minnesota. The samples from Minnesota also follow trends from the K = 4 plot, where most of the isolates form their own group away from other samples. The samples from New York in the PCA also match the structure results where the samples seem to be a mix and of different populations. The results from the phylogeny follow the same trend In the K = 4 STRUCTURE plot, where a singular sample from Minnesota does not cluster with the rest of the samples but clusters with samples from Michigan and New York. The phylogeny also clusters the Canadian samples together but are still closely related to samples from Michigan (Figure 5.4).

In the K = 6 plot the clustering stays relatively the same as the K = 4 plot for the Minnesota and New York populations. While Canadian and Michigan samples have more diversity in the clustering, in the K = 6 plots most samples have their ancestry contributed to

multiple clusters at low levels (> 10 %). While the K = 6 plot is statically correct for the STRUCUTRE analysis, the K = 4 clusters seem to make more biological sense as the samples were collected from 4 different locations and K = 4 is the lower value of K (Evanno et al. 2005; Pritchard et al. 2010). This does not mean that the K = 6 clusters are not valid, the uneven sampling may reduce the power of the test to separate under sampled populations into different cluters. Although greater genetic diversity may exist among isolates at that location, it was not captured in our sampling (Phillips et al. 2019). In the Michigan samples there are multiple farms represented in different parts of the state, and therefore more genetic diversity was sampled from that state. While samples from the other regions were sampled from induvial or yards that are geographically close to each other. When looking at the PCAs for just Michigan isolates, it seems that there is substructure to this population, and there seems to be three to four different clusters in the individual population. (Figure 5.11b, 5.11f, 5.11j). The Michigan isolates also have the largest nucleotide diversity (π), indicating that the Michigan samples included more genetic variation than the sampling of other locations (Table 5.2). Among the Canada samples there was also high nucleotide diversity (π) and in the PCA plots isolates are spread out and do not cluster tightly like the other populations. (Table 5.2, Figure 5.11a, 5.11e, 5.11f). In the PCA plots for the New York and Minnesota samples, at least one isolate did not cluster with the other isolates, indicating that the SNPs collected from the isolate differ from the rest of the isolates, while the remaining isolates form tight clusters. These populations also have lower nucleotide diversity than the other two populations (Table 2, Figure 5.11c, 5.11g, 5.11k, 5.11d, 5.11h, 5.11l).

While samples from New York and Minnesota have lower nucleotide diversity they both have high amounts of segregating sites in these populations. (Table 5.8) The New York

population Tajima's D is not significantly different from zero, indicating that there is no evidence of selection for that population. In the Minnesota samples there is also a high number of segregating sites with a negative Tajima's D indicating that there could have been a recent bottleneck in the population, and that rare alleles are abundant. The LD for the two populations have higher R^2 values and have slower decay than the populations in Michigan and Canada. The New York and Minnesota populations also have higher index of association indicating that the populations could have a high rate of clonal reproduction, thus explaining the loss of nucleotide diversity and higher LD. In other fungi as populations move towards complete clonal reproduction nucleotide diversity decreases, and LD increases (Flanagan et al. 2021, de Meeûs and Balloux 2004). The isolates from Canada and Michigan have index of association that are close to zero indicating that the populations could have recombination in their individual populations allowing for higher amounts of diversity and rapid LD.

In all populations both mating type loci were detected but the sexual state has not be identified for this pathogen (Allan-Perkins et al. 2020). In other *Diaporthe* species homothallic fungi will have both loci or variations of both of the loci on the same genome (Gomes et al. 2013; Santos et al. 2010). This was not the case in *D. humulicola*, where only one loci was found per isolate. The New York and Minnesota populations have higher index of association indicating that the populations could have a high rate of clonal reproduction, while the population have both mating type loci present. This could be due to sample bias as samples collected were in sites experiencing high levels of disease, and it has been hypothesized that samples in active disease outbreaks will be biased towards clonal isolates (Drenth et al. 2019).

There is a high amount of genetic variation between the different populations. The Minnesota population has relatively high F_{st} values with the other populations, this denotes that

there are high amounts of genetic differences between the two populations and that there could be lower amounts of geneflow between the populations. As the isolates collected from Minnesota are the most geographically distant of the isolates there could local adaptations that are not present in the other populations (Rúa et al. 2016). The Minnesota isolates were collected from a breeding population of hop plants, therefor, another explanation to the amount of diversity when compared to Minnesota is that some isolates in the population has become adapted to the new cultivars, that are not in in the other regions. (Amezrou et al. 2024; Burdon and Silk 1997; Havill et al. 2023). The high F_{st} values could also be explained by the low amounts of samples that are in the Canada and Minnesota isolates as F_{st} has been shown to be inflated with low sample size, but high amounts of SNPs have also been shown to mitigate this issue (Bashalkhanov et al. 2009, Nazareno et al. 2017). The Canadian and New York pair also have high F_{st} values, but this trend does not follow with the Michigan and Canadian/New York pair where the lowest F_{st} values were detected. There could be gene flow between the three populations causing the lower F_{st} values, through the movement of isolates by nurseries and though trade (Rodrigues et al. 2022).

In summary *D. humulicola* was frequently recovered in our study and is responsible for significant yield loss on hops in Michigan (Higgins et al. 2021). Genetic diversity seems to be determined by location for populations in the eastern United States and Canada, but there is some mixing of genetic materials between three of the sampling locations. Some populations appear to be clonal and have not had recent recombination events. We also determined that the fungi *D. humulicola* is heterothallic, but the sexual state has not been determined. Further research is need to determine the sexual state of the fungi and how likely is recombination is possible in field environments.

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TABLES

Table 5.1. Isolates used for population genetics experiments with location of collection, year of collection, and accession numbers for both ITS and TEF sequences.

Isolate	Location	Year	ITS	TEF	SRA
Cd_1	Canada	2020	PQ962927	PV068677	SAMN46923680
Cd_2	Canada	2020	PQ962924	PV068676	SAMN46923682
Cd_3	Canada	2020	PQ962933	PV068675	SAMN46923683
Cd_4	Canada	2020	PQ962925	PV068674	SAMN46923684
Cd_5	Canada	2020	PQ962930	PV068673	SAMN46923685
Cd_6	Canada	2020	PQ962932	PV068672	SAMN46923686
Cd_7	Canada	2020	MZ934713.1	MT909101	SAMN46923687
Cd_8	Canada	2020	PQ962928	PV068670	SAMN46923688
Mi_1	Michigan	2020	PV018902	PV068669	SAMN46923690
Mi_2	Michigan	2021	PQ962923	PV068668	SAMN46923701
Mi_3	Michigan	2020	PQ962918	PV068667	SAMN46923712
Mi_4	Michigan	2020	PV018901	PV068666	SAMN46923722
Mi_5	Michigan	2020	PQ962887	PV100839	SAMN46923723
Mi_6	Michigan	2021	PQ962896	PV068665	SAMN46923724
Mi_7	Michigan	2021	PQ962897	PV068664	SAMN46923725
Mi_8	Michigan	2020	PQ962920	PV068663	SAMN46923726
Mi_9	Michigan	2020	PQ962899	PV068662	SAMN46923727
Mi_10	Michigan	2020	PV101259	PV068661	SAMN46923691
Mi_11	Michigan	2021	PQ962922	PV068660	SAMN46923692
Mi_12	Michigan	2020	PQ962894	PV068621	SAMN46923693
Mi_13	Michigan	2021	PQ962903	PV068659	SAMN46923694
Mi_14	Michigan	2020	PQ962931	PV068658	SAMN46923695
Mi_15	Michigan	2020	PQ962906	PV068657	SAMN46923696
Mi_16	Michigan	2020	PQ962890	PV068656	SAMN46923697
Mi_17	Michigan	2020	PQ962892	PV068655	SAMN46923698
Mi_18	Michigan	2021	PQ962889	PV068654	SAMN46923699
Mi_19	Michigan	2021	PQ962888	PV068653	SAMN46923700
Mi_20	Michigan	2020	PQ962929	PV068652	SAMN46923702
Mi_21	Michigan	2020	PQ962905	PV068651	SAMN46923703
Mi_22	Michigan	2020	PV018900	PV068650	SAMN46923704
Mi_23	Michigan	2021	PV018899	PV068649	SAMN46923705
Mi_24	Michigan	2021	PV018898	PV068648	SAMN46923706
Mi_25	Michigan	2021	PQ962902	PV068647	SAMN46923707
Mi_26	Michigan	2021	PQ962926	PV068646	SAMN46923708
Mi_27	Michigan	2021	PQ962898	PV068645	SAMN46923709
Mi_28	Michigan	2021	PQ962893	PV068644	SAMN46923710
Mi_29	Michigan	2021	PQ962891	PV068643	SAMN46923711
Mi_30	Michigan	2021	PQ962900	PV068642	SAMN46923713
Mi_31	Michigan	2020	PQ962901	PV100840	SAMN46923714
Mi_32	Michigan	2020	PQ962921	PV068641	SAMN46923715
Mi_33	Michigan	2020	PQ962919	PV068640	SAMN46923716
Mi_34	Michigan	2020	PQ962895	PV100841	SAMN46923717
Mi_35	Michigan	2020	PQ962904	PV068639	SAMN46923718
Mi_36	Michigan	2021	PV018897	PV068619	SAMN46923719
Mn_1	Minnesota	2020	PV101258	PV068638	SAMN46923728
Mn_2	Minnesota	2020	PQ962936	PV068637	SAMN46923732
Mn_3	Minnesota	2020	PQ962935	OQ256245	SAMN46923733

Table 5.1. (cont'd)

Mn_4	Minnesota	2020	OQ144379.1	OQ256245.1	SAMN46923734
Mn_5	Minnesota	2020	PQ962907	PV100842	SAMN46923735
Mn_6	Minnesota	2020	PQ962886	PV068622	SAMN46923736
Mn_7	Minnesota	2020	PV101261	PV068636	SAMN46923737
Mn_8	Minnesota	2020	PQ962934	PV068635	SAMN46923738
Ny_1	New York	2020	PQ962910	PV068634	SAMN46923740
Ny_2	New York	2020	PQ962912	PV068633	SAMN46923744
Ny_3	New York	2020	PQ962913	PV068632	SAMN46923745
Ny_4	New York	2020	PV018895	PV068631	SAMN46923746
Ny_5	New York	2020	PQ962914	PV068630	SAMN46923747
Ny_6	New York	2020	PQ962915	PV068629	SAMN46923748
Ny_7	New York	2020	PQ962916	PV068628	SAMN46923749
Ny_8	New York	2020	PQ962917	PV068627	SAMN46923750
Ny_9	New York	2020	PQ962908	PV068626	SAMN46923751
Ny_10	New York	2020	PV101260	PV068625	SAMN46923741
Ny_11	New York	2020	PQ962909	PV068624	SAMN46923742
Ny_12	New York	2020	PQ962911	PV068623	SAMN46923743
Cd_9[A]	Canada	2020	PV069118	PV158023	SAMN46923689
Cd_10[A]	Canada	2020	PV069119	PV158024	SAMN46923681
Mn_9[A]	Minnesota	2020	PV069122	PV158027	SAMN46923739
Mn_10[A]	Minnesota	2020	PV069123	PV158028	SAMN46923729
Mn_11[A]	Minnesota	2020	PV069124	PV158029	SAMN46923730
Mn_12[A]	Minnesota	2020	PV069125	PV158030	SAMN46923731
Mi_37[A]	Michigan	2020	PV069120	PV158025	SAMN46923720
MI_38[A]	Michigan	2021	PV069121	PV158026	SAMN46923721

[A] Samples that were removed from population genetics analysis due to clonality

Table 5.2. Mean Weir and Cockerham weighted F_{st} values between each sampling population using the 95% data set. Values were calculated in a patched version of VCFtools (v0.1.17) for use with haploid data sets.

	Mi	**Mn**	**Cd**
Mi	-	-	-
Mn	0.22	-	-
Cd	0.08	0.33	-
Ny	0.11	0.43	0.28

Table 5.3. Population statistics calculated for individual populations. Mean nucleotide diversity (π) and mean Tajima's D were both calculated in 100kb windows using the 95% data set, and index of association was calculated using a random assortment of 1000 SNPs with 1000 replication each using the 95% data set.

Population	nucleotide diversity (π)[A]	Standard error	Mean Tajima's D[A]	95% confidence interval	Index of association
CD	1.11×10^{-03} A	2.50×10^{-05}	0.38 A	0.35 , 0.40	0.0021
MI	1.24×10^{-03} B	2.74×10^{-05}	0.87 B	0.85 , 0.89	0.0059
MN	7.78×10^{-04} C	2.15×10^{-05}	-0.76 C	-0.71 , -0.82	0.1700
NY	9.03×10^{-04} D	2.16×10^{-05}	0.03 D	-0.02 , 0.08	0.0560

[A] Datasets were analyzed with a one-way ANOVA. Fisher's LSD was conducted and letters next to mean values denote significance ($p < 0.05$). Values with the different letters are significantly different from other letters
[B] One sample t test was conducted for each value and all datasets had a p value of $< 2.2 \times 10^{-16}$

Table 5.4. Percentage of *D. humulicola* (N = 64) isolates with either Mat-1-1-1 or Mat-1-2-1 derived from alignments of reads to each region.

Population	Mat-1-1-1 (%)	Mat-1-2-1(%)
CD	25.0	75.0
MI	45.0	55.0
MN	50.0	50.0
NY	30.0	70.0
Total	37.5	65.2

Table 5.5. Counts of *Diaporthe humulicola* isolates collected in a fungal diseased cone survey in the lower peninsula of Michigan from 2019-2022 by farm. Fungal isolates were isolated from necrotic hop cone tissue and identified using culture and pycnidia morphology.

Farm	Location[A]	Diaporthe 2019	Diaporthe 2020	Diaporthe 2021
1	Southeast, MI	5	1	2
2	Southeast, MI[B]	21	8	4
3	Southwest, MI[B]	17	0	21
4	Southwest, MI[B]	12	7	0
5	Southwest, MI	1	-	0
6	Southwest, MI	2	10	-
7	Southwest, MI	2	-	-
8	Southwest, MI	1	-	-
9	Southern, MI	10	6	-
10	Southern, MI	3	8	3
11	Northeast, MI	0	3	-
12	Northern, MI	1	-	2
13	Northern, MI	-	-	9
14	Northern, MI	2	-	-
15	Northern, MI	-	2	-
16	Northern, MI	-	-	9
17	Eastern, MI	-	-	3
18	Eastern, MI[B]	-	12	9
19	Eastern, MI	-	9	0
20	Central, MI[C]	5	-	-
Total		82	66	54

[A] All farms are in the lower peninsula of Michigan

[B] Hop farms that had more than one geographically distinct hopyard and ten samples were collected from each of the geographically distinct yard

[C] Farm 21 is a research yard at Michigan state university all other farms are commercial hop growers

Table 5.6. Identity of subset of isolates from cone disease survey using sequencing of the ITS region.

Isolate	Location	year	Identity	ITS
CD_dia_1	Canada	2020	*Diaporthe humulicola*	PV069127
CD_dia_2	Canada	2020	*Diaporthe humulicola*	PV069128
CD_dia_3	Canada	2020	*Diaporthe humulicola*	PV069129
CD_dia_4	Canada	2020	*Diaporthe humulicola*	PV069130
CD_dia_5	Canada	2020	*Diaporthe humulicola*	PV069131
CD_dia_6	Canada	2020	*Diaporthe humulicola*	PV069132
CD_dia_7	Canada	2020	*Diaporthe humulicola*	PV069133
MI_20_1	Michigan	2019	*Fusarium sp.*	PV061186
MI_20_2	Michigan	2019	*Alternaria sp.*	PV061187
MI_20_3	Michigan	2019	*Fusarium sporotrichioides*	PV061188
MI_20_4	Michigan	2019	*Epicoccum sp.*	PV061189
MI_20_5	Michigan	2019	*Alternaria alternata*	PV061190
MI_20_6	Michigan	2019	*Alternaria alternata*	PV061191
MI_20_7	Michigan	2019	*Alternaria alternata*	PV061192
MI_20_8	Michigan	2019	*Alternaria alternata*	PV061193
MI_20_9	Michigan	2019	*Alternaria alternata*	PV061194
MI_20_10	Michigan	2019	*Alternaria alternata*	PV061195
MI_20_11	Michigan	2019	*fusarium sp*	PV061196
MI_20_12	Michigan	2019	*Epicoccum sp.*	PV061197
MI_20_13	Michigan	2019	*Alternaria alternata*	PV061198
MI_20_14	Michigan	2019	*Alternaria alternata*	PV061199
MI_20_15	Michigan	2019	*Epicoccum sp.*	PV061200
MI_20_16	Michigan	2019	*Alternaria alternata*	PV061201
MI_20_17	Michigan	2019	*Fusarium equiseti*	PV061202
MI_20_18	Michigan	2019	*Alternaria alternata*	PV061203
MI_20_19	Michigan	2019	*Fusarium sporotrichioides*	PV061204
MI_20_20	Michigan	2019	*Alternaria alternata*	PV061205
MI_20_21	Michigan	2019	*Fusarium sporotrichioides*	PV061206
MI_20_22	Michigan	2019	*Alternaria alternata*	PV061207
MI_20_23	Michigan	2019	*Fusarium citri*	PV061208
MI_20_24	Michigan	2019	*Alternaria alternata*	PV061209
MI_20_25	Michigan	2019	*Alternaria alternata*	PV061210
MI_20_26	Michigan	2019	*Epicoccum sp.*	PV061211
MI_20_27	Michigan	2019	*Alternaria alternata*	PV061212
MI_20_28	Michigan	2019	*Alternaria alternata*	PV061213
MI_20_29	Michigan	2019	*Alternaria alternata*	PV061214
MI_20_30	Michigan	2019	*Periconia byssoides*	PV061215
MI_20_31	Michigan	2019	*Fusarium equiseti*	PV061216
MI_20_32	Michigan	2019	*Fusarium sporotrichioides*	PV061217
MI_20_33	Michigan	2019	*Alternaria sp.*	PV061218
MI_20_34	Michigan	2019	*Alternaria alternata*	PV061219
MI_20_35	Michigan	2019	*Alternaria alternata*	PV061220
MI_20_36	Michigan	2019	*Alternaria alternata*	PV061221
MI_20_37	Michigan	2019	*Fusarium equiseti*	PV061222
MI_20_38	Michigan	2019	*Alternaria alternata*	PV061223
MI_20_39	Michigan	2019	*Uncultured Psiloglonium*	PV061224
MI_20_40	Michigan	2019	*Periconia byssoides*	PV061225
MI_20_41	Michigan	2019	*Alternaria alternata*	PV061226
MI_dia_42	Michigan	2020	Diaporthe humulicola	PV069174

Table 5.6. (cont'd)

Mi_dia_1	Michigan	2020	*Diaporthe humulicola*	PV069126
MI_dia_4	Michigan	2020	*Diaporthe humulicola*	PV069136
MI_dia_5	Michigan	2020	*Diaporthe humulicola*	PV069137
MI_dia_6	Michigan	2020	*Diaporthe humulicola*	PV069138
MI_dia_7	Michigan	2020	*Diaporthe humulicola*	PV069139
MI_dia_8	Michigan	2020	*Diaporthe humulicola*	PV069140
MI_dia_18	Michigan	2020	*Diaporthe humulicola*	PV069150
MI_dia_19	Michigan	2020	*Diaporthe humulicola*	PV069151
MI_dia_20	Michigan	2020	*Diaporthe humulicola*	PV069152
MI_dia_21	Michigan	2020	*Diaporthe humulicola*	PV069153
MI_dia_22	Michigan	2020	*Diaporthe humulicola*	PV069154
MI_dia_36	Michigan	2020	*Diaporthe humulicola*	PV069168
MI_dia_37	Michigan	2020	*Diaporthe humulicola*	PV069169
MI_dia_38	Michigan	2020	*Diaporthe humulicola*	PV069170
MI_dia_39	Michigan	2020	*Diaporthe humulicola*	PV069171
MI_dia_40	Michigan	2020	*Diaporthe humulicola*	PV069172
MI_dia_41	Michigan	2020	*Diaporthe humulicola*	PV069173
MI_dia_46	Michigan	2020	*Diaporthe humulicola*	PV069178
MI_dia_47	Michigan	2020	*Diaporthe humulicola*	PV069179
MI_dia_48	Michigan	2020	*Diaporthe humulicola*	PV069180
MI_dia_49	Michigan	2020	*Diaporthe humulicola*	PV069181
MI_dia_50	Michigan	2020	*Diaporthe humulicola*	PV069182
MI_dia_51	Michigan	2020	*Diaporthe humulicola*	PV069183
MI_dia_52	Michigan	2020	*Diaporthe humulicola*	PV069184
MI_dia_69	Michigan	2020	*Diaporthe humulicola*	PV069206
MI_dia_53	Michigan	2020	*Diaporthe humulicola*	PV069207
MI_dia_54	Michigan	2020	*Diaporthe humulicola*	PV069208
MI_dia_55	Michigan	2020	*Diaporthe humulicola*	PV069209
MI_dia_56	Michigan	2020	*Diaporthe humulicola*	PV069210
MI_dia_58	Michigan	2020	*Diaporthe humulicola*	PV069212
MI_dia_59	Michigan	2020	*Diaporthe humulicola*	PV069213
MI_dia_60	Michigan	2020	*Diaporthe humulicola*	PV069214
MI_dia_61	Michigan	2020	*Diaporthe humulicola*	PV069215
MI_dia_62	Michigan	2020	*Diaporthe humulicola*	PV069216
MI_dia_63	Michigan	2020	*Diaporthe humulicola*	PV069217
MI_dia_64	Michigan	2020	*Diaporthe humulicola*	PV069218
MI_dia_65	Michigan	2020	*Diaporthe humulicola*	PV069219
MI_dia_2	Michigan	2021	*Diaporthe humulicola*	PV069128
MI_dia_3	Michigan	2021	*Diaporthe humulicola*	PV069129
MI_dia_9	Michigan	2021	*Diaporthe humulicola*	PV069141
MI_dia_10	Michigan	2021	*Diaporthe humulicola*	PV069142
MI_dia_11	Michigan	2021	*Diaporthe humulicola*	PV069143
MI_dia_12	Michigan	2021	*Diaporthe humulicola*	PV069144
MI_dia_13	Michigan	2021	*Diaporthe humulicola*	PV069145
MI_dia_14	Michigan	2021	*Diaporthe humulicola*	PV069146
MI_dia_15	Michigan	2021	*Diaporthe humulicola*	PV069147
MI_dia_16	Michigan	2021	*Diaporthe humulicola*	PV069148
MI_dia_17	Michigan	2021	*Diaporthe humulicola*	PV069149
MI_dia_23	Michigan	2021	*Diaporthe humulicola*	PV069155
MI_dia_24	Michigan	2021	*Diaporthe humulicola*	PV069156
MI_dia_25	Michigan	2021	*Diaporthe humulicola*	PV069157
MI_dia_26	Michigan	2021	*Diaporthe humulicola*	PV069158
MI_dia_27	Michigan	2021	*Diaporthe humulicola*	PV069159

Table 5.6. (cont'd)

MI_dia_28	Michigan	2021	*Diaporthe humulicola*	PV069160
MI_dia_29	Michigan	2021	*Diaporthe humulicola*	PV069161
MI_dia_30	Michigan	2021	*Diaporthe humulicola*	PV069162
MI_dia_31	Michigan	2021	*Diaporthe humulicola*	PV069163
MI_dia_32	Michigan	2021	*Diaporthe humulicola*	PV069164
MI_dia_33	Michigan	2021	*Diaporthe humulicola*	PV069165
MI_dia_34	Michigan	2021	*Diaporthe humulicola*	PV069166
MI_dia_35	Michigan	2021	*Diaporthe humulicola*	PV069167
MI_dia_43	Michigan	2021	*Diaporthe humulicola*	PV069175
MI_dia_44	Michigan	2021	*Diaporthe humulicola*	PV069176
MI_dia_45	Michigan	2021	*Diaporthe humulicola*	PV069177
MI_dia_57	Michigan	2021	*Diaporthe humulicola*	PV069211
MI_dia_66	Michigan	2021	*Diaporthe humulicola*	PV069220
MI_dia_67	Michigan	2021	*Diaporthe humulicola*	PV069221
MI_dia_68	Michigan	2021	*Diaporthe humulicola*	PV069222
MN_dia_1	Minnesota	2020	*Diaporthe humulicola*	PV069205
NY_dia_1	New York	2020	*Diaporthe humulicola*	PV069185
NY_dia_2	New York	2020	*Diaporthe humulicola*	PV069186
NY_dia_3	New York	2020	*Diaporthe humulicola*	PV069187
NY_dia_4	New York	2020	*Diaporthe humulicola*	PV069188
NY_dia_5	New York	2020	*Diaporthe humulicola*	PV069189
NY_dia_6	New York	2020	*Diaporthe humulicola*	PV069190
NY_dia_7	New York	2020	*Diaporthe humulicola*	PV069191
NY_dia_8	New York	2020	*Diaporthe humulicola*	PV069192
NY_dia_9	New York	2020	*Diaporthe humulicola*	PV069193
NY_dia_10	New York	2020	*Diaporthe humulicola*	PV069194
NY_dia_11	New York	2020	*Diaporthe humulicola*	PV069195
NY_dia_12	New York	2020	*Diaporthe humulicola*	PV069196
NY_dia_13	New York	2020	*Diaporthe humulicola*	PV069197
NY_dia_14	New York	2020	*Diaporthe humulicola*	PV069198
NY_dia_15	New York	2020	*Diaporthe humulicola*	PV069199
NY_dia_16	New York	2020	*Diaporthe humulicola*	PV069200
NY_dia_17	New York	2020	*Diaporthe humulicola*	PV069201
NY_dia_18	New York	2020	*Diaporthe humulicola*	PV069202
NY_dia_19	New York	2020	*Diaporthe humulicola*	PV069203
NY_dia_20	New York	2020	*Diaporthe humulicola*	PV069204

Table 5.7. Number of SNPs in each dataset and usage of each data set.

Data set	Number of SNPs	Analysis
95 percent	249,585	Population stats, PCA, phylogeny
LD pruned	35,916	PCA, STRUCTURE
100 percent	193,877	PCA

Table 5.8. Average segregating sites per population.

Population	Average segregating sites
CD	68,071.8
MI	68,897.5
MN	74,590.4
NY	74,348.0

FIGURES

Figure 5.1. Examples of variation of symptoms of halo blight on hop cones from the fungal cone survey. A) Desiccation in the lower half of the hop cone. B) singular bracts of hop cone with desiccation, C) hop cone with spotted lesions and half of the cone is desiccated, D) girding or the hop cone and desiccation at the tip.

Figure 5.2. Bar plots of the percentage of isolates of different genera collected in a fungal disease cone survey from 2019-2021 in the lower peninsula of Michigan, in 2019 there was 400 isolates collected, in 2020 there was 261 isolates collected and finally in 2021 there was 217 isolates collected.

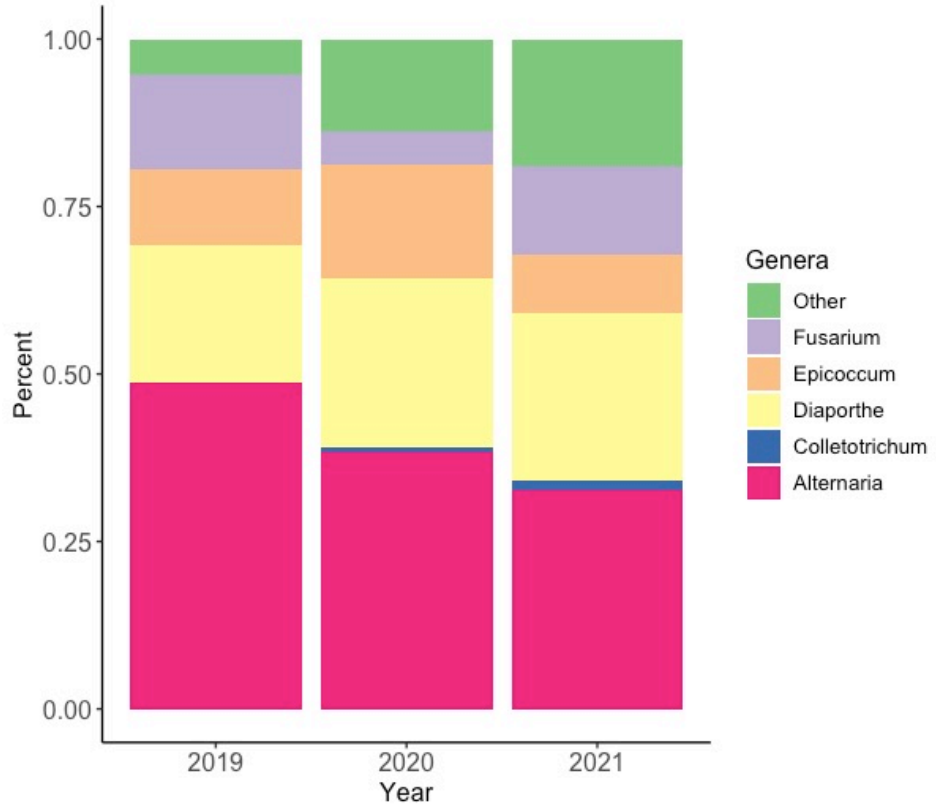

Figure 5.3. A) Delta K plot of the STRUCTURE Bayesian clustering analyses for Ks of 2–9 from 20 STRUCTURE runs using the LD pruned data set. B) Principal component analysis of genetic differentiation among isolates, using the LD pruned data set used for the Bayesian cluster analysis.

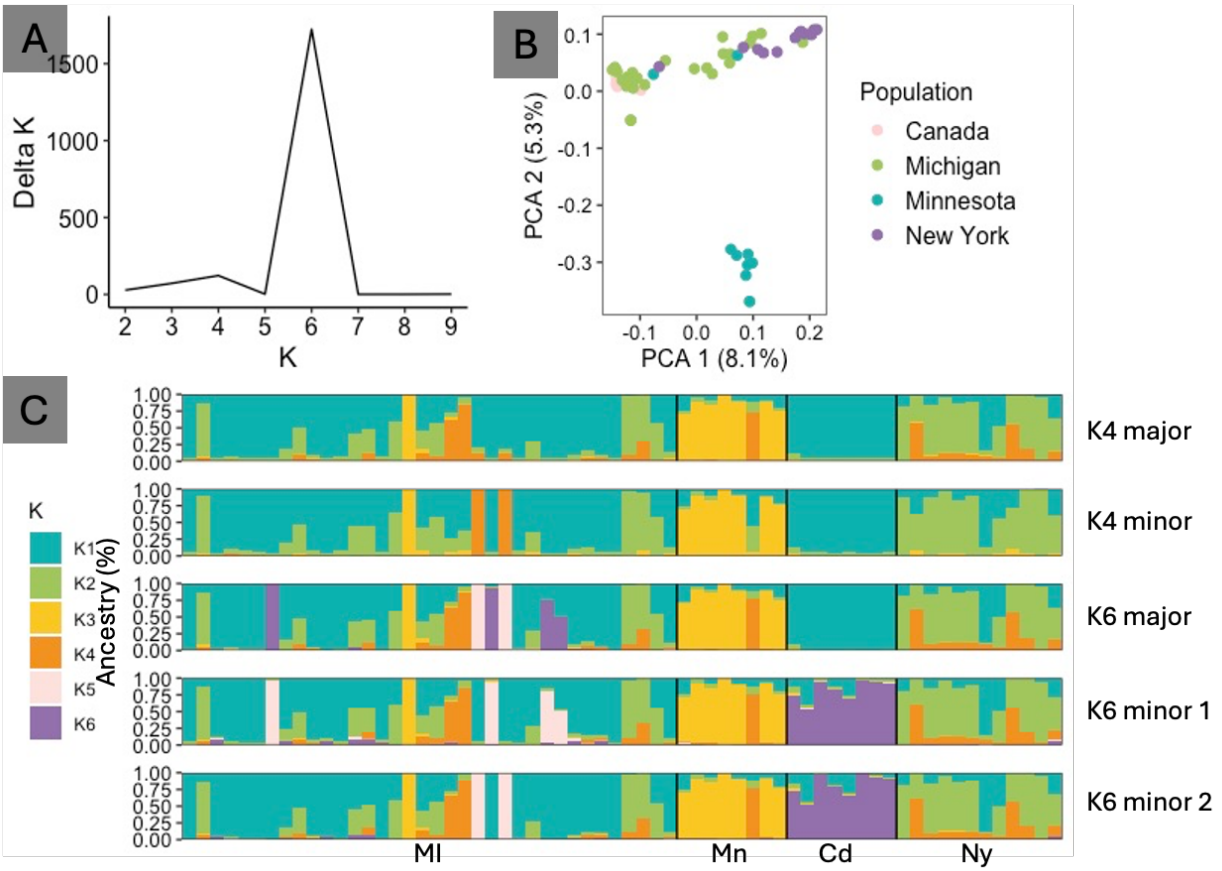

Figure 5.3. (cont'd)

The percentage of variance explained by the first two principal components is shown in parentheses. Samples are colored according to their sampling location. C) Bar plots of ancestry membership proportions (*y*-axis) as inferred by STRUCTURE for K = 4 and K = 6. Each bar represents a different sample, and each color represents a different cluster. K = 4 has a major and minor clusters that appeared in 10/20 and 9/20 runs, respectively. A single run produced a different set of clusters but is not shown. For K=6 there is one set of major clusters that was present in 14/20 runs. There is also 2 minor clusters that appeared in 4/20 and 2/20 runs, respectively. Samples from each sampling location are group together and location names are on the top. Mi = Michigan, Mn = Minnesota, Cd = Canada, and Ny = New York.

Figure 5.4. Consensus maximum likelihood phylogenetic tree obtained using the SNPs from the 95% data set. The tree was run with 10,000 replications using the program IqTree2 (v2.2.2.7) with the model TVM + F + ASC + R3. the tree was rooted using isolate Mi_23 and tree length connecting that branch is not to scale with the rest of the tree. The tree was visualized in FigTree (v1.4.4).

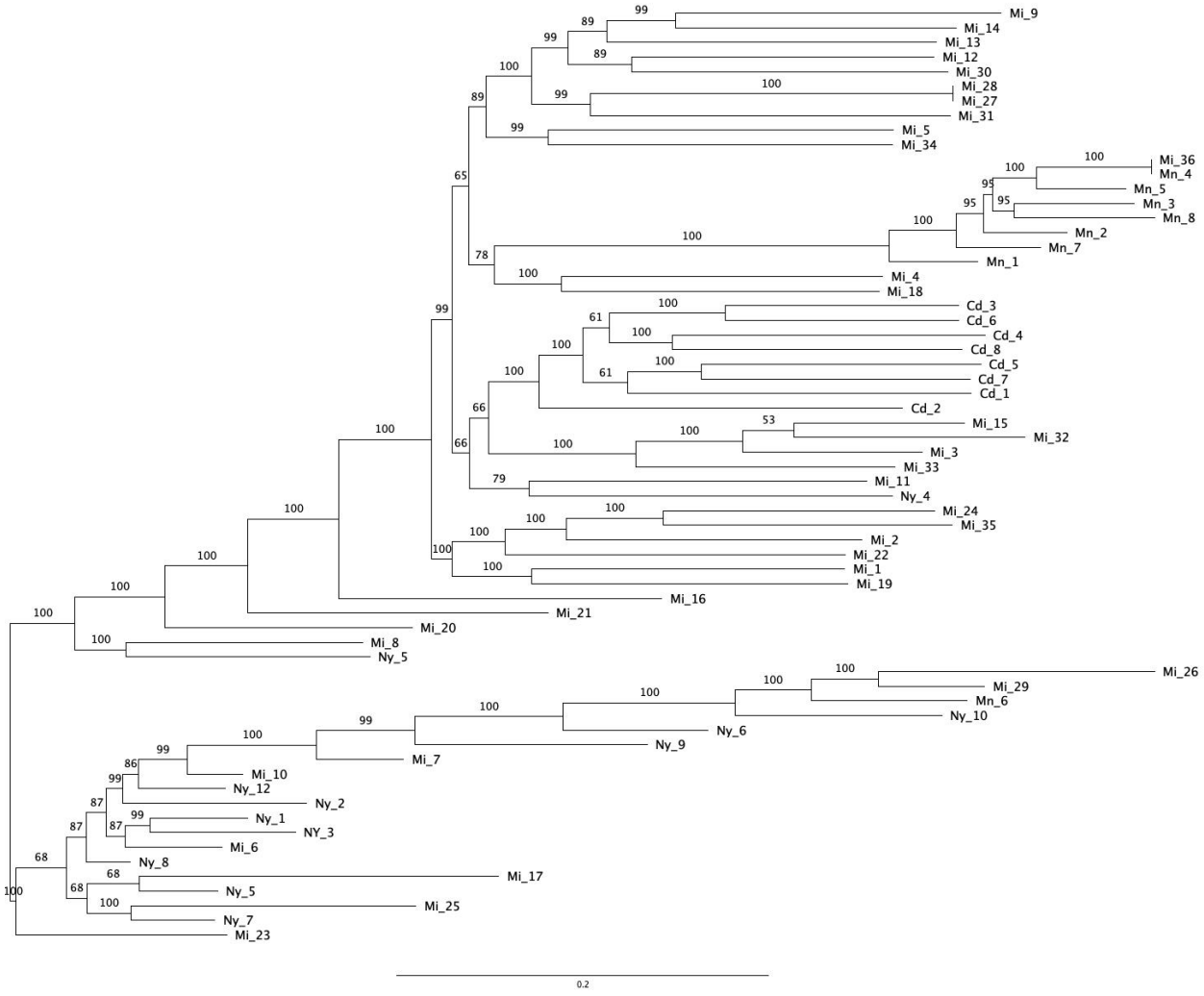

Figure 5.5. Mean of probabilities (lnP(K)) and their standard deviation of posterior probability from 20 STRUCTURE runs using the LD pruned data set.

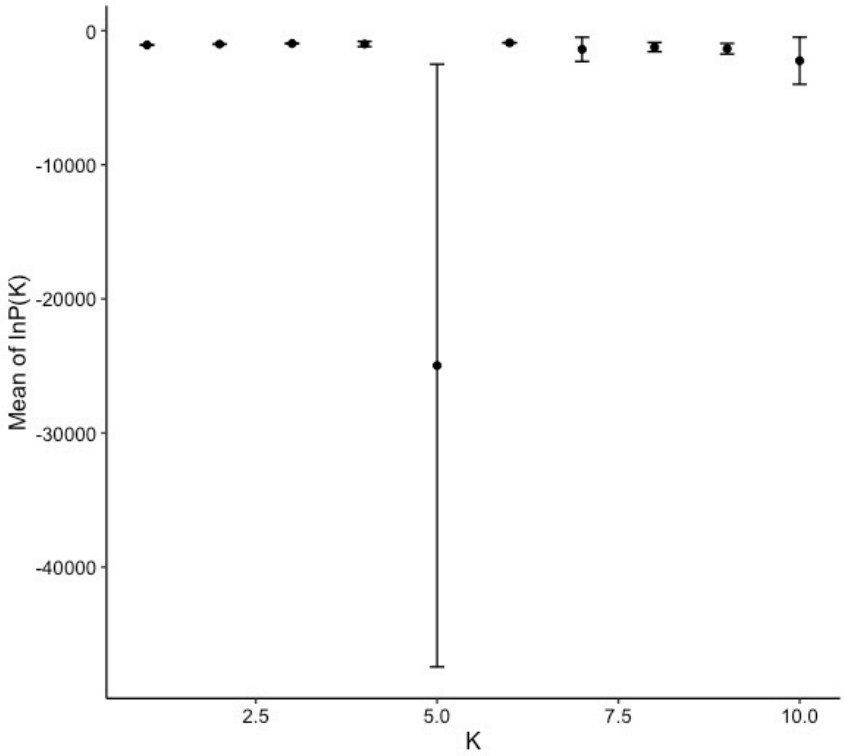

Figure 5.6. Alignments of reads for *Diaporthe* samples against mating loci *MAT-1-1-1*, *Mat-1-1-2, Mat-1-1-3*, and alignments for Tef. A) Alignment for *Mat-1-1-1, Mat-1-1-2*, and *Mat-1-1-3* from isolate Mn_1, Mn_1 has reads at all three genes and has high coverage in the tef region, isolates that matched this pattern were denoted as matting type 1-1-1. B) Alignment for *Mat-1-1-1, Mat-1-1-2,* and *Mat-1-1-3* for isolate Mi_6 isolate Mi_6 does not have reads in the *MAT-1-1-1* region and high coverage in the tef gene, isolates that followed this pattern were denoted as matting type 1-2-1. reads were aligned to the sequence *Mat-1-2-1* to confirm the identity of matting type 1-2-1, but data is not shown.

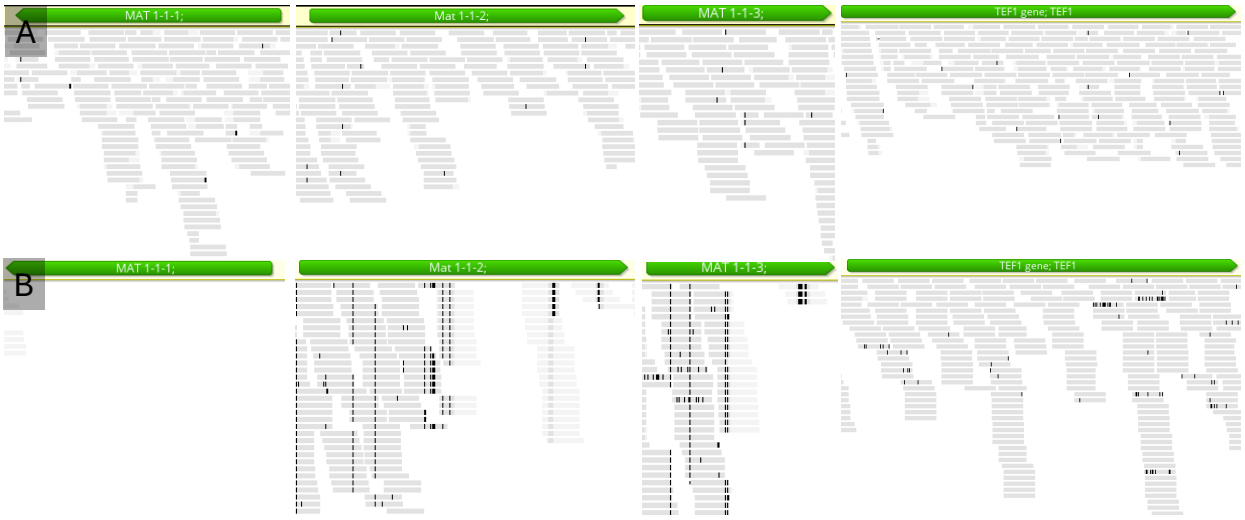

Figure 5.7. Decay of linkage disequilibrium (r2) with physical distance for each population using contig 1.

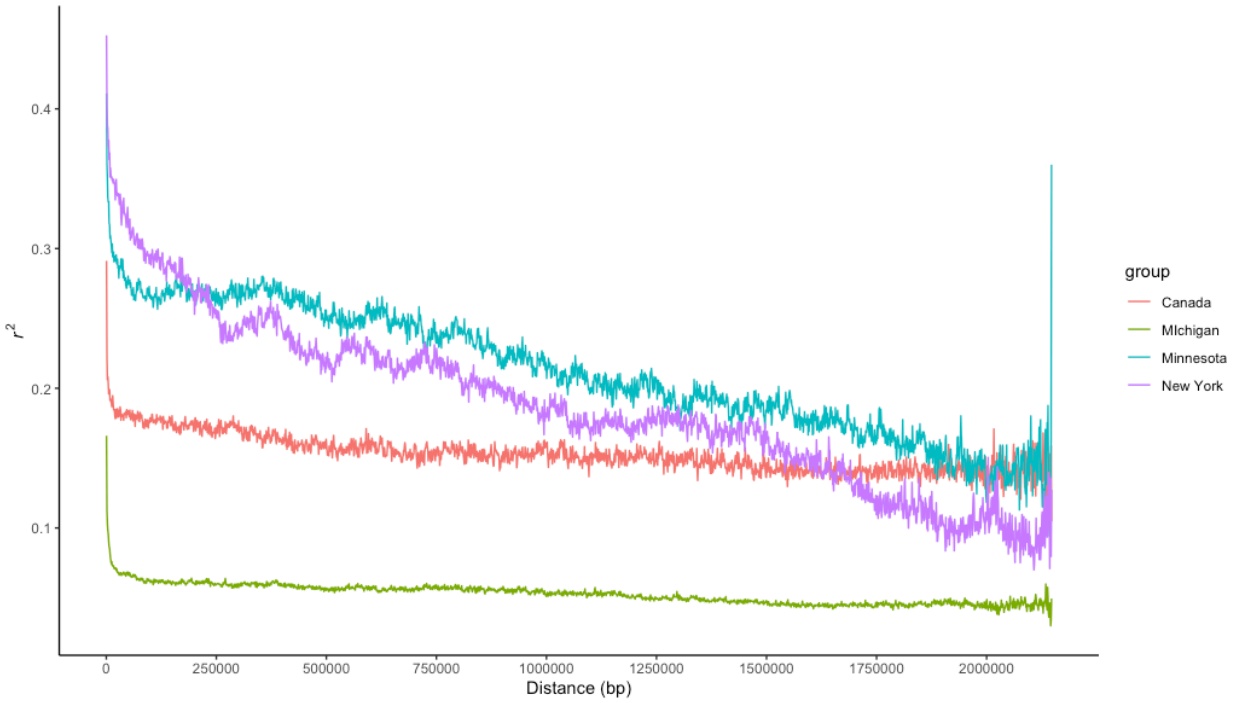

Figure 5.8. Decay of linkage disequilibrium (r^2) with physical distance for all population using contig 1.

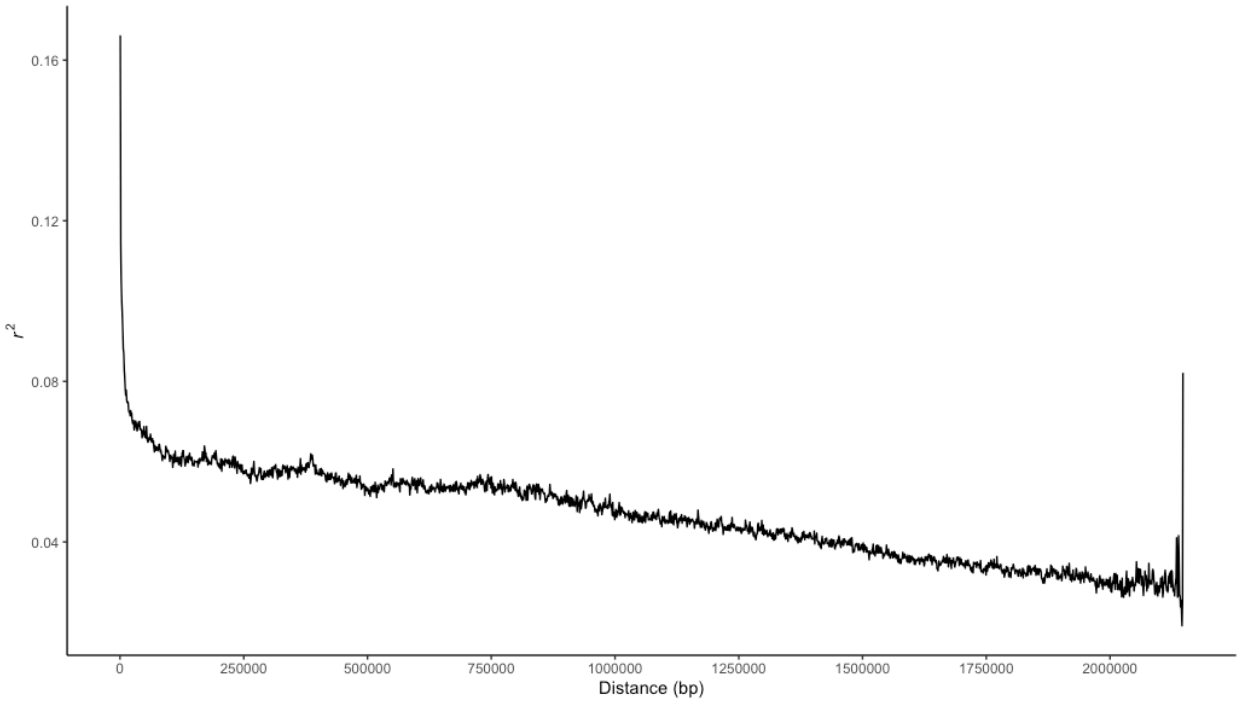

Figure 5.9. Decay of linkage disequilibrium (r^2) with physical distance for all population in the whole genome.

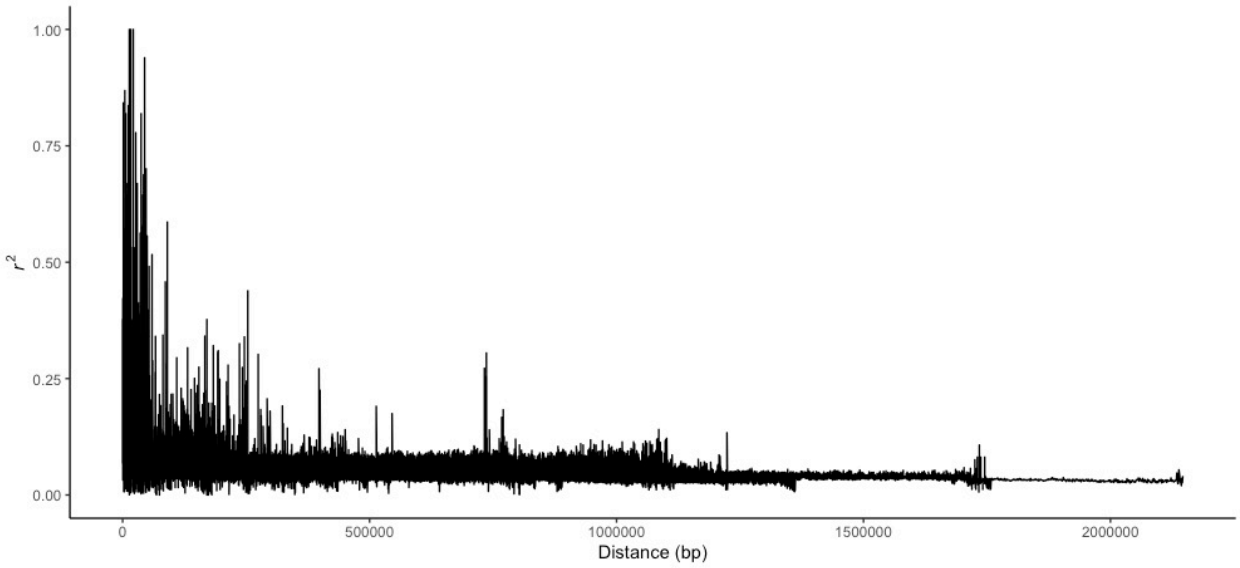

Figure 5.10. Principal component analysis of genetic differentiation among all populations. A) Using 95% data set, B) Using 100% data set.

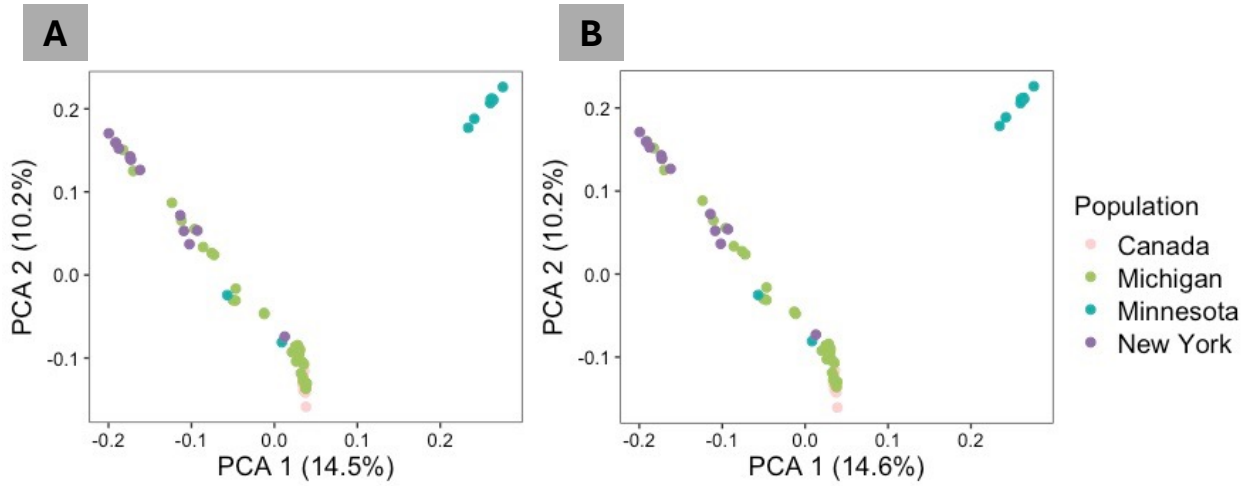

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Figure 5.11. Principal component analysis of genetic differentiation among each population using each dataset. A) PCA for Canada isolates using ld pruned dataset, B) PCA for Michigan isolates using ld pruned dataset, C) PCA for Minnesota isolates using ld pruned dataset, D) PCA for New York isolates using ld pruned dataset. E) PCA for Canada isolates using the 95% dataset, F) PCA for Michigan isolates using 95% dataset, G) PCA for Minnesota isolates using 95% dataset, H) PCA for New York isolates using 95% dataset, I) PCA for Canada isolates using the 100% dataset, J) PCA for Michigan isolates using 100% dataset, K) PCA for Minnesota isolates using 100% dataset, L) PCA for New York isolates using 100% dataset.

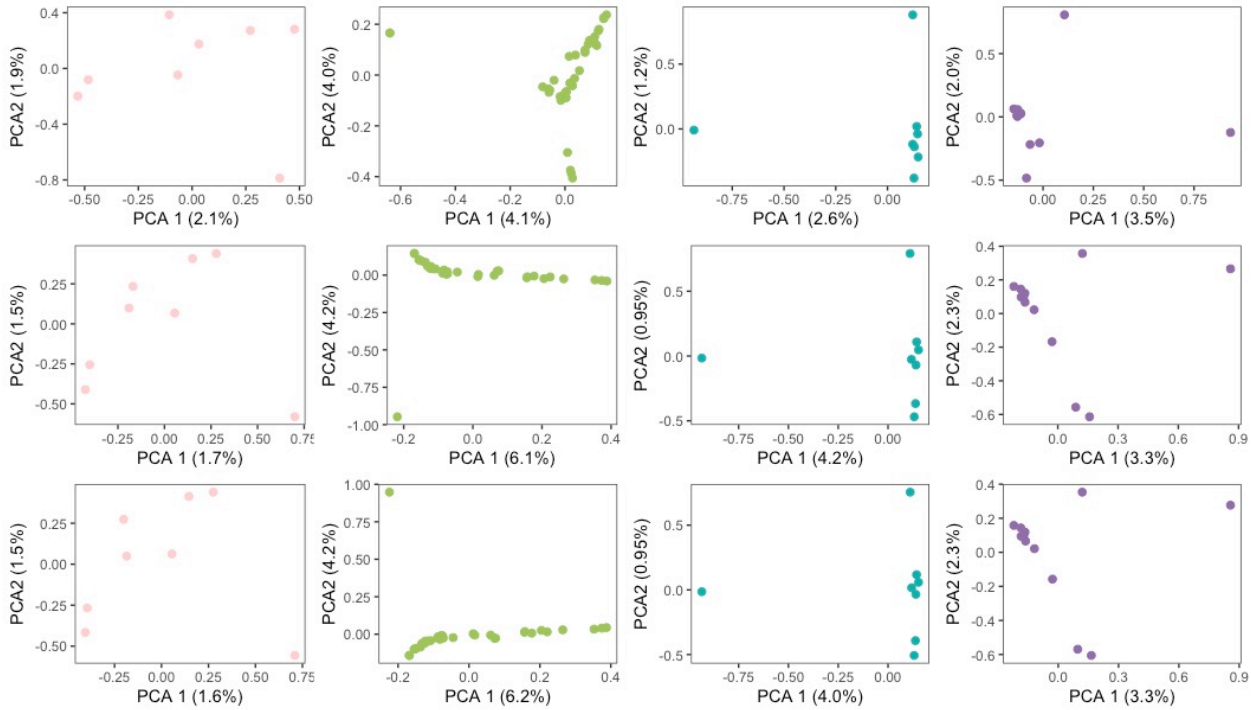


Figure 5.12. Bar plots of ancestry membership proportions (*y*-axis) as inferred by STRUCTURE for K = 1-K = 10. Each bar represents a different sample, and each color represents a different cluster. K = 1, 2 , 3, and 10, all have clusters that appear in all 20 runs. K = 4 has a major cluster that appeared in 10/20 runs. For K = 5 the major cluster appears in 14/20 runs. For K = 6 the major clusters were present in 14/20 runs. K = 7 and K = 8 both have major clusters that appear in 18/20 runs. K = 9 as a major cluster that appears in 10/20 runs. Samples from each sampling location are group together and location names are on the top. Mi = Michigan, Mn = Minnesota, Cd = Canada, and Ny = New York.

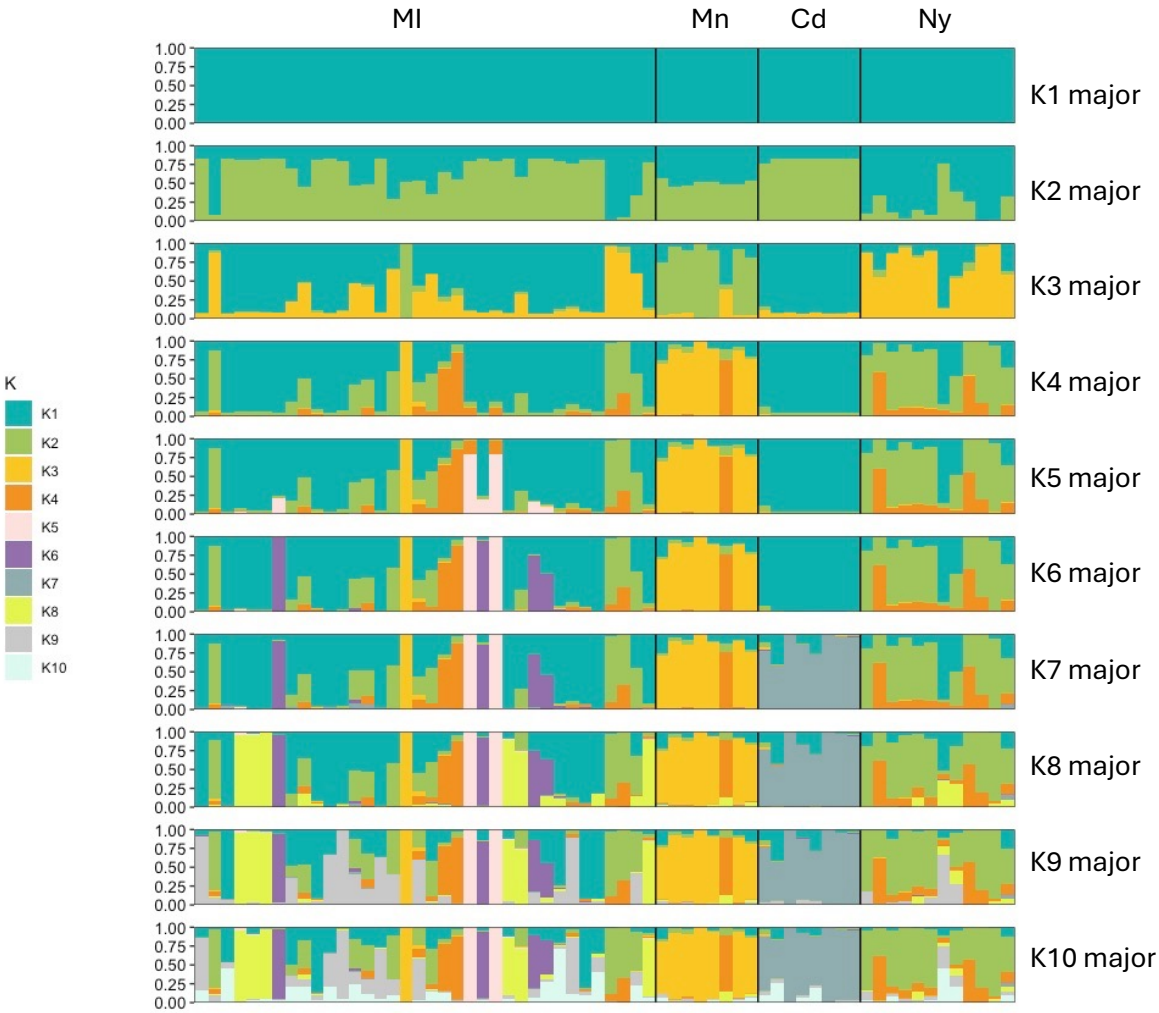


Figure 5.13. Bar plots of ancestry membership proportions (*y*-axis) as inferred by STRUCTURE. Each bar represents a different sample, and each color represents a different minor cluster K = 4 has a minor cluster that appeared in 9/20 runs. For K=5 there are two minor clusters that appear in 4 and 2 runs respectively. For K = 6 there are two minor clusters that appear in 4 and 2 runs respectively. K = 9 as a minor cluster that appears in 10/20 runs. Samples from each sampling location are group together and location names are on the top. Mi = Michigan, Mn = Minnesota, Cd = Canada, and Ny = New York.

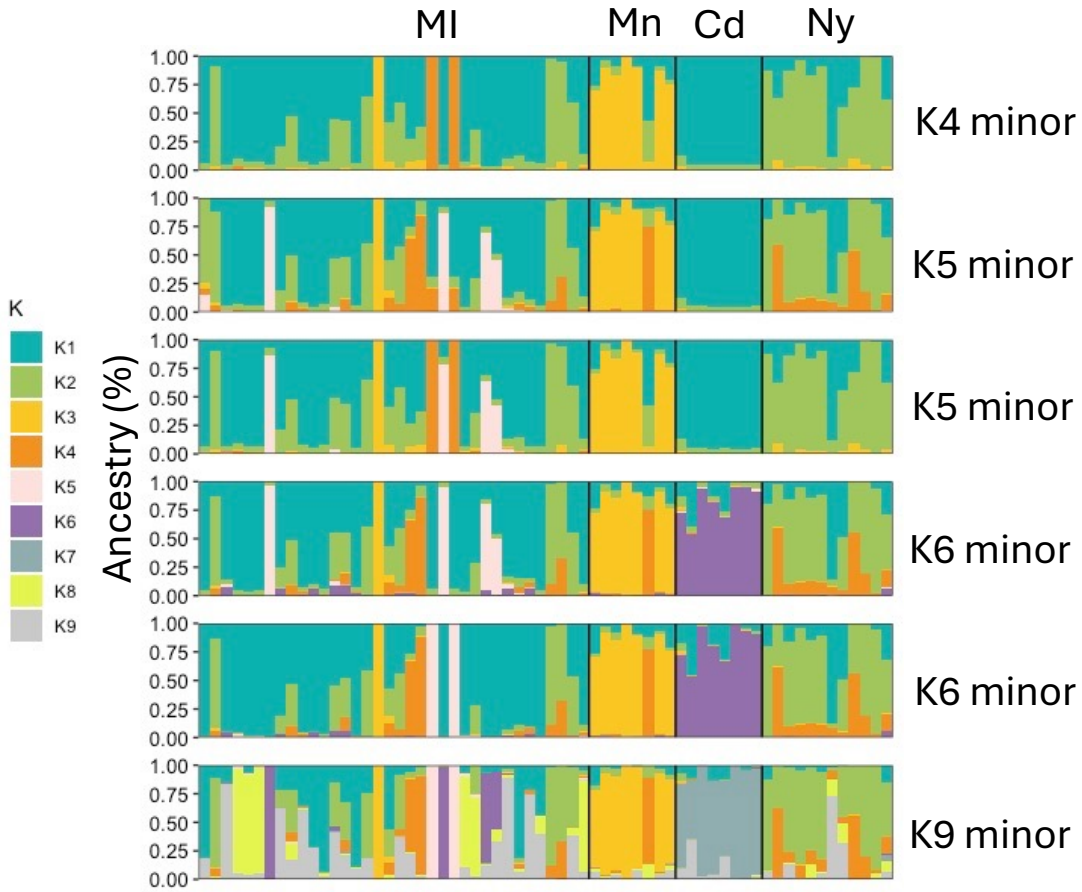


Figure 5.14. Bar plots of ancestry membership proportions (*y*-axis) as inferred by STRUCTURE. Each bar represents a different sample, and each color represents a different cluster that appeared in a single run out of twenty. Samples from each sampling location are group together and location names are on the top. Mi = Michigan, Mn = Minnesota, Cd = Canada, and Ny = New York.

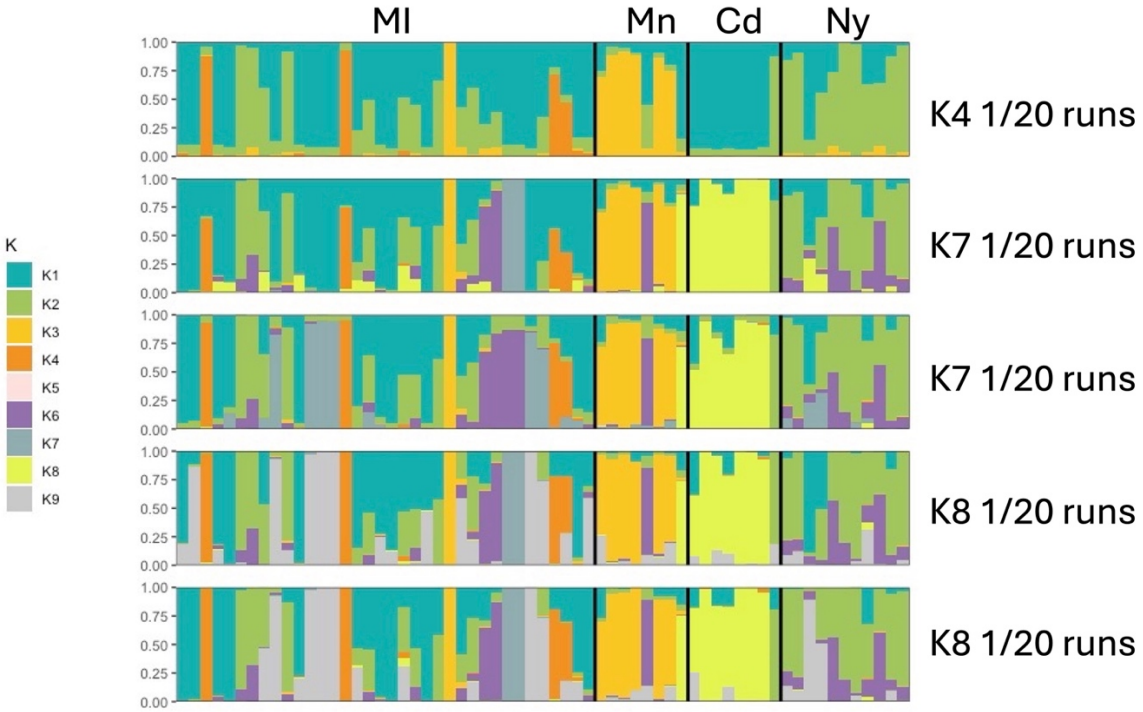


Figure 5.15. Nucleotide diversity per population. Nucleotide diversity (π) calculated in 100kb sliding windows along genomes.

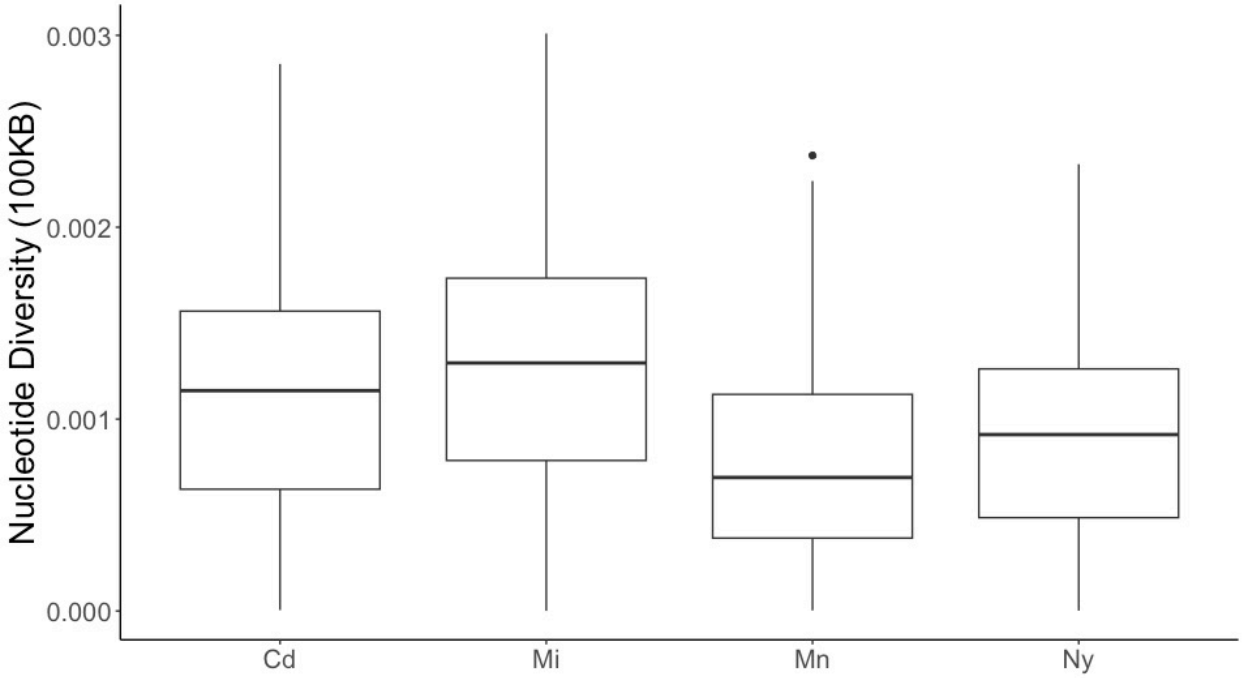
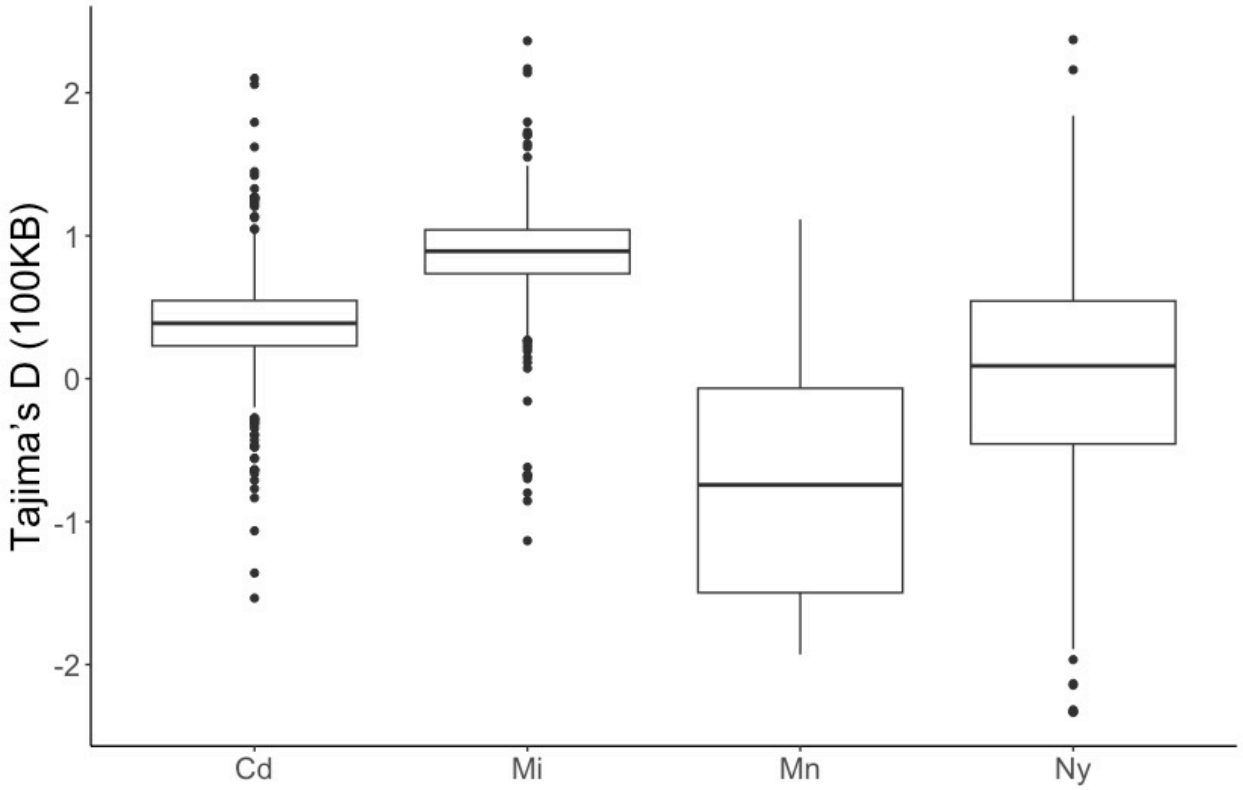


Figure 5.16. Tajima's D per population. Tajima's D was calculated in 100kb sliding windows along genomes.



LITERATURE CITED

Agricultural Research Service (USDA). 2025. Fungal databases: U.S. culture collection. Beltsville (MD): United States Department of Agriculture. [accessed 2025 Jan 9]. https://fungi.ars.usda.gov.

Allan-Perkins, E., Li, D., Schultes, N., Yavuz, S., and LaMondia, J. 2020. The identification of a new species, *Diaporthe humulicola*, a pathogen causing diaporthe leaf spot on common hop. Plant Dis. 104:2377-2390.

Ahmed, A. M., Mahmoud, B. A., Millán-Aguiñaga, N., Abdelmohsen, U. R., and Fouad, M. A. 2023. The endophytic *Fusarium* strains: a treasure trove of natural products. RSV adv. 13(2):1339–1369.

Amwzrou, 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., and Marcel, T. C. 2024 Quantitative pathogenicity and host adaptation in a fungal plant pathogen revealed by whole-genome sequencing. Nat Commun 15.

Bashalkhoanov, S., Pandey, M., and Rajora, O. P. 2009. A simple method for estimating genetic diversity in large populations from finite sample sizes. BMC. 10:84.

Bienaplf, J. C. 2004. Hop cone tip blight : a new disease in the Pacific Northwest. M.S. Thesis. Oregon State University.

Burdon, J. J. and Silk J., 1997. Sources and patterns of diversity in plant-pathogenic fungi. Phytopath.87(7):664-669.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Padadopoulos, J., Bealer. K., Madden., T. L., 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421.
Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia. 91(3):553–556.

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohanm, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., and Li, H. 2021. Twelve years of SAMtools and BCFtools. Gigascience 10(2).

Darby, P. 1984. Department of hop research annual report 1983, Wye College. Invicta Press, Ashford, UK.

Darby, P. 1988. *Alternaria alternata* infection of hop (*Humulus lupulus*) cones. Mycol. Res. 90:650-653.

De Meeûs, T., and Balloux, F., 2004. Clonal reproduction and linkage disequilibrium in diploids: a simulation study. Infect. Genet. Evol. 4(4):345-351.

Drenth, A., McTaggart, A. R., and Wingfield, B. D. 2019. Fungal clones win the battle, but recombination wins the war. IMA Fungus. 10(18).

Earl, D. A., and von Holdt, B. M. 2011. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4:359-361.

Evanno, G., Regnaut, S., and Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 14(8):2611-2020.

Falush, D., Stephens, M., and Pritchard J. K. 2003. Inference of population structure using multilocus genotype data:linked loci and correlated allele frequencies. Genetics. 164: 1567–1587.

Flanagan, B. A., Krueger-Hadfield, S. A., Murren, C. J., Nice, C. C., Strand, A. E., and Sotka, E. E. 2021. Founder effects shape linkage disequilibrium and genomic diversity of a partially clonal invader. Mol. Ecol. 30(9):1972-1978.

Foster, A., Mills, A., and Mccallum, J. 2024. First report of *Diaporthe humulicola* causing halo blight of hop (*Humulus lupulus L.)* in Prince Edward Island, Canada Can. J. Plant Pathol. Preprint.

Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., and Crous, P. W. 2013. *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia 31:1–41.

Hatlen, R. J., Higgins, D. S., Venne, J., Rojas, J. A., Hausbeck, M. K., and Miles, T. D. 2022. First report of halo blight of hop (*Humulus lupulus*) caused by *Diaporthe humulicola* in Quebec, Canada. Plant Dis. 106:1750.

Hatlen, R. J., Swift., R. M., Miles, L. A., Byrne, J. M., and Miles, T. D. 2023. First Report of *Colletotrichum fioriniae* Infecting Hop (*Humulus lupulus*) in Michigan. 107(10): 3280.

Hatlen, R. J., Hausbeck, M. K., Anthony, M. J., Sysak, R., Smith, R., Miles, T. D. 2025. Chemical management strategies for halo blight of hop and in vitro sensitivity of *Diaporthe humulicola* populations to various fungicide classes. Plant Dis. In first look.

Hatlen, R. J., Szymanski, S. L., Adair, N. L., Fan, Q., Panwar. P., Sysak, R., Miles, L. A., Higgins, D. S., Rojas, J. A., Gent, D. H., and Miles, T.D., 2025 Development of a molecular diagnostic tool for *Diaporthe humulicola*, the causal agent of halo blight of hop. Unpublished, submitted for review in Plant Disease.

Havill, J. S, Hatlen, R. J., Muehlbauer, G. J., and Miles, T. D. 2023. First report of halo blight on hop (Humulus lupulus) caused by *Diaporthe humulicola* in Minnesota. Plant Dis. 107:2523.

Higgins, D. S., Hatlen, R. J., Byrne, J. M., Sakalidis, M. L., Miles, T. D., and Hausbeck, M. K. 2021a. Etiology of halo blight in Michigan hopyards. Plant Dis. 105:859-872.

Hop growers of America, 2024. USA Hops 2023 Statistical Report, https://www.usahops.org/img/blog_pdf/474.pdf.

Hubisz M. J., Falush, D., Stephens, M., and Pritchard J. K. 2009. Inferring weak population structure with the assistance of sample group information. Mol. Ecol. Resour. 9:1322-1332.

Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A., and Mayrose, I. 2015. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. Mol. Ecol. Resour. 15(5):1179-1191.

Lahlali, R., Taoussi, M., Laasli, S., Grachara, G., Ezzouggari, R., Belbess, Z., Aberkani, K., Assouguem, A., Meddich, A., El Jarroudi M., and Ait Barka, E. 2024. Effects of climate change on plant pathogens and host-pathogen interactions. Crop Env. 3(3):159-1770.

Laraba, I., McCormick, S. P., Vaughan, M. M., Geiser, D. M., and O'Donnell, K. 2021. Phylogenetic diversity, trichothecene potential, and pathogenicity within *Fusarium sambucinum* species complex. PLoS One 16:e0245037.

Li, H., and Durbin, R. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinform. 25(14):1754-1760.

Litrico, I., and Violle C. 2015. Diversity in Plant Breeding: A new conceptual framework. Trends Plant Sci. 20(10) 604-613.

Lou, R. N., Jacobs, A., Wilder, A. P., and Therkildsen, N. O. 2021. A beginner's guide to low-coverage whole genome sequencing for population genomics. Mol. Ecol., 30(23):5966–5993.

Macchioni, V., Picchi, V., and Carbone, K. 2022. Hop leaves as an alternative source of health-active compounds: effect of genotype and drying conditions. Plants 11:99.

Miles, T. D., Day, B., and Schilder, A. C. 2011. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. Mol. Plant Pathol. 12:463-477.

Minh, B. Q., Schmidt, H. A., Chernomer, O., Schrempf, D., Woodhams, M., von Haesler. A., and Lanfear, R. 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. MBE 37(5):1530-1534.

Nazareno, A. G., Bemmels, J. B., Dick, C. W., and Lohmann, L. G. 2017. Minimum sample sizes for population genomics: an empirical study from an Amazonian plant species. Mol. Ecol. Resour. 17(6):1136-1147.

Ortiz, E.M. 2019. vcf2phylip v2.0: convert a VCF matrix into several matrix formats for phylogenetic analysis. DOI:10.5281/zenodo.2540861.

Pethybridge, S. J., Hay, F. S., Wilson, C. R., Sherriff., L. J., and Leggett, G. W. 2001a.First report of infection of hop cones by *Alternaria alternata* in Australia. Pant dis. 85(7):804.

Pethybridge, S. J., Hay, F. S., Wilson, C. R., Sherriff., L. J., and Leggett, G. W. 2001b. First Report of *Fusarium crookwellense* Causing Tip Blight on Cones of Hop. Pant dis. 85(11):1208.

Phalip, V., Hatsch, D., Laugel, B., and Jeltsch, J. M., 2006. An overview of fungal community diversity in diseased hop plantations. FEMS Microbiol. Ecol. 56(2):321-329.

Phillips, J. D., Gillis, D. J., and Hanner, R. H. 2019. Incomplete estimates of genetic diversity within species: Implications for DNA barcoding. Ecol Evol. 9(5):2996–3010.

Porras-Hurtado, L., Ruiz, Y., Santos, C., Philips, C., Carracedo, Á., and Lareu, M. V. 2013. An overview of STRUCTURE: applications, parameter settings, and supporting software Sec. Statistical Genetics and Methodology 4.

Pritchard, J. K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. GSA. 945-959.

Rodrigues, A. S. B., Silva, D. N., Várzea, V., Paulo, O. S., and Bastista, D. 2022. Worldwide population structure of the coffee rust fungus *Hemileia vastatrix* is strongly shaped by local adaptation and breeding history. Phytopath. 112(9): 1998-2011.

Rossini, F., Virga, G., Loreti, P., Lacuzzi, N., Ruggeri, R., and Provenzano M. E., 2021, Hops (*Humulus lupulus L.)* as a novel multipurpose crop for the mediterranean region of Europe: challenges and opportunities of their cultivation. Agriculture. 11(6), 484.

Rúa, M. A., Wilson. E. C., Steele. S., Munters, A. R., Hoeksema, J. D., and Frank, A. C. 2016. Associations between ectomycorrhizal fungi and bacterial needle endophytes in *Pinus radiata*: implications for biotic selection of microbial communities. Front. Microbiol. 7.

Sabo, J., Duric, T., and Jasnic, S. 2007. *Fusarium* fungi as a pathogen causing hop wilt. Plant Prot. Sci. 38:308–310.

Santos, J. M., Correla, V. G., and Phillips, A. J. L. Primers for mating-type diagnosis in *Diaporthe* and *Phomopsis*: their use in teleomorph induction in vitro and biological species definition. 2010. Fungal Biol. 114(2-3):255-270.

Schönberger, C., and Kostelecky, T. 2012 125th Anniversary Review: The Role of Hops in Brewing, JIB 117:259-267.

Sharma, S., Strickland, D. A., Hay, F., and Pethybrige, S. J. 2023. First report of halo blight on hop caused by *Diaporthe humulicola* in New York. Plant Dis. 107:216.

Sirrine, R., Lizotte, E., Brown, D., O'Brien, T., and Leach, A. 2014. Michigan State University Extension, https://www.canr.msu.edu/uploads/resources/pdfs/estimated_costs_of_producing_hops_in_michigan_(e3236).pdf.

Stamborski, N., Jung. G., and Back, C. G, 2022. First report of *Diaporthe humulicola* infecting stems in hop plants in Massachusetts (Abstr.) Phytopathology 112:S2.20. https://doi.org/10.1094/PHYTO-112-8-S2.20
Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 123(3):585-595.

Takahata, N., and Nei, M. 1985. Gene genealogy and variance of interpopulational nucleotide differences. Genetics. 110(2):325-344.

Taylor, N. P., And Cunniffe, N. J. 2023. Optimal resistance management for mixtures of high-risk fungicides: robustness to the initial frequency of resistance and pathogen sexual reproduction. Phytopathol. 113(1):55-69.

Tomlan, M. A. 2013. Tinged with Gold: Hop Culture in the United States. The University of Georgia Press. Athens, GA.

Torres-Cruz, T. J., Whitaker, B., Proctor, R. H., Broders, K., Laraba, I., Kim, H. S., Brown, D. W., O'Donnell, K., Estrada-Rodríguez, T. L., Lee Y. H., Cheong, K., Wallace, E. C. McGee, C. T., Kang, S., and Geiser, D. M. 2022. FUSARIUM-ID v.3.0: An updated downloadable resource for *Fusarium* species identification. Plant Dis. 106:1610-1616.

Toyama, K. S., Crochet, P. A., and Lebiois, R. 2020 Sampling schemes and drift can bias admixture proportions inferred by structure. Mol. Ecol. Resour. 20(6)1769-1785.
Weir, B. S., and Cockerham, C. C., 1984. Estimating f-statistics for the analysis of population structure. Evolution. 38(6):1358-1370.

White, T. J., Bruns, T., Lee, J., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR protocols: A guide to methods and applications. Vol. 18. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

CONCLUSION AND FUTURE DIRECTIONS

In summary, this dissertation expands the knowledge of *Diaporthe humulicola*, the causal agent of halo blight of hop, through fungicide testing, the development of a diagnostic tool, genome assembly, and population genetic analysis of different pathogen populations. Currently, there are no registered fungicides for controlling *D. humulicola*. However, our fungicide trials revealed that fungicides classified as sterile biosynthesis inhibitors (FRAC 3), quinone outside inhibitors (FRAC 11) and succinate dehydrogenase inhibitors (FRAC 7) can reduce the incidence and severity of symptoms in the field and inhibit *D. humulicola* growth in vitro. Additionally, some fungal isolates exhibited reduced sensitivity to the fungicide trifloxystrobin.

The qPCR-based diagnostic tool developed in this study significantly reduced the detection time for *D. humulicola* from weeks to hours. This tool also provided evidence that the fungus can colonize hop rhizomes and below-ground tissues. Furthermore, this research presents the first confirmed genome assembly *of D. humulicola*, providing valuable genetic resources for studies on the genus *Diaporthe*. The assembled genome also enabled a population genetics study using *Diaporthe* isolates from across the eastern United States and Canada, revealing evidence of recombination among different populations and confirming that *D. humulicola* is a heterothallic.

Future researchers can utilize the tools developed in this dissertation to monitor the Pacific Northwest for the pathogen in hopyards. The qPCR tool could also be applied to assess whether wild hop populations serve as pathogen hosts and should be used for pathogen detection in nursery systems. Additionally, research should focus on determining whether wild hops or existing cultivars exhibit resistance to *D. humulicola*. Finally, this qPCR tool may provide helpful in understanding the overall epidemiology of this pathogen in the field and allow researchers to refine the management of halo blight of hop.

APPENDIX A: MANAGING HALO BLIGHT OF HOP

Abstract

Halo blight of hop is caused by the fungus *Diaporthe humulicola* and is a major concern for hop growers in Michigan and in other parts of the Midwest. The pathogen was first discovered in 2018 but was not formally described until 2021. The disease has been reported in four different hop producing states in the northeastern United States. The pathogen can cause high amounts of yield loss and can change the chemical composition in the hop cone.

Symptoms

Halo blight of hop first appears in late spring to early summer as brown to gray lesions that develop on the hop leaves that are surrounded by a chlorotic (yellow to light green) halo (Figure 1A.1). The lesions may have concentric rings radiating from the center of the leaf lesion, but rings may not be present (Figure 1A.1).

Smaller lesions or lesions that are early in development may be mistaken as another fungus *Colletotrichum fioriniae* (Figure 1A.2). Brown to black fungal, spore producing structures (pycnidia) may appear on the surface of the leaf lesion, resembling pimples. Hop leaves may have multiple lesions at different stages of development on a single leaf. The pathogen also seems to be able to colonize dead or discarded leaf tissue during the growing season.

The pathogen can also infect hop cones and burrs. Hop burrs become necrotic and desiccated. The cone's bracts become desiccated, leaving bract tissue a light to dark brown color. The desiccation can occur on single bracts or whole sections of the hop cone (Figure 1A.3). Brown to black pycnidia can develop on the surface of the hop cone. Late in the season, white mycelia can be seen on the hop rachis (strig), but this is not a consistent stage of the disease

development (Figure 1A.3). Infected hop cones with desiccated cone tissue often shatter upon harvest, resulting in yield losses up to 50%. Cones that survive harvest with moderate levels of halo blight seem to have an altered aroma profile; growers report that infected cones often have a "cheese like" aroma. It is important to note that other diseases can also cause cone desiccation such as downy mildew, powdery mildew, Alternaria cone disorder and fusarium cone tip blight. After harvest in the late fall to winter, *D. humulicola* can colonize the left-over bine tissue and produce pycnidia on the surface of the tissue (Figure 1A.4).

Disease cycle

The pathogen overwinters on dried hop tissue as pycnidia (small black fungal structures). When conditions are warm (>60 degrees Fahrenheit, or 15.6 degrees Celsius) and humid, the pycnidia will sporulate and produce conidia. The conidia are released from the pycnidia and dispersed by weather events. The pathogen causes lesions on the surface of the hop leaf that give rise to new pycnidia. Pycnidia produce conidia that can infect the burrs/cones and leaves, causing lesions on the leaves and desiccation of the hop cone. This portion of the life cycle seems to be polycyclic and if environmental conditions are right, the hop plant can go through multiple infection cycles per year. In the late summer or early fall, the pathogen seems to colonize hop bines that have been left after harvest, where the pathogen remains dormant until late spring early summer (Figure 1A.5).

Management

Cultural

In early fall, post-harvest dead material and new shoots should be removed to prevent colonization and overwintering by *D. humulicola*. Post-harvest hop plants can also have the top layer of their crown removed in a process called crown pruning, which can reduce inoculum of

the pathogen that can infect the hop crown. Extensive research has been done on crown pruning for downy and powdery mildew control, and anecdotal evidence from hop growers in Michigan suggest it may offer some control of halo blight as well.

There appears to be a correlation between the severity of downy mildew symptoms and the severity of halo blight symptoms, therefore early season downy mildew disease management can be a preventive measure against halo blight of hops.

Chemical

The disease is present throughout the hop growing season, so multiple chemical applications for halo blight will likely be needed per season. At the time of this publication, no fungicides are registered for this disease, but there is evidence that fungicides registered for powdery mildew with the active ingredients trifloxystrobin (FRAC 11), flutriafol (FRAC 3), tebuconazole (FRAC 3) and fluopyram (FRAC 7) have been shown to cause a reduction in the incidence and severity of halo blight symptoms. These products include the commercial fungicides Flint Extra (Bayer), Rhyme (FMC) and Luna Experience (Bayer). Biological fungicides like Lifegard (*Bacillus mycoides* Isolate J – Certis USA) have also been shown to reduce foliar symptoms of halo blight. Furthermore, we have noticed that controlling downy mildew with a chemical management strategy can reduce the incidence and severity of halo blight. Downy mildew is typically controlled with entirely different modes of action (FRAC classes 4, 11, 21, 27, 40, 43, 45 and 49).

It is important to manage fungicide resistance and avoid applying similar products back-to-back. This is particularly important with site-specific systemic fungicides. To reduce the development of resistance with systemic fungicides:

- Do not make more than three applications per season of the same FRAC code.

- Do not make two consecutive applications of the same FRAC code.
- Rotate with unrelated fungicides in a different FRAC code that have efficacy on the target pathogen.

FIGURES

Figure 1A.1 Hop leaves with various symptoms of halo blight (caused by *D. humulicola*) on multiple hop leaves.

Figure 1A.2. A) Small lesions on a hop leaf caused by *C. fioriniae* (Laura Miles, MSU). B) Hop leaf with smaller lesions caused by *D. humulicola.*

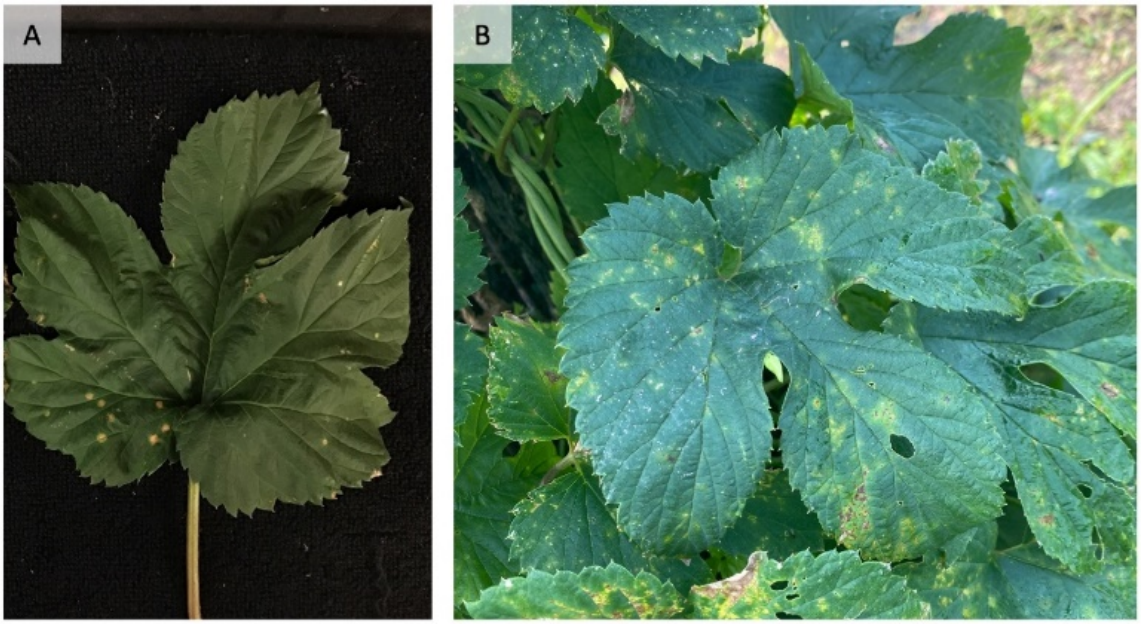


Figure 1A.3. A) Hop cones with various symptoms of halo blight of hop. B) Hop cone with halo blight symptoms (necrosis and desiccation of the internal bracts and bracteoles) and signs of *D. humulicola* (white mycelia colonizing the strig of the hop cone).

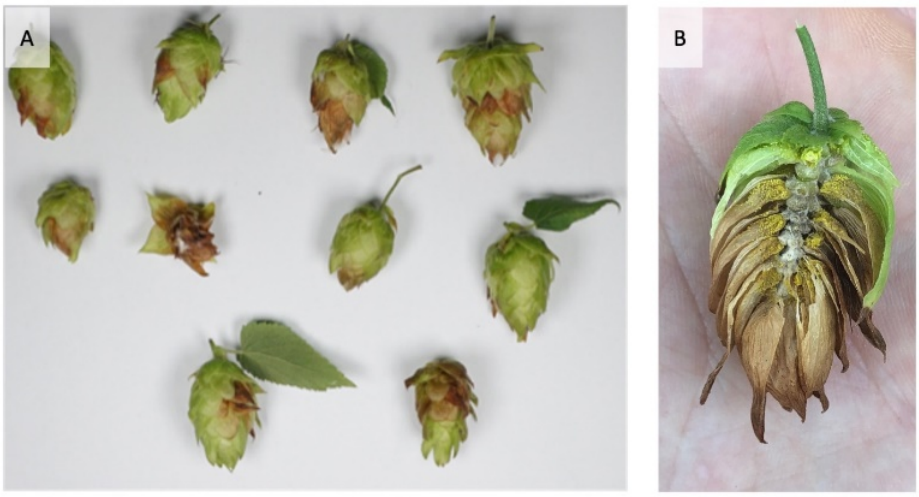
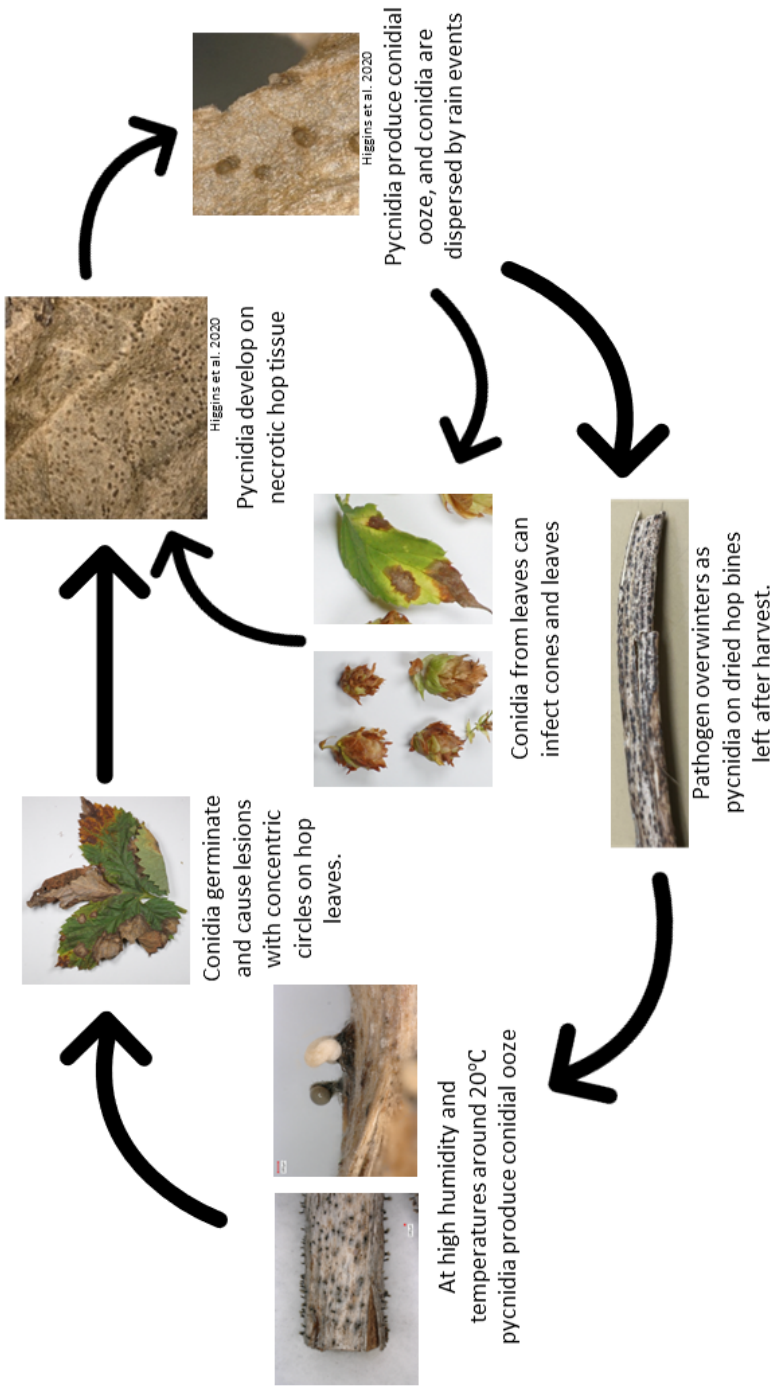


Figure 1A.4. A) Hop twig covered in *D. humulicola* pycnida that have not germinated. B) Hop twig with covered in *D. humulicola* pycnidia that have started to germinate. C) Hop twig with *D. humulicola* pycnidia with conidial ooze.

Figure 1A.5. Disease cycle for halo blight of hop.



APPENDIX B: OTHER PUBLISHED PEER-REVIEWED ARTICLES DURING PH.D. PROGRAM

Hatlen, R. J., Higgins, D.S., Venne, J., Rojas, J.A., Hausbeck M.K., and Miles, T.D. 2022. First report of halo blight of hop (*Humulus lupulus*) caused by *Diaporthe humulicola* in Quebec, Canada. Plant Dis. 106:6: 1750.

Hatlen, R.J., Swift, R.M., Miles, L.A., Byrne, J.M., and Miles T.D. 2023. First report of *Colletotrichum fioriniae* infecting hop (*Humulus lupulus*) in Michigan. Plant Dis. 107:10:3280.

Havill J.S, **Hatlen R.J.,** Muehlbauer G.J., and Miles, T.D. 2023. First report of halo blight on hop (Humulus lupulus) caused by *Diaporthe humulicola* in Minnesota. Plant Dis. 107:8: 2523.

Higgins D.S., **Hatlen R.J.,** Byrne, J.M., Sakalidis, M.L., Miles, T.D., and Hausbeck, M.K. 2021. Etiology of halo blight in Michigan hopyards. Plant Dis. 105:859-872.

Kelin-Gordon J. M., **Hatlen, R. J**., Miles T. D., 2023. First Report of Stem Blight Caused by *Diaporthe eres* on Highbush Blueberry (*Vaccinium corymbosum*) in Michigan, Plant Dis. 107 2529.

Szymanski S., Longley, R., **Hatlen, R. J.,** Heger, L., Sharma, N., Bonito, G., Miles, T. 2023. The Blueberry Fruit Mycobiome Varies by Tissue Type and Fungicide Treatment. Phytobiomes 7:2: 208-219.

Abbey, J. A., Alzohariy, S. A., Neugebauer, K. A., **Hatlen, R. J.,** Miles, T. D. 2024. Fungicide resistance in Botrytis cinerea and identification of Botrytis species associated with blueberry in Michigan. Front. Microbiol. 15.

Gent, D. H., Adair, N. L., **Hatlen, R. J.,** Miles, T. D., Richardson, B. J., Rivedal, H. M., Ross, C., Wiseman, M. S. 2024. Detection of *Podosphaera macularis* in Air Samples by Quantitative PCR. Plant Dis. 108:9.