Characterization of *Passalora fulva* and tomato leaf mold-associated fungi in Minnesota high-tunnels and the management of common high-tunnel tomato diseases

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Abstract

High-tunnels are used in locations with short seasons to extend tomato production both before and after the traditional season as well as to provide protection from extreme temperatures and weather events. However, on average, high-tunnels have increased humidity as well as decreased airflow, which can increase the prevalence of certain tomato diseases. Tomato leaf mold, caused by Passalora fulva, is one disease that is prevalent in the humid high-tunnel environment. Tomato leaf mold lesions contain both P. fulva and other fungal species in the genus Cladosporium. In this research, the diversity of a collection of 93 P. fulva and Cladosporium spp. isolates was assessed by molecular and functional techniques. DNA sequences from the internal transcribed spacer (ITS), β-tubulin, and translation elongation factor-1α (TEF-1α) regions were used to separate P. fulva from Cladosporium spp. and to differentiate species within the Cladosporium genus. Better resolution may be obtained through other techniques such as multilocus sequence analysis or genotype-by-sequencing marker technology. DNA sequences of Avr loci were used to identify mutations within collected P. fulva isolates, relative to corresponding reference sequences. A two base-pair deletion was detected in the Avr2 locus, resulting in a presumed non-functional protein. Additionally, synonymous and non-coding mutations were detected in the Avr4E locus and non-coding mutations and a non-synonymous

mutation were detected in the Avr9 locus. The temperature growth optima for a selection of isolates were assessed to functionally characterize the predominant species in the collection. In this study, the area of growth varied as a result of incubator temperature, growth media, and isolate. In addition to tomato leaf mold, gray mold and early blight are very prevalent in high-tunnel tomato production. In this research, ten pesticide spray programs were assessed for their efficacy in managing tomato leaf mold, gray mold, and early blight in high-tunnel tomato production, relative to an untreated control. Spray program did not have a significant effect on the incidence and severity of the diseases, the overall health of tomato plants, or the yield. Location had a significant effect on marketable and non-marketable yields. These results may raise concerns about the experimental design of the study, rather than implying that pesticides have no effect on plant health, yield, or disease severity. In the future, experimental plots, laid out across uniform but distinct locations, should be effectively spaced to reduce the chance of pesticide drift between treatments.

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Chapter 1. Literature Review

1.1 Overview

Leaf mold, caused by the fungus *Passalora fulva* (Cooke) U. Braun & Crous (syn. *Cladosporium fulvum, Fulvia fulva* (Cooke)), is a common disease of tomato. Tomato is the only known symptomatic host of the disease (Johnson, Orshinsky, and Grabowski 2015). Leaf mold is most commonly found in tomatoes grown in high tunnels or greenhouses (protected culture) due to the high humidity requirement for infection (Winspear, Postlethwaite, and Cotton 1970; Jones et al. 2014). The increase in protected tomato production has likely led to an increase in disease incidence in areas, such as the Midwest, where tomato leaf mold was not previously a major problem (Thomma et al. 2005).

The infection process begins with a germinated spore of *P. fulva* entering the tomato via the stomata. Once inside the plant, *P. fulva* grows through the apoplast, never entering the plant cells, and using apoplastic nutrients to survive rather than using haustoria to penetrate the cells (de Wit 2016). At the beginning stages of the disease, the plant develops irregularly shaped yellow lesions on the

adaxial leaf surface. The fungus then emerges from the stomata, producing a dense mat of brown to grey spores on the abaxial leaf surface, corresponding to the area of the chlorotic lesions (Blancard et al. 2012). As the disease progresses, the lesions move from the bottom foliage of the plant, to the top foliage. The lesions may coalesce and the area surrounding lesions become necrotic. Leaves often curl as the disease becomes more severe (Jones et al. 2014). The lesions decrease the photosynthetic ability of the plant and make plant respiration more difficult (Thomma et al. 2005). This necrotic tissue also allows secondary invaders to colonize the plant tissue (G. Agrios 2005). In rare cases, tomato leaf mold can affect the fruit, causing leather black rot on the stem end (Jones et al. 2014).

Some genetic resistance to tomato leaf mold exists but it is largely qualitative as the fungus and plant exhibit a gene-for-gene relationship, causing the resistance to be race dependent. For resistance to be effective, the product of a resistance gene in the plant, called a “Cf protein”, must recognize the corresponding gene product in the fungus, called an “Avr protein” (Van den Ackerveken, Van Kan, and De Wit 1992). Other methods of managing the disease include fungicide applications, removal of crop residue, proper sanitization of all equipment, including high tunnel plastic, adequate spacing of plants, staking or trellising tomatoes, managing night time temperatures, providing sufficient ventilation of greenhouses or high tunnels, and other activities

that promote airflow and reduce humidity (Jones et al. 2014; Johnson, Orshinsky, and Grabowski 2015; Belina et al. 2012).

1.1.1 History of the tomato

The cultivated tomato is a perennial (though often grown as an annual), diploid dicotyledon. The origin of wild tomatoes is thought to be the central west coast of South America (near Ecuador, Peru and northern Chile). This area is also considered to be the center of diversity for tomatoes (Davis, Pernezny, and Broome 2012). Domestication may have originally occurred in Mexico or Peru, as there is evidence pointing to both locales (Bergougnoux 2014). Following domestication, tomatoes were brought to Europe in the early 16th century, where they were initially used solely as ornamentals due to fears that they might be poisonous, like other plants in the nightshade family (Bergougnoux 2014). From Europe, tomatoes were transported first to China and South and South-east Asia in the 17th century and later to the United States and Japan in the 18th century (Heuvelink 2005).

The taxonomy of the tomato has changed over time and has been a subject of debate. Prior to formal nomenclature, tomatoes had names such as

“pomi d’oro” and “pomme d’Amour” (Peralta, Iris, Knapp, and Spooner, David 2006) which roughly translate to “golden apple” and “apple of love” respectively. The cultivated tomato was originally categorized into the Latin binomial system by Carl Linnaeus in 1753, as *Solanum lycopersicum*. In 1986 the cultivated tomato was reclassified into the *Lycopersicon* genus (species: *esculentum*) by Phillip Miller based on morphological characters (Peralta, Iris, Knapp, and Spooner, David 2006). Following phylogenetic studies, the domesticated tomato has returned to its original binomial name: *Solanum lycopersicum* L. (Olmstead et al. 2008; Bohs and Olmstead 1997).

Tomatoes are grown for two distinct purposes: for eating fresh and for processing. Processing tomatoes tend to be determinate, dwarf plants yielding tough-skinned fruit and with a high soluble solids content. Processing tomatoes are field-grown. Fresh market tomatoes can be either determinate or indeterminate and are generally grown from transplants, often in greenhouses or high tunnels. Unlike processing tomatoes, fresh tomatoes are harvested by hand (Heuvelink 2005). The majority of tomatoes (about 4 to 1) grown in the United States are processed (Davis, Pernezny, and Broome 2012). Processed foods include tomato preserves (canned whole tomatoes, juice, etc.), dried tomatoes, and tomato-based foods (soups, sauces, etc.) (Heuvelink 2005).

In 2011, 160 million tons of tomatoes were produced worldwide, with production currently increasing (Bergougnoux 2014). As of 2013, the United States was one of the top three tomato producers in the world (13,950,973 tons),

following China and India (FAOstat 2017). All 50 states produce tomatoes in varying quantities but about 96% of the processing tomatoes and 33% of the fresh tomatoes grown in the U.S. are produced in California. Florida ranks second for production (USDA ERS 2016). Combined, fresh and processing tomatoes account for more than $2 billion in revenue for U.S. tomato producers (USDA ERS 2016).

1.1.2 High tunnel production

High tunnels, at their most basic, are non-permanent structures, covered with a single or double layer of plastic (usually 6-mil polyethylene). While some high tunnels do use electricity for ventilation or heating, most use no electricity (Belina et al. 2012). In addition to built-in ventilation, the sides of high tunnels can be rolled up to allow for some control of temperature and humidity, which is essential for the health of the plants inside the structure as well as the health of pollinators used by growers. Most high tunnels use drip irrigation to efficiently deliver water and allow for fertigation and chemigation (Everhart et al. 2010). High tunnels come in Gothic or Quonset style. The Gothic style is pointed near the top and has sides that are less rounded; this style holds up better against the

weight of snow. The Gothic style also helps with ventilation and provides the height needed for some crops, such as trellised tomatoes. Quonset high tunnels, by contrast, are rounded. While the Quonset style is generally cheaper, it may not be appropriate for some crops and does not hold up as well to snow (Everhart et al. 2010).

High tunnels are popular worldwide, with the highest rates of use in China (360,000 ha) and Spain (55,000 ha) (Lamont 2009). The use of high tunnels has more recently become popular in the United States. As of 2003, the U.S. produced around 5,000 ha of high value crops in high tunnels or plastic greenhouses; this number has since grown (Lamont 2009). The benefits of using high tunnels are greater in cooler climates, and in locations with a shorter warm season, as they can increase the growing environment by a full hardiness zone (Everhart et al. 2010). In Minnesota, high tunnels are an important part of production for high value crops such as tomatoes, peppers, melons, cucumbers and berries because they allow for an extended growing season on either end of the traditional season. In addition to reducing temperature extremes, high tunnels allow for earlier planting dates and lower the likelihood of frost damage (Belina et al. 2012). Another factor that has increased the use of high tunnels in the United States is conservation programs that provide funding for their construction, such as the Environmental Quality Incentives Program (EQIP) through the United States Department of Agriculture (USDA) (USDA-NRCS 2017)

While there are clear advantages to growing certain crops in high tunnels,

there are also some considerations. It can be costly to set up a high tunnel. Per an estimation by the University of Minnesota extension service, a 20’x96’ high tunnel could cost between $7,000-$11,000 to set up (Belina et al. 2012). Some of the initial cost can be offset with government funding and through strategic pricing down the line (Foord 2009). Generally, high tunnels also require more maintenance than field plots. Plants must be watered more frequently as the plants do not benefit from rain. Since plants can be more productive, often growing quickly in a high tunnel, they also require more nutrient inputs. In addition, it is very important to maintain proper ventilation by rolling up the sides, and properly spacing and trimming plants. Improper ventilation can lead to plant stress due to both excessive heat and humidity, as well as increased pressure from certain pests (Belina et al. 2012). Despite the high maintenance required, when done properly, high tunnels can produce an abundance of high value crops.

1.1.3 Common diseases of tomatoes in the USA

Many diseases affect tomatoes in the USA. Some diseases are more prevalent in field-grown tomatoes while others are more problematic in the humid high tunnel environment. A few of the most prevalent fungal and bacterial

diseases of tomatoes are described here, including symptoms, signs of the pathogen, and management of the disease.

Fungal

Leaf mold of tomato is caused by the fungal pathogen *Passalora fulva*. *P. fulva* is a pathogen that thrives in high humidity and primarily causes damage in greenhouse or high tunnel-grown tomatoes (Jones et al. 2014). Symptoms begin on the foliage at the base of the plant, in the form of irregular, chlorotic lesions. As the disease progresses, the pathogen and symptomatic lesions move up the plant and develop green-to-brown patches of conidia on the abaxial side of the leaf, corresponding to the chlorotic lesions, as shown in Figure 1A (Gleason and Edmunds 2006). As the disease becomes more severe, lesions may coalesce, decreasing photosynthetic area and allowing more opportunistic pathogens to invade. In addition, leaves and infected blossoms may drop (Johnson, Orshinsky, and Grabowski 2015). In rare cases fruit may develop a dry, black rot near the stem (Jones et al. 2014). Some fungicides may be effective in managing leaf mold. Cultural practices include planting of resistant varieties, proper sanitation of all tools and structures, management of night temperatures, plowing under plant debris in the fall, staking plants, proper ventilation, and pruning (Jones et al. 2014; Johnson, Orshinsky, and Grabowski 2015; Egel et al. 2017).

Early blight, caused by the fungal pathogens *Alternaria solani* (Sorauer) and *Alternaria tomatophila* (Simmons), is one of the most common diseases of tomato, causing damage particularly in the east and central United States in both

high tunnel and field settings (Babadoost 2011). Symptoms include brown-to-black lesions that have a characteristic target or concentric circle appearance. Chlorotic borders on the lesions are a key diagnostic feature of *Alternaria* spp. All above-ground plant parts, including the fruit may display these features as shown in Figure 1B (Blancard et al. 2012). Lesions begin on older foliage and move up the plant, reaching the fruit last. In addition, early blight can cause fruit to drop, causing yield losses (Jones et al. 2014). Signs of the disease include black, beaked conidia, which form in conspicuous concentric rings, as described above (Babadoost 2011). Disease control includes crop rotation, genetic resistance, fungicide sprays, and cultural practices such as removing plant debris and Solanaceous weed hosts, and using UV-resistant plastic on high tunnels to reduce sporulation (Jones et al. 2014; Egel et al. 2017).

Gray mold, another fungal pathogen of tomato, is caused by the pathogen *Botrytis cinereal* (Pers). Gray mold causes damage to tomatoes grown in high tunnels or fields all over the United States and throughout most of the world. Infection tends to occur following some form of mechanical damage, due to the opportunistic nature of the fungus (Jones et al. 2014). Additionally, germination of the conidia is aided by continuous leaf wetness (Orshinsky and Grabowski 2016) .Symptoms first develop on leaflets, in the form of necrotic lesions, then move progressively to petioles and stem tissue. Stem cankers caused by gray mold result in most of the damage to the plant by inducing wilt (Blancard et al. 2012). Fruit may also be affected as the disease progresses. Fruit may either

develop white decayed lesions, or in some cases small halos, referred to as ghost spots, as shown in Figure 1D (Blancard et al. 2012). Signs of *B.cinerea* are easily recognizable. Gray-brown sporophores grow abundantly from necrotic tissue and give tissue a fuzzy appearance, as shown in Figure 1C. Dark, irregular, sclerotia may also form in plant tissue (Jones et al. 2014). Fungicides may be used for management but caution is advised due to the pathogen's rapid development of resistance to several fungicide classes (Hahn 2014; Rupp et al. 2016; Fernández-Ortuño et al. 2014). Cultural practices include crop rotation, maintenance of appropriate calcium levels, and practices which promote air flow such as pruning in the late morning or early afternoon, after the dew has dried, and proper ventilation of high tunnels (Egel et al. 2017; Babadoost 2011)

Septoria leaf spot damages leaf tissue on tomatoes grown throughout most of the United States, with the most severe damage occurring in humid areas and locales with heavy rainfall (Jones et al. 2014). Although septoria can occur in either high tunnels or in the field, transmission of spores from soil to leaves by rain splash makes outdoor tomatoes more prone to the disease (Babadoost 2011). Symptoms of septoria leaf spot begin to appear following fruit set. Round yellow spots initially appear on the lower leaves of the plant. These spots develop into circular lesions, with brown margins, tan centers and, chlorotic halos (Floyd 1999). As the disease progresses, these lesions may also be found on stems, petioles and calyxes (Babadoost 2011). Fungal signs include abundantly produced pycnidia, which may become visible in the tan centers of

lesions (Blancard et al. 2012). Management of septoria leaf spot include 2-3 year crop rotation, beginning with pathogen-free propagules, staking plants, watering only the base of plants in the morning, removal of plant debris at the end of the season, and fungicide applications (Floyd 1999; Babadoost 2011; Egel et al. 2017).

Fusarium oxysporum f. sp*. lycopersici* (Snyder and Hansen) is the causal fungus of fusarium wilt, which affects high tunnel and field-grown tomatoes in most of the United States. Infected plants begin to wilt during the hottest part of the day, but often recover overnight (Jones et al. 2014). As the disease progresses, leaflets on half of the plant begin to turn yellow. Eventually, the entire plant will become chlorotic and wilt irreversibly (Johnson, Grabowski, and Orshinsky 2016b). Additionally, leaves may drop from the plant (Gleason and Edmunds 2006). If the disease is present, a reddish brown vascular discoloration in the base of the stem will be visible (Babadoost 2011). Management strategies include planting resistant varieties, beginning with pathogen-free seeds and transplants, removal of plant debris, avoiding the use of wooden stakes, sanitation of all equipment, and raising the pH of the soil to around 7.0 (Gleason and Edmunds 2006; Jones et al. 2014; Egel et al. 2017).

Bacterial

Bacterial speck, caused by the bacterial pathogen *Pseudomonas syringae* van Hall pv. *tomato* (Okabe) Young, Dye & Wilkie, is most prevalent in field-grown tomatoes in areas with cool, humid climates (Jones et al. 2014).

Symptoms begin as small, dark, round lesions on the leaflets. These lesions may coalesce and will eventually develop chlorotic halos (Jones et al. 2014). Similar lesions may develop on stems and petioles (Blancard et al. 2012). Characteristic lesions that develop on the fruit are very small, slightly raised, and do not penetrate deeply. Because of the way these fruit lesions form, bacterial speck primarily affects fruit quality (Gleason and Edmunds 2006). Management once the pathogen is present is limited, therefore practices focus on preventing the introduction of the pathogen. Strategies include using only certified pathogen-free seed, resistant tomato cultivars, sanitizing all farming implements, and the use of copper sprays (Blancard et al. 2012; Jones et al. 2014).

Bacterial spot, which can result in large yield losses through defoliation and production of unsalable fruit, is caused by four *Xanthomonas* species: *X. vesicatoria* (Doidge) Vauterin et al*., X. euvesicatoria* Jones et al., *X. gardneri* (Šutic) Jones et al*.,* and *X. perforans* Jones et al (Jones et al. 2014). Bacterial spot is most commonly found in tomatoes which were transplanted from greenhouses due to the high humidity and temperature which are present in the enclosed structures (Jones et al. 2014). Lesions on the leaflets are similar to bacterial speck; they are brown, circular to slightly angular but with a lighter chlorotic halo. Leaflet lesions may become desiccated in the center and fall out, causing a shot-hole appearance. Similar lesions may appear on petioles, stems and pedicels, though they often appear streaked due to the coalescing of the lesions. Fruit lesions may be differentiated from bacterial speck lesions because

they are larger, have a scab-like appearance, are rough to the touch, and may have a light white halo. Control measures include sanitization of all materials, starting with clean seed or transplants, preventative treatment of seeds with copper and macozeb, removal of debris, rotation away from solanaceous crops, and limited use of resistant varieties (Jones et al. 2014; Blancard et al. 2012; Johnson, Grabowski, and Orshinsky 2016a).

1.2 Tomato leaf mold-associated fungi

Tomato leaf mold is caused by the ascomycete fungus *Passalora fulva*. *P. fulva* enters the plant via stomata, grows intercellularly, then emerges as conidiophores from stomata, as shown in appendix figure A3 (Thomma et al. 2005). Leaf mold lesions contain many conidia, which help to spread the pathogen and begin a new infection cycle (Jones et al. 2014). While *P. fulva* is the known pathogen, other fungi exist in, and can be isolated from, leaf mold lesions. Despite the minimal literary discussion on the topic, *Cladosporium* spp. appear to live in close contact with *P. fulva* in infected tomato tissue (Medina et al. 2015).

Cladosporium spp. are ubiquitous and can be found in the air, soil, marine

environments, and in homes (Udochukwu et al. 2016; Bensch et al. 2012; Aihara, Tanaka, and Takatori 2001; Zalar et al. 2007). They are also found as endophytes in herbaceous plants and in the apple phyllosphere (Gange et al. 2007; He et al. 2012). In some cases *Cladosporium* spp. also act as a pathogen such as with blossom blight of strawberry, cucumber scab, and leaf blotch of onion and leek (Walker 1952; Nam et al. 2015; Kirk and Crompton 1984). Additionally, *Cladosporium* spp. have been evaluated for their use in biocontrol (Köhl et al. 2015).

Despite the taxonomic history of *P. fulva*, the taxon is no longer considered to be part of the *Cladosporium* genus. *P. fulva* and *Cladosporium* spp. can be differentiated by their distinct conidiogenous scars, viewable by scanning electron microscopy (SEM), the size of their spores, as well as through molecular differences (Crous and Braun 2003; Braun et al. 2003). Though these taxa are not as closely related as once thought, it seems that they live in close contact within leaf mold lesions (Medina et al. 2015). It is currently unknown whether this arrangement is due to the ubiquitous nature of *Cladosporium* spp. or if a more complex relationship between *P. fulva* and *Cladosporium* spp. exists.

1.2.1 The Taxonomy of *Passalora fulva*

Passalora fulva, the pathogen causing tomato leaf mold, has gone through several taxonomic reclassifications since its discovery. Initially, the fungus was referred to as a species of the genus *Cladosporium* (Cooke 1883). The *Cladosporium* genus grew to be one of the most heterogenous, in part due to the ambiguity of morphological descriptions of the included taxa (Ogórek et al. 2012).

Taxonomic revisions of the *Cladosporium* genus continued for many years in an effort to reduce some of the ambiguity inherent in the initial description of the genus. In 1952, the tomato leaf mold fungus was re-classified as *Fulvia fulva*, by Ciferri. Then, in 1983 it was proposed by van Arx that the taxon be re-classified as *Mycovelosiella fulvum* (Curtis, Gore, and Oliver 1994). *Mycovelosiella fulvum* was not widely accepted and was eventually rejected on the basis of molecular studies of the taxon (Curtis, Gore, and Oliver 1994).

In 2000 the taxonomic name returned to the *Cladosporium* genus as *Cladosporium fulvum* (Bhalla and Sarbhoy 2000). While *C. fulvum* was not accepted as a taxonomic name until 2000, it was being used in the 1990's in phylogenetic literature to compare this taxon to *Cladosporium* species. In one of these early studies it was noted that *C. fulvum* appeared to be the least closely related of all Cladosporia used in the study (Curtis, Gore, and Oliver 1994). Much of the initial classification resulted from morphological comparisons, such as

variations in mycelium, conidiophores, conidial shape, size, and septation, conidiogenous scars, and pigmentation (Crous, Kang, and Braun 2001). As phylogenetic analyses became popular, more precise differentiation of taxa within the *Cladosporium* genus became possible. Many taxa within the *Cladosporium* genus were re-assigned to different genera, including the change from *Cladosporium fulvum* to *Passalora fulva*, in 2003 (Crous and Braun 2003; Braun et al. 2003). *P. fulva* is the currently accepted name of the fungus, though it is still commonly referred to as *C. fulvum* in scientific literature. Based upon phylogenetic analyses we now know that *P. fulva* is in the phylum: Ascomycota, the class: Dothideomycetes, the order: Capnodiales, and the family: *Mycosphaerellaceae*.

1.2.2 Biology of *Passalora fulva*

P. fulva is a non-obligate biotroph, for which no sexual stage has ever been observed (M. H. A. J. Joosten and de Wit 1999). Although no sexual stage is known, the mating type genes, MAT1-1-1 and MAT1-2-1, are both found in populations of *P. fulva.* However, evaluation of the relative frequency and distribution of these genes suggests asexual reproduction (Stergiopoulos,

Groenewald, et al. 2007). Conidia form on unbranched, pigmented conidiophores, which are about 200 µm in length (Jones et al. 2014). Conidia are highly pigmented, one to two celled, often septate (0-3 septa possible), and can form in branched chains (Jones et al. 2014; M. H. A. J. Joosten and de Wit 1999). Hyphae of *P. fulva* are septate, branched, and have cell walls primarily composed of glucan and chitin (M. H. A. J. Joosten and de Wit 1999). *P. fulva* grows relatively slowly on simple medium and forms colonies that are green to brown in color (Thomma et al. 2005).

P. fulva is very host specific, as are most species within the Mycosphaerellaceae family, and is only known to cause symptoms on tomato (Thomma et al. 2005). High humidity (≥85%) increases the frequency spore germination on host tissue (Jones et al. 2014). The germ tube runs over the leaf surface until it encounters a stoma, through which it can enter the host tissue. Hyphae then populate the apoplast of the host, between spongy mesophyll cells, never entering the plant cells, as the fungus lacks penetration structures (Thomma et al. 2005). Growth of the fungus is often greatest near vascular tissue, most likely due to the availability of sucrose as it is transported via the apoplast to the phloem (Joosten, Hendrickx, and De Wit 1990). After at least 10 days, hyphae aggregate in the substomatal space, then conidiophores form on aerial mycelia and emerge from the stomata. In the final stage of the disease cycle conidia form on conidiophores and spread to new tissue to begin the next infection cycle (Thomma et al. 2005).

P. fulva can survive for at least one year in a high tunnel or greenhouse in the form of conidia or sclerotia, due to its saprophytic potential (Blancard et al. 2012). The fungus can be spread through contaminated seed, crop residue, tools, clothing, high tunnel plastic, stakes, and possibly insects (Jones et al. 2014). Cultural management of the disease primarily focuses on keeping plants free from standing water and excess humidity and includes staking plants, pruning, properly spacing plants, and keeping night and day temperatures relatively consistent. Additionally, proper sanitation should be maintained by removal of crop residue, sterilization of all plastic and tools, and the use of clean propagative material (Jones et al. 2014; Blancard et al. 2012). Fungicide applications may also aid in the control of leaf mold (Egel et al. 2017).

1.3 Races of *Passalora fulva* and tomato resistance genes

The interaction between *P. fulva* and tomato follows the gene-for-gene model first described by Flor in 1942, wherein the product of a resistance gene (*R* gene) in the plant recognizes the product of a matching avirulence gene (*Avr* gene) in the fungus and results in a hypersensitive response (HR) (Keen 1990; van den Ackerveken, van Kan, and de Wit 1992; Wang and Wang 2018). The *R*

genes associated with the tomato-*P. fulva* pathosystem are termed *Cf* genes, titled after the synonymous name of the fungus, *Cladosporium fulvum* (Thomma et al. 2005). The effector genes in this system are termed either *Avr* or *Ecp* (extracellular protein) genes, originally differentiated by whether the corresponding *Cf* gene was available in a near isogenic tomato line at the time the gene was cloned. If there was a near-isogenic line, the gene was termed *Avr* and otherwise it was termed *Ecp* (Stergiopoulos, De Kock, et al. 2007). In addition, *Ecp* genes are considered to be present in all races, whereas *Avr* genes are race-specific (Thomma et al. 2005; de Wit et al. 2002).

An interaction which results in an HR is termed an incompatible reaction, whereas a successful infection of tomato by *P. fulva* is called a compatible interaction (Keen 1990; van den Ackerveken, van Kan, and de Wit 1992). Races of *P. fulva* are defined by their compatible or incompatible reactions on near-isogenic lines of tomato, each containing a functional *Cf* allele at a single *Cf* locus, which results from the pattern of *Avr* genes present in each biotype (Keen 1990). Races are named according to the resistance gene(s) that they can overcome to cause infection; therefore a race 2 fungus would cause disease in a plant containing a *Cf-2* gene (Thomas et al. 1998). So far 13 *P. fulva* effectors and their coding genes have been identified and cloned (Mesarich et al. 2018).

1.3.1 *Avr* and *Ecp* Genes in *Passalora fulva*

Despite the presumed asexual nature of the fungus, *P. fulva* evolves quickly to overcome *Cf* genes used in commercial tomato plants (Iida, van ’t Hof, et al. 2015; Iida et al. 2010; Leski 1977; Lindhout et al. 1989). The selection pressure is expected to be a direct result of *Cf* genes used in commercial plants (Stergiopoulos, De Kock, et al. 2007). Accordingly, an understanding of the effectors involved in the tomato-*P. fulva* pathosystem is essential for the management of durable resistance.

All known effector genes in this pathosystem code for small secreted proteins that have cysteine residues in an even number. These cysteine residues form disulfide bridges and aid in the stability of the structures (Stergiopoulos, De Kock, et al. 2007). So far five *Avr* and seven *Ecp* genes have been cloned (van Esse et al. 2008; Bolton et al. 2008; Mesarich et al. 2014; de Wit et al. 2002; Lauge et al. 2000). There is more variation present in the *Avr* genes than in the *Ecp* genes, which is thought to be a result of selection pressure, on the former, from the use of corresponding *Cf* genes in commercial tomato plants (Stergiopoulos, De Kock, et al. 2007; Medina et al. 2015).

Avr2, *Avr4*, *Avr4E*, *Avr5*, and *Avr9* have been cloned (de Wit et al. 2002; Mesarich et al. 2014; Joosten, Cozijnsen, and De Wit 1994). Avr2 functions as a cysteine protease inhibitor, protecting the fungus from basal plant defenses (van

Esse et al. 2008; Shabab et al. 2008). The plant cysteine protease Rcr3 plays an important role in the detection of Avr2 by Cf2. In the absence of Avr2, Cf2 associates with Rcr3. Avr2 targets Rcr3 for inhibition through the formation of a protein complex, thereby changing the conformation of Rcr3; the change in conformation is then recognized by the Cf2 protein, triggering an HR (Rooney et al. 2005; van Esse et al. 2008; Kruger et al. 2002). Generally *Avr2* avoids detection through the accumulation of small gene mutations, mostly single nucleotide insertions or deletions, resulting in a truncated protein which is presumed to be unstable and non-functional (Luderer et al. 2002; Stergiopoulos, De Kock, et al. 2007).

The *P. fulva*-produced Avr4 protein protects fungal cell walls from hydrolysis by binding to chitin (van den Burg et al. 2006). This mechanism has also been found to protect cell walls of *Trichoderma viride* and *Fusarium solani* f. sp. *phaseoli* from chitin hydrolysis (van den Burg et al. 2006). Detection of surplus unbound Avr4 protein induces an HR (van den Burg et al. 2006). Mutations in the *Avr4* gene result in a protein with one Cysteine to Tyrosine substitution at position 64, 70, or 109 (Joosten et al. 1997). This substitution disrupts one of three disulfide bonds, allowing Avr4 to avoid detection by Cf4 (van den Burg et al. 2003; Stergiopoulos, De Kock, et al. 2007; Joosten, Cozijnsen, and De Wit 1994). These isoforms still bind chitin but instability leads to more rapid turnover of the surplus protein (van den Burg et al. 2003).

No studies have determined the function of the *Avr4E, Avr5, or Avr9*

genes but all three generally avoid detection through gene deletion (van den Ackerveken, van Kan, and de Wit 1992; Stergiopoulos, De Kock, et al. 2007; Mesarich et al. 2014). In some cases, rather than deletion, *Avr4E* accumulates mutations which result in a phenylalanine to leucine substitution at position 82 and a methionine to leucine substitution at position 93 in the protein (Westerink et al. 2004). In a single case *Avr5* was found to have produced a mutated protein (Mesarich et al. 2014).

In all races of *P. fulva Ecp* genes are expressed *in planta* and are considered virulence factors as their loss results in a less virulent infection (de Wit et al. 2002; Laugé et al. 1997; Lauge et al. 2000; Wubben, Joosten, and de Wit 1994). *Ecp* genes have remained relatively constant over time with most mutations occurring in non-coding regions. This consistency is most likely due to a lack of selection pressure (Stergiopoulos, De Kock, et al. 2007). *Ecp1*, *Ecp2*, *Ecp3*, *Ecp4*, *Ecp5*, *Ecp6*, and *Ecp7* have been cloned (van den Ackerveken et al. 1992; Lauge et al. 2000; Bolton et al. 2008; Joosten and de Wit 1988). Less is known about *Ecp* genes than *Avr* genes, but a few studies have been conducted. In one study mutants lacking *Ecp1* and *Ecp2* were found to be significantly reduced in virulence, including a reduction in spore count, but were still able to infect plants (Laugé et al. 1997). Another study found that Ecp2 can induce an HR in the non-host *Nicotiana paniculate* (Lauge et al. 2000). Finally, one mechanism of action for an Ecp protein is known. Ecp6 has multiple Lysine motifs (LysM) that combine to form a binding groove that has an ultra-high chitin

binding affinity. This groove allows Ecp6 to outcompete the plant's chitin binding, thereby avoiding detection by the host (Thomma, Nürnberger, and Joosten 2011; Sánchez-Vallet et al. 2013).

Interestingly, homologs exist for the Avr4, Ecp2, and Ecp6 proteins. Orthologs of Ecp6 are found in many different fungi, including *Aspergillus niger, Botrytis cinerea, Sclerotinia sclerotiorum,* and species in the genus *Mycosphaerella*, all of which contain LysM domains (Bolton et al. 2008; Stergiopoulos et al. 2010; Sánchez-Vallet et al. 2013). Avr4 homologs are found in *Mycosphaerella fijiensis* as well as in several *Cercospora* species. These homologs also have a functional chitin binding domain that protects the fungal cell walls from chitinases (Stergiopoulos et al. 2010). In addition, when the *M. fijiensis* homolog was inoculated onto tomato plants, using the *Potato Virus X* based expression system, it was detected by Cf4, resulting in an HR (Stergiopoulos et al. 2010). Ecp2 homologs have been found in *M. fijiensis* and *M. graminicola*. When inoculated onto a tomato plant containing the *Cf-Ecp2* gene, the *M. fijiensis* homolog was able to induce an HR (Stergiopoulos et al. 2010). These results suggest that *Avr4* and *Ecp2* are not species-specific. However, no homologs of the other *P. fulva* effectors have been found, suggesting that most of the effectors in this pathosystem should still be considered species-specific.

1.3.2 Cf Genes in Tomato

Cf genes, named according to the *P. fulva* synonym *Cladosporium fulvum*, produce proteins which recognize the proteins from corresponding *Avr*/*Ecp* genes. *Cf* genes encode receptor-like proteins (RLP) which contain leucine-rich repeats (LRRs) in differing quantities as well as trans-membrane domains and short cytoplasmic tails (de Wit et al. 2002; Kruijt, De Kock, and De Wit 2005; Mesarich et al. 2014, 2018). The genes are considered to be organized into two gene families called *Hcr9* and *Hcr2*, representing homologs of *Cladosporium fulvum* resistance gene 9 and 2, respectively (Thomas et al. 1998; M. Joosten and de Wit 1999; Kruijt, De Kock, and De Wit 2005). These gene families differ in part by the number of LRRs they encode; *Hcr9* genes encode 25 or 27 LRRs, while *Hcr2* genes encode variable numbers between 25 and 38 (De Wit et al. 2009). *Hcr2* genes are located on chromosome 6, whereas *Hcr9* genes are located on chromosome 1 (Dickinson, Jones, and Jones 1993; Jones et al. 1993; De Wit et al. 2009). Some *Cf* genes present in commercial tomato cultivars were introgressed from wild species such as *Cf-9* and *Cf-2* from *Solanum pimpinellifolium and Cf-4* from *Solanum habrochaites*, while others were found in *Solanum lycopersicum* (Stevens and Rick 1986; Dickinson, Jones, and Jones 1993; Thomas et al. 1998). Although *Cf* genes corresponding to the *Ecp* genes have been found in wild tomato accessions and cloned, none are used in

commercial tomato cultivars (Stergiopoulos, De Kock, et al. 2007).

There are 24 known *Cf* genes which are named *Cf-1* to *Cf-24* (Kanwar, Kerr, and Harney 1980a, 1980b). *Cf-1* to *Cf-6*, *Cf-9*, and *Cf-11* have been introduced into commercial tomatoes from wild species (Leski 1977; Thomma et al. 2005; Iida et al. 2010). Initially only one or two resistance genes were bred into each tomato variety and the pathogen overcame resistance in as little as 2 years (Stevens and Rick 1986; de Wit 2016). In the late 1970's *Cf-9* was introduced into commercial tomato varieties and was considered to be effective against all races of the fungus known at the time (Thomma et al. 2005). The resistance afforded by *Cf-9* was relatively stable for a long period of time but was also eventually overcome (Iida et al. 2010; Iida, van 't Hof, et al. 2015; de Wit 2016). Currently, resistant tomato varieties generally have 3-4 stacked resistance genes, a strategy thought to enhance durability (de Wit 2016).

Despite the prevalent use of multiple *Cf* genes in commercial tomato varieties, races of *P. fulva* are able to overcome this resistance, sometimes overcoming as many as 5 genes (Lindhout et al. 1989; Stergiopoulos, De Kock, et al. 2007; Iida et al. 2010; Iida, van 't Hof, et al. 2015; de Wit 2016). The rapid pace of the pathogen's ability to overcome resistance genes, has resulted in ongoing research into new *Cf* genes that could be effectively employed in commercial cultivars (Zhao et al. 2016; Mesarich et al. 2018). Additionally, research is being conducted to determine the race structure of *P. fulva* present in different geographic locations to aid in the proper use of existing resistant tomato

varieties (Medina et al. 2015; Li et al. 2015; Iida, van ’t Hof, et al. 2015; Nedim 2016).

1.3.3 Worldwide distribution of *Passalora fulva*

The tomato leaf mold pathogen, *Passalora fulva*, was first described by Mordecai Cooke in 1883 from a sample collected in South Carolina (Makemson 1918; Jones et al. 2014). Since the initial description, tomato leaf mold has been found on every continent except for Antarctica (Plantwise 2018). Due to the pathogen’s high humidity requirement, leaf mold is primarily a problem in areas where tomatoes are grown in an enclosed area such as a high tunnel or a greenhouse, though it has been found on field-grown tomatoes in years with high humidity averages (Jones et al. 2014). *P. fulva* can cause disease in conditions ranging from 4-32°C and can therefore survive in many different climates, though the pathogen does better in temperate areas (Jones et al. 2014; Iida, Van ’T Hof, et al. 2015). The presence of leaf mold in tomato growing areas can vary depending upon the resistance genes present in the varieties grown (de Wit 2016). However, once present in a growing region, leaf mold can spread at a rapid pace due to the efficient dissemination of the pathogen’s many conidia by

wind and rain and the ability of the conidia to survive for at least 1 year (Jones et al. 2014).

1.3.4 Project Justification and Objectives

Tomato leaf mold is present worldwide in all tomato-growing areas (Jones et al. 2014; Plantwise 2018). Much of the research conducted in the past few decades has focused on the molecular interaction of tomato *Cf* genes and the *Avr* genes of the tomato leaf mold pathogen *Passalora fulva* (Kanwar, Kerr, and Harney 1980a, 1980b; Stergiopoulos, De Kock, et al. 2007; de Wit 2016; Mesarich et al. 2018). Our current research has uncovered a topic which has only rarely been discussed previously. We found that *Cladosporium* species are found alongside *P. fulva* in leaf mold lesions. The presence of these *Cladosporium* species has been mentioned previously but never discussed in detail (Medina et al. 2015). My first objective is to characterize isolates of *P. fulva* and *Cladosporium* spp. collected from high tunnels in Minnesota, New York, and Indiana. In chapter 2, the phylogenetic relationship of the isolates of *Cladosporium* spp. and *P. fulva* collected from leaf mold lesions in Minnesota, New York, Indiana, and Argentina will be presented to determine the variation

present among and between species. In addition, I will characterize the *Avr* genes present in all *P. fulva* isolates collected from Minnesota high tunnels. I will examine the variation of the *Avr* genes relative to a reference isolate to evaluate the diversity of isolates from different tomato-growing areas in Minnesota. Finally, the temperature range and optima of isolates will be evaluated to determine when the species are most likely to be active in tomato-growing areas.

In chapter 3, organic and non-organic spray programs will be evaluated for their efficacy against *P. fulva*, *Alternaria* spp., and *B. cineria* on high tunnel-grown tomatoes. In addition, differences in general plant health, and marketable and non-marketable yield will be determined.

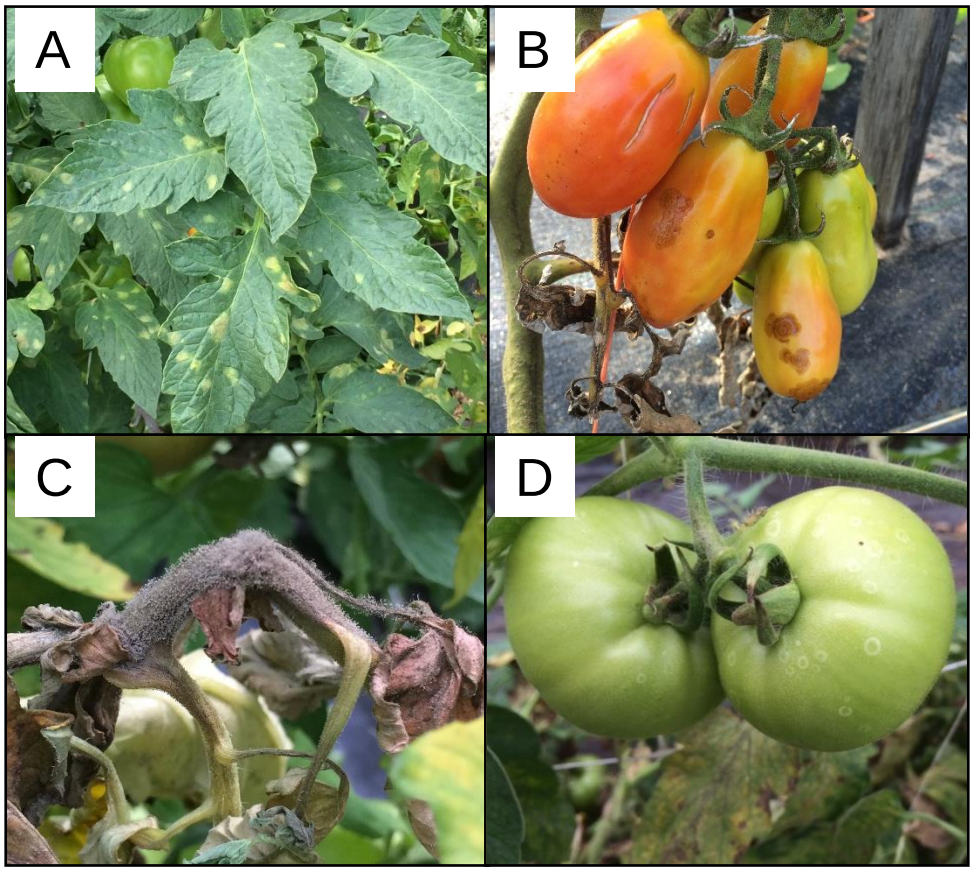

Figure 1.

Disease symptoms on tomato (A) Yellow lesions with irregular margins characteristic of tomato leaf mold infection (B) Fruit symptoms characteristic of early blight infection (C) B. cinerea *sporophores growing from tomato tissue (D) Ghost spots characteristic of gray mold infection.*

Chapter 2. Characterization of tomato leaf mold-associated fungi

2.1 Preface

A collection of *Cladosporium* spp. and *Passalora fulva* isolates, collected from tomato leaf mold lesions in Minnesota, New York, and Indiana, was characterized in this study. The internal transcribed spacer (ITS), β-tubulin, and translation elongation factor-1α (TEF-1α) regions of the genomes of each isolate were sequenced and variation between and among the genera was examined using phylogenetic approaches. ITS, β-tubulin, and TEF-1α markers each resolved isolates from the two genera and separated *C. sphaerospermum* from other species. The TEF-1α marker also allowed separation of one additional species. The agriculturally relevant *Avr2, Avr4, Avr4E, and Avr9* loci from the *Passalora fulva* isolates were sequenced and compared to corresponding reference sequences. In general, *Avr* loci displayed little variation. However, compared to reference sequences, a two base pair deletion (AT) was noted in the *Avr2* locus, resulting in a presumed non-functional protein that might allow

the pathogen to evade detection by the cognate resistance gene. Other observed variation included synonymous and non-coding mutations (T>C or C>T) in the *Avr4E* locus, and non-coding and a non-synonymous mutation in the *Avr9* locus. The effects of temperature on *in vitro* growth of *C. cladosporioides*, *C. sphaerospermum*, and *P. fulva* isolates were also assessed to determine their potential viability at different points in the growing season. Differences in growth of isolates for the temperature assay were related to isolate, incubator temperature, and media type.

2.2 Introduction

Tomato leaf mold disease, caused by the fungus *Passalora fulva* (Cooke) U. Braun & Crous (syn. *Cladosporium fulvum, Fulvia fulva* (Cooke)), is one of the most prevalent diseases of tomatoes grown in high-tunnels, an environment with reduced airflow and high humidity (Winspear, Postlethwaite, and Cotton 1970; Jones et al. 2014). *Passalora fulva* was once classified as a *Cladosporium* species, but it has been moved to the genus *Passalora* based on both biological differences, such as conidiogenous scars, and on molecular variation (Crous and Braun 2003; Braun et al. 2003). Interestingly, it has been observed recently that

Cladosporium spp. are frequently associated with *Passalora fulva* in tomato leaf mold lesions (Medina et al. 2015). Little is known about the interaction between these genera or whether their association can alter the tomato leaf mold disease phenotype.

Diversity within and between *Cladosporium* spp. has been studied using DNA sequences for β-tubulin, actin, translation elongation factor-1α, and the internal transcribed spacer regions (Braun et al. 2003; Bensch et al. 2010, 2015). However, the focus of molecular work for *P. fulva* has been sequence variation at *Avr* loci, which have a gene-for-gene association with tomato resistance genes (*Cf* genes) (Keen 1990; van den Ackerveken, van Kan, and de Wit 1992; Wang and Wang 2018). In combination, sequence analysis of β-tubulin, actin, translation elongation factor-1α, and *Avr* loci can be useful in distinguishing between and among the *Cladosporium* spp. and *P. fulva* isolates.

The functional diversity of *Cladosporium* spp. and *P. fulva* isolates can also be compared through biological assays. The activity of fungi and their role as pathogens are regulated by a number of environmental factors including light, humidity, nutrient availability, and temperature (G. N. Agrios 2005). Functional comparison of fungi has therefore focused on their ability to grow and reproduce within specific parameters of these environmental factors. Variations in the growth optima of *Cladosporium* spp. and *P. fulva* isolates in response to temperature, in particular, could have an effect on their potential interaction within the tomato host.

In order to characterize the diversity between and among the fungal genera present in the tomato leaf mold system, we generated a set of 78 *Cladosporium* spp. and 15 *P. fulva* isolates. The molecular diversity of the collection was examined using DNA sequences for β-tubulin, translation elongation factor-1α, the internal transcribed spacer region, and *Avr* loci. Additionally, a subset of isolates was tested for their functional diversity by assessment of their relative ability to grow at a range of temperatures. This study provides insight into the diversity of fungi present within the tomato leaf mold pathosystem, which can be used in the development of future management strategies.

2.3 Materials and Methods

2.3.1 Isolate collection and purification

Passalora fulva and *Cladosporium* spp. isolates were collected from high tunnels in Minnesota, Indiana, and New York (Table 3). Tomato leaves

containing leaf mold lesions were collected between May and October in 2015, 2016, and 2017. The leaves were either surface sterilized for 1 minute in 10% bleach and rinsed with sterile water, then cut with a sterilized scalpel, or spores were swabbed from the lesion. For either method, collected material was plated onto water agar [15g agar/L (Difco™, Franklin Lakes, NJ)], 0.125 strength potato dextrose agar (PDA) [3g/L potato dextrose broth (Difco™) + 15g/L agar], and full-strength PDA [39g/L potato dextrose agar (Difco™)] and grown at 25°C. When fungal growth appeared, spores were swabbed only from colonies that appeared circular to irregular, raised, and green to brown in color. Spores were diluted in sterile water and plated on PDA at a concentration of 100 spores/plate. After 2 days of growth at 25°C, a single spore from each plates was transferred to a new PDA plate with the aid of a dissecting scope. Isolates were preserved as small agar cubes in a 50/50 solution of 17% w/v skim milk powder solution and 20% w/v glycerol at − 80°C for long-term storage.

2.3.2 Fungal DNA extractions

DNA extractions were carried out following a modified DNAzol® (Thermo Fischer Scientific, Waltham, MA) protocol. Specifically, isolates were grown, from

spores collected on a cotton swab, on cellophane topped PDA for 2 days. 1x1 mm pieces of mycelia were scraped from the cellophane using a scalpel and placed into 50 µl of nuclease-free water in microcentrifuge tubes. The tubes were then incubated at 95°C for 20 minutes. Tubes were allowed to cool, then 1 ml of DNAzol® and 5 µl of 20mg/ml proteinase K (Fischer Scientific™, Waltham, MA) were added to each tube. The tubes were incubated at room temperature for 4 hours.

The tubes were then centrifuged (5430 R, Eppendorf, Hauppauge, New York) at 10°C for 10 minutes at 10,000 rcf. The supernatant was transferred to new microcentrifuge tubes and 0.3 ml of Chloroform:Isoamyl alcohol 24:1 (VWR, Radnor, PA) was added to each tube. The tubes were agitated by hand then centrifuged at 10°C for 10 minutes at 10,000 rcf. The top 0.5-0.6 ml of supernatant was transferred to a new tube. The DNA was precipitated by a 10 minute, room temperature incubation in 0.5 ml of 95% ethanol. The tubes were then centrifuged at 10°C for 2 minutes at 4,000 rcf. The 95% ethanol was removed, and the DNA was washed twice with 1 ml of 75% ethanol. The tubes were centrifuged at 10°C for 2 minutes at 4,000 rcf and the ethanol was discarded between washes. In the final step, the ethanol was removed completely and the DNA pellet solubilized in 50-100µl of 8mM NaOH (Fischer Scientific, Waltham, MA). The DNA concentration and quality were evaluated by electrophoresis on a 0.8% agarose gel and with the Nanodrop 2000 (Thermo Scientific™, Waltham, MA). DNA was stored at 4°C and -20°C.

2.3.3 DNA amplification and sequencing

The molecular diversity of the isolate collection, described in section 2.3.1, was examined by PCR amplification of portions of the internal transcribed spacer (ITS), the translation elongation factor 1-α (TEF-1α), and the β-tubulin regions. Additionally, the diversity of *Avr* gene sequences was examined using primers for the partial amplification of *Avr2*, *Avr4*, *Avr4E*, and *Avr9*. PCR primers are described in Table 1. Primers were obtained from Integrated DNA Technologies (Skokie, IL).

PCRs were completed in 25 µl volumes containing 1µl (~50 ng) of template DNA, 12.5 µl of Gotaq® G2 green mastermix (Promega, Madison, WI), 1 µl of each primer (previously diluted to a 10 µM stock), and 9.5 µl of nuclease-free water. All reactions were run on a C1000™ or T100™ Thermal Cycler (Bio-Rad, Hercules, CA).

The PCR conditions for the ITS region were a denaturation step of 30 seconds at 94°C, 29 cycles of [94°C for 30 seconds, 57°C for 1 minute, and 68°C for 1 minute], followed by a final extension step of 5 minutes at 68°C.

The PCR conditions for the EF-1α region were an initial denaturation step

of 5 minutes at 94°C, 35 cycles of [94°C for 30 seconds, 54°C for 1 minute, 72°C for 1 minute], followed by a final extension step of 7 minutes at 72°C. Seven isolates (Pf39, Pf47, Pf60, Pf63, Pf71, Pf72, and Pf73) were amplified under altered conditions of 30 cycles at an annealing temperature of 58°C, with other conditions remaining unchanged

The PCR conditions for the β-tubulin region were an initial denaturation step of 5 minutes at 94°C, 35 cycles of [94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute], followed by a final extension step of 5 minutes at 72°C.

The *Avr2*, *Avr4*, *Avr4E*, and *Avr9* PCR conditions were an initial denaturation step of 5 minutes at 94°C, 35 cycles of [94°C for 30 seconds, 30 seconds at the annealing temperatures of 54°C, 58.2°C, 59°C, or 55°C for *Avr2*, *Avr4*, *Avr4E*, and *Avr9* respectively, 72°C for 1 minute], followed by a final extension step of 7 minutes at 72°C.

For all PCRs, amplification was verified by agarose gel electrophoresis. Successful reactions were subsequently sent to Molecular Cloning Laboratories (South San Francisco, CA) for an ExoSAP-IT™ enzymatic PCR clean-up (Thermo Fischer Scientific, Waltham, MA) and Sanger sequencing on the ABI 3730X DNA analyzer (Thermo Fischer Scientific, Waltham, MA).

Table 1. Primer sequences used for the amplification of loci from Cladosporium *spp. and* Passalora fulva *isolates*

Locus	Primer	Primer DNA sequence (5'-3')	Source
ITS	ITS-1F	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns 1993)
	ITS4	TCCTCCGCTTATTGATATGC	(White et al. 1990)
EF-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG	(Carbone and Kohn 1999)
	EF2	GGARGTACCAGTSATCATGTT	(O'Donnell et al. 1998)
β-tubulin	Bt1a	TTCCCCCGTCTCCACTTCTTCATG	(Glass and Donaldson 1995)
	Bt1b	GACGAGATCGTTCATGTTGAACTC	(Glass and Donaldson 1995)
Avr2	Avr2F	CATCAGCATATCCTCTTCCATCC	(Medina et al. 2015)
	Avr2R	CAGTACGTTCAAAAGCAGATAACG	(Medina et al. 2015)
Avr4	Avr4F	ACGGTAGGTCTGTACACGAGCC	(Medina et al. 2015)
	Avr4R	ACCGAACTGGGTCATGGAATG	(Medina et al. 2015)
Avr4E	Avr4EF	GCCCGGTATATCGCTGTGC	(Medina et al. 2015)
	Avr4ER	CGGAACCCCTGGCTGAGA	(Medina et al. 2015)
Avr9	Avr9F	AATACAACCTTGAAACAGCTAGG	(Medina et al. 2015)
	Avr9R	GGACTCTACGGGGCTTGG	(Medina et al. 2015)

Table 2. Accession and origin information for all reference sequences used for ITS, β-tubulin, TEF-1α, and Avr markers.

Marker	Species	Strain ID	GenBank Accession No.	Origin
ITS	*Cercospora beticola*	CPC 5123	AY752134	The Netherlands
ITS	*Cladosporium acalyphae*	CBS 125982	HM147994	The Netherlands
ITS	*Cladosporium basiinflatum*	CBS 822.84	HM148000	The Netherlands
ITS	*Cladosporium chalastosporoides*	CBS 125985	HM148001	The Netherlands
ITS	*Cladosporium cladosporioides*	AAS2 A	JQ768317	Argentina
		AAS16 A	JQ768318	Argentina
		AAS17 A	JQ768319	Argentina
		CFOPC A	JQ768321	Argentina
		CFOT7 A	JQ768322	Argentina
		CFP14 A	JQ768323	Argentina
		HD10 A	JQ768327	Argentina
		CPC 10150	HM148062	The Netherlands
		CBS 113746	HM148061	The Netherlands
		CPC 11664	HM148058	The Netherlands
		CPC 14292	HM148046	The Netherlands
ITS	*Cladosporium colocasiae*	CBS 386.64	HM148067	The Netherlands
ITS	*Cladosporium cucumerinum*	CBS 176.54	HM148078	The Netherlands
ITS	*Cladosporium delicatulum*	CPC 14372	HM148089	The Netherlands
		CPC 14360	HM148087	The Netherlands
ITS	*Cladosporium exasperatum*	CBS 125986	HM148090	The Netherlands
ITS	*Cladosporium exile*	CBS 125987	HM148091	The Netherlands
ITS	*Cladosporium flabelliforme*	CBS 126345	HM148092	The Netherlands
ITS	*Cladosporium funiculosum*	CBS 122129	HM148094	The Netherlands
ITS	*Cladosporium globisporum*	CBS 812.96	HM148096	The Netherlands
ITS	*Cladosporium hillianum*	CPC 15458	HM148098	The Netherlands
ITS	*Cladosporium inversicolor*	CPC 14368	HM148109	The Netherlands
ITS	*Cladosporium iranicum*	CBS 126346	HM148110	The Netherlands
ITS	*Cladosporium licheniphilum*	CBS 125990	HM148111	The Netherlands
ITS	*Cladosporium myrtacearum*	CBS 126350	HM148117	The Netherlands
ITS	*Cladosporium oxysporum*	CBS 126351	HM148119	The Netherlands
ITS	*Cladosporium perangustum*	CPC 14911	HM148148	The Netherlands
ITS	*Cladosporium phyllactiniicola*	CBS 126355	HM148153	Netherlands
ITS	*Cladosporium phyllophilum*	CPC 13873	HM148155	Netherlands
ITS	*Cladosporium*	CBS 117153	HM148157	The Netherlands

	pseudocladosporioides	CPC 14382	HM148190	The Netherlands
		CPC 14992	HM148192	The Netherlands
ITS	*Cladosporium rectoides*	CBS 126357	HM148194	The Netherlands
ITS	*Cladosporium scabrellum*	CBS 126358	HM148195	The Netherlands
ITS	*Cladosporium sphaerospermum*	HD8 A	JQ768326	Argentina
ITS	*Cladosporium subuliforme*	CBS 126500	HM148196	The Netherlands
ITS	*Cladosporium tenuissimum*	CPC 12795	HM148209	The Netherlands
ITS	*Cladosporium tenuissimum*	CPC 14370	HM148221	The Netherlands
ITS	*Cladosporium xylophilum*	CBS 113749	HM148228	The Netherlands
ITS	*Passalora fulva*	ELH	JQ768324	Argentina
		EMP	JQ768325	Argentina
		ALH A	JQ768320	Argentina
		CH6 A	KC132842	Argentina
		COA A	KC132843	Argentina
		CK813 A	KM488552	Argentina
		ColA A	KM488553	Argentina
		ComA A	KM488554	Argentina
		ELS A	KM488556	Argentina
		EOP A	KM488557	Argentina
β-tubulin	*Cercospora beticola*	-	AY856373	United States
β-tubulin	*Cladosporium sphaerospermum scaffold 13:453099-454730*[1]	JGI UM843	-	Malaysia
β-tubulin	*Passalora fulva scaffold 186931*[2]	JGI V1.0	-	The Netherlands
β-tubulin	*Passalora fulva*	Cf2-1	EF432762	China
TEF-1α	*Cladosporium sphaerospermum*	JGI UM843	-	Malaysia
TEF-1α	*Cladosporium asperlatum*	CBS 113744	HM148237	The Netherlands
TEF-1α	*Cladosporium basiinflatum*	CBS 822.84	HM148241	The Netherlands
TEF-1α	*Cladosporium chalastosporoides*	CBS 125985	HM148242	The Netherlands
TEF-1α	*Cladosporium cladosporioides*	CPC 14355	HM148289	The Netherlands
		CPC 15457	HM148302	The Netherlands
TEF-1α	*Cladosporium cucumerinum*	CBS 176.54	HM148322	The Netherlands
TEF-1α	*Cladosporium delicatulum*	CBS 126342	HM148323	The Netherlands
TEF-1α	*Cladosporium funiculosum*	CBS 122129	HM148338	The Netherlands
TEF-1α	*Cladosporium lycoperdinum*	CBS 574.78	HM148359	The Netherlands
TEF-1α	*Cladosporium oxysporum*	CBS 126351	HM148363	The Netherlands
TEF-1α	*Cladosporium phyllophilum*	CPC 13873	HM148399	The Netherlands
TEF-1α	*Cladosporium pseudocladosporioides*	CBS 117134	HM148400	The Netherlands
		CBS 126356	HM148403	The Netherlands
		CPC 5100	HM148437	The Netherlands

TEF-1α	*Cladosporium subuliforme*	CBS 126500	HM148441	The Netherlands
TEF-1α	*Cladosporium tenuissimum*	CBS 125995	HM148442	The Netherlands
		CPC 14370	HM148466	The Netherlands
TEF-1α	*Cladosporium varians*	CBS 126362	HM148470	The Netherlands
TEF-1α	*Cladosporium vignae*	CBS 121.25	HM148473	The Netherlands
TEF-1α	*Passalora fulva scaffold 7180000130789*	CBS 131901	JH932235	The Netherlands
Avr2	*Passalora fulva*		AJ421628	The Netherlands
Avr4	*Passalora fulva*		Y08356	The Netherlands
Avr4E	*Passalora fulva*		AY546101	The Netherlands
Avr9	*Passalora fulva*		X60284	The Netherlands

[1] (Ng et al. 2012)
[2] (Ohm et al. 2012; de Wit et al. 2012)

Table 3. Isolate identification code, county of collection, collection date, and tomato variety (if known) for all collected isolates.

Isolate ID	County of Origin	Date Collected	Tomato Variety
Pf1	McLeod County, MN	June, 2015	Big Beef
Pf2	McLeod County, MN	June, 2015	Sun Gold
Pf3	McLeod County, MN	June, 2015	Unknown
Pf4	McLeod County, MN	June, 2015	Unknown
Pf5	Anoka County, MN	July, 2015	Unknown
Pf6	Anoka County, MN	July, 2015	Unknown
Pf7	Anoka County, MN	July, 2015	Unknown
Pf8	Anoka County, MN	July, 2015	Unknown
Pf9	St. Louis County, MN	July, 2015	Yellow Cherry
Pf10	St. Louis County, MN	July, 2015	Cherry
Pf11	Wabasha County, MN	August, 2015	Sweet Golden Cherry
Pf12	McLeod County, MN	August, 2015	Unknown
Pf13	McLeod County, MN	August, 2015	Unknown
Pf14	Le Sueur County, MN	August, 2015	Unknown
Pf15	Wabasha County, MN	August, 2015	Rose
Pf16	McLeod County, MN	August, 2015	Unknown
Pf17	Wabasha County, MN	August, 2015	Paul Robeson
Pf18	Le Sueur County, MN	August, 2015	Unknown
Pf19	St. Louis County, MN	August, 2015	Unknown
Pf20	Wabasha County, MN	August, 2015	Rose
Pf21	St. Louis County, MN	August, 2015	Cherokee Purple
Pf22	St. Louis County, MN	August, 2015	Cherry
Pf23	Le Sueur County, MN	August, 2015	Unknown
Pf24	McLeod County, MN	August, 2015	Unknown

Pf25	McLeod County, MN	September, 2015	Rocky Top
Pf26	St. Louis County, MN	September, 2015	Amish Paste
Pf27	McLeod County, MN	September, 2015	Unknown
Pf28	Kanabec County, MN	September, 2015	Carmella
Pf29	St. Louis County, MN	September, 2015	Unknown
Pf30	McLeod County, MN	September, 2015	Unknown
Pf31	McLeod County, MN	September, 2015	Roma
Pf32	McLeod County, MN	September, 2015	Big Beef
Pf33	Meeker County, MN	September, 2015	Big Beef
Pf34	Anoka County, MN	October, 2015	Unknown
Pf35	Schuyler County, NY	October, 2015	Unknown
Pf36	Orleans County, NY	September, 2015	Unknown
Pf37	Orleans County, NY	September, 2015	Unknown
Pf38	Schuyler County, NY	October, 2015	Unknown
Pf39	Unknown County, NY	August, 2015	Sun Gold
Pf40	Orleans County, NY	September, 2015	Unknown
Pf41	Erie County, NY	June, 2015	Yellow Brandywine
Pf42	Essex County, NY	August, 2015	Unknown
Pf43	Essex County, NY	August, 2015	Sun Sugar
Pf44	Columbia County, NY	August, 2015	Unknown
Pf45	Columbia County, NY	August, 2015	Unknown
Pf46	Columbia County, NY	August, 2015	Unknown
Pf47	Columbia County, NY	August, 2015	Unknown
Pf48	Orleans County, NY	September, 2015	Unknown
Pf49	Schuyler County, NY	October, 2015	Unknown
Pf50	Essex County, NY	August, 2015	Sun Sugar

Pf51	Orleans County, NY	September, 2015	Unknown
Pf52	Washington County, NY	September, 2015	Unknown
Pf53	Suffolk County, NY	September, 2015	Unknown
Pf54	Orleans County, NY	September, 2015	Unknown
Pf55	Clinton County, NY	August, 2015	Red Mountain
Pf56	Saratoga County, NY	Unknown, 2015	BHN 589
Pf57	Schuyler County, NY	October, 2015	Unknown
Pf58	Columbia County, NY	Unknown, 2015	Unknown
Pf59	Unknown County, NY	July, 2015	Native Bites
Pf60	Unknown County, NY	August, 2015	Sun Gold
Pf61	Essex County, NY	August, 2015	Sun Gold
Pf62	Columbia County, NY	Unknown, 2015	Unknown
Pf63	Essex County, NY	August, 2015	Sun Gold
Pf64	Essex County, NY	August, 2015	Sun Gold
Pf65	Schuyler County, NY	October, 2015	Unknown
Pf66	Unknown County, NY	July, 2015	Native Bites
Pf67	Saratoga County, NY	Unknown, 2015	Unknown
Pf68	Washington County, NY	Unknown, 2015	Cherry
Pf69	Unknown County, WI	Unknown, 2014	Unknown
Pf70	Unknown County, IN	May, 2016	Unknown
Pf71	Unknown County, IN	May, 2016	Unknown
Pf72	Daviess County, IL	June, 2016	Carolina Gold
Pf73	Daviess County, IL	June, 2016	Carolina Gold
Pf74	Daviess County, IL	June, 2016	Carolina Gold
Pf75	Anoka County, MN	July, 2016	BHN 589
Pf76	Anoka County, MN	July, 2016	BHN 589

Pf77	Anoka County, MN	July, 2016	BHN 589
Pf78	Anoka County, MN	July, 2016	BHN 589
Pf79	Anoka County, MN	July, 2016	BHN 589
Pf80	St. Louis County, MN	July, 2016	Estonia
Pf81	Mower County, MN	July, 2016	Unknown
Pf82	St. Louis County, MN	July, 2016	Sweet Cherry
Pf83	Benton County, MN	July, 2016	BHN 589
Pf84	Benton County, MN	July, 2016	BHN 589
Pf85	Benton County, MN	July, 2016	BHN 589
Pf86	Anoka County, MN	August, 2016	Unknown
Pf87	Mower County, MN	October, 2016	Unknown
Pf88	McLeod County, MN	October, 2016	Unknown
Pf89	McLeod County, MN	October, 2016	Unknown
Pf90	Douglas County, MN	July, 2017	Big Beef
Pf91	Anoka County, MN	August, 2017	Unknown
Pf92	McLeod County, MN	August, 2017	Unknown
Pf93	Douglas County, MN	August, 2017	Big Beef

2.3.4 Phylogenetic analysis

The ITS, EF-1α, and β-tubulin sequences were trimmed and assembled in CLC Genomics Main Workbench 8.0 (https://www.qiagenbioinformatics.com/ n.d.). For each marker, MUSCLE alignments with a maximum of 8 iterations were performed in Geneious 11.1.2 (Kearse et al. 2012). Model testing, subsequent maximum-likelihood tree building, and assessment were done in CLC Main Workbench 8.0. The Hierarchical Likelihood Ratio Test (hLRT), Bayesian information criterion (BIC), Akaike information criterion (AIC), and the sample size corrected AIC (AICc) were used for model testing to determine appropriate maximum likelihood models and parameters for each data set. A separate maximum likelihood phylogeny was made for each gene marker, using parameters obtained through model testing. The EF-1α sequences were fragmented into “high” and “low” diversity segments on the basis of visual inspection of sequence alignments. The low diversity regions were used to construct a maximum likelihood phylogeny. Additionally, a maximum likelihood phylogeny was constructed based upon the full EF-1α sequences (i.e., both low and high diversity segments in a single analysis).

2.3.5 Variation in *Avr* markers relative to reference sequence

The *Avr2*, *Avr4*, *Avr4E*, and *Avr9* gene sequences were each aligned, as described in section 2.3.4, with the full gene and coding sequences from the corresponding gene of a “reference” isolate (Table 2). Mutations in the nucleotide sequences relative to the reference isolate were recorded for both the coding and non-coding regions. The coding sequences were then translated and any protein differences, relative to the reference isolate, were noted.

2.3.6 Determination of temperature growth optima and limits

An experiment was conducted to determine the temperature(s) at which the *P. fulva* and *Cladosporium* spp. isolates can grow and the temperature(s) at which they grow best. Two isolates each of *P. fulva* (Pf90 and Pf93), *C. cladosporioides* (Pf14 and Pf27), and *C. sphaerospermum* (Pf6 and Pf7), the species most frequently represented in the isolate collection, were used in this experiment. *Cladosporium* spp. and *P. fulva* isolates were grown at 25°C on PDA

for 1 or 2 weeks, respectively. Isolates were then sub-cultured onto PDA and grown for an additional week at 23°C. Spores were harvested using sterile cotton swabs, then diluted in sterile water to make a spore solution. For the experiment, the isolates were grown on both V8 medium (HiMedia®, West Chester, PA) and PDA with initial inoculum comprising 10 µl of a 10,000 spore/ml concentration applied as a droplet near the center of each plate. The plates were dried until the droplets absorbed into the agar and were then sealed with parafilm.

Preliminary data (not shown) were used to determine temperature extremes. Growth rates were ultimately tested at 4°C, 15°C, 20°C, 25°C, and 30°C +/- 2°C. Inoculated plates were randomized within each temperature treatment and incubated upside down to decrease moisture build up. Temperature and humidity were recorded throughout the experiment using Onset® HOBO U12-012 Temp/RH/Light/External Data Loggers (Bourne, MA) or Fisherbrand™ Traceable™ Thermometer/Clock/Humidity Monitor (Waltham, MA). Individual data collection devices were synchronized to ensure uniformity. Each isolate/medium/temperature combination was replicated three times.

The plates were rated for colony growth every two days for two weeks. Ratings consisted of two right angle measurements, in cm, of colony growth from the bottom side of the plate, according to a previously developed protocol (Brancato and Golding 1953). Variations in growth, color, or texture were noted.

The response variable, area of growth, was analyzed relative to the explanatory variables of isolate, growth medium, and temperature. The analysis

was conducted in Rstudio (Rstudio team 2016). The residuals were assessed for heteroscedasticity, and distribution using Levene's test, and through residual vs. fitted, normal Q-Q, scale-location, and constant leverage: residual vs. factor level plots. The area of growth data were log-transformed using the equation: Log area=log(A+0.5) and the residuals were re-assessed as described. An analysis of variance for area of growth was conducted using a model which included the explanatory variables of isolate, growth medium, and temperature, as well as all interactions between them. Variables showing significant differences (<0.5) were then assessed using a Tukey's honest significant difference (HSD) test (Steel, Dickey, and Torrie 1997).

2.4 Results

2.4.1 Phylogenetic analysis

ITS, β-tubulin, and TEF-1α sequences were assembled separately and trimmed to a length of 443, 455, and 372 base pairs, respectively. ITS, β-tubulin, and TEF sequence alignments included sequences from 145, 88, and 101

isolates and were 474, 455, and 648 nucleotides in length, respectively, including any introduced gaps. Separate maximum likelihood trees were constructed for each marker due to discrepancies between available reference sequences corresponding to the marker locations. References sequences are listed in Table 2.

The ITS maximum likelihood tree (Figure 2) was constructed using the Kimura 80 (K80) model, accounting for variations in rate and topology. The β-tubulin (Figure 3) and the full TEF-1α sequence (Figure 4) trees were constructed with the Generalised time-reversible (GTR) model, accounting for variations in rate and topology. A maximum likelihood tree was also constructed for the low variation region of the TEF-1α sequences (not shown), however, it was determined that the full TEF-1α sequence tree was more biologically informative. Analyses with each marker separated the *P. fulva* isolates from the *Cladosporium* spp. isolates and differentiated *C. sphaerospermum* and the other *Cladosporium* spp. Only the TEF-1α marker separated *C. pseudocladospoirioides* from other *Cladosporium* spp. isolates. Alone, none of the markers were able to fully resolve the *Cladosporium* spp. isolates.

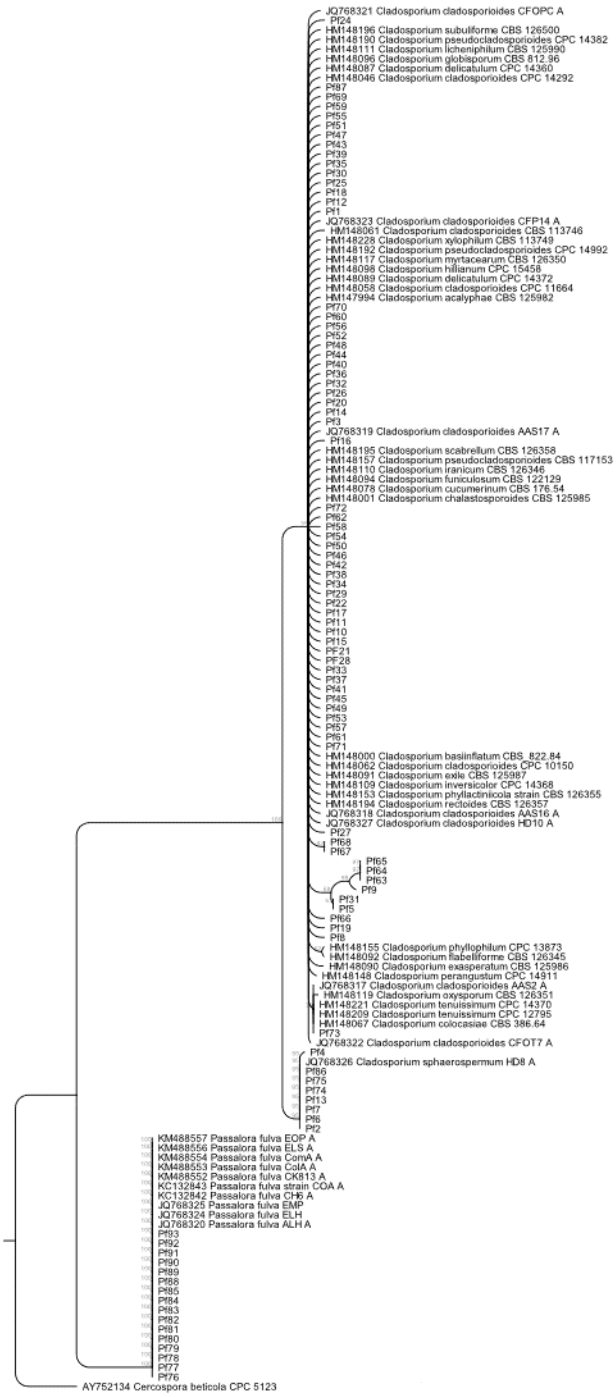

Figure 2. *ITS, neighbor joining, maximum likelihood tree, with gaps. The tree was constructed using the Kimura80 model, accounting for rate and topology (+G+T). ITS sequence from Cercospora beticola (AY752134) was used as the outgroup. Nodes supported by 60% bootstrap values, based on 1,000 replications, are shown. Separation of* Passalora fulva, *and* Cladosporium sphaerospermum *isolates from all other C. spp. is evident.*

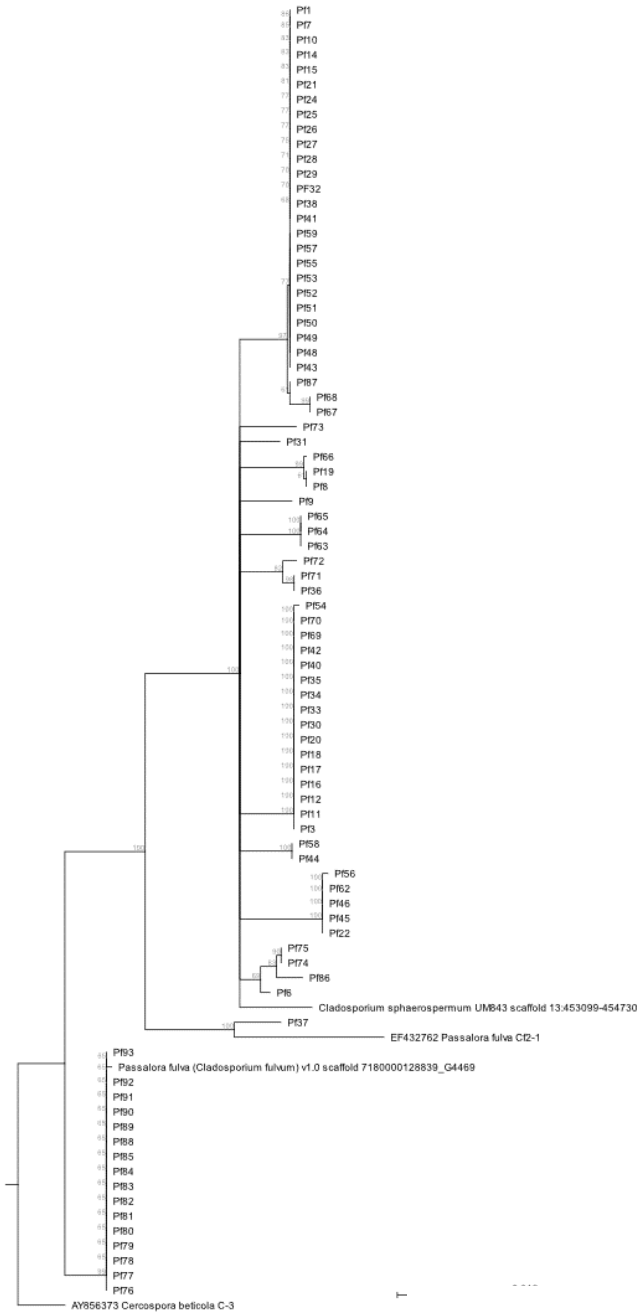

Figure 3. β-tubulin, neighbor joining, maximum likelihood tree, with gaps. The tree was constructed using the Generalised time-reversible model, accounting for rate and topology variations (+G+T). β-tubulin sequence from Cercospora beticola *(AY856373) was used as the outgroup. Nodes supported by 60% bootstrap values, based on 1,000 replications, are shown. Note clear separation of the* P. fulva *isolates from the* Cladosporium *spp. isolates is strong. A weaker separation exists between* C. sphaerospermum *and other* Cladosporium *spp. isolates.*

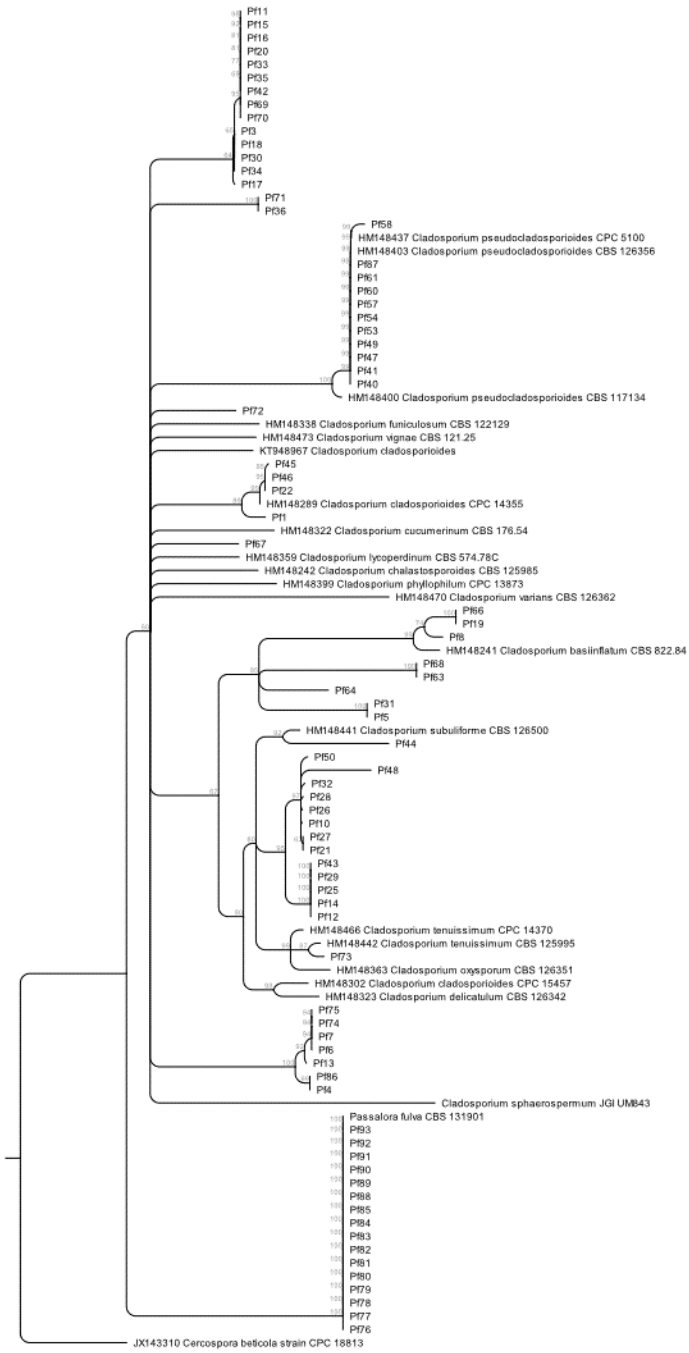

Figure 4. TEF 1-α, neighbor joining, maximum likelihood tree, with gaps. The tree was constructed using the Generalised time-reversible model, accounting for rate and topology variation (+G+T). TEF 1-α sequence from Cercospora beticola *(JX143310) was used as the outgroup. Nodes supported by 60% bootstrap values, based on 1,000 replications, are shown. Separations are present between* Passalora fulva *isolates and* Cladosporium *spp. isolates, as well as between* Cladosporium sphaerospermum *and* C. pseudocladosporioides *isolates and all other* C. *spp.*

2.4.2 Variation in *Avr* markers relative to reference sequence

Avr sequence data are summarized in Table 4. *Avr2*, *Avr4*, *Avr4E*, and *Avr9* amplicons were trimmed to 536, 774, 611, and 630 bp, respectively. Individual amplicon sequences were compared to designated reference reference sequences (Table 2). Sequence variation was low. No sequence variation was discovered for *Avr4* (not shown). For other Avr amplicons, any mutations discovered were verified by triplicate independent PCRs and sequencing reactions. Sequences with verified mutations were then translated, *in silico*, to assess effects on the corresponding protein sequence.

One isolate (Pf80) displayed a two base-pair deletion (AT at position 375-376) in its *Avr2* sequence. The deletion was in the coding sequence, resulting in a frame shift mutation, which produces a purportedly non-functional Avr2 protein (Figure 5).

In the *Avr4E* sequences, three isolates (Pf88, Pf89, and Pf92) displayed a single base pair mutation, relative to the reference reference, from T>C, at position 580, in a non-coding region. These same isolates also displayed two mutations (both C>T, at positions 422 and 426) in their coding sequences. These DNA sequence mutations were synonymous changes, causing no change in the predicted Avr4E protein sequence (not shown).

For the *Avr9* sequences, all isolates (Pf76, Pf77, Pf78, Pf79, Pf80, Pf81,

Pf82, Pf88, Pf89, Pf90, Pf91, Pf92, Pf93) contained two mutations in their non-coding sequences, relative to the reference reference (A>G at position 126 and A>C at position 526). Additionally, all isolates had one non-synonymous mutation in their coding sequences (T>C at position 251). This mutation resulted in a change from valine to alanine in the Avr9 protein (Figure 6).

Table 4. Summary of Avr sequence data. Expected and trimmed sizes of amplicons for each marker, polymorphisms relative to reference reference sequence by location and type of polymorphisms present in coding sequence.

Gene	Size (bp)		Number of polymorphisms		Types of polymorphisms in coding sequence			
	Expected	Trimmed	Coding region	Non-coding region	SNP	Indels (bp)	Synonymous[1]	Nonsynonymous[2]
Avr2	570	536	2/235	0/301	0	1 (2)	N/A	N/A
Avr4	806	774	0/408	1/366	0	0	0	0
Avr4E	641	611	2/366	1/275	2	0	2	0
Avr9	712	630	1/192	2/438	1	0	0	1

[1] Causing no change in the protein sequence
[2] Causing a change in the protein sequence

Figure 5. Avr2 *in silico translated sequences compared to the reference sequence AJ421628. Pf80 displayed a two base-pair deletion, resulting in the boxed, putative non-functional protein sequence. All other isolates produced protein sequences identical to that of the reference.*

Figure 6. Avr9 *in silico translated sequences compared to the reference sequence X60284. All isolate DNA sequences displayed one non-synonymous mutation from thymine to cytosine, relative to the reference sequence. The observed mutation resulted in the boxed change from valine to alanine in the protein sequence.*

2.4.3 Temperature and growth optima and limits

The results of the two temperature growth optima replicate trials were independently analyzed, as statistically significant ($p<0.05$) differences existed between trials. With each replicate trial, there were significant ($p<0.05$) differences in the area of growth associated with isolate, incubator temperature, growth medium, and all interactions between isolates, temperature, and growth medias. Effects of isolate, incubator temperature and growth mediums are summarized in Figure 7.

While differences in growth exist between different isolates of the same species, larger differences exist between isolates of different species. *Cladosporium cladosporioides* isolates (Pf14 and Pf27) had a significantly ($p<0.05$) larger area of growth than the *C. sphaerospermum* isolates (Pf6 and Pf7), which had a significantly ($p<0.05$) larger area of growth than the *P. fulva* isolates (Pf90 and Pf93) (Figure 7). In both trials, Pf27 had the largest area of growth overall, followed by Pf14. In one trial Pf6 had a larger area of growth than Pf7; in the other trial no difference was found between the area of growth for Pf6 and Pf7. There were no significant differences between the growth of Pf90 and Pf93.

There was a significant ($p<0.05$) effect of incubator temperature on the area of growth for all isolates. Overall, the growth of isolates was highest in the

mid-range of temperatures (20°C and 25°C). Only Pf27 and Pf14 (both *C. cladosporioides*) were able to grow at 4°C and only Pf27, Pf14, and the *C. sphaerospermum* isolate Pf6 were able to grow at 30°C (Figure 7). In one trial isolates grew equally at 20°C and 25°C; in the other trial isolates grew faster, by a small but statistically ($p<0.05$) significant margin, at 25°C. In general, isolates grew best at 20°C or 25°C, moderately at 15°C, and poorly at 30°C and 4°C.

The effect of medium type was significant ($p<0.05$), with isolates growing better on PDA, in both trials (Figure 7).

Pf27 and Pf14 (*C. cladosporioides*) grew better than all other isolates on both media types, while Pf90 and Pf93 (*P. fulva*) grew least robustly on both medias. For Pf27, Pf14, Pf6, and Pf7 there was a small (but not statistically significant) advantage of growth on PDA. Pf90 and Pf93 displayed a small (but not statistically significant) growth advantage on V8. Across all isolates, the interaction between isolate and medium type was significant ($p<0.05$) (data not shown).

The effect of the interaction of incubator temperature and growth medium was significant ($p<0.05$) (data not shown). Regardless of media type, isolates grew the least at 30°C and 4°C. There was a small, but non-significant, growth advantage on PDA at 15°C, 20°C, and 25°C and on V8 at 30°C and 4°C.

The interaction between temperature and isolate was also significant ($p<0.05$) (Figure 8). Both isolates of *C. cladosporioides* (Pf27 and Pf14) grew best at 20°C, followed by 15°C, 25°C, 30°C, then 4°C. In one trial, both isolates

of *C. sphaerospermum* (Pf6 and Pf7) grew best at 20°C, followed by 25°C, 15°C, 30°C, then 4°C. In the other trial, Pf7 performed similarly but Pf6 grew best at 25°C. Both *P. fulva* isolates (Pf90 and Pf93) grew best at 25°C, followed by 20°C, 15°C, 30°C, then 4°C.

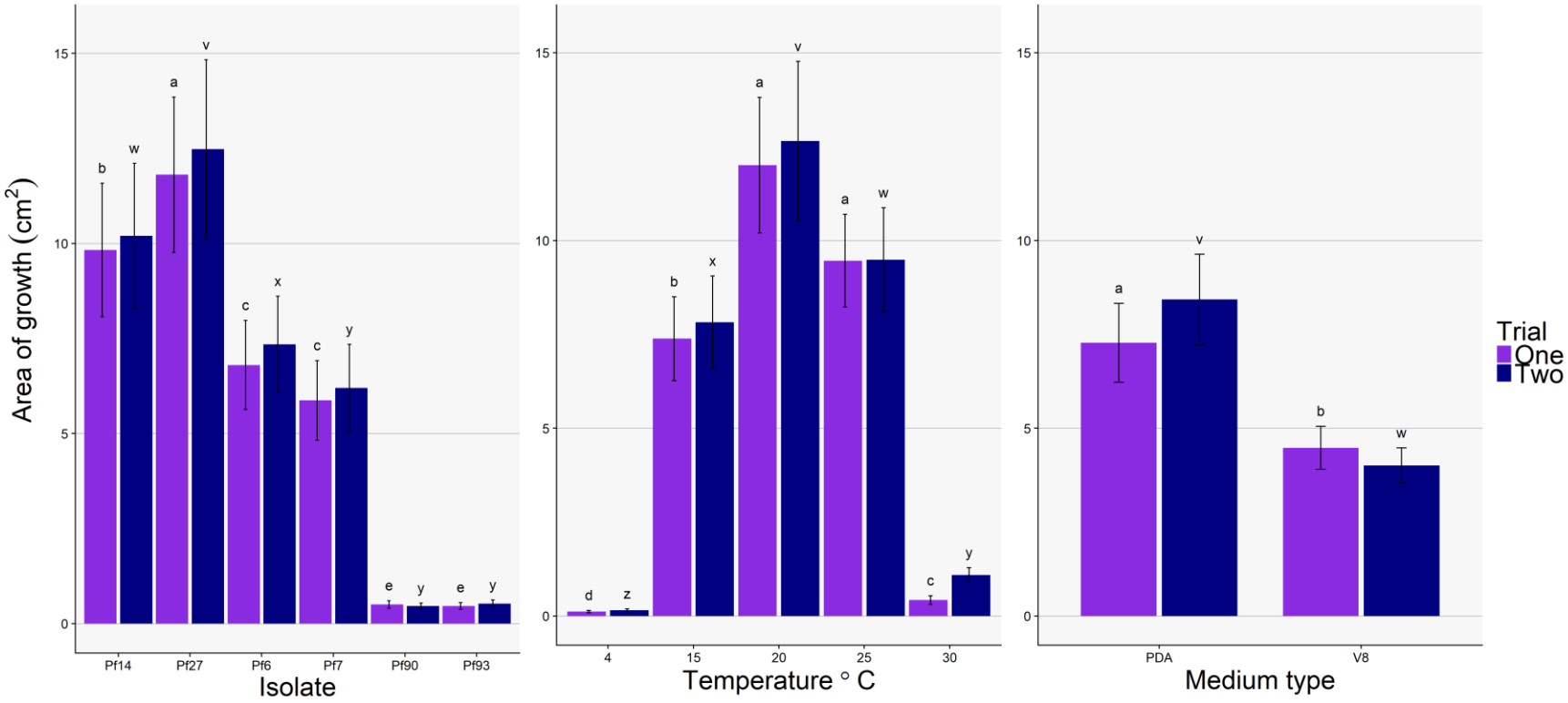

Figure 7. Area of growth relative to isolate, temperature, and media type. Replicate Trials 1 and 2 are graphed. Error bars were calculated as the mean +/- standard error and lettering was based upon statistical grouping of the data. Mean separations in Trial 1 are indicated by a-e and in Trial 2 by v-z. Cladosporium cladosporioides *isolates are Pf14 and Pf27;* C. sphaerospermum *isolates are Pf6 and Pf7;* Passalora fulva *isolates are Pf90 and Pf93.*

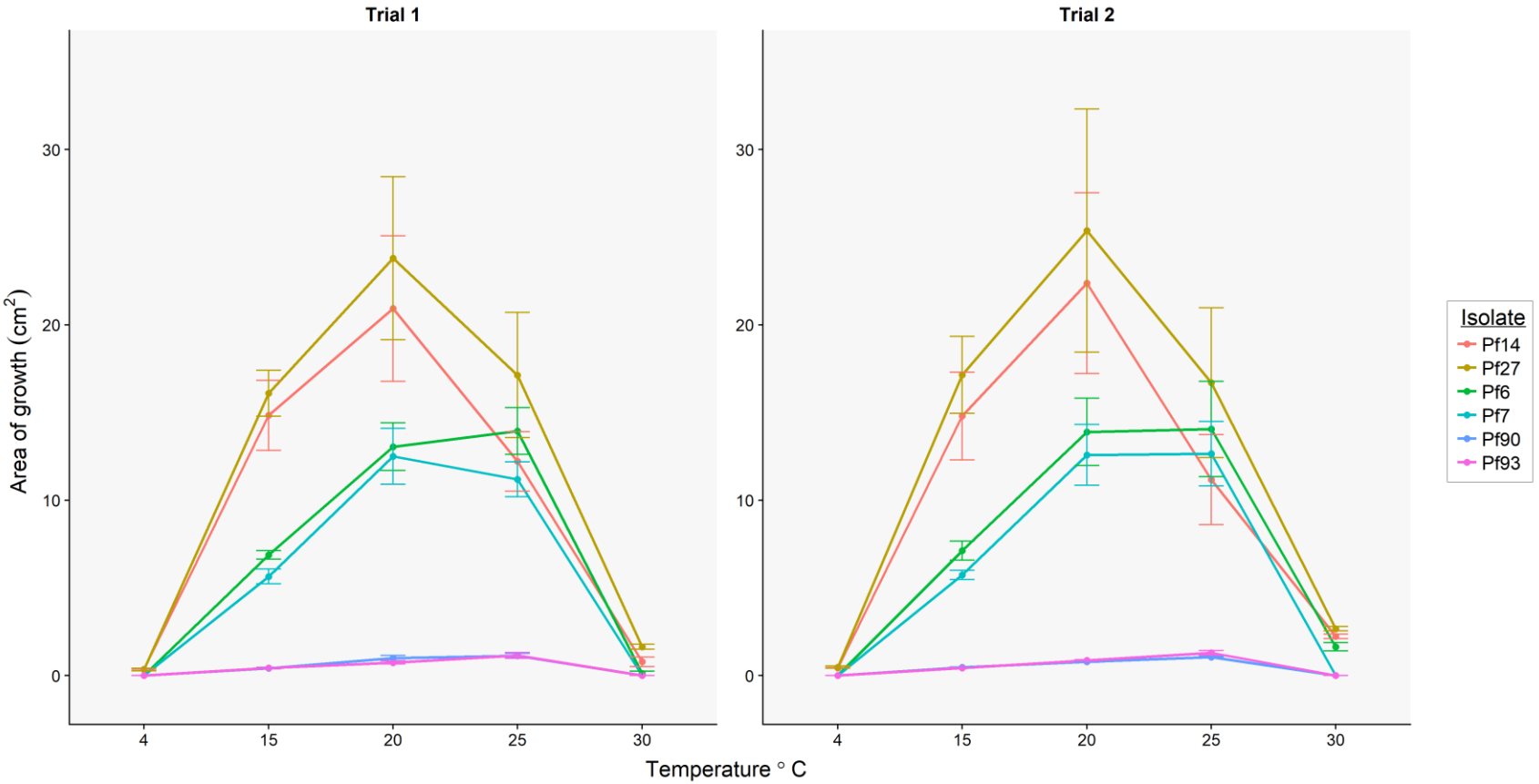

Figure 8. Area of growth relative to temperature and isolate for Trial 1 and Trial 2. Error bars were calculated using mean +/- SE. Cladosporium cladosporioides *isolates are Pf14 and Pf27;* C. sphaerospermum *isolates are Pf6 and Pf7;* Passalora fulva *isolates are Pf90 and Pf93.*

2.5 Discussion

The prevalence of *Cladosporium* spp., in addition to the causal pathogen *P. fulva*, in tomato leaf mold lesions has been recently noted (Medina et al. 2015). *Cladosporium* spp. are ubiquitous and known to fill different ecological roles, including that of a pathogen (Kirk and Crompton 1984; Lorenzini and Zapparoli 2015; Liu et al. 2016). It is unknown whether interaction between species of the two genera affect the ability of *P. fulva* to infect tomatoes or the severity of disease development. The possibility of such an interaction underscores the need for research on the identification of molecular and functional differences that exist between the genera. Additionally, because of the known gene-for-gene structure of the tomato leaf mold pathosystem, it is important to identify the variation within isolates of the pathogen, *P. fulva* (van den Ackerveken, van Kan, and de Wit 1992).

In this study, ITS, β-tubulin, and TEF-1α markers were successful in separating *P. fulva* and *Cladosporium* spp. isolates. In addition, *C. sphaerospermum* isolates were separated from all other *Cladosporium* spp. by all three markers, suggesting that this species is unique, on a molecular level, from other species in the genus. As suggested in previous studies, TEF-1α markers may serve as an alternative to ITS barcoding (Mirhendi et al. 2015). In this study

the TEF-1α markers were able to separate an additional species (*C. pseudocladosporioides*) (Figure 4).

None of the markers alone were able to fully resolve the *Cladosporium* spp. isolates or to separate *P. fulva* isolate sequences by small scale molecular differences. The development of more precise and consistent amplification methods would facilitate the assessment of differences between and among these genera. A multi-locus sequence analysis using a combination of ITS, actin, β-tubulin, and/or TEF-1α would likely provide an increased resolution. A similar method has been used previously, however, no standardized approach using three or more markers has been developed for these genera (Bensch et al. 2010, 2015). A possible alternative, genotype-by-sequencing (GBS) marker technology, has not been used previously in either *Cladosporium* or *Passalora fulva,* but has potential to yield accurate results and higher resolution distinctions. There was also some variation among the *Passalora fulva* isolates, relative to the reference references at *Avr* loci, important to the tomato leaf mold pathosystem. Within *Avr2* sequences, a two-base pair deletion was detected in one isolate. Indels within *Avr2*, resulting in a non-functional protein, have been noted previously (Stergiopoulos, De Kock, et al. 2007). A non-functional Avr2 protein would no longer alter its plant target, the cysteine protease Rcr3, thereby evading detection by the tomato Cf2 protein, which detects changes in the Rcr3 conformation (Rooney et al. 2005; van Esse et al. 2008). Such a change in *P. fulva* would be expected to render the *Cf2* tomato leaf mold resistance gene

ineffective.

Avr4E sequences for three isolates contained a T>C mutation in their non-coding sequences, as well as two C>T synonymous mutations in their coding sequences. Mutations have been previously reported in both non-coding and coding regions of *Avr4E,* with both synonymous changes and phenylalanine to leucine and methionine to tyrosine transitions noted (Westerink et al. 2004; Stergiopoulos, De Kock, et al. 2007).

All isolate *Avr9* sequences were differentiated from the reference sequence by the presence of two mutations (A>G and A>C) in their non-coding sequences, as well as one non-synonymous mutation (T>C), resulting in an amino acid change from valine to alanine in the expected Avr9 sequence. Alanine and valine are very non-reactive and are therefore rarely involved in protein function (J. Betts and Russell 2003). The amino acid change from valine to alanine observed in the Avr9 protein has been previously described, and is not expected to have a strong effect on the function of the protein (Stergiopoulos, De Kock, et al. 2007).

On average, with the exception of deletions reported for *Avr2,* the *Avr* mutations present in this study are unlikely to have a strong effect on disease management. However, it would be beneficial to monitor both the race-types present across the world, as well as the functionality of the Avr genes present in the isolates. At present there are very few studies which have evaluated the race structure of isolates within the United States. This research will be valuable given

the increase in high-tunnel usage in many parts of the United States, which facilitates tomato leaf mold development (Jones et al. 2014).

Biological differences between *P. fulva* and *Cladosporium* spp. could also have direct effects on any potential interactions present between the genera during the infection process as well as during symptom development of tomato leaf mold. One major factor influencing the development of disease and the growth rates of fungi, is the temperature range at which the microbes can grow and temperatures at which they are most active.

In this study, the ability of isolates to grow was significantly ($p<0.05$) affected by the isolate (and species), medium type, and temperature. In general, isolates of *C. cladosporioides* grew most vigorously, *C. sphaerospermum* displayed moderate growth, and *P. fulva* had the least vigorous growth. Overall, the isolates grew better on PDA, however this was likely skewed by the 4:2 ratio of *Cladosporium* spp. isolates (which grew preferentially on PDA) to *P. fulva* isolates (which grew preferentially on V8). The temperature range of all isolates was limited, with extreme temperatures (4°C and 30°C) yielding little or no growth. Growth was greatest at mid-range temperatures (20°C and 25°C) but still reasonably robust at 15°C. The range of temperatures for tomato growth in high-tunnels is approximately 18-27°C, with an optimum range of 21-24°C (Belina et al. 2012). Functionally, this means that the *Cladosporium* spp. and *P. fulva* isolates thrive at roughly the same optimum temperature range as the tomatoes themselves, and in fact can grow reasonably well below the common range of

tomato production temperatures. This suggests that the management of temperature in high-tunnels should focus primarily on the requirements of the tomatoes, rather than on the potential cultural management of tomato leaf mold. Future work should assess other biological factors such as humidity, leaf wetness, and light intensity, all of which fluctuate within high-tunnels. Additionally, the effect of daily variations in temperature, similar to fluctuations which occur in a high-tunnel, should be evaluated.

Little is known about the potential interaction between *Cladosporium* spp. and *P. fulva*, which are found together in tomato leaf mold lesions. Attempts to induce disease development in susceptible tomatoes using only the known pathogen, *P. fulva*, were met with complications (Appendix A.2). It would be valuable to develop a more consistent protocol for the successful inoculation of tomato plants with *P. fulva*. This would facilitate future research to determine whether the interaction of these fungi have any effect on tomato leaf mold initiation, development, or severity. Additionally, further research into the molecular and biological differences between these genera may reveal more effective strategies for the management of tomato leaf mold.

Chapter 3. Evaluation of pesticide spray programs

3.1 Preface

Tomato leaf mold, gray mold, and early blight are three of the most prevalent diseases of high-tunnel grown tomatoes. The efficacy of ten organic or conventional pesticide spray programs was assessed for use on high-tunnel tomatoes, relative to an untreated control. The programs were evaluated in three locations across Minnesota: Oak Grove, Foley, and Duluth. The marketable and non-marketable yields, disease incidence and severity, and overall plant health were used to evaluate the efficacy of the spray programs. In this study, there was no effect of treatment on either the marketable or non-marketable yield. However, location had a significant effect on both yields. Disease severity was significantly affected by location but not by treatment. The overall plant health ratings were unaffected by treatment or location. The results appear to suggest no benefit of pesticides for the management of tomato leaf mold, early blight, and gray mold. However, the results may instead point to the ineffectiveness of the experimental design, which may have allowed for pesticide drift. Future studies would benefit

from larger research plots, which would allow for more adequate spacing between treatments. In addition, the use of more consistent experimental locations is suggested.

3.2 Introduction

High-tunnels are used to extend the season for high value crops in areas, such as Minnesota, where the vegetable season is ordinarily short (Belina et al. 2012). While high-tunnels can provide favorable conditions for crops, they can also increase the prevalence of pathogens that thrive under conditions of high humidity (Belina et al. 2012; Jones et al. 2014). Tomato leaf mold (*Passalora fulva*), gray mold (*Botrytis cinerea*), and early blight (*Alternaria solani* and *Alternaria tomatophila)* are some of the most prevalent diseases in high-tunnel tomatoes (Babadoost 2011; Jones et al. 2014; Grabowski, Orshinsky, and Johnson 2015).

Cultural practices such as regular plant trimming, adequate spacing of plants, proper ventilation, and sanitation of equipment can help to ease disease pressure in the high-tunnel system (Jones et al. 2014; Johnson, Orshinsky, and

Grabowski 2015; Egel et al. 2017). However, in many cases it is necessary to use some form of chemical or biological management to maintain adequate yield and quality.

From a regulatory perspective, high-tunnels are subject to the same pesticide application rules as greenhouses, restricting pesticides available for use in high-tunnels and dictating safety precautions that must be observed (Grabowski, Orshinsky, and Johnson 2015). There are many pesticides, organic and conventional, which are approved for high-tunnel tomato production. A number of studies have assessed the ability of individual pesticides, or biocontrols, to inhibit mycelial growth of tomato pathogens *in vitro* (Shengming, Fei, and Jia 2016; Walke et al. 2014). However, the efficacy of these pesticides has not been adequately assessed *in vivo*. Additionally, the practice of using pesticide spray programs, which reduces the rate of pesticide resistance, has not been sufficiently evaluated for use in high-tunnel tomatoes. Pesticide spray programs, which rotate chemistries, can decrease the rate of pathogen acquired resistance, a concern particularly for *B. cinerea* (Leroux 2007; Rodríguez, Acosta, and Rodríguez 2014).

3.3 Materials and Methods

3.3.1 Locations and experimental design

Treatments included four organic pesticide spray programs (made up of combinations of the pesticides: Regalia®, Kocide® 3000, Actinovate®, Rootshield®, Milstop®, and OxiDate® 2.0) and six conventional pesticide spray programs (made up of combinations of the pesticides: Gavel® 75DF, Kocide® 3000, Switch® 62.5WG, Tanos®, Quadris Top®, and Revus Top®). Complete treatment details are provided in Appendix A.3. The tomatoes were treated about every three weeks with one of the fungicides in the treatment program. Untreated plants served as controls.

High-tunnel tomatoes (variety: BHN 589) from three Minnesota locations (Oak Grove, Foley, and Duluth) were used in this study. Farm locations are shown in Figure 9. The experimental design varied by location.

In the Oak Grove and Duluth high-tunnels, a randomized block design was employed. Each high-tunnel comprised three rows, with each row being a block containing one set of tomatoes for each treatment. Rows included two plants per treatment, with two spacer plants between each treatment. Tomato plants were

planted eighteen inches apart within rows and two feet between rows.

The high-tunnels in Foley were laid out differently, with the tomato plants organized as two row plots, with spacing similar to the Oak Grove and Duluth locations. While treatments were the same at Foley, a completely randomized design was employed, with three replicates of each treatment distributed across both rows. Two additional high-tunnels were employed in Foley, with the same experimental design. Both were fumigated high-tunnel sites. One high-tunnel contained grafted tomatoes and one contained non-grafted tomatoes.

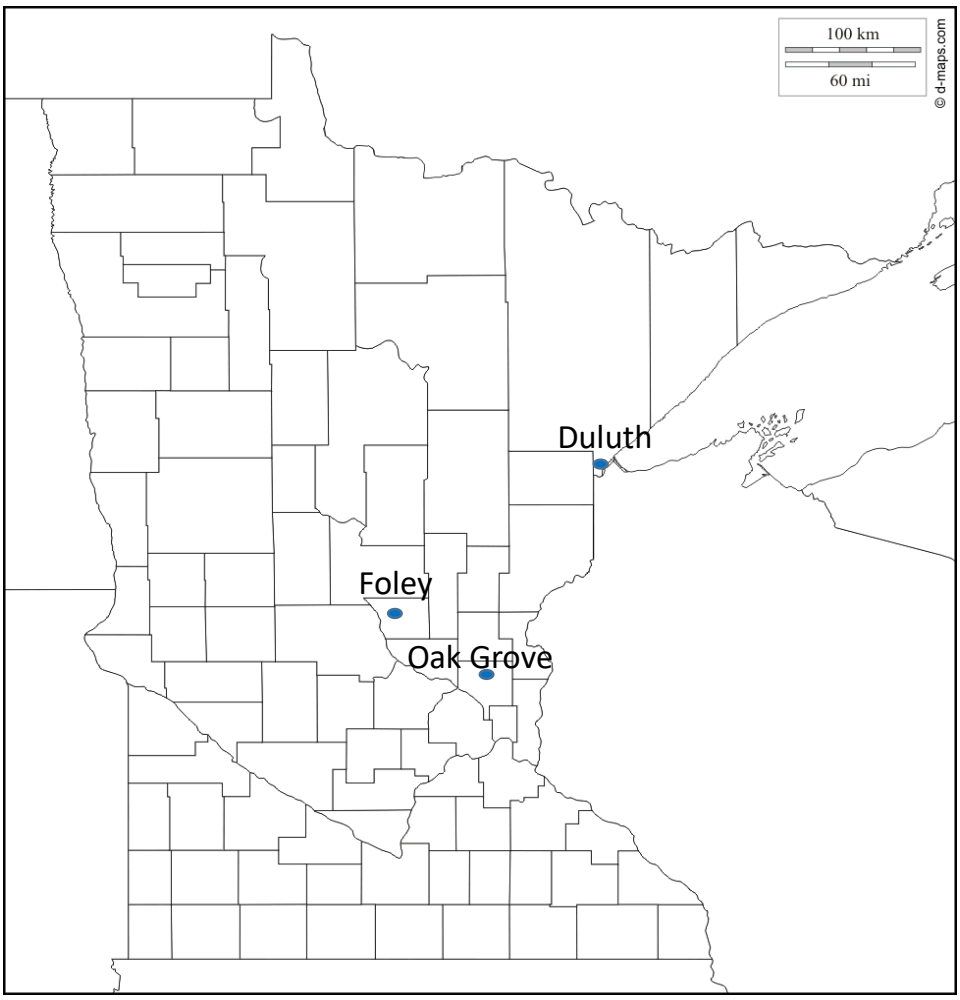

Figure 9. Pesticide spray program locations. Pesticide programs were tested for their efficacy in managing diseases on high-tunnel tomatoes in three cities: Duluth, Foley, and Oak Grove, MN. Each location is a single farm.

3.3.2 Evaluation of the pesticide spray programs

Response variables were measured throughout the tomato growing season. Each week the yield from tomatoes within each treatment were recorded. First the tomato fruits were separated into “marketable” and “non-marketable” categories based on visual inspection. Tomato fruits were categorized as non-marketable if physical damage, as a result of biotic or abiotic stress, mechanical impacts, or age, was visible. The marketable and non-marketable yields were measured, separately, and recorded. For the non-marketable category, reasons for non-marketability were recorded. In Duluth the yield was measured twice per week and the data were combined into a single weekly yield measurement.

The tomatoes were rated biweekly for measurements of plant health and disease. Plant health was rated on a visual 1-5 scale. A rating of 1 was given to a healthy plant (lacking signs of environmental or biotic stress). A rating of 2 indicated the presence of biotic or abiotic damage, such as a chlorotic or necrotic tissue on only portions of the plant (<50% coverage). A rating of 3 was given when chlorotic, or necrotic tissue or disease symptoms were present on most of the tomato plant (>50% coverage). A rating of 4 indicated tissue and fruit damage present on most of the plant. A rating of 5 was given if a plant was dead or clearly approaching death.

The presence of diseases on the tomatoes was also recorded at this time.

The severity of three of the most common diseases of high-tunnel tomatoes, tomato leaf mold, gray mold, and early blight, was rated on a visual 0-5 scale. A rating of 0 indicated no visual disease symptoms. A rating of 1 indicated a presence of visual disease symptoms, such as the formation of lesions, on less than 10% of the plant. A rating of 2 was given when symptoms, including lesions and chlorotic tissue, were present on 10-40% of the plant. A 3 indicated lesions, chlorosis, and/or necrosis on 40-60% of the plant. A 4 was given when chlorotic and necrotic tissue and disease signs were visible on 60-80% of the plant. A 5 indicated that the disease was severe enough that disease signs and symptoms were present on more than 80% of the plant.

Data were analyzed in Rstudio (Rstudio-team 2016). The marketable and non-marketable yields were analyzed separately by analysis of variance. In these analyses, yield was considered the response variable and analyzed relative to the explanatory variables of location and treatment. Statistical differences were then evaluated using Tukey's honest significant difference (HSD) test (Steel, Dickey, and Torrie 1997). The plant health and disease severity ratings were analyzed using a generalized linear model with a Poisson error distribution. Means were further differentiated by multivariate t-distributions using the "multcomp" package in Rstudio (Hothorn, Bretz, and Westfall 2008). The model was evaluated using a Chi-square goodness of fit test.

3.4 Results

3.4.1 Yield

Total marketable and non-marketable yields across all farms are displayed in Figures 10 and 11, respectively. Analysis of variance for marketable yields showed no significant differences between spray programs (treatments) and unsprayed controls. The interaction of treatments and locations was also non-significant. There was, however, a significant ($p<0.05$) difference between locations. Tukey's HSD post-hoc test showed a significant decrease in marketable yield at the Oak Grove farm relative to the other two farms. There were no differences in marketable yield between the Duluth and Foley farms.

The analysis of variance for the non-marketable yields showed no significant differences between treatments or the interaction of locations and treatments. There was a significant ($p<0.05$) difference in non-marketable yield by location. Tukey's HSD post-hoc test indicated a significant increase in non-marketable yield at the Foley farm relative to the other two farms. No differences were present between the Duluth and Oak Grove farms.

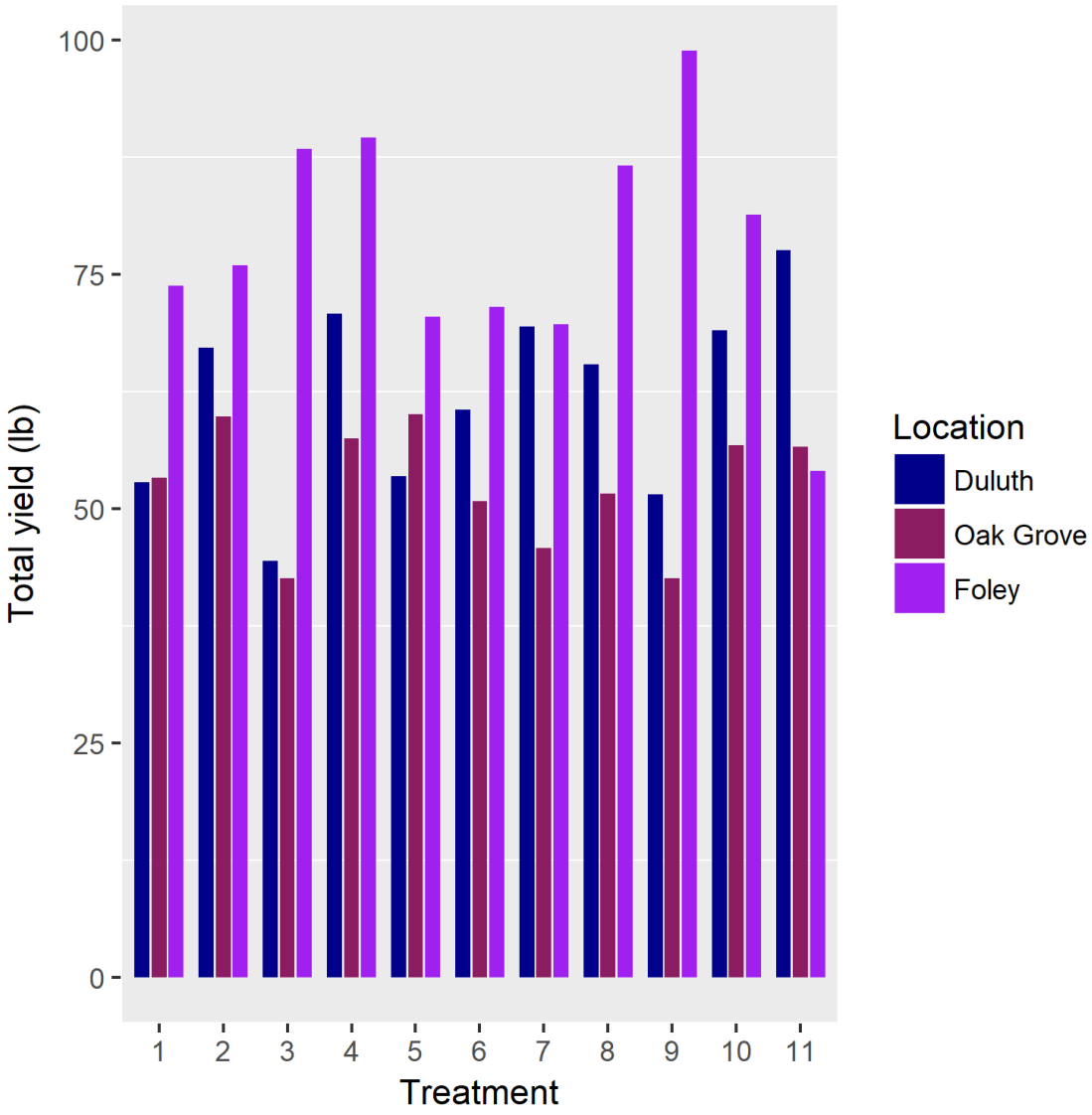

Figure 10. Total marketable yield by location and treatment. No significant differences were found between treatments. Oak Grove had a significantly ($p<0.05$) lower marketable yield than Duluth and Foley. Treatments are described in Appendix A.3.

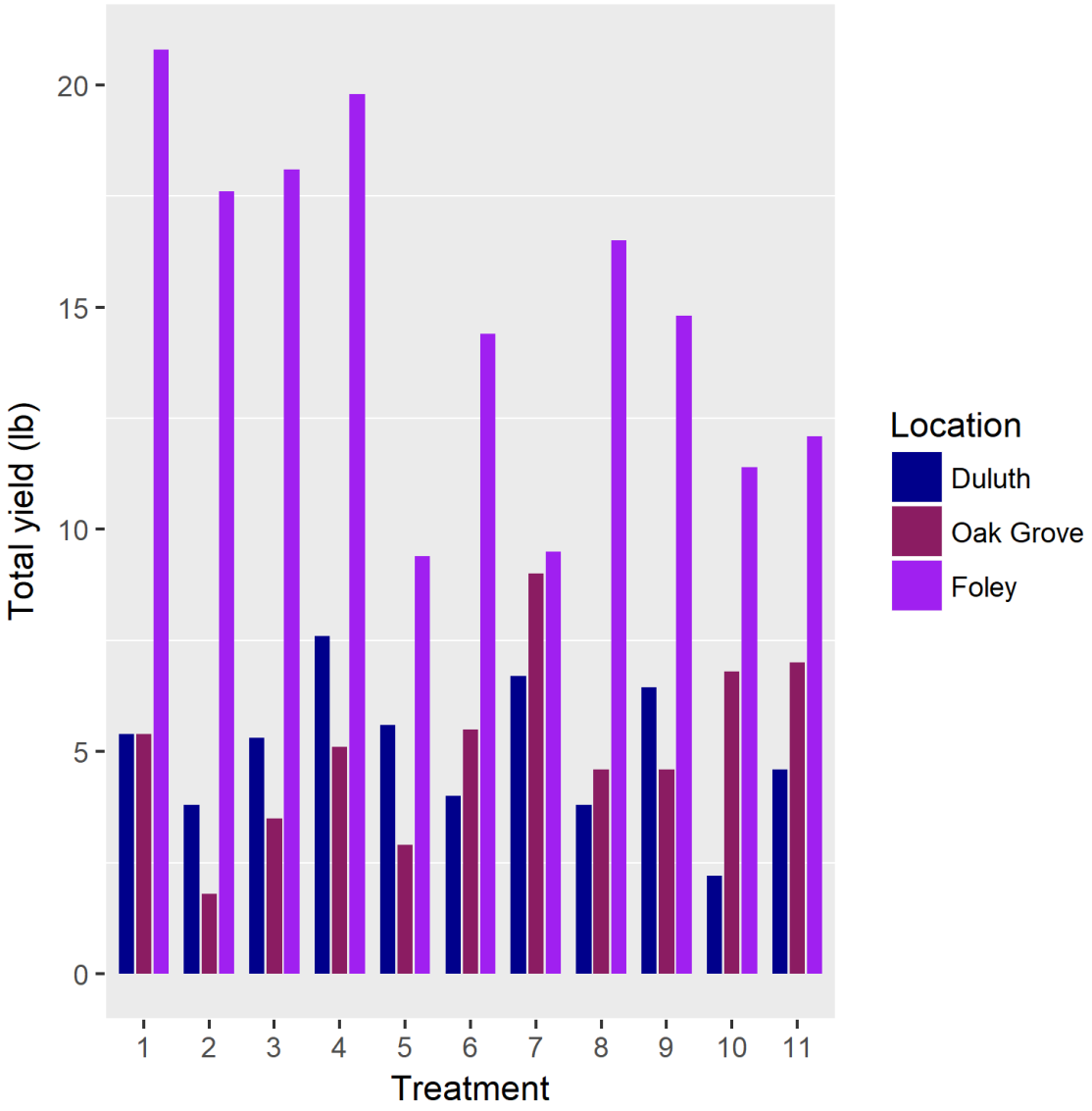

Figure 11. Total non-marketable yield by location and treatment. No significant differences were found between treatments. Foley had a significantly ($p<0.05$) higher non-marketable yield than Duluth or Oak Grove. Treatments are described in Appendix A.3.

3.4.2 Health and Disease Ratings

Health and disease rating data were analyzed using a general linearized model (GLM) with a Poisson distribution, assessed for model fit by a Chi-square goodness of fit test. The ratings of overall health, leaf mold severity, gray mold severity, and early blight severity were analyzed separately as response variables in the GLM, using the explanatory variables of treatment and location. Means were further differentiated by multivariate t-distributions.

Disease incidence (Figure 12) and severity (Figure 13) for leaf mold, gray mold, and early blight were recorded for each plant within each treatment. Incidence was rated as either present or absent, therefore no formal analysis was pursued. Treatments had no significant effect on the severity of leaf mold, early blight, or gray mold. However, location did have a significant ($p<0.05$) effect on disease severity. Leaf mold was most severe in Oak Grove and least severe in Duluth. Early blight was more severe in Foley than in Duluth. No early blight was found in Oak Grove. Gray mold was equally severe in Duluth and Foley but significantly ($p<0.05$) less severe in Oak Grove.

In addition to disease incidence and severity, each plant in this study was assigned a composite plant health score based on visual observations (Figure

14). Neither treatment nor location had an effect on the composite plant health score.

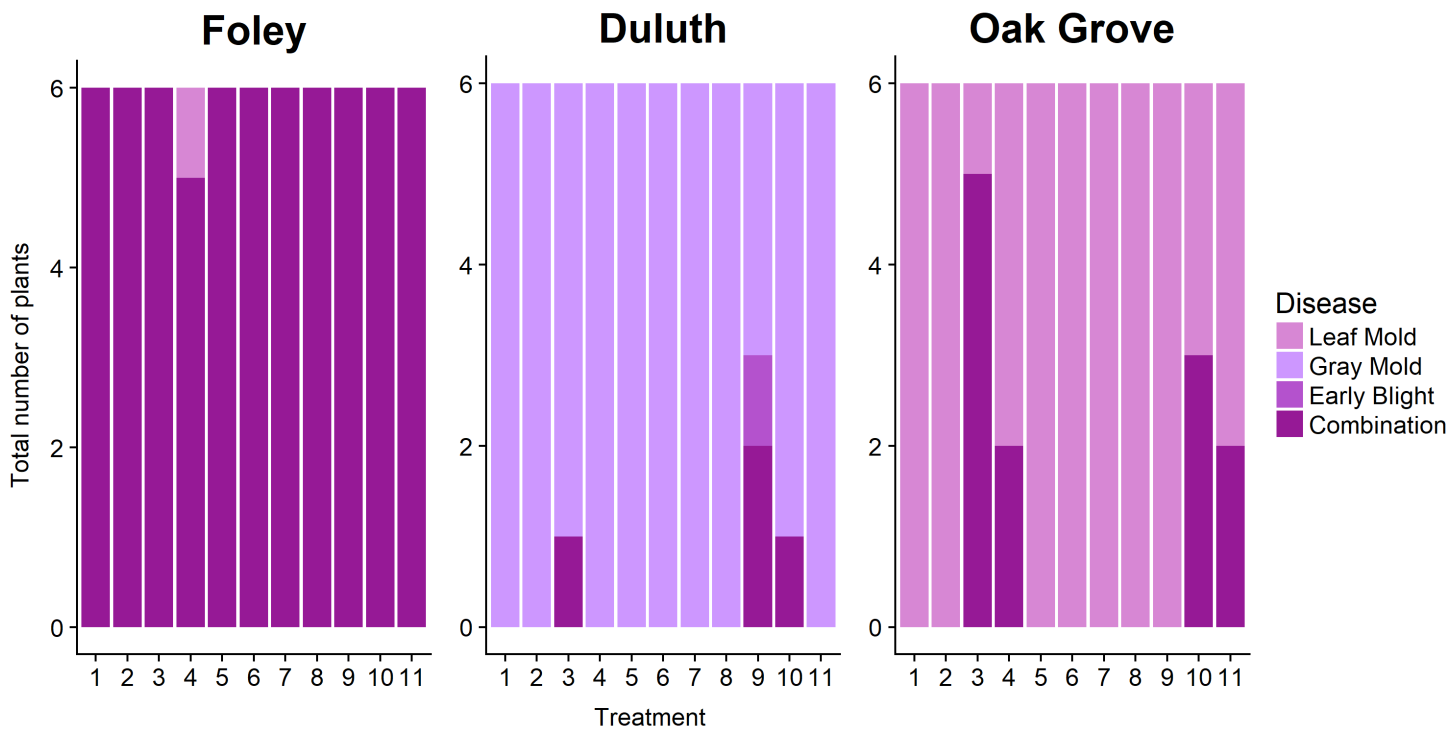

Figure 12. Disease incidence for leaf mold, gray mold, and early blight by treatment and farm. The category "combination" indicates that two or more diseases were present on the same plant. Treatments are described in Appendix A.3.

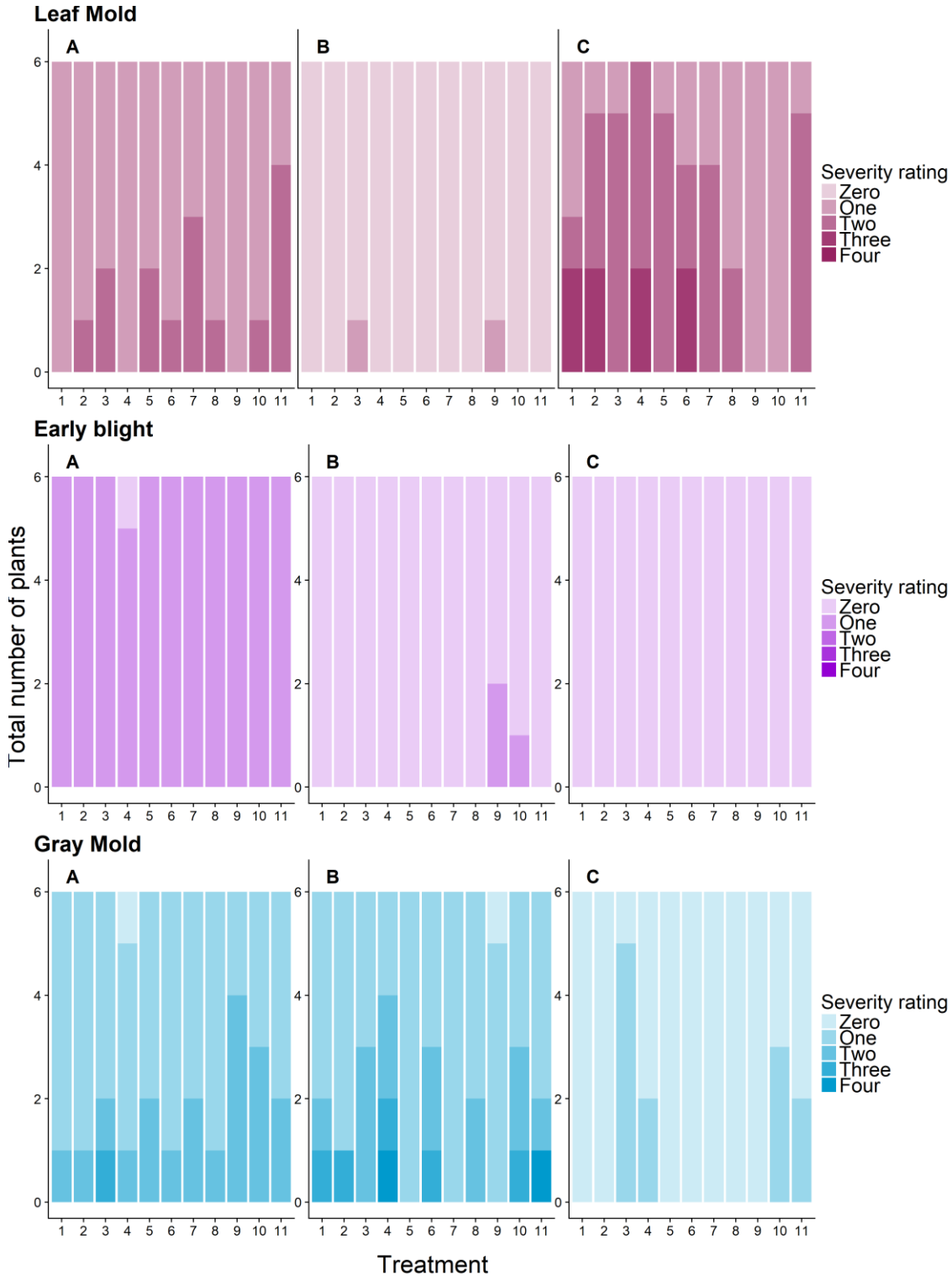

Figure 13. Disease severity of leaf mold, early blight, and gray mold. Ratings were from 0-5 for each disease, with a lower rating indicating less severe symptoms, as indicated. No ratings of 5 were observed. "A"-Foley, "B"-Duluth, "C"-Oak Grove. Treatments are described in Appendix A.3.

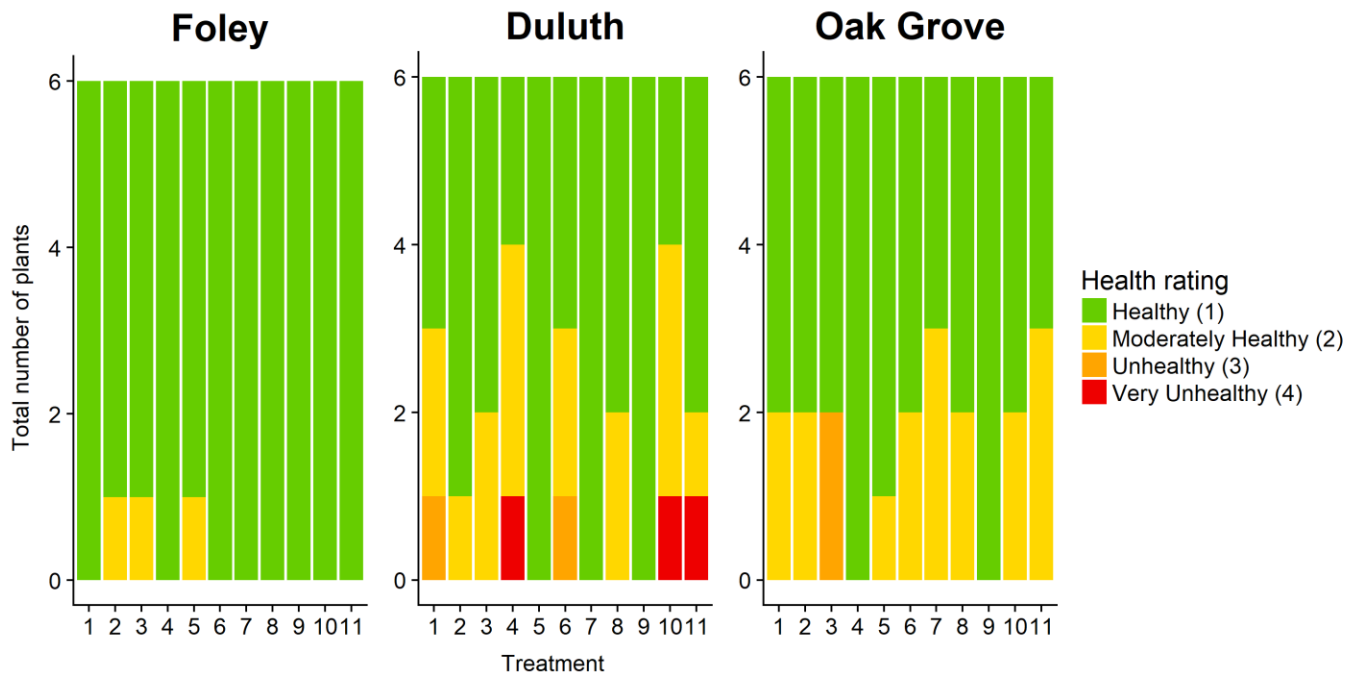

Figure 14. Plant health was rated using a 1-5 visual ranking [healthy (1), moderately healthy (2), unhealthy (3), very unhealthy (4), or dead (5)]. No plants in this experiment were assigned a plant health rating of 5. Treatments are described in Appendix A.3.

3.5 Discussion

Chemical treatments can be an important component of disease management programs in high-tunnel tomatoes. A number of *in vitro* studies have assessed fungicide efficacy for mycelial inhibition of the tomato pathogens *Botrytis cinerea, Alternaria solani,* and *Passalora fulva* (Walke et al. 2014; Rautela and Singh 2017). However, few studies have thoroughly analyzed the *in vivo* efficacy of spray programs, using commercial fungicides approved for the management of early blight, gray mold, and tomato leaf mold, in high-tunnel tomatoes.

In this study, there was no significant effect of fungicide spray program on the marketable or non-marketable yield of the high-tunnel tomatoes, relative to untreated controls (Figures 10 and 11). The fungicide treatments also had no significant effect on the severity of symptoms from early blight, gray mold, or tomato leaf mold, or on the overall health of tomato plants, relative to untreated controls (Figures 13 and 14). However, the location of the high-tunnels had a significant effect on marketable and non-marketable yield, as well as disease severity, for all rated diseases. There was no effect of location on overall plant health.

At the surface, the implication of these results is that using either an organic or conventional spray program will result in no yield or disease severity

benefit, relative to not treating tomatoes. In addition, the location at which tomatoes are grown should impact yield and disease severity.

But the observed results likely reflect limitations of experimental design rather than a lack of treatment efficacy *per se.* Due to resource limitations, each treatment contained few reps per location. A larger data set would have had more statistical power and would have allowed for variation to be more accurately established. In addition, only two spacer plants were present between treatments, including between the untreated controls. The proximity of spray treatments could have led to pesticide drift between treatments and control plants. Future research should be done on a larger scale, with adequate spacing, in order to establish true differences between treatments.

Variation between locations was significant. Many factors contributed to this variation. The soil type at each location varied, with Foley having very sandy soil, compared to Duluth and Oak Grove. In addition, the high-tunnel style was different between locations. Foley had very long, connected high-tunnels, whereas Duluth and Oak Grove had small, stand-alone, high-tunnels. Cultural practices also varied by location. In Duluth and Oak Grove tomatoes were grown to a height of approximately 4-5 ft, supported by small stakes, and trimmed regularly to maintain adequate spacing. In Foley, tomatoes were grown on hanging trellises, which allowed tomatoes to reach approximately 6-7 ft in height and decreased the space between plants. The design and setup of these high-tunnels could have had an impact on two important disease determinants,

humidity and airflow. Long high-tunnels are more likely to restrict airflow and increase humidity relative to shorter tunnels. Additionally, tomatoes that are grown taller instead of wider should have increase airflow between plants, which should also decrease humidity. Fluctuations in weather, disease pressure, timing of high-tunnel venting, and plant maintenance also contributed to variation between locations.

Future studies would benefit from large-scale, adequately spaced experimental plots. Plots should be preferentially located at sites which are as similar as possible, aside from inherent differences in climate. In this study, it was not possible to inoculate the tomatoes as the high-tunnels were the property of the growers, however, disease pressure was consistently high in the locations chosen for the study. It may be beneficial to artificially inoculate tomatoes for future studies to increase the consistency of disease pressure. Additionally, assessing a wider range of tomato varieties could provide more broad-scale results.

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Appendices

A.1 Methods for the isolation of *Passalora fulva*

The isolation of *Passalora fulva* from tomato leaf mold lesions is more involved than one would infer from the information available in the tomato leaf mold literature. Isolation is complicated by the close association of *P. fulva* with *Cladosporium* spp. in the lesions of infected tomato leaves. *Cladosporium* spp. may be isolated and misidentified as *P. fulva* if proper attention is not paid to the single spore selected for the purification of an isolate. A dissecting microscope is essential for the proper selection of spores for purification. The appearance of germinated spores from *P. fulva* (A) and *Cladosporium spp.* (B) is shown in figure A1. Germinated spores of *P. fulva* have a vertical growth pattern, whereas *Cladosporium* spp. have a radial growth pattern. By comparing the growth pattern of germinated spores, a proper spore can be transferred, using a small scalpel blade, to a new agar plate for the final step of isolate purification.

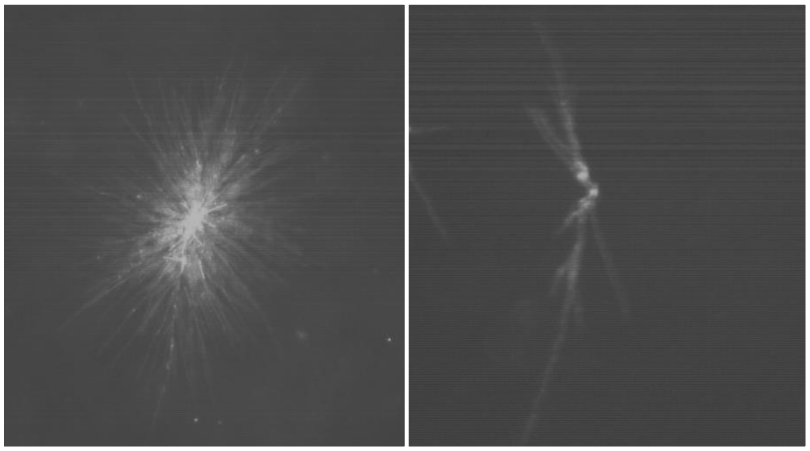

Figure A.1. Growth pattern of germinated spores from tomato leaf mold lesions. A) A germinated spore of P. fulva*, magnified at 25x exhibiting a vertical growth pattern. B) A germinated spore from* C. pseudocladosporioides*, magnified at 63x exhibiting a radial growth pattern.*

A.2 Methods for the inoculation of tomato leaves with *Passalora fulva*

Very little mention of the difficulties of successfully inoculating tomatoes with *Passalora fulva* is present in the available tomato leaf mold literature. Despite the lack of a formal discussion of these difficulties, researchers from NY, PA, and WI have been unsuccessful in their attempts to infect tomatoes with *P. fulva* (personal communication with Angela Orshinsky). The inoculation methods which were attempted as part of this research, all yielding no symptoms, are illustrated in figure A2, below. All inoculations were followed by a period of high humidity (80-100%), as is customary in the literature.

While inoculations with either *P. fulva* or *Cladosporium* spp. isolates did not result in visible symptoms on the tomato plants, there is evidence to suggest that the spores did germinate and grow within the tomato leaves. Figure A2.2 below illustrates the ability of these spores to germinate, grow within the leaves and in some cases even produce a small number of spores.

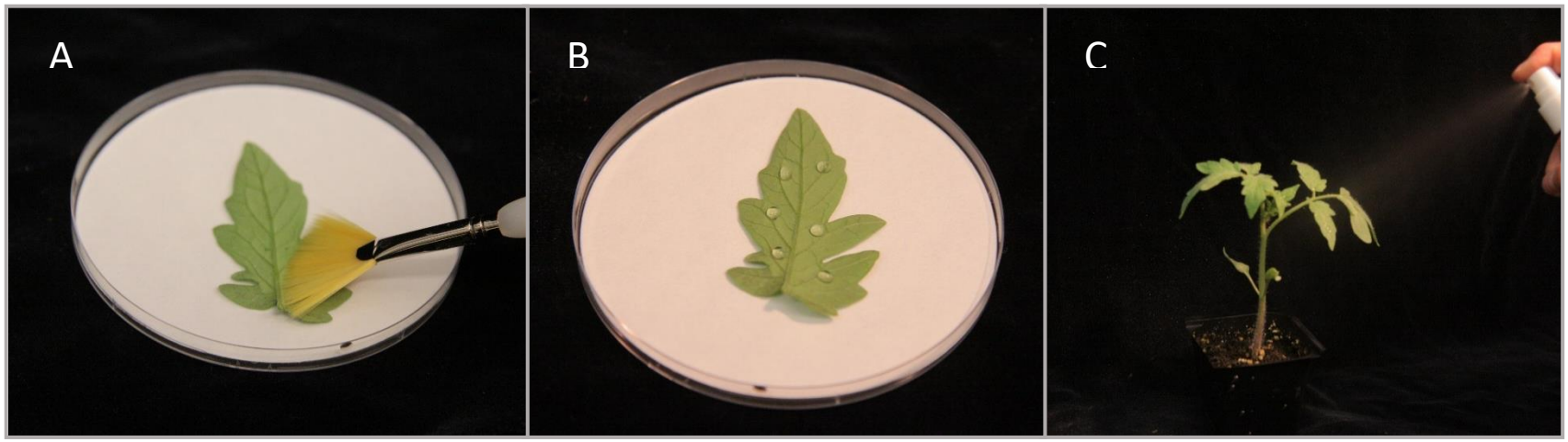

Figure A.2. Methods for the Inoculation of tomato with Passalora fulva. *A) Dry spores applied with a soft brush to the abaxial side of tomato leaves. B) Droplets of spores at a concentration of* $1x10^5$-$1x10^6$ *spores/ml applied to the abaxial side of tomato leaves. C) Spores at a concentration of* $1x10^5$-$1x10^6$ *spores/ml applied by a sprayer to both sides of all leaves on tomato plants with 2-4 true leaves. Photo credit: Angela Orshinsky.*

A.3 Fungicide spray programs

Figure A.3.1. Fungicides and biological controls used in this study (Chapter 3).

Trade name	Common name	Company	Location
Regalia®	Reynoutria sachalinensis	Marrone® Bio Innovations	Davis, CA
Kocide® 3000	Copper Hydroxide	Dupont™	Wilmington, DE
Actinovate® AG	Streptomyces lydicus WYEC 108	Valent BioSciences®	Libertyville, IL
Rootshield® WP	Trichoderma harzianum Rifai strain KRL-AG2	Bioworks®	Victor, NY
Milstop®	Potassium bicarbonate	Bioworks®	Victor, NY
OxiDate® 2.0	Hydrogen Dioxide (27.1%) and Peroxyacetic Acid (2.0%)	BioSafe Systems	Hartford, CT
Gavel® 75DF	Mancozeb (66.7%) and Zoxamide (8.3%)	Gowan®	Yuma, AZ
Switch® 62.5WG	Cyprodinil (37.5%) and Fludioxonil (25%)	Syngenta®	Greensboro, NC
Tanos®	Famoxadone (25%) and Cymoxanil (25%)	Dupont™	Wilmington, DE
Quadris Top®	18.2% Azoxystrobin and 11.4% Difenoconazole	Syngenta®	Greensboro, NC
Revus Top®	Mandipropamid (21.9%) and Difenoconazole (21.9%)	Syngenta®	Greensboro, NC

Table A.3.1. Spray programs used in field fungicide study (Chapter 3). Note that treatment 1 is the untreated control. Organic programs are listed first. FRAC codes are included where applicable.

Treatment	Application Dates	Fungicide/Biocontrol	Rate	Volume	FRAC code
Treatment 1	All	Untreated control	N/A	N/A	N/A
Treatment 2 (Organic 1)	May 23rd	Regalia (soil drench)	3 qt/100 gal	500 ml/plant	P5
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	June 13th	Actinovate (foliar)	1 tsp/ gal	20 gal/acre	N/A
	July 5th	Regalia (foliar)	1 qt/acre	20 gal/acre	P5
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	July 26th	Actinovate (foliar)	1 tsp/ gal	20 gal/acre	N/A
	August 16th	Regalia (foliar)	1 qt/acre	20 gal/acre	P5
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	August 30th	Actinovate (foliar)	1 tsp/ gal	20 gal/acre	N/A
Treatment 3 (Organic 2)	May 2nd	Rootshield (at planting)	5 oz/ 100 gal	500 ml/plant	BM02
	May 23rd	Milstop	1 tbsp/ gal	20 gal/acre	N/A
	June 13th	Oxidate 2.0	32 oz/ 100 gal	20 gal/acre	N/A
	July 5th	Milstop	1 tbsp/ gal	20 gal/acre	N/A
	July 26th	Oxidate 2.0	32 oz/ 100 gal	20 gal/acre	N/A
	August 16th	Milstop	1 tbsp/ gal	20 gal/acre	N/A
	August 30th	Oxidate 2.0	32 oz/ 100 gal	20 gal/acre	N/A
Treatment 4 (Organic 3)	June 13th	Regalia (foliar)	1 qt/acre	20 gal/acre	P5
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	July 5th	Actinovate (foliar)	1 tsp/ gal	20 gal/acre	N/A

	July 26th	Regalia (foliar)	1 qt/acre	20 gal/acre	P5
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	August 16th	Actinovate (foliar)	1 tsp/ gal	20 gal/acre	N/A
	August 30th	Regalia (foliar)	1 qt/acre	20 gal/acre	P5
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01

Treatment	**Application Dates**	**Fungicide/Biocontrol**	**Rate**	**Volume**	**FRAC code**
Treatment 5 (Organic 4)	June 13th	Milstop	1 tbsp/ gal	20 gal/acre	N/A
	July 5th	Oxidate 2.0	32 oz/ 100 gal	20 gal/acre	N/A
	July 26th	Milstop	1 tbsp/ gal	20 gal/acre	N/A
	August 16th	Oxidate 2.0	32 oz/ 100 gal	20 gal/acre	N/A
	August 30th	Milstop	1 tbsp/ gal	20 gal/acre	N/A
Treatment 6 (Conventional 1)	May 2nd	Gavel	1.5 lb/acre	20 gal/acre	M22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	May 23rd	Switch	12 oz/acre	20 gal/acre	9, 12
	June 13th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	July 5th	Switch	12 oz/acre	20 gal/acre	9, 12
	July 26th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	August 16th	Switch	12 oz/acre	20 gal/acre	9, 12
	August 30th	Gavel	1.5 lb/acre	20 gal/acre	M, 22

		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
Treatment 7 (Conventional 2)	May 2nd	Switch	12 oz/acre	20 gal/acre	9, 12
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	May 23rd	Tanos	8 oz/acre	20 gal/acre	27, 11
	June 13th	Switch	12 oz/acre	20 gal/acre	9, 12
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	July 5th	Tanos	8 oz/acre	20 gal/acre	27, 11
	July 26th	Switch	12 oz/acre	20 gal/acre	9, 12
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	August 16th	Tanos	8 oz/acre	20 gal/acre	27, 11
	August 30th	Switch	12 oz/acre	20 gal/acre	9, 12
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01

Treatment	**Application Dates**	**Fungicide/Biocontrol**	**Rate**	**Volume**	**FRAC code**
Treatment 8 (Conventional 3)	May 2nd	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	May 23rd	Quadris Top	8 fl oz/acre	20 gal/acre	3, 11
	June 13th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq	20 gal/acre	M01

			ft		
	July 5th	Quadris Top	8 fl oz/acre	20 gal/acre	3, 11
	July 26th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	August 16th	Quadris Top	8 fl oz/acre	20 gal/acre	3, 11
	August 30th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
Treatment 9 (Conventional 4)	May 23rd	Switch	12 oz/acre	20 gal/acre	9, 12
	June 13th	Revus Top	6 oz/acre	20 gal/acre	3, 40
	July 5th	Switch	12 oz/acre	20 gal/acre	9, 12
	July 26th	Revus Top	6 oz/acre	20 gal/acre	3, 40
	August 16th	Switch	12 oz/acre	20 gal/acre	9, 12
	August 30th	Revus Top	6 oz/acre	20 gal/acre	3, 40
Treatment 10 (Conventional 5)	June 13th	Quadris Top	8 fl oz/acre	20 gal/acre	3, 11
	July 5th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	July 26th	Quadris Top	8 fl oz/acre	20 gal/acre	3, 11
	August 16th	Gavel	1.5 lb/acre	20 gal/acre	M, 22
		Kocide 3000	1.5 tbsp/1000 sq ft	20 gal/acre	M01
	30-Aug	Quadris Top	8 fl oz/acre	20 gal/acre	3, 11
Treatment 11 (Conventional 6)	July 5th	Switch	12 oz/acre	20 gal/acre	9, 12
	July 26th	Revus Top	6 oz/acre	20 gal/acre	3, 40
	August 16th	Switch	12 oz/acre	20 gal/acre	9, 12

	30-Aug	Revus Top	6 oz/acre	20 gal/acre	3, 40