ABSTRACT

Title of Dissertation:	CUCURBIT DISEASE MANAGEMENT WITH REDUCED CHLOROTHALONIL AND IMPROVED UNDERSTANDING OF *PSEUDOPERONOSPORA CUBENSIS* POPULATION DYNAMICS
	Jake Gardner Jones, Doctor of Philosophy, 2020
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Research has linked chlorothalonil exposure to declines in pollinator health due to an increased likelihood of *Nosema ceranae* infection, altered gut microbial community, and a reduction in colony fitness and survival of honey bees (*Apis mellifera*). Therefore, a reduction in use of chlorothalonil, a large component of cucurbit disease management, may be needed. Without chlorothalonil, a widely used, broad-spectrum fungicide, the fungal and oomycete pathogens in cucurbit cropping systems can more quickly evolve resistance to targeted fungicides due to a limited number of efficacious modes of action and frequent sprays. *Pseudoperonospora cubensis*, the causal agent of cucurbit downy mildew, for example, has a short life cycle, experiences repeated applications of fungicides, and has a wide host range making it a

high risk for fungicide resistance development. Our research focused on the development of an alternative fungicide spray program in melons to reduce the use of chlorothalonil, identifying the fungicide insensitivities of local *P. cubensis* populations and determining the efficacy of fungicides used to manage cucurbit downy mildew, and investigating the clade-host relationship and formation of oospores in regional *P. cubensis* samples. Efficacy on two important diseases in melon, powdery mildew and gummy stem blight, can be largely maintained without chlorothalonil but anthracnose control was not adequate without the inclusion of chlorothalonil. Currently, there are a number of highly effective targeted fungicides available to growers for management of cucurbit downy mildew including oxathiapiprolin, zoxamide + chlorothalonil, chlorothalonil, and cyazofamid. Our research shows evidence of *P. cubensis* clade-host associations, with clade 1 preferentially infecting acorn and summer squash (*Cucurbita pepo*), butternut squash (*Cucurbita moschata*), and watermelon (*Citrullus lanatus*), while clade 2 preferentially infects cucumber (*Cucumis sativus*). Melons (*Cucumis melo*) and pumpkin (*Cucurbita maxima*) are hosts to both clade 1 and clade 2 *P. cubensis.* Using these findings, producers can choose the fungicide that most appropriately targets the more virulent clade 2 or less virulent clade 1 infections.

CUCURBIT DISEASE MANAGEMENT WITH REDUCED CHLOROTHALONIL AND IMPROVED UNDERSTANDING OF *PSEUDOPERONOSPORA CUBENSIS* POPULATION DYNAMICS

by

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Preface

Included in this thesis is research published as journal articles, which constitute Chapter 2 and Chapter 3, respectively.

1. Jones, J. G., Korir, R. C., Walter, T. L., and Everts, K. L. 2020. Reducing chlorothalonil use in fungicide spray programs for powdery mildew, anthracnose, and gummy stem blight in melons. Plant Dis. https://doi.org/10.1094/PDIS-04-20-0712-RE
2. Jones, J. G., Everts, K. L., McGrath, M. T., and Gugino, B. K. *under review*. Efficacy of fungicides for *Pseudoperonospora cubensis* determined using bioassays over multiple years in the Mid-Atlantic and Northeastern United States. Plant Heal. Prog.

Dedication

I would like to dedicate this work to my dogs: Bessie, Denali, and Bindi.

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I would first like to thank my advisor, Dr. Everts who gave me this opportunity. Her recommendations and advice have been extremely helpful throughout this process. I have become a much better researcher while under her guidance.

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Finally, my family and friends have supported me during my entire educational career, and I am grateful to them for pushing me, especially my mother, Lisa and wife, Courtney.

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Chapter 1: Introduction

Cucurbit production, regionally and nationally

In 2019, the United States grew $1.6 billion worth of cucurbit crops on over 156,000 hectares (USDA 2020). The Mid-Atlantic region of the U.S. is a concentrated area of production, due to their proximity to major East Coast cities. Maryland alone grows more than 2,900 hectares of cucurbits: cantaloupes and honeydew melon (*Cucumis melo*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita maxima*), summer and winter squash (*Cucurbita pepo* and *Cucurbita moschata*), and watermelon (*Citrullus lanatus*) (USDA 2017b). Nearly 25% of vegetable acreage in Maryland is devoted to cucurbit crops, making them a vital part of local economies (USDA 2017b). All cucurbit crops (except for a small amount of parthenocarpic cucumbers) are insect-pollinated, mainly by native bees (*Bombus* and *Peponapis* spp.) and honey bees (*Apis mellifera*).

Major diseases of cucurbit crops and management strategies

Production of cucurbit crops in the field exposes the plants to annual disease outbreaks (Marine et al. 2016). There are more than 200 diseases of cucurbit crops, some of the most important include powdery mildew, anthracnose, gummy stem blight, and downy mildew (McGrath 2009).

Gummy stem blight is caused by complex of three morphologically identical fungal species: *Stagonosporopsis citrulli*, *S. cucurbitacearum* (formerly *Didymella bryoniae*), and *S. caricae* (Keinath 2016a; Stewart et al. 2015). The ascomycete

pathogen was responsible for reducing the average yield of untreated watermelon to 43% of the maximum yield in research plots for a decade (Keinath and Duthie 1998). *Stagonosporopsis* spp. can reproduce on all plant parts: leaves, tendrils, pedicles, peduncles, petioles, vines, and fruit, with watermelon, honeydew melon, and cantaloupe being the most susceptible cucurbit hosts, likely due to genetic resistance in squashes (Keinath 2014a,b).

Powdery mildew is a common disease of cucurbits worldwide and is caused by the ascomycete, *Podosphaera xanthii*. Powdery mildew causes foliar lesions that reduce photosynthesis and can reduce yields (McGrath 1996). Powdery mildew severity was higher in diploid watermelons than the more widely grown triploid watermelons (Keinath and Hassell 2000). But the benefits of fungicide on yield remained important in triploid cultivars, as fungicides increased yields more than 75% compared to the untreated control in South Carolina (Keinath 2015b). If severe enough to cause loss of foliage, powdery mildew can directly threaten fruit quality via sun-scalding (Barickman et al. 2017).

One of the most important threats to watermelon fruit is anthracnose, caused by the ascomycete, *Colletotrichum orbiculare*. Anthracnose causes lesions on all aboveground plant parts, including the watermelon fruit (Keinath 2017). Maintaining fruit quality is vital, as anthracnose is included in the USDA standards for grades of watermelon. Anthracnose can cause significant losses, with an average of 46% yield reduction in the untreated control in research trials over a decade and occurring on as many as 69% of fruit in untreated research plots in a separate trial (Damicone and Pierson 2014; Keinath and Duthie 1998).

Cucurbit downy mildew is caused by the oomycete, *Pseudoperonospora cubensis* and causes foliar lesions which can result in devastating yield losses of up to 100% in untreated, early infected crops (Cohen et al. 2015). *P. cubensis* has a wide host range, with all pathotypes virulent on cucumber (Cohen et al. 2015; Holmes et al. 2015). The pathogen cannot overwinter in areas with a killing frost and its asexual sporangia are spread by wind, northward, every year from the southern U.S. (Ojiambo et al. 2011).

Disease forecasting models were designed to better schedule fungicide applications in cucurbits. The annual spread of *P. cubensis* throughout the U.S. is tracked and forecasted with the website cdm.ipmpipe.org, along with a network of sentinel plots, which are non-sprayed plots with a set of cucurbit species, monitored for the onset of cucurbit downy mildew (Holmes et al. 2015; Ojiambo et al. 2011). Melcast is another disease forecasting system and uses leaf wetness and temperature data to help growers make fungicide timing decisions to manage anthracnose and gummy stem blight (Latin and Egel 2001). Preventative fungicide sprays are key to effectively managing the diseases (Holmes et al. 2015; Keinath 2000; Keinath 2018). Fungicide sprays too early negatively impact growers' finances but sprays too late can result in a lack of disease control and yield loss (Everts et al. 2019; Keinath 2018; Ojiambo et al. 2011). Often times, gummy stem blight, powdery mildew, anthracnose, and downy mildew occur at similar times during the growing season of cucurbits. If a fungicide has activity on more than one disease, it may reduce the overall amount of fungicides applied. Challenges in finding targeted fungicides that are efficacious on the different disease is complicated further by fungicide resistance

development. For example, currently the Fungicide Resistance Action Committee (FRAC) Group 1 fungicide, thiophanate-methyl, is efficacious on anthracnose but has reduced efficacy on powdery mildew and gummy stem blight due to previous resistance development (Keinath 2015a,b; Keinath 2018). Broad-spectrum fungicides, like chlorothalonil and mancozeb, are efficacious on multiple diseases and important components of fungicide resistance management strategies as tank mix companions with fungicides that have a high risk of resistance development (Brent and Hollomon 2007; Keinath 2000; Keinath 2018).

Dependence on fungicides, specifically chlorothalonil and its effect on pollinators

Fungicides are critical to producing high yield and high-quality fruit, with 77% of watermelon acres, 93% of honeydew, and 83% of cucumber acres receiving treatment in the United States (USDA 2019). One of the most-important protectant, broad-spectrum fungicides, chlorothalonil, was the most widely used fungicide in the United States in 2012, and the 10th most applied active ingredient of all pesticides (Atwood and Jones 2017). Chlorothalonil is often used as a tank-mix with targeted, systemic fungicides to manage fungicide resistance (Hobbelen et al. 2011). It has shown efficacy on gummy stem blight, powdery mildew, anthracnose, and downy mildew (Keinath et al. 2007; Keinath 2015b, 2016, 2018). Chlorothalonil and other fungicides are applied during flowering, while honey bees are pollinating the crops, exposing them to fungicide residues. In one study, chlorothalonil was detected at a sublethal level in pollen, but infection rates were twice as high in bees that consumed chlorothalonil than those that did not, to the endoparasitic fungal pathogens *Nosema apis* and *Nosema ceranae* (Pettis et al. 2013). Another study showed the negative

effects of chlorothalonil exposure to honey bee nutrition, growth and development, social immunity, and survivability when challenged with a viral infection (O'Neal et al. 2019). The interactions of pesticides with each other, with acaricides, and with diseases needs further research as synergistic effects can increase toxicity (Johnson et al. 2013; Pettis et al. 2013; Zhu et al. 2014). The European Union recently banned chlorothalonil over its high toxicity in aquatic ecosystems and carcinogenic concerns (EFSA 2018). With increased regulatory scrutiny and risks to pollinator health, alternatives to the chemical, which was applied to nearly half the U.S. watermelon acreage in 2018, are needed (USDA 2019).

Management of downy mildew in cucumber

Fungicide sprays every 5-7 days are needed to properly manage downy mildew in cucumber and protect yield (Holmes et al. 2015). This was not always the case, however, as host resistance was very successful and the disease was managed largely without fungicides in cucumber, until 2004 (Sitterly 1972). From the 1940's to the 1960's, Barnes developed and released multiple cucumber varieties with high levels of resistance to downy mildew derived from Chinese Long and Plant Introduction (PI) 197087 varieties (Sitterly 1972). In 2004, an epidemic started in North Carolina from a new, more aggressive population of *P.* cubensis, which overcame the host-resistance of cucumbers and devastated growers in North Carolina, Delaware, Maryland, Virginia, Georgia, South Carolina, and New Jersey who were unprepared for the outbreak (Holmes et al. 2015). Late applied, ineffective fungicides, for example, mefenoxam, were sprayed and did not protect yields in a year which saw estimated losses of $20 million (Holmes et al. 2015; Wallace et al.

2020). Growers were slow to adopt new fungicide programs, but extension researchers conducted more than 140 fungicide trials over the next ten years on downy mildew in cucumber (Holmes et al. 2015). In recent years, varieties of pickling cucumbers with moderate resistance to *P. cubensis* have been commercially released, allowing growers to use an integrated pest management approach, with fungicides, to manage downy mildew (Everts et al. 2019; McGrath et al. 2018).

Management of downy mildew in other cucurbits

Before the 2004 epidemic, cucurbit downy mildew outbreaks on crop hosts other than cucumbers occurred late in the season. Late season outbreaks seldom warranted fungicide sprays in the Mid-Atlantic and Northern U.S. (Holmes et al. 2015). When downy mildew did appear on the other cucurbit crops earlier in the season and yield was threatened, it was controlled with mefenoxam, the same fungicide ineffective on the outbreak in pickling cucumbers, tank-mixed with chlorothalonil or mancozeb (Holmes et al. 2015). During this time, cucurbit downy mildew in the Southern U.S. commonly affected squash and cantaloupes and fungicide efficacy trials took place in these crops (Holmes et al. 2015). Propamocarb, was one of the fungicides recommended to squash and cantaloupe growers based on efficacy but was not widely used in the 2004 cucumber outbreak (Holmes et al. 1998). Coincidentally, propamocarb was first labeled for cucurbit downy mildew in 2004 and was one of the most efficacious fungicides used in cucumber for downy mildew, until 2012, when resistance first developed (Ojiambo and Holmes 2010; Thomas et al. 2018). Since the 2004 epidemic in cucumber, the opposite scenario is now occurring, fungicide efficacy trials occur largely in cucumber and efficacy is

conveyed to other cucurbit crops (Goldenhar and Hausbeck 2019; Keinath et al. 2019). Today, like before the epidemic, downy mildew in crops other than cucumbers often appears later in the season, sometimes not occurring at all. It also, does not always require the most efficacious fungicides to protect yields (Everts *personal communication*; Wyenandt et al. 2017). Attempts to explain the differences in virulence on different cucurbit crops and the 2004 resurgence in the U.S. of the disease have focused on a better understanding of the pathogen causing the disease, *P. cubensis*.

P. cubensis **mating types**

A study of the *P. cubensis* mating types in the U.S. found significant associations with host; the A1 mating type was found on *C. sativus* and *C. maxima*, the A2 mating type was found on *C. pepo*, *C. moschata*, and *C. lanatus*, and both A1 and A2 mating types were found on *C. melo* (Thomas et al. 2017). Israeli research suggests that *C. maxima* and *C. melo* are hosts of both mating types (Cohen et al. 2013; Thomas et al. 2017). Oospores, the survival structures of oomycetes, serve as evidence of sexual reproduction in *P. cubensis* (Cohen et al. 2011; Thomas et al. 2017). *P. cubensis* is reported to be heterothallic and hosts such as *C. melo* should be monitored for the production of oospores, since it serves as a host to both mating types (Thomas et al. 2017). Unlike in other countries, oospores remain undetected in nature in the U.S. (Cohen and Rubin 2012; Cohen et al. 2003; Zhang et al. 2012). The impacts of oospore formation in the U.S. could lead to recommendations of crop rotation and spatial separation of different cucurbit hosts to minimize the likelihood of oospore production (Cohen et al. 2013). Sexual recombination in *P. cubensis* could

also lead to broadened host range and altered fungicide sensitivities, compared to the parents (Cohen et al. 2011; Thomas et al. 2017). Researchers suggest in 2004, one of the two mating types could have been introduced to the U.S. via anthropogenic spread, perhaps sexually reproducing, and caused the epidemic on cucumbers (Cohen et al. 2011; Cohen et al. 2015; Thomas et al. 2017).

***P. cubensis* clades**

Genetics based studies of *P. cubensis* have resulted in the separation of two clades (Kitner et al. 2015; Runge et al. 2011; Wallace et al. 2020). In the U.S. a clade-host relationship exists: clade 1 was found primarily on *C. pepo*, *C. moschata*, and *C. lanatus*, while clade 2 preferentially infected *C. sativus* (Wallace et al 2020). Research from Europe also showed an association of clade 2 and *C. sativus* (Kitner et al. 2015; Runge et al. 2011). *C. melo* and *C. maxima* are hosts to both clades (Wallace et al. 2020). Historically, clade 1 samples originated mainly in North America, while those in clade 2 were indigenous to East Asia (Runge et al. 2011). Clade researchers suggest clade 2 was introduced via anthropogenic spread to the U.S. and Europe, causing epidemics in 2004 and 1984, respectively (Runge et al. 2011). Researchers have also found evidence of sexual recombination in *P. cubensis* genetics (Kitner et al. 2015; Wallace et al. 2020).

***Pseudoperonospora humuli*, a sister species**

Phylogenetic studies show *Pseudoperonospora humuli* and *P. cubensis* are closely related (Kitner et al. 2015; Runge et al. 2011). The two species are morphologically similar, with limited cross infectivity of *P. cubensis* on *Humulus*

lupulus (hop) and *P. humuli* on *C. sativus* (Kitner et al. 2015; Runge and Thines 2012). *P. humuli* perhaps gave rise to *P. cubensis* after a host jump (Runge et al. 2011). Separation of these two species based on molecular tools is now possible (Summers et al. 2015; Withers et al. 2016). A comparative genomics approach between *P. cubensis* and *P. humuli* eventually gave rise to a useful clade marker based on polymorphisms in a single copy gene from *P. cubensis* (Withers et al. 2016). This marker-based assay will be deployed in *P. cubensis* spore traps to determine the cucurbit crops at risk of infection and focus fungicides on only those cucurbit species (Rahman et al. 2020).

Importance of Extension

The Smith-Lever Act of 1914 created a Cooperative Extension Service to be associated with each land-grant institution. Extension was originally designed to disseminate research from institutions' experimental stations. The relationship between extension and clientele quickly evolved, as the clientele became more educated and had more access to information, including agricultural chemical and seed company research (Everts et al. 2012). The shift from a one-way knowledge transfer to a dialogue was quick, as extension personnel now collaborate with clientele via demonstrations and on-farm research and build relationships with information flowing both-ways (Everts et al. 2012; Holmes et al. 2015). North Carolina State University extension personnel, Holmes and Thornton were the first to identify *P. cubensis* in the 2004 epidemic, which was originally mistaken for pesticide damage on cucumbers (Holmes et al. 2015). Extension personnel in other cucurbit growing states were warned of the disease and used previously built

relationships and communication tools to spread news of the outbreak and fungicide efficacy results with growers and consultants. Since 2004, research on *P. cubensis* from extension specialists around the U.S. has greatly increased the understanding of cucurbit downy mildew. L. M. Quesada-Ocampo's research on *P. cubensis* has provided breakthroughs in genetics, diagnostic markers, and relationships with cucurbit hosts, as previously discussed (Quesada-Ocampo et al. 2012; Rahman et al. 2020; Wallace et al. 2020; Withers et al. 2016). A. P. Keinath developed a bioassay to quickly determine fungicide efficacy and disseminate results in-season (Keinath 2016b). With an extension focus, our project was developed and evolved to answer immediate applied questions and provide the groundwork for future research to improve recommendations made to cucurbit growers on disease management.

Justification

Cucurbit downy mildew was ranked among the highest priority areas by stakeholders in the Delaware and Maryland Eastern Shore pickle industry in 2005, New England Winter Squash Pest Management Strategic Plan (PMSP) in 2006, and along with anthracnose and gummy stem blight by watermelon stakeholders in Delaware, Maryland, New Jersey, and North Carolina in 2008. The devastating yield loss caused by cucurbit diseases along with the economic expense of weekly fungicide applications, drive research on management of the diseases. Identifying efficacious fungicides, those that have efficacy on more than one disease, and alternatives to chlorothalonil are vital to protecting cucurbit crops and their production. A call to protect pollinator health by both Northern and Southern IPM regions as well as growing public concern for pollinator well-being highlight the

importance of protecting pollinators and investigating the link between chlorothalonil and declines in pollinator health and survival.

The results from this dissertation will benefit cucurbit growers in the Mid-Atlantic region and other growing areas, since fungicide resistance is not a localized issue. This research will assist in other production systems that rely on a cornerstone chemical that loses its label or is forced to reduce its use. This dissertation will provide important efficacy information, an alternative fungicide program for chlorothalonil-based programs, and information on the population dynamics of *P. cubensis* in order to improve decision making by growers and consultants on which fungicides to apply.

Research objectives

The goals of this dissertation were to conduct applied field and laboratory experiments in order to address multiple issues threatening production of cucurbit crops in the Mid-Atlantic. This cross-disciplinary, multi-state approach allowed for cooperation with growers throughout the region as well as partner universities to research these important issues. The first objective was to develop an alternative fungicide spray program that reduced the use of chlorothalonil and compare the efficacy to programs that are typically reliant on chlorothalonil for control of common melon and watermelon diseases. The second objective was to identify the fungicide insensitivities of local *P. cubensis* populations and determine the efficacy of fungicides used to manage cucurbit downy mildew. The third objective was to use molecular and genetic tools to improve our understanding of *P. cubensis* population dynamics and clade-host relationships. The final objective was to disseminate our

research findings at scientific meetings, in the scientific press, and perhaps most importantly, at local extension meetings to improve the decision-making abilities of growers to manage cucurbit diseases.

Chapter 2: Reducing Chlorothalonil Use in Fungicide Spray Programs for Powdery Mildew, Anthracnose, and Gummy Stem Blight in Melons

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Abstract

Fungicides are applied to nearly 80% of U.S. melon acreage to manage the numerous foliar and fruit diseases that threaten yield. Chlorothalonil is the most widely used fungicide but has been associated with negative effects on human and bee health. We designed alternative fungicide programs to examine the impact of reducing chlorothalonil use (Bravo Weather Stik) on watermelon, cantaloupe, and honeydew melon in 2016, 2017, and 2018 in Maryland. Chlorothalonil was replaced in the tank-mix of weekly sprays of targeted fungicides, with either polyoxin D zinc salt (Oso) or an extract of *Reynoutria sachalinensis* (Regalia). Powdery mildew (PM-*Podosphaera xanthii*), gummy stem blight (GSB-*Stagonosporopsis* spp.), and anthracnose (*Colletotrichum orbiculare*) were the most prevalent diseases to occur in the three years. Replacing chlorothalonil with the biopesticides, as the tank mix component of the fungicide spray program was successful in reducing GSB and PM severity in cantaloupe, honeydew melon, and watermelon compared to the untreated control, with the exception of GSB in 2017 in cantaloupe and similar to the program including chlorothalonil in all cases, except anthracnose in watermelon. Anthracnose disease severity was not significantly reduced compared to the untreated control when chlorothalonil was replaced with the biopesticides and yields were not improved over the chlorothalonil alone treatment in any of the trials. Therefore, replacement of chlorothalonil may not fully address its loss as a fungicide resistance management tool, but efficacy can be maintained when polyoxin D is alternated with *R. sachalinensis* as a tank-mix with targeted fungicides to manage PM and GSB.

Introduction

The Cucurbitaceae family contains five vegetable crops of worldwide importance: pumpkin, squash, watermelon, melon, and cucumber, with utilized production in the United States valued over $1.6 billion in 2017 (Paris 2016; USDA 2020). Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai), honeydew (*Cucumis melo* L. Inodorus group) and cantaloupe (*Cucumis melo* L. Reticulatus group) were grown on an estimated 1,600 hectares in Maryland in 2015 (USDA 2016). Field production exposes plants to annual disease outbreaks (Marine et al. 2016). Gummy stem blight is a fungal disease that can affect the leaves, stems, and fruit of melons and watermelons and is caused by a complex of three morphologically identical species: *Stagonosporopsis citrulli* M.T. Brewer and J.E. Stewart, *S. cucurbitacearum* (Fr.) Aveskamp, Gruyter and Verkley [formerly *Didymella bryoniae* (Auersw.) Rehm], and *S. caricae* (Sydow & P. Sydow) Aveskamp, Gruyter and Verkley (Keinath 2016a; Stewart et al. 2015). Cucurbit powdery mildew is a foliar fungal disease of melon and watermelon, caused by *Podosphaera xanthii* (Castag.) Braun and Shishkoff [formerly *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Pollacci] (McGrath and Thomas 1996). Anthracnose, is a leaf, stem, and fruit disease caused by the fungal pathogen *Colletotrichum orbiculare* (Berk.) Arx. (Keinath 2016a). Anthracnose damage on fruit can directly reduce both yield and quality and is the sole disease mentioned in the United States Standards for Grades of Watermelon (Keinath 2018; USDA 2006b). These three diseases are widespread and can cause serious yield losses in cantaloupe, watermelon, and honeydew melon (Guan et al. 2013; Keinath 2000, 2014, 2015b, 2018; Wasilwa et al. 1993).

Fungicides are integral to producing high yield and high-quality fruit, with 77% of watermelon acres and 93% of honeydew acres receiving treatment in the United States (USDA 2019). Often, especially when genetic resistance and cultural control options are limited, fungicides are the main management tool used to protect crop yield but problems can arise if they are overused or misused. Fungicides from the same Fungicide Resistance Action Committee (FRAC) group have the same mode of action, and when applied in succession can lead to the development of resistance within the pathogen population, especially when single-site modes of action are used (Brent and Hollomon 2007). *Stagonosporopsis* spp. have shown insensitivity to fungicides in FRAC groups 1 (methyl benzimidazole carbamates [MBC]), 3 (demethylation inhibitors [DMI]), 7 (succinate-dehydrogenase inhibitors [SDHI]), and 11 (quinone outside inhibitors [QoI]) (Keinath 2009; Keinath and Zitter 1998; Newark et al. 2020; Thomas et al. 2012). The most important active ingredients for gummy stem blight are now in FRAC groups 12 (phenylpyrroles [PP]) and 9 (anilino pyrimidines [AP]), and 3 even with resistance detected (Keinath 2015a; Newark et al. 2020). Some *P. xanthii* populations also have resistance to FRAC groups 1, 7, 11, 13 (aza-naphthalenes), as well as FRAC group 3 (Bost 2010; Keinath 2015b; McGrath 2017; McGrath and Wyenandt 2017; Wyenandt et al., 2010). Fungicides in FRAC groups 3, 13, and U06 (phenyl-acetamide) are still used with resistance management practices, in addition to FRAC groups 50 (aryl-phenyl-ketones) and U13 (thiazolidine) to replace those lost to insensitivity (Keinath 2015b, McGrath and Sexton 2019). *C. orbiculare* has shown resistance to fungicides in FRAC group 7 (Ishii et al. 2016). FRAC group 11 fungicides currently remain the most effective

chemistries against *C. orbiculare*, along with FRAC group 1, M05 (chloronitriles) and M03 (dithiocarbamates) fungicides (Damicone and Pierson 2013; Keinath 2018).

Gummy stem blight, powdery mildew, and anthracnose infections often occur at similar times during the growing season of watermelon, cantaloupe and honeydew melon. If a fungicide has activity against more than one of them, it may reduce the overall amount of fungicides applied. A premixture of 24.1% cyprodinil (FRAC group 9) and 8.4% difenoconazole (FRAC group 3) (Inspire Super; Syngenta Crop Protection) is one of the main products used to manage gummy stem blight (Keinath 2015a). A major tool for managing powdery mildew has been 10% cyflufenamid (FRAC group U06) (Torino; Gowan Co.). Cyflufenamid was applied to 36% of honeydew acres in the United States (USDA 2019). FRAC group 11 fungicides 22.9% azoxystrobin (Quadris; Syngenta Crop Protection), 20% pyraclostrobin (Cabrio; BASF Ag Products), and 50% trifloxystrobin (Flint; Bayer Crop Science) along with the FRAC group 1 fungicide 45% thiophanate-methyl (Topsin; United Phosphorous, Inc.) were effective for management of anthracnose in watermelon (Keinath 2018). FRAC group 7 penthiopyrad 20.4% (Fontelis; Corteva Agriscience) was not effective on gummy stem blight or anthracnose but was moderately effective for management of powdery mildew in watermelon (Avenot et al. 2012; Egel and Hoke 2011; Keinath 2015b; Keinath and DuBose, 2014). FRAC group 3 tebuconazole 38.7% (Folicur; Bayer Crop Science) was not effective for management of powdery mildew in watermelon but in rotation with chlorothalonil, was effective in reducing gummy stem blight in watermelon, and moderately effective against anthracnose in watermelon either alone or as part of a rotation (Adams et al. 2015;

Keinath 2015a, 2015b; Price et al. 2013). It is important to note that some of the most heavily relied upon fungicides controlling gummy stem blight, powdery mildew, and anthracnose come from FRAC groups in which the pathogen has already developed resistance. The importance of using the fungicides in a responsible manner to minimize resistance development in the pathogen population is especially clear in these cases.

One fungicide resistance management strategy is to tank mix a broad spectrum/multisite fungicide with the targeted fungicide. Copper, mancozeb, and chlorothalonil, FRAC group M01, M03, and M05, respectively are three multisite fungicides with low risk of resistance (FRAC 2020). These fungicides are applied extensively in the United States, with 53% of watermelon acreage receiving mancozeb, 49% receiving chlorothalonil, and 12% receiving copper hydroxide (USDA 2019). Chlorothalonil was the most widely used fungicide in the United States in 2012, and the tenth most applied active ingredient of all pesticides (Atwood and Jones 2017). Chlorothalonil is efficacious on gummy stem blight, powdery mildew, and anthracnose (Keinath 2015a, 2015b, 2018; Keinath et al. 2007). All three fungicides are facing increased scrutiny due to environmental and health concerns. Repeated applications of copper products can lead to negative impacts on soil invertebrates and fungi, as well as the occurrence of phytotoxicity (Adrees et al. 2015; Chu et al. 2010; Van-Zweiten et al. 2004). Mancozeb is currently being re-evaluated in Canada due to dietary risks, occupational risks, and environmental risks to birds and small mammals all found to be unacceptable, with a proposal of cancellation for all uses of mancozeb except in greenhouse tobacco (PMRA 2020).

Certain formulations of chlorothalonil may cause eye and skin irritations in people and it was recently banned by the European Union for its high toxicity in aquatic ecosystems and carcinogenic concerns (EFSA 2018).

Chlorothalonil and other fungicides are applied during flowering, while Western honey bees (*Apis mellifera* L.) are pollinating the crops, possibly exposing them to fungicide residues. Chlorothalonil is characterized as "relatively non-toxic" to honey bees with an LD_{50} of >1,414.06 ppm (EPA 1999). The Environmental Impact Quotient for chlorothalonil includes a bee health component which shows chlorothalonil as relatively safe, compared to other fungicides (Kovach et al. 1992). In a survey of honey bee colonies pollinating field crops, including the cucurbits cucumber, pumpkin, and watermelon, chlorothalonil was detected at a mean value of 4,491.2±2,130.7 ppb (Pettis et al. 2013). Although chlorothalonil was detected at a sublethal level, the infection rates to the endoparasitic fungal pathogens *Nosema apis* and *Nosema ceranae* were twice as high in bees that consumed chlorothalonil than those that did not (Pettis et al. 2013). *Nosema* spp. can adversely affect honey bee colony health (Higes et al. 2008). Chlorothalonil has also been shown to alter the structure and function of honey bee gut bacterial communities (Kakumanu et al. 2016). In addition, honey bee larval survival can be affected by chlorothalonil, with chronic toxicity tests showing that field relevant exposure levels should not impact developing bee survival, but that synergistic toxic effects of chlorothalonil and frequently used in-hive miticides can occur (Dai et al. 2018; Zhu et al. 2014). Despite the concerns surrounding chlorothalonil and its limited availability due to manufacturing problems that have plagued chlorothalonil in recent years, growers

continue to rely on the chemical and efforts need to be taken to identify suitable alternatives for growers to adopt.

One option to manage resistance is to include biopesticides, which use living organisms, natural compounds from living organisms, or certain minerals to suppress pathogen populations (Marrone 2009). There were 366 biopesticide active ingredients registered in the United States, as of April 2016 (EPA 2018). One of the benefits of biopesticides is their generally shorter re-entry and pre-harvest restrictions compared to conventional fungicides (Marrone 2009). Some commercially available products for cucurbit production contain the active ingredients *Bacillus subtilis* (Serenade ASO; Bayer Crop Science), hydrogen dioxide (Oxidate; BioSafe Systems LLC.), potassium bicarbonate (Armicarb; Helena Chemical Company), polyoxin D zinc salt (Oso; Certis USA, LLC.), and plant extract of *Reynoutria sachalinensis* (Regalia; Marrone Bio Innovations) (Keinath 2016a; Marine et al. 2016). *R. sachalinensis* alternated with copper fungicides reduced powdery mildew in a Maryland study but was not efficacious when used to control gummy stem blight or anthracnose in a greenhouse (Keinath 2016a; Marine et al. 2016). Polyoxin D was effective in the same greenhouse trial against gummy stem blight but not against anthracnose (Keinath 2016a). We chose polyoxin D and *R. sachalinensis* as alternatives to chlorothalonil in our study because they were not implicated in the Pettis et al. 2013 study showing increased *Nosema* spp. infections in bees and are less toxic to bees than chlorothalonil (EFSA 2015; EPA 1999; USDA 2017a). The re-entry interval of the biopesticides was also appealing, with polyoxin D and *R. sachalinensis* having 4 h and chlorothalonil having 12 h. Biopesticides with lower potentials for resistance

development, like *R. sachalinensis*, with its resistance not known, can play an important role in resistance management plans, with their value not solely based upon their direct effect on disease reduction (FRAC 2020; Matheron and Porchas 2013). *R. sachalinensis* could possibly serve as a replacement in a resistance management strategy for chlorothalonil, which has a low risk of resistance development without any signs of resistance developing and is used extensively as a tank mix to protect targeted fungicide efficacy (FRAC 2020; Tucker et al. 2015). Polyoxin D has a medium risk of resistance development and resistance management is required for this biopesticide, similar to targeted synthetic fungicides and its value in a fungicide program should be placed solely on its efficacy on diseases or its use in organic operations (FRAC 2020).

The objective of this study was to evaluate the effect of reducing chlorothalonil use in fungicide rotations by replacing it with biopesticides, on the control of the fungal diseases gummy stem blight, powdery mildew, and anthracnose on field-grown melons in Maryland.

Materials and Methods

Fungicide treatments

Two rotational fungicide programs, three solo fungicides, and an untreated control were trialed in this study. The rotational programs included: Inspire Super 2.8F, active ingredient (AI) difenoconazole + cyprodinil (Syngenta Crop Protection, Greensboro, NC), Fontelis 1.67SC, AI penthiopyrad (Corteva Agriscience, Wilmington, DE), Folicur 3.6F, AI tebuconazole (Bayer Crop Science, Research

Triangle Park, NC), and Torino 0.85SC, AI cyflufenamid (Gowan Co., Yuma, AZ), tank mixed with Bravo Weather Stik 6SC, AI chlorothalonil (Syngenta Crop Protection), or tank mixed with Regalia 5%, AI plant extract of *Reynoutria sachalinensis* (Marrone Bio Innovations, Davis, CA), alternated with Oso 5%, AI polyoxin D zinc salt (Certis USA, LLC., Columbia, MD). The two rotational fungicide programs were named 'commercial standard' for the one with chlorothalonil and 'chlorothalonil alternatives' for the one with *R. sachalinensis* and polyoxin D. The solo fungicides were chlorothalonil, polyoxin D, and *R. sachalinensis* (Table 2.1).

Field experiments

Small-plot, replicated field trials were conducted in 2016, 2017, and 2018 at the University of Maryland Lower Eastern Shore Research and Education Center in Salisbury (UMD-LESREC) to evaluate the impact of fungicide programs on cucurbit diseases and yield. The soil type for all three fields was loamy sand with a history of mixed vegetable production. The fields were broadcast fertilized with 17-03-16 (N-P-K) + S(12%), Mg(1.1%), Ca(1.5%), Zn(0.05%), and B(0.14%) at 841 kg/ha in 2016 and with 785 and 757 kg/ha of 16-03-15 (N-P-K) + S(10%), Mg(1%), Ca(1.5%), Zn(0.05%), and B(0.1%) respectively, in 2017 and 2018. The experimental design for the trials was a split-plot, randomized complete block design, with 4 replications. The 6 fungicide treatments were the main plot, which were applied the length of the row, and the 3 cucurbit species were the subplot. Each trial consisted of 24 rows of raised beds covered with black polyethylene mulch, each 30.5 m long on 2.1 m centers. Three cucurbit crops were included in the experiments. The triploid watermelon

cultivar was ‘Crunchy Red’ (Harris Seeds, Rochester, NY). The cantaloupe cultivar used was ‘Athena’ (Johnny‘s Selected Seeds, Winslow, ME), which has intermediate resistance to cucurbit powdery mildew. The honeydew melon cultivar was ‘Snow Leopard’ (Johnny’s Selected Seeds). All were seeded into 72-cell flats in the greenhouse between late April and early May and allowed to grow to the two to three true-leaf stage. Prior to transplanting, seedlings were removed from the greenhouse to “harden-off.” Seedlings were treated with Admire Pro 4.6SC, AI imidacloprid (Bayer Crop Science), prior to transplanting at a rate of 2.188 ml over 12 72-cell trays to manage cucumber beetles. The cucurbit plants were spaced 0.9 m apart within the row. A total of 10 plants of each cucurbit were transplanted in one of the three 10.2 m subplots in each row. The seedlings were transplanted into the field on 31 May 2016, 31 May 2017 for the cantaloupe and honeydew melons, 16 June 2017 for the watermelon due to poor emergence in the greenhouse, and 24 May 2018. Overhead irrigation was used in combination with drip tape as needed. Fungicide treatments were applied before disease symptoms were visible in the research plots, on a weekly schedule, with a tractor mounted boom sprayer calibrated to deliver 421 liter/ha at 296 kPa, starting 30 June 2016, 3 July 2017, 20 June 2018. Six TeeJet hollow cone nozzles (D4 core, #45 disc, #50 stainless steel mesh screen, TeeJet, Chicago) were situated to form three sides of a square and allow for maximum coverage of the raised beds. Insects were managed in 2016 with Admire Pro 4.6SC at a rate of 146 ml/ha on 18 June and Warrior 1CS, AI lambda-cyhalothrin (Syngenta Crop Protection), at a rate of 256 ml/ha on 21 June. Insecticides applied in 2017 were Admire Pro 4.6SC (146 ml/ha) on 14 July and Sniper 2EC, AI bifenthrin (Loveland Products, Inc.

Greely, CO) at a rate of 365 ml/ha on 19 July. Sniper 2EC (365 ml/ha) was applied on 8 June 2018.

Data collection

Disease epidemics resulted from natural inoculum and varied in severity among years. Gummy stem blight (GSB) was visually rated for disease severity on a 100-point scale. Three ratings were made per plot, after examining the stems and leaves of plants in three, 1m sections. Anthracnose was visually rated for severity on a 100-point scale, as well. Three anthracnose ratings were made per plot, on the stem, leaves, and fruit of the plants in three, 1m sections. Powdery mildew was rated visually as percent of leaf surface with the signs of the disease on a 100-point scale. Five, similarly aged leaves were rated per plot, separately for the adaxial and abaxial sides. Ratings were performed approximately weekly depending on if the diseases were present between 1 August and 25 August 2016, 20 July and 6 September 2017, and 19 July and 31 August 2018. Yield was recorded as number and weight of melon fruits. Harvests were performed as needed between 28 July and 8 September 2016, 24 July and 18 September 2017, and 23 July and 4 September 2018.

Rainfall for May, June, July, August, and September was 13.59, 26.19, 6.65, 12.65, and 30.30 cm, respectively, in 2016, 14.05, 5.54, 23.27, 32.13, and 4.17 cm, respectively, in 2017, and 31.60, 14.76, 12.67, 4.11, and 21.44 cm, respectively, in 2018. The 30-year average rainfall in Salisbury, MD for May, June, July, August, and September is 9.19, 9.42, 11.13, 11.25, and 10.11 cm, respectively (National Weather Service 2020). Rainfall was higher than the 30-year average in all cases except, July 2016, June and September 2017, and August 2018. During the three years of

experiments, rainfall between May and September was 38.28, 28.06, and 33.48 cm above the 30-year average in 2016, 2017, and 2018, respectively.

Statistical analyses

Area under the disease progress curve (AUDPC) values were calculated for powdery mildew on the abaxial (lower leaf) surface, using mean severity values of the five leaves per plot. Without sufficient ratings to create AUDPC values for GSB and anthracnose, critical disease dates were selected for comparison directly before the diseases started to kill the untreated control plots. The season total number of fruit per plot as well as the total weight of fruit were analyzed for yield effects of the fungicide programs. Model fit and data transformation were evaluated based on residual plots. PM AUDPC, number of fruit, and fruit weight values did not need transformation before analysis, which was done with SAS PROC GLIMMIX (SAS Studio, SAS, Inc., Cary, NC). Anthracnose and GSB mean severity values from the critical dates were transformed by adding a constant value of one before specifying a 'lognormal' distribution in PROC GLIMMIX. Fungicide treatment, year, and crop were fixed effects and block-by-year and block-by-year within treatment were random effects in PROC GLIMMIX. When treatment-by-year or treatment-by-crop interactions were significant, block-by-treatment was the random effect, and the 'by' option in PROC GLIMMIX was used to compare treatments within years, or within crops. LS-Means were separated using Tukey Test at $P = 0.05$.

Results

Anthracnose symptoms were not widely observed in cantaloupe and honeydew melon and therefore only rated in the watermelon plots in our study. Treatment-by-year interactions were not significant ($P = 0.1481$) but treatment had a significant effect ($P < 0.0001$) (Table 2.2). *R. sachalinensis* and the chlorothalonil alternatives treatment were both similar to the untreated control. Polyoxin D and chlorothalonil were significantly better than the untreated control, reducing anthracnose severity 86% and 96%, respectively. The commercial standard treatment provided the best level of control for anthracnose, with mean severity below 1%.

Powdery mildew symptoms were highly variable between years in our study. Treatment-by-year and treatment-by-crop interactions were significant ($P = 0.0002$ and $P = 0.0026$, respectively) (Table 2.3). Therefore, treatments were compared within crop and years not across. Powdery mildew in cantaloupe remained at very low severity in 2016 and 2018, and treatment effect was not significant with $P = 0.4013$ and $P = 0.2964$, respectively. In 2017, treatment effect was significant ($P < 0.0001$) as the disease was much more severe in cantaloupe that year. *R. sachalinensis*, polyoxin D, and chlorothalonil were all statistically similar to the untreated control. The commercial standard and chlorothalonil alternatives treatments significantly reduced powdery mildew AUDPC by over 99% compared to the untreated control.

Powdery mildew in watermelon did not occur at a sufficient level in 2016 to permit rating. In both 2017 and 2018, treatment effect was significant ($P = 0.0039$ and $P = 0.0006$, respectively) (Table 2.3). The untreated control treatment AUDPC

was highest in 2017 and was statistically similar to polyoxin D and chlorothalonil. *R. sachalinensis* and the commercial standard provided statistically significant control and were similar to all treatments except the untreated control. The lowest AUDPC powdery mildew value was the chlorothalonil alternatives treatment, significantly reducing AUDPC by 75% compared to the untreated control. In 2018, the untreated control again had the highest AUDPC value, with *R. sachalinensis* and chlorothalonil statistically similar to the untreated control. Polyoxin D and the commercial standard treatments significantly reduced AUDPC to an intermediate level, with the chlorothalonil alternatives providing the greatest reduction in AUDPC at 76%, compared to the untreated control.

Honeydew melon was the most susceptible crop to powdery mildew and was rated all three years, with treatments significantly different in 2016, 2017, and 2018 at $P < 0.0001$, $P = 0.0011$, and $P < 0.0001$, respectively (Table 2.3). In 2016, *R. sachalinensis*, chlorothalonil, chlorothalonil alternatives, and the commercial standard significantly reduced AUDPC between 75% and 95%, compared to the untreated control and were significantly lower than the untreated control and polyoxin D. In 2017, *R. sachalinensis*, polyoxin D, and chlorothalonil were similar to the control. Chlorothalonil alternatives and the commercial standard treatment significantly reduced AUDPC by 65% and 66%, respectively, compared to the untreated control. In 2018, chlorothalonil had the numerically highest powdery mildew AUDPC, similar to the untreated control. The untreated control was also similar to *R. sachalinensis* and polyoxin D. The commercial standard and chlorothalonil alternatives treatments

provided the greatest reduction in AUDPC relative to the control at 85% and 94%, respectively.

Gummy stem blight severity was variable among years in our study, with significant treatment-by-year and treatment-by-crop interactions ($P = 0.0001$ and $P = 0.0088$, respectively) (Table 2.4). Therefore, treatments were again compared within crop and years not across. Treatment effects on GSB severity in cantaloupe were significant in 2016 and 2017 ($P = 0.0003$ and $P = 0.0052$, respectively) but not in 2018 ($P = 0.1036$). In 2016, the untreated control was similar to *R. sachalinensis* and chlorothalonil. Similar to chlorothalonil, but with significantly lower severity than the untreated control were polyoxin D, chlorothalonil alternatives and commercial standard treatments, which reduced GSB severity compared to the untreated control by 81% to 89%. In 2017, *R. sachalinensis* had the numerically highest GSB severity, similar to the untreated control, polyoxin D, and the chlorothalonil alternatives treatments. Only the commercial standard treatment was statistically lower than the untreated control, providing a 56% reduction in GSB severity.

Gummy stem blight severity in watermelon was on average the lowest among the three crops, with treatment effects significant in 2016 ($P = 0.0002$) and 2017 ($P = 0.0006$) but not in 2018 ($P = 0.8256$) (Table 2.4). In 2016, the untreated control treatment was similar only to *R. sachalinensis*. Polyoxin D provided intermediate reduction in GSB severity, compared to the untreated control. Chlorothalonil alternatives, chlorothalonil, and the commercial standard treatments reduced GSB severity between 90% and 94%, compared to the untreated control. In 2017, *R. sachalinensis* had the numerically highest GSB severity and was similar only to the

untreated control. The untreated control was also similar to chlorothalonil and the commercial standard treatments. Only polyoxin D and chlorothalonil alternatives were different from the untreated control and reduced GSB severity 86%.

Honeydew melon was the most susceptible crop to GSB all three years, with treatment effects significant in 2016 and 2017 but not 2018 at P <0.0001, P <0.0001, and P = 0.3743, respectively (Table 2.4). In 2016, *R. sachalinensis* was the only treatment similar to the untreated control. Polyoxin D, chlorothalonil, chlorothalonil alternatives, and commercial standard treatments all performed significantly better than the untreated control, with GSB severity reductions ranging from 62% to 89%. The 2017 trial year had the highest GSB severity of the study and again *R. sachalinensis* was similar to the untreated control treatment. Polyoxin D and chlorothalonil provided an intermediate level of reduction in GSB severity of 71% and 75%, respectively, compared to the untreated control. Chlorothalonil alternatives and the commercial standard treatment provided a GSB severity reduction of 93% and 94%, respectively.

Treatment-by-year interactions were significant for number of fruit (P <0.0001) in cantaloupe, and treatment effects were significant in 2017 on the number of fruit (P <0.0001), but not 2016 (P = 0.3619) and 2018 (P = 0.0998) (Table 2.5). Cantaloupe treated with polyoxin D, chlorothalonil, chlorothalonil alternatives, and commercial standard had the highest number of fruit, between 45% and 68% more compared to the untreated control. Treatment-by-year interactions were significant on total weight (P <0.0001) in cantaloupe, while treatment effects were not significant in 2016 (P =0.1188) but were significant in 2017 (P = 0.0077) and 2018 (P <0.0001)

(Table 2.5). In 2017, the commercial standard was similar to chlorothalonil, chlorothalonil alternatives, and polyoxin D and increased total weight by 32% compared to the untreated control. The untreated control and *R. sachalinensis* had the lowest total weight in 2017 but in 2018 along with polyoxin D and chlorothalonil had the highest. The commercial standard and chlorothalonil alternatives had the lowest total weights, a reduction of 33% and 42%, respectively compared to the untreated control, in 2018.

Treatment-by-year interactions were significant for the number of fruit (P = 0.0120) and total weight (P = 0.0176) in watermelon. Treatment effects were not significant in 2016 (P = 0.0825) or 2018 (P =0.8247), but were in 2017 (P = 0.0002) on the number of watermelon fruit (Table 2.6). The commercial standard, chlorothalonil, and polyoxin D had significantly higher numbers of fruit compared to the untreated control, with increases ranging between 180-200% in 2017. Chlorothalonil alternatives and *R. sachalinensis* were both similar to the untreated control. Treatment effects were significant in 2017 (P = 0.0003) but not 2016 (P = 0.0503) or 2018 (P = 0.4105) for the total weight of watermelon harvested (Table 2.6). Similar to the number of fruit, chlorothalonil, the commercial standard, and polyoxin D were significantly better than the untreated control, with increases of 205%, 159%, and 147%, respectively in 2017 on the total weight of watermelon fruit. *R. sachalinensis* and chlorothalonil alternatives were similar to the untreated control in total weight.

Treatment-by-year interactions were again significant for the number of fruit (P = 0.0002) and total weight (P = 0.0007) in honeydew melon. Treatment effects

were significant in 2017 (P <0.0001) but not in 2016 (P = 0.1907) or 2018 (P = 0.8506) on the number of fruit (Table 2.7). The commercial standard had the most fruit per plot, a significant increase of 113% compared to the lowest yielding untreated control in 2017. Chlorothalonil alternatives had an intermediate number of fruit, similar to polyoxin D and chlorothalonil, but greater than *R. sachalinensis* and the untreated control. Treatment effects were significant on total weight of honeydew melon fruit in 2017 (P <0.0001) but not 2016 (P = 0.3996) or 2018 (P = 0.3336) (Table 2.7). The commercial standard treatment had the highest total weight in 2017, similar to the chlorothalonil alternatives treatment. The commercial standard and chlorothalonil alternatives treatments increased the total weight in 2017 compared to the untreated control by 50% and 26%, respectively. Chlorothalonil, polyoxin D, and *R. sachalinensis* were similar to the untreated control.

Discussion

Replacing chlorothalonil with the biopesticides polyoxin D and *R. sachalinensis*, as the tank mix component of the fungicide spray program was successful in reducing gummy stem blight and powdery mildew severity in cantaloupe, honeydew melon, and watermelon compared to the untreated control, with one exception of gummy stem blight in 2017 in cantaloupe. Anthracnose disease severity was not significantly reduced compared to the untreated control when chlorothalonil was replaced with the biopesticides. Growers and regulators may seek to reduce chlorothalonil use in the future due to environmental, human health, or pollinator health concerns and replacement with biopesticides appears to be a viable option for gummy stem blight and powdery mildew, but not anthracnose control.

However, the chlorothalonil alternatives treatment did not improve yields over the chlorothalonil alone treatment in any of the crops or years. This is in agreement with multiple other studies in cucurbits where biopesticides did not improve yield as part of a fungicide program (Marine et al. 2016; Rideout et al. 2010; Zhang et al. 2011).

Chlorothalonil is a contact fungicide and proper spray coverage is vital to disease control. With less fungicide coverage on the abaxial leaf surface, control of powdery mildew with chlorothalonil was limited and similar to the untreated control. When paired with a powdery mildew targeted fungicide, chlorothalonil will help control the disease and assist in fungicide resistance management (Coolong and Seebold 2011). Chlorothalonil reduced gummy stem blight severity compared to the untreated control in all cases except in honeydew melon in 2018. These results of an often-intermediate level of gummy stem blight reduction are in agreement with a study in South Carolina (Keinath 2015a). Chlorothalonil was very effective in controlling anthracnose and improving watermelon yield in our study, similar to another study in South Carolina (Keinath 2018). In that study, chlorothalonil provided similar control to mancozeb while in a study in Oklahoma, mancozeb was more effective than chlorothalonil (Damicone and Pierson 2013; Keinath 2018). Mancozeb was also effective in controlling gummy stem blight on cantaloupe seedlings in a greenhouse trial and could be used as an alternative to chlorothalonil in a tank mix to prevent anthracnose and gummy stem blight (Keinath 2016a), however mancozeb is not labeled for powdery mildew.

The performance of the two biopesticides in our study can be evaluated using different metrics. Polyoxin D has a medium risk of resistance development and must

be valued solely on efficacy, while *R. sachalinensis* can have its value based on both its efficacy and as a component of a resistance management strategy (FRAC 2020; Matheron and Porchas 2013). In our study we observed an 86% reduction of anthracnose severity with polyoxin D but no disease reduction occurred with *R. sachalinensis*, similar to a study by Keinath in 2016, who saw limited efficacy with both products in the greenhouse. *R. sachalinensis* was not effective in reducing anthracnose or gummy stem blight severity and was similar to the untreated control in all cases. Powdery mildew control by *R. sachalinensis* was irregular but a 63% reduction in 2017 watermelon and 75% reduction in 2016 honeydew melon was observed compared to the untreated control. *R. sachalinensis* was not effective in years with higher powdery mildew severity in our study, which was similar to greenhouse trials in South Carolina, where *R. sachalinensis* efficacy faltered under higher gummy stem blight severity (Keinath 2016a). *R. sachalinensis* did provide intermediate powdery mildew control in greenhouse cucumbers under high disease pressure in Greece (Konstantinidou-Doltsinis and Schmitt 1998) though, as Milsana (Marrone Bio Innovations). Sakalia, AI *R. sachalinensis* (Syngenta Crop Protection) was combined with the wetting agent Yuccah, a plant extract from *Yucca schidigera* (Plant Health Care, Inc. Pittsburg, PA) and provided the best control of powdery mildew among the biopesticides tested in Sweden (Rur et al. 2018). Control of powdery mildew with polyoxin D was variable, although in 2018 in watermelon and honeydew melon it was more effective than chlorothalonil alone. In a study in Mississippi, chlorothalonil outperformed polyoxin D in reducing powdery mildew on pumpkin, differing from our study with the exception of 2016 in honeydew melon,

likely due to their method of rating of all leaf tissue not just the underside of leaves as we did (Barickman et al. 2017). Complementing biopesticides by choosing resistant crop varieties is an integrated pest management approach. The cantaloupe variety we chose, which had an intermediate resistance to powdery mildew, kept the disease to a minimum in two of the three trial years.

The targeted chemicals used in our trial were not from FRAC group 1 or 11, the most effective chemistries for anthracnose (Damicone and Pierson 2013; Keinath 2018). Some of the targeted fungicides were from FRAC group 3 (tebuconazole and difenoconazole) and 9 (cyprodinil), groups that contain the most effective fungicides for gummy stem blight and powdery mildew (Keinath 2015b). This helps explain the poor performance of the chlorothalonil alternatives treatment on anthracnose. In addition, the difenoconazole and cyprodinil mixture could be responsible for the majority of the gummy stem blight reduction, as seen in a study in South Carolina (Keinath 2015a). It is difficult to estimate the efficacy of the targeted synthetic fungicides, as they were not included as separate treatments.

The commercial standard treatment provided the greatest reduction or was similar to the treatment with the greatest reduction in disease severity of gummy stem blight, powdery mildew, and anthracnose in each of the crops. The yield effects were also consistent, providing yields that were the highest or similar to the highest treatment in every crop and year, except cantaloupe in 2018. The chlorothalonil alternatives treatment also protected yields and was similar to the commercial standard in all cases, except the number of honeydew melons harvested in 2017. This high level of disease control and yield protection supports the recommendations

found on most fungicide labels, to tank mix targeted fungicides and protectant fungicides and rotate FRAC groups. The economic effect of replacing the lower-cost chlorothalonil with a biopesticide was not taken into consideration.

Anthracnose and gummy stem blight foliar symptoms are similar and can be misidentified (Kalischuk et al. 2018; Zitter 1992). An issue that arose during the trials, was the difficulty in separating gummy stem blight and anthracnose, which might have affected the precision with which the efficacy of the fungicides was determined, especially late in the season (Keinath 2018). By choosing critical disease dates to compare, instead of season long averages, we avoided including late season ratings, where the diseases were more frequently confused due to the coalescence of symptoms. Similarly, growers need to accurately identify the disease before choosing which fungicides to apply, preferably as preventative applications for maximum efficacy. Anthracnose can cause significant damage to watermelon fruits, reducing quality and overall yield (Keinath 2018). In our study, yield was recorded as number and weight of healthy fruits. The number and weight of cull fruit was not recorded, which could have provided valuable information on the control of anthracnose symptoms on fruits.

In conclusion, the substitution of biopesticides for chlorothalonil can maintain efficacy on gummy stem blight and powdery mildew but was ineffective at controlling anthracnose in watermelon. Biopesticide efficacy can be variable under field conditions and biopesticides should be part of a tank mix, not stand-alone sprays. Polyoxin D provided intermediate disease control and *R. sachalinensis* provided little to no control in most cases, with the exception of powdery mildew,

although *R. sachalinensis* may be useful as part of a resistance management strategy. The causation link between exposure of chlorothalonil and *Nosema* spp. infection in bees needs further investigation. Farmers should be proactive and take steps to protect pollinators, such as spraying chlorothalonil containing mixtures after sunset, when bees are not active, in order to limit exposure.

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Table 2.1. Fungicide treatments applied to watermelon, cantaloupe, and honeydew melon to manage gummy stem blight, powdery mildew, and anthracnose.

Treatment	Active Ingredient	FRAC [a] code	Rate (liter/ha)	Application schedule 2016	2017	2018
Untreated Control	-	-	-	-	-	-
Commercial Standard	Difenoconazole + Cyprodinil + Chlorothalonil	3 + 9 M5	1.46 2.34	1,3	1,3,8,10	1,3,7,9
Chlorothalonil Alternatives	Penthiopyrad + Chlorothalonil	7 M5	1.17 2.34	4,6	4,6	4,6
	Tebuconazole + Chlorothalonil	3 M5	0.58 2.34	2	2,9	2,8
	Cyflufenamid + Chlorothalonil	U6 M5	0.25 2.34	5,7	5,7	5
	Difenoconazole + Cyprodinil + *Reynoutria sachalinensis*	3 + 9 P5	1.46 9.35	1,3	1,3,8,10	1,3,7,9
	Penthiopyrad + *Reynoutria sachalinensis*	7 P5	1.17 9.35	4,6	4,6	4,6
	Tebuconazole + Polyoxin D zinc salt[b]	3 19	0.58 0.95	2	2,9	2,8
	Cyflufenamid + Polyoxin D zinc salt	U6 19	0.25 0.95	5,7	5,7	5
Chlorothalonil	Chlorothalonil	M5	2.34	1-7	1-10	1-9
R. sachalinensis	*Reynoutria sachalinensis*	P5	9.35	1-7	1-10	1-9
Polyoxin D	Polyoxin D zinc salt	19	0.95	1-7	1-10	1-9

[a]FRAC codes from the Fungicide Resistance Action Committee.

[b]Adjuvant Silwet L-77 (Helena Chemical Co., Collier, TN) at rate 106 ml/ha used with polyoxin D treatments.

Table 2.2. Anthracnose disease severity on watermelon in 2016-2018.

Treatment	Disease Severity[w] (%)
Untreated Control	23.6 a[x]
R. sachalinensis	25.9 a
Polyoxin D	3.4 b
Chlorothalonil	0.8 bc
Chlorothalonil Alternatives[y]	15.5 a
Commercial Standard[z]	0.5 c
Treatment *P* value	<0.0001

[w]Anthracnose severity was rated visually as the percent infection of stems, leaves, and fruit of plants in three, 1m sections per plot. Diseases were rated on 14 Aug. 2016, 4 Aug. 2017, 9 Aug. 2018.

[x]Means in a column connected by the same letter are not significantly different $P = 0.05$, according to Tukey's HSD.

[y]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with either polyoxin D or *R. sachalinensis*.

[z]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with chlorothalonil.

Table 2.3. Area under disease progress curve values of powdery mildew on cantaloupe, watermelon, and honeydew melon in 2016-2018.

	Area Under Disease Progress Curve[w]								
	Cantaloupe			Watermelon			Honeydew Melon		
Treatment	2016	2017	2018	2016	2017	2018	2016	2017	2018
Untreated Control	0.2	1425.7 a[x]	1.4	0.0	272.4 a	379.9 a	194.1 a	1755.3 a	1050.9 ab
R. sachalinensis	0.0	1148.1 a	0.0	0.0	101.7 bc	263.9 ab	48.2 b	1224.5 ab	920.2 b
Polyoxin D	0.3	862.4 a	0.0	0.0	203.5 abc	196.4 bc	184.8 a	1612.4 a	897.0 b
Chlorothalonil	1.7	995.8 a	0.3	0.0	238.0 ab	241.4 abc	19.5 b	1324.4 ab	1554.4 a
Chlorothalonil Alternatives[y]	0.0	0.6 b	0.0	0.0	67.4 c	92.3 c	24.9 b	623.2 b	59.7 c
Commercial Standard[z]	2.1	2.4 b	0.0	0.0	85.5 bc	138.5 bc	9.9 b	590.7 b	158.8 c
Treatment *P* value	0.4013	<0.0001	0.2964	N/A	00.0039	0.0006	<0.0001	0.0011	<0.0001

[w]Powdery mildew severity was rated visually as the percent infection on the lower surface of five leaves per plot. Area Under Disease Progress Curves were calculated from four severity ratings in 2016 (zero on watermelon), five ratings in 2017, and five ratings for watermelon and honeydew melon and four ratings for cantaloupe in 2018.

[x]Means in a column connected by the same letter are not significantly different $P = 0.05$, according to Tukey's HSD.

[y]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with either polyoxin D or *R. sachalinensis*.

[z]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with chlorothalonil.

Table 2.4. Gummy stem blight disease severity on cantaloupe, watermelon, and honeydew melon under field conditions in 2016-2018.

	Disease Severity[w] (%)								
	Cantaloupe			Watermelon			Honeydew Melon		
Treatment	2016	2017	2018	2016	2017	2018	2016	2017	2018
Untreated Control	7.0 a[x]	6.8 ab	3.4	8.0 a	1.4 ab	0.1	11.8 a	39.2 a	3.3
R. sachalinensis	5.8 a	8.1 a	1.5	6.0 ab	2.5 a	0.2	10.8 ab	26.2 a	2.9
Polyoxin D	1.3 b	4.7 abc	2.1	2.3 bc	0.2 c	0.1	4.5 c	11.2 b	1.9
Chlorothalonil	2.6 ab	3.7 bc	0.8	0.5 c	0.5 bc	0.1	4.0 bc	9.9 b	2.5
Chlorothalonil Alternatives[y]	1.1 b	4.2 abc	3.9	0.8 c	0.2 c	0.3	2.8 c	2.8 c	0.5
Commercial Standard[z]	0.8 b	3.0 c	2.0	0.5 c	0.8 abc	0.2	1.3 c	2.4 c	1.8
Treatment *P* value	0.0003	0.0052	0.1036	0.0002	0.0006	0.8256	<0.001	<0.001	0.3743

[w]Gummy stem blight severity was rated visually as the percent infection of stems and leaves of plants in three, 1m sections per plot. Diseases were rated on 1 Aug. 2016, 27 July 2017, 19 July 2018.

[x]Means in a column connected by the same letter are not significantly different $P = 0.05$, according to Tukey's HSD.

[y]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with either polyoxin D or *R. sachalinensis*.

[z]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with chlorothalonil.

Table 2.5. Effects of fungicides on cantaloupe yield in 2016-2018.

	Cantaloupe					
	Number Fruit/plot[v]			Fruit Weight/plot[w](kg)		
Treatment	2016	2017	2018	2016	2017	2018
Untreated Control	18	22 c[x]	21	43.4	44.4 b	49.4 a
R. sachalinensis	18	24 bc	17	45.4	45.4 b	43.5 ab
Polyoxin D	20	33 a	19	50.5	55.5 ab	43.7 a
Chlorothalonil	19	32 ab	17	44.8	56.2 ab	41.1 ab
Chlorothalonil Alternatives[y]	16	34 a	16	35.4	55.4 ab	28.6 c
Commercial Standard[z]	19	37 a	17	43.6	58.7 a	33.0 bc
Treatment *P* value	0.3619	0.0001	0.0998	0.1188	0.0077	<0.0001

[v]Plots consisted of ten plants and were 10.2 m in length.

[w]Season total fruit weight harvested per plot.

[x]Means in a column connected by the same letter are not significantly different $P = 0.05$, according to Tukey's HSD.

[y]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with either polyoxin D or *R. sachalinensis*.

[z]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with chlorothalonil.

Table 2.6. Effects of fungicides on watermelon yield in 2016-2018.

	Watermelon					
	Number Fruit/plot[v]			Fruit Weight/plot[w](kg)		
Treatment	2016	2017	2018	2016	2017	2018
Untreated Control	19	5 c	17	104.1	22.9 c	98.4
R. sachalinensis	12	8 bc	16	73.2	32.8 bc	95.8
Polyoxin D	18	14 ab	17	108.1	59.2 ab	100.4
Chlorothalonil	24	15 a	17	141.1	69.8 a	107.5
Chlorothalonil Alternatives[y]	15	10 abc	17	94.7	37.9 bc	98.0
Commercial Standard[z]	21	15 a	17	125.4	56.5 ab	108.3
Treatment *P* value	0.0825	0.0002	0.8247	0.0503	0.0003	0.4105

[v]Plots consisted of ten plants and were 10.2 m in length.

[w]Season total fruit weight harvested per plot.

[x]Means in a column connected by the same letter are not significantly different $P = 0.05$, according to Tukey's HSD.

[y]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with either polyoxin D or *R. sachalinensis*.

[z]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with chlorothalonil.

Table 2.7. Effects of fungicides on honeydew melon yield in 2016-2018.

	Honeydew Melon					
	Number Fruit/plot[v]			Fruit Weight/plot[w](kg)		
Treatment	2016	2017	2018	2016	2017	2018
Untreated Control	32	30 c	37	37.9	39.3 c	33.4
R. sachalinensis	38	32 c	36	41.6	40.0 bc	34.3
Polyoxin D	43	37 bc	38	46.0	43.1 bc	34.1
Chlorothalonil	37	40 bc	41	43.3	43.2 bc	38.5
Chlorothalonil Alternatives[y]	46	48 b	38	49.0	49.6 ab	30.2
Commercial Standard[z]	49	64 a	39	50.3	58.8 a	30.7
Treatment *P* value	0.1907	<0.0001	0.8506	0.3996	<0.0001	0.3336

[v]Plots consisted of ten plants and were 10.2 m in length.

[w]Season total fruit weight harvested per plot.

[x]Means in a column connected by the same letter are not significantly different $P = 0.05$, according to Tukey's HSD.

[y]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with either polyoxin D or *R. sachalinensis*.

[z]Difenoconazole + cyprodinil, penthiopyrad, tebuconazole, cyflufenamid, each rotated and tank mixed with chlorothalonil.

Chapter 3: Efficacy of Fungicides for *Pseudoperonospora cubensis* Determined using Bioassays over Multiple Years in the Mid-Atlantic and Northeastern United States

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Abstract

In the United States, fungicides are the primary management option for cucumber growers to protect their crops from *Pseudoperonospora cubensis*, the causal agent of downy mildew. Fungicide resistance in some fungicide classes can quickly develop with the repeated applications needed to protect yield. In order to identify the most effective fungicides and monitor fungicide resistance, nine bioassays were conducted from 2016 to 2019 in Delaware, Maryland, Pennsylvania, and New York. Potted cucumber plants were sprayed with fungicides or not treated, placed next to field-grown plants with downy mildew for up to 2 days, then kept in a greenhouse until symptoms developed. Severity of symptoms was compared to determine fungicide efficacy. Azoxystrobin (Quadris) was ineffective in seven of the nine bioassays, while mandipropamid (Revus) was ineffective in six of seven bioassays. Dimethomorph (Forum) and fluopicolide (Presidio) were ineffective in three of eight and four of nine bioassays, respectively. The most effective fungicides were chlorothalonil (Bravo), zoxamide + chlorothalonil (Zing!), and oxathiapiprolin (Orondis), all of which consistently suppressed disease severity more than 90% when compared with the untreated control. Propamocarb (Previcur Flex) and cyazofamid (Ranman) were also effective in every bioassay.

Introduction

Cucurbit downy mildew is one of the most significant diseases of an important crop group in the United States. Cucurbit crops including cucumber (*Cucumis sativus*), pumpkin and squash (*Cucurbita maxima, C. pepo, C. moschata*), watermelon (*Citrullus lanatus*), and melon (*Cucumis melo*) were grown on over

156,000 hectares in the U.S. and had a utilized production value of more than $1.6 billion in 2019 (USDA 2020). An annual threat to production is the foliar disease cucurbit downy mildew, caused by *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev. Epidemics have occurred annually in the Eastern U.S. since 2004, when a more aggressive population of *P. cubensis* overcame the host-resistance of cucumbers and devastated growers (Holmes et al. 2015). Commercially available, partially resistant cucumber cultivars were only recently released (Adams et al. 2020a,b; Everts et al. 2019, Keinath 2019; McGrath et al. 2018). Therefore, fungicides became the primary tool to manage cucurbit downy mildew and were applied to 83% of cucumber acreage in the U.S. in 2018 (Cohen et al. 2015; Holmes et al. 2015; USDA 2019; Wyenandt et al. 2017). Fungicide resistance is a major concern as the most effective fungicides are at risk due to their single site mode of action with any one active ingredient applied between two and four times to a cucumber crop to manage downy mildew, thereby increasing selection pressure on *P. cubensis* populations for resistant mutants (Brent and Hollomon 2007). Additionally, *P. cubensis* spreads throughout the Eastern U.S. via wind-dispersed spores, thus the pathogen experiences more selection events than just those in a crop. For example, fluopicolide (Presidio) and propamocarb (Previcur Flex) were first labeled for cucurbit downy mildew in 2007 and 2004, respectively, and were among the most efficacious fungicides in the U.S., until reduced efficacy was observed starting in 2012 and resistance confirmed in 2018 (Keinath 2016b; Langston and Sanders 2013; Ojiambo et al. 2010; Thomas et al. 2018). Fungicides with novel modes of action, such as oxathiapiprolin (Orondis) have been released but have not kept pace with the

loss of older chemistries (Salas et al. 2019). Rotation of Fungicide Resistance Action Committee (FRAC) groups, that numerically group active ingredients by modes of action, helps reduce selection pressure on *P. cubensis* to develop resistance. Newer efficacious chemicals including ethaboxam (Elumin), fluazinam (Omega), and oxathiapiprolin have not shown apparent signs of resistance within the pathogen populations (Thomas et al. 2018). Fluazinam, mancozeb + zoxamide (Gavel), cyazofamid (Ranman), and oxathiapiprolin were highly effective in reducing cucurbit downy mildew severity in multiple trials across Ohio, New York, and South Carolina (Keinath et al. 2019). While in Michigan, ethaboxam, cyazofamid, ametoctradin + dimethomorph (Zampro), mancozeb + zoxamide, mancozeb (Koverall), chlorothalonil (Bravo), and oxathiapiprolin, oxathiapiprolin + chlorothalonil (Orondis Opti), and oxathiapiprolin + mandipropamid (Orondis Ultra) were all effective (Goldenhar and Hausbeck 2019). However, rotating the most efficacious active ingredients can be expensive for a grower and the development of fungicide resistance within *P. cubensis* populations is well documented (Olaya et al. 2009; Thomas et al. 2018). Incorporation of host-resistant cultivars into downy mildew management strategies in cucumber allowed for lower efficacy fungicides to result in higher yields than the most efficacious fungicides applied to the most susceptible cultivars (Call et al. 2013). In New Jersey, moderate and lower efficacy fungicides provided adequate control of downy mildew in winter squash (Wyenandt et al. 2017). *P. cubensis* population dynamics and movement are important to the efficacy of fungicides. In the Mid-Atlantic and Northeast, cucurbit downy mildew generally spreads northward from states to the south, as host availability increases. Host

availability, rather than favorable weather conditions or dispersal of inoculum, is the main constraint for long distance spread (Ojiambo et al. 2015).

Population studies have divided *P. cubensis* into two clades and although each clade can infect different cucurbit hosts, host-preference is apparent with clade 1 mostly found infecting pumpkins, squash, and watermelons, while clade 2 occurs mainly on cucumbers and cantaloupe (Crandall et al. 2018; Wallace et al. 2020). The limited number of U.S. samples prior to 2004 from cucumber align closely with the clade 1 grouping, and post 2004 align with clade 2 (Runge et al. 2011; Kitner et al. 2015). Pre-epidemic clade 2 samples originate in East Asia, suggesting clade 2 is indigenous to that region (Runge et al. 2011). Clade 2 is now the prominent genotype in the post 1984 and 2004 epidemics infecting cucumbers in Europe and the U.S., respectively (Runge et al. 2011; Kitner et al. 2015; Wallace et al. 2020). It has been hypothesized that the 2004 epidemic was caused by a new biotype of *P. cubensis* introduced to the U.S. that was resistant to mefenoxam and strobilurin fungicides, as well as virulent on the previously resistant cucumbers (Holmes et al. 2015). It is possible this new and aggressive pathotype in 2004, was clade 2 *P. cubensis* (Runge et al. 2011).

Materials and Methods

Fungicide bioassays

A bioassay was developed to determine the efficacy of fungicides on cucurbit downy mildew (Keinath 2016b) and has been successfully used on different *P. cubensis* populations from multiple states over multiple years (Keinath et al. 2019).

The speed and ease of the bioassay are key benefits: it can be completed in as little as four weeks, from seeding to data collection, it can be repeated multiple times during the growing season, and a large number of fungicides can be included for testing. Variability in fungicide efficacy has been observed both in time and location using the bioassay (Keinath 2016b; Keinath et al. 2019). The objective of this study was to determine the efficacy of select fungicides on cucurbit downy mildew, monitor for insensitivities to the fungicides, and compare the results across multiple states and years to examine variability of fungicide efficacy across the Mid-Atlantic and Northeast Regions.

Nine bioassays were successfully completed across four states (Delaware, Maryland, Pennsylvania, and New York) over four years 2016-2019 (Table 3.1). 'Silver Slicer' cucumber was used, which has no resistance to *P. cubensis* but is resistant to powdery mildew caused by *Podosphaera xanthii*. Resistance to *P. xanthii* reduced competition on the leaf surface for infection by *P. cubensis* and helped avoid confusion when rating disease symptoms. Greenhouse grown cucumber seedlings with two to three true leaves, in 10 cm square pots, were arranged in a randomized complete block design, with four replications. Each replication consisted of single seedlings in pots receiving either fungicide or water in the untreated controls (Table 3.2). Fungicides were mixed in 200 ml water at full label rates. Fungicide applications were made to leaves with a backpack sprayer (New York and Pennsylvania) or a handheld spray bottle (Delaware and Maryland) until runoff. The following day, 12 to 24 hours post-treatment, seedlings were exposed to natural inoculum by placement in downy mildew infected cucumber research plots or a commercial cucumber field. The

growing tip of each seedling was removed to slow the senescence of the treated leaves. There were two water control seedlings for each replication in case one was damaged, rendering that replicate set of treatments invalid. Seedlings were arranged in replications and left in the field for 24 to 48 hours to allow for infection to occur before they were returned to the greenhouse for 7 to 14 days to allow for symptoms to develop.

Data collection and analysis

Disease severity was rated on a scale of 0-100% in New York and Pennsylvania by estimating percent coverage of disease symptoms on the second leaf, while lesions were counted on the second leaf in Delaware and Maryland. Relative disease severity (RDS) was calculated as percent severity in fungicide treated plants divided by average percent severity of the water control plants in the same replication (Keinath 2016b). Relative disease severity was used as opposed to disease severity to reduce the variability in disease pressure due to timing or location (Keinath et al. 2019). Fungicides were considered effective when RDS values were below a threshold of 35% (Thomas et al. 2018). Relative disease severity values were transformed by adding a constant value of one before specifying a 'lognormal' distribution in PROC GLIMMIX. Fungicide treatment, date, and state were considered fixed effects and block nested within state or date was a random effect in PROC GLIMMIX. The 'by' statement was used to analyze RDS within state and year. LS-Means were separated using Student's T LSD P =0.05. The water control treatment was excluded from the datasets used in analyses to reduce the inequality of variances.

Results and Discussion

Azoxystrobin (Quadris) was ineffective in seven of the nine bioassays, but even when it was effective in 2016 Delaware and 2017 Berks County Pennsylvania, its RDS was close to the 35% cutoff at 34.5% and 31.4%, respectively (Table 3.3). Resistance and consequent poor efficacy of azoxystrobin is well known and widely reported (Ishii et al. 2001; Keinath 2016b; Keinath et al. 2019; Miller et al. 2020). Mandipropamid (Revus) and dimethomorph (Forum) are both FRAC Group 40 fungicides (carboxylic acid amides [CAA]) and were ineffective in six out of seven and three out of eight bioassays they were included in, respectively (Table 3.3). Mandipropamid was previously reported as ineffective at controlling downy mildew in cucumber (Goldenhar and Hausbeck 2019; Keinath 2016b, Keinath et al. 2019, Salas et al. 2019). Dimethomorph performed better in our bioassays than in field trials in Michigan, Ohio, and South Carolina (Goldenhar and Hausbeck 2019; Keinath et al. 2019; Miller et al. 2020). But dimethomorph response in our bioassays was similar to that in bioassays conducted in South Carolina and Ohio (Keinath 2016b; Miller et al. 2020), where dimethomorph insensitivity was observed less often than insensitivity to mandipropamid. Ametoctradin + dimethomorph was only ineffective in one bioassay out of the eight it was included in and was similar to the most efficacious products in three of the eight bioassays. Ametoctradin + dimethomorph was among the most efficacious products on cucurbit downy mildew in Michigan but in separate studies was ineffective in Ohio and South Carolina (Goldenhar and Hausbeck 2019; Keinath et al. 2019). Ametoctradin + dimethomorph was effective in two of the three bioassays that dimethomorph was ineffective, reducing RDS from 37.0% to 13.6% in

Maryland in 2017 and 69.9% to 0% in Berks County Pennsylvania in 2017, compared to dimethomorph alone.

Fluopicolide was included in all nine bioassays and was ineffective in New York for three consecutive years in 2017-2019, as well as in Maryland in 2019 (Table 3.3). Failure to reduce cucurbit downy mildew severity has also been seen in South Carolina and Michigan (Goldenhar and Hausbeck 2019; Keinath et al. 2019). Propamocarb was effective in our bioassays (Table 3.3), albeit close to the 35% threshold in 2017 in both locations in Pennsylvania and in New York, although it was ineffective in an earlier field trial in Pennsylvania (Gugino and Grove 2016). Variation from year to year in the efficacy of propamocarb has been observed in South Carolina and Ohio but not in Michigan, where propamocarb was ineffective over three consecutive years (Baysal-Gurel et al. 2015; Goldenhar and Hausbeck 2019; Keinath 2016b; Keinath et al. 2019). Resistance to fluopicolide and propamocarb has been found in approximately 65% and 26%, respectively, of samples collected in the eastern U.S. from as early as 2008 (Thomas et al. 2018). Seven of the 31 isolates from Thomas et al. 2018, showed multiple resistance to the unrelated fungicides, fluopicolide (FRAC group 43) and propamocarb (FRAC group 28). Even so, a combination of fluopicolide + propamocarb was effective when used either as a preventative or curative treatment by Salas et al. 2019.

Cymoxanil (Curzate) was included in all nine bioassays and was effective seven times, except 2017 New York, when its RDS was 36.0%, just above the effective RDS value, and 2019 Maryland when its RDS was 41.0% (Table 3.3). Similar results of mixed efficacy were seen in Keinath et al. 2019, where cymoxanil

was ineffective in three of the six trials. But reports of cymoxanil failing to control cucurbit downy mildew are common, with two out of three years in Michigan and half of the trials in South Carolina not reducing severity compared to the untreated control and the majority of isolates screened in the Czech Republic from 2005-2010 were resistant to cymoxanil (Goldenhar and Hausbeck 2019; Keinath 2016b; Pavelková et al. 2014).

Zoxamide was only examined as a standalone treatment in two trials and was highly effective in 2018 but ineffective in 2019 in New York (Table 3.3). In a separate study, zoxamide reduced cucurbit downy mildew severity in New York and South Carolina in 2017 (Keinath et al. 2019). Zoxamide is marketed in premixtures with mancozeb or chlorothalonil. Zoxamide + mancozeb was among the most effective fungicides in 50% of the trials in New York, South Carolina, and Ohio, and 100% of the trials in Michigan, but was not included in our bioassays (Goldenhar and Hausbeck 2019; Keinath et al. 2019). Zoxamide + chlorothalonil (Zing!) was included in seven of the nine bioassays in our study and was similar to the fungicide with the lowest RDS or had the lowest RDS six times (Table 3.3). Zoxamide + chlorothalonil was effective in North Carolina and one of the most effective fungicides in a trial in Michigan (Adams et al. 2019; Hausbeck et al. 2017).

Chlorothalonil as a standalone treatment was consistently effective and included in eight of the nine bioassays (Table 3.3). It was similar to the fungicide with the lowest RDS or had the lowest RDS in all bioassays except 2019 in New York, when it had an RDS value of 7.3%. Chlorothalonil was effective in both New York and South Carolina in 2015 and 2017 but ineffective in Ohio both years

(Keinath et al. 2019). Chlorothalonil was among the most efficacious fungicides in a separate study in Ohio and seven of eight bioassays in South Carolina (Goldenhar and Hausbeck 2019; Keinath 2016b). Chlorothalonil and mancozeb, which was not included in this study but is efficacious on cucurbit downy mildew, are both broad spectrum fungicides (FRAC group M05 and M03, respectively) with a low risk of resistance development (FRAC 2020; Keinath 2016b). Their roles in tank mixes help maximize the effective life of higher risk fungicides (Hobbelen et al. 2011). Chlorothalonil and mancozeb are protectant fungicides and efficacious if applied with proper spray coverage and before infection occurs, but when applied season-long, both control of downy mildew and protection of cucumber yield becomes inadequate (Colucci et al. 2007; Adams et al. 2020a,c). Therefore, mixtures with newer chemistries that are single-site inhibitors are common, in order to improve disease management and reduce the risk of fungicide resistance.

Oxathiapiprolin was included in six of the nine trials and had the lowest RDS in five of the six bioassays (Table 3.3). The average RDS for oxathiapiprolin across all bioassays was 0.1%. Oxathiapiprolin was also among the most efficacious fungicides in Michigan, New York, Ohio, North Carolina, and Delaware (Adams et al. 2020b; Goldenhar and Hausbeck 2019; Keinath et al. 2019; Miller et al. 2020; Salas et al. 2019). Oxathiapiprolin is a FRAC group 49 fungicide with medium to high resistance risk (FRAC 2020). In order to manage resistance development, it is only marketed as a premixture in the U.S.

Cyazofamid was included in every bioassay and was consistently efficacious with RDS grouping with the most efficacious fungicides in six of the nine bioassays

(Table 3.3). These results are similar to those seen across multiple states and years (Adams et al. 2020b; Goldenhar and Hausbeck 2019; Keinath 2016b; Keinath et al. 2019).

Ethaboxam was the most recently registered product tested and was highly effective in the single bioassay in which it was included. In Michigan, ethaboxam was among the most effective fungicides in two out of three years and significantly better than the untreated control the third year (Goldenhar and Hausbeck 2019). In North Carolina, ethaboxam was effective but not grouped with the most effective fungicides, but in Ohio was among the best treatments (Adams et al. 2019, 2020b; Miller et al. 2020). Isolates screened by Thomas et al. 2018 were highly sensitive to ethaboxam, although baseline sensitivity varied depending on where the samples were collected.

Fluazinam was only included in two bioassays but was highly effective in Maryland in 2019 and effective in New York in 2019 (Table 3.3). Fluazinam was among the most effective fungicides in New York, Ohio, South Carolina, North Carolina, and Michigan (Adams et al. 2020b; Goldenhar and Hausbeck 2019; Keinath et al. 2019).

Active ingredients from 11 different FRAC groups were evaluated for efficacy in the nine bioassays conducted in four states. Oxathiapiprolin, zoxamide + chlorothalonil, chlorothalonil, and cyazofamid were among the most effective fungicides in the majority of bioassays across all states (Table 3.4). While azoxystrobin, mandipropamid, fluopicolide, dimethomorph, and cymoxanil were all ineffective in more than one bioassay (Table 3.4). Treatments at half the label rates of

fungicides were also included in our bioassays to monitor for insensitivity of *P. cubensis* to the fungicides. Ametoctradin + dimethomorph, dimethomorph, cymoxanil, and chlorothalonil all performed significantly worse at the half rate than at the full rate in one of the nine bioassays and the half rate of propamocarb performed worse in two bioassays (data not shown). Rotating among the most efficacious fungicides and mixing with multisite fungicides is expected to provide the best control and protect the efficacy of single-site fungicides over time. The cucurbit hosts with the most acreage vary among the states in our study, with Maryland and Delaware acreage dominated by cucumbers and watermelon and Pennsylvania and New York acreage dominated by squash and pumpkins. Host availability plays an important role in the spread of cucurbit downy mildew and the population dynamics of *P. cubensis*. The availability of host resistance in commercial cucumber cultivars provides growers with an additional tool and a more integrated approach to manage downy mildew. Bioassays provide a fast and easy way to determine the efficacy of fungicides in-season and can be used to make in-season decisions, as well as see trends in efficacy over time.

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Table 3.1. Locations and dates of cucumber bioassay experiments used to evaluate the efficacy of fungicides applied preventatively on cucurbit downy mildew.

Year	State	County	Exposed	Rated
2016	Delaware	Sussex	8/03/16	8/10/16
2016	New York	Suffolk	9/15/16	9/29/16
2017	New York	Suffolk	8/26/17	9/7/17
2017	Pennsylvania	Berks	8/29/17	9/8/17
2017	Pennsylvania	Blair	8/16/17	8/25/17
2017	Maryland	Wicomico	8/1/17	8/11/17
2018	New York	Suffolk	9/15/18	9/25/18
2019	New York	Suffolk	9/13/19	9/23/19
2019	Maryland	Wicomico	8/16/19	8/27/19

Table 3.2. List of fungicides evaluated for control of cucurbit downy mildew across four states.

Active Ingredient	Trade Name	FRAC[a] Code	Full Rate (per hectare)[b]
Chlorothalonil 82.5%	Bravo Ultrex 82.5WDG	M5	1.57 kg
Cymoxanil 60%	Curzate 50DF	27	0.35 kg
Ethaboxam 42.5%	Elumin 4SC	22	0.58 L
Dimethomorph 43.5%	Forum 4.17SC	40	0.44 L
Fluazinam 40%	Omega 500F	29	1.75 L
Oxathiapiprolin 10.2 - 18.7%	Orondis Formulations[c]	49	0.15-0.67 L
Propamocarb 66.5%	Previcur Flex 6SL	28	1.40 L
Fluopicolide 39.5%	Presidio 4SC	43	0.29 L
Azoxystrobin 22.9%	Quadris 2.08F	11	1.13 L
Cyazofamid 34.5%	Ranman 400SC	21	0.20 L
Mandipropamid 23.3%	Revus 2.08SC	40	0.58 L
Ametoctradin 26.9% + dimethomorph 20.2%	Zampro 525SC	45 + 40	1.02 L
Zoxamide 6.8% + chlorothalonil 40%	Zing! 4.9SC	22 + M5	2.63 L
Zoxamide	Zoxamide (Technical Grade)	22	400 ppm

[a]Fungicide Resistance Action Committee.

[b]Maximum rate given on fungicide label for use on cucurbit downy mildew.

[c]Orondis Gold 200SC (18.7% oxathiapiprolin) in New York, Orondis Opti A 0.83OD (10.2% oxathiapiprolin) in Pennsylvania, and Plenaris 200FS (18.7% oxathiapiprolin) in Maryland.

Table 3.3. Relative downy mildew severity values for fungicides tested on cucumbers in bioassays across four states and four years.

	2016		2017				2018	2019	
Fungicide	DE	NY	MD	NY	PA[v]	PA[w]	NY	MD	NY
Azoxystrobin	34.5a[x]	70.7a	35.7ab	85.7ab	86.2a	31.4ab	107.5a	117.5a	88.2ab
Mandipropamid	13.5b	81.5a	105.9a	75.8abc	ND[y]	ND	62.3a	44.2bc	122.7a
Fluopicolide	0c	13.0b	4.1de	99.6a	17.2ab	1.1c	66.0a	76.5ab	36.2bc
Dimethomorph	ND	74.7a	37.0abc	22.2cde	13.4bc	69.9a	4.0b	0.8d	30.8bc
Ametoctradin + dimethomorph	16.9ab	59.4a	13.6bcd	20.9de	7.0bcd	0c	1.6bc	11.0c	ND
Cymoxanil	3.1c	3.2bc	2.6de	36.0bcd	7.6bcd	28.4a	1.0bc	41.0c	0.6f
Zoxamide	ND	ND	ND	ND	ND	ND	0c	ND	44.3ab
Zoxamide + chlorothalonil	0c	7.5b	2.6cde	7.6ef	0d	0c	ND	0d	ND
Chlorothalonil	ND	0.3c	6.5cde	4.2f	5.6cd	8.3bc	0.8bc	0d	7.3de
Propamocarb	0c	4.1b	ND	30.7cde	26.1ab	27.9a	1.9bc	0d	4.5ef
Fluazinam	ND	ND	ND	ND	ND	ND	ND	0d	24.2de
Cyazofamid	0c	10.3b	2.3de	21.9de	1.1cd	8.3bc	0.2c	0d	13.3cd
Ethaboxam	ND	ND	ND	ND	ND	ND	ND	0d	ND
Oxathiapiprolin[z]	ND	ND	0e	ND	0d	0c	0.7bc	0d	0f
Fungicide p value	<0.0001	<0.0001	0.0013	0.0001	0.0008	0.0015	<0.0001	<0.0001	<0.0001

[v]Blair County, Pennsylvania

[w]Berks County, Pennsylvania

[x]State mean by year within each column followed by the same letter are not significantly different, Student's T LSD, *P*=0.05.

[y]ND = no data.

[z]Orondis Gold 200SC in NY, Orondis Opti A 0.83OD in PA, and Plenaris 200FS in MD.

Table 3.4. Ranking of fungicides for effectiveness in reducing *Pseudoperonospora cubensis* infection across nine bioassays in four states.

	2016		2017				2018	2019	
Fungicide	DE	NY	MD	NY	PA[a]	PA[b]	NY	MD	NY
Azoxystrobin	E	I	I	I	I	E	I	I	I
Mandipropamid	E	I	I	I	ND	ND	I	I	I
Fluopicolide	*	E	H	I	E	H	I	I	I
Dimethomorph	ND	I	I	E	E	I	E	H	E
Cymoxanil	H	H	H	I	H	E	H	I	H
Zoxamide	ND	ND	ND	ND	ND	ND	*	ND	I
Ametoctradin + dimethomorph	E	I	E	E	H	*	H	E	ND
Propamocarb	*	E	ND	E	E	E	H	*	H
Fluazinam	ND	ND	ND	ND	ND	ND	ND	*	E
Cyazofamid	*	E	H	E	H	H	H	*	E
Chlorothalonil	ND	*	H	*	H	H	H	*	E
Zoxamide + chlorothalonil	*	E	H	H	*	*	ND	*	ND
Ethaboxam	ND	ND	ND	ND	ND	ND	ND	*	ND
Oxathiapiprolin[c]	ND	ND	*	ND	*	*	H	*	*

[a]Blair County, Pennsylvania

[b]Berks County, Pennsylvania

[c]Orondis Gold 200SC in NY, Orondis Opti A 0.83OD in PA, and Plenaris 200FS in MD.

I = RDS >35% (Thomas et al. 2018).

E = RDS <35% but significantly higher than treatment with the lowest RDS.

H = highly effective, RDS similar to best treatment.

* = lowest RDS in the bioassay.

ND = no data.

Chapter 4: Biology and Population Dynamics of *Pseudoperonospora cubensis* in the Mid-Atlantic United States

Abstract

The foliar disease cucurbit downy mildew caused by *Pseudoperonospora cubensis*, threatens cucurbit crops worldwide but is especially challenging to manage in cucumbers. In 2004, the host resistance of pickling cucumbers was overcome by a more virulent strain of *P. cubensis* than had existed previously in the United States. Post-2004, research to better understand the pathogen, as well as fungicide use to manage the disease, has increased dramatically. In this research, we sought to improve the understanding of *P. cubensis* populations in the Mid-Atlantic by establishing clade-host relationships and examining the possibility of sexual reproduction by this pathogen. A qPCR assay that detects specific clades of *P. cubensis* was used to examine samples from across the region. The separation of *P. cubensis* clades helps researchers and extension personnel better understand the differences in virulence based on cucurbit host and make targeted fungicide recommendations. The clade 1 genotype was identified from diseased samples of *Cucurbita pepo*, *Cucurbita moschata*, and *Citrullus lanatus*, while the clade 2 genotype was identified from diseased samples of *Cucumis sativus*. Both *P. cubensis* clades were identified from diseased samples of *Cucurbita maxima* and *Cucumis melo*. Oospores were observed in infected *C. moschata* samples, the first known naturally formed oospores described from the U.S. Inoculation experiments with oospores did not yield any disease symptoms, in agreement with Thomas et al. 2017. A fluorescent *in situ* hybridization assay was developed to visualize *P. cubensis in*

planta, but high levels of background fluorescence and non-specific staining precluded distinct visualization of the pathogen structures. Together these results show that *P. cubensis* clades preferentially infect certain cucurbit hosts and oospores play a limited role in current epidemics.

Introduction

Pseudoperonospora cubensis (Berk. & M.A. Curtis) Rostovzev, is an oomycete pathogen that causes the foliar disease cucurbit downy mildew (Savory et al. 2011). Over 60 species and 20 genera of Cucurbitaceae are hosts of *P. cubensis* (Lebeda et al. 2016). In the United States, over 156,000 hectares of cucurbits are grown, with a value of more than $1.6 billion (USDA 2020). The cucurbit crops grown include: *Cucumis sativus* (cucumber), *Cucumis melo* (cantaloupe), *Citrullus lanatus* (watermelon), *Cucurbita maxima* (pumpkin), *Cucurbita pepo* (acorn and summer squash), and *Cucurbita moschata* (butternut squash). Prior to 2004, cucurbit downy mildew was effectively controlled with host resistance in cucumber and minimal fungicide inputs (Holmes et al. 2015). The resurgence of cucurbit downy mildew in 2004 led to widespread crop losses. For example, pickling cucumber yield per hectare in 2003-2004 versus 2005-2006 showed a reduction of 8.4% nationwide (Holmes et al. 2015). Cucumber acreage was reduced 19% between 2003 and 2008 in the U.S., in part due to disease management challenges associated with downy mildew driving growers to raise different crops (USDA 2006a; USDA 2009). Similar outbreaks of downy mildew in cucurbits that previously were not impacted by the disease have occurred in Israel and Europe (Cohen and Rubin 2012; Kitner et al. 2015).

The resurgence of cucurbit downy mildew was caused by a breakdown of the host resistance derived from Plant Introduction (PI) 197087 (Holmes et al. 2015; Sitterly 1972). Partially resistant commercial cucumber cultivars were only recently released (Adams et al. 2020; Everts et al. 2019, Keinath 2019; McGrath et al. 2018). As a result, following the epidemic onset in 2004, fungicides became the primary management tool for cucurbit downy mildew and were applied to 83% of cucumber acreage in the U.S. in 2018 (Cohen et al. 2015; Holmes et al. 2015; USDA 2019). Fungicide efficacy trials help inform growers on which fungicides to spray and when, based on efficacy results and the cost of the fungicide relative to the crop (Everts et al. 2019, Jones et al. *under review*; Keinath et al. 2019). The resistant cucumber line PI 197088 was found to achieve the highest yields with lower fungicide inputs compared to the most susceptible cucumber varieties with the highest fungicide inputs in a North Carolina study (Call et al. 2013). In New Jersey, lower efficacy fungicides were successful in managing downy mildew in squash, but not in cucumber (Wyenandt et al. 2017). The timing of initial downy mildew outbreaks varies with host, as seen in the cucurbit downy mildew forecasting and tracking network (cdm.ipmpipe.org), where cucumbers account for the majority of early reports and other cucurbit hosts are often reported later in the season.

Two lines of research attempt to explain the observed differences in *P. cubensis* virulence and host specificity that led to the resurgences of cucurbit downy mildew: mating type and genetic differentiation of *P. cubensis* into two phylogenetic clades (Cohen et al. 2003; Kitner et al. 2015; Lebeda and Cohen 2011; Thomas et al. 2017; Wallace et al. 2020). Mating type research suggests that *P. cubensis* is a

heterothallic organism with two mating types, A1 and A2 (Cohen and Rubin 2012). Mating type association with host was found in both Israel and the U.S., with the A1 mating type primarily associated with *Cucumis* spp. and the A2 mating type primarily associated with *Cucurbita* spp. (Cohen et al. 2013; Thomas et al. 2017). In the U.S., *C. melo* hosted both *P. cubensis* mating types (Thomas et al. 2017). A regional association was observed with the A1 mating type, found in the Northern U.S. only, while both A1 and A2 mating types were found in the Southern U.S. (Thomas et al. 2017). It is unknown how widespread the observed distribution of mating type is, as the relatively small sample size (40 samples) could explain the regional associations, especially since all but two from the Northern region were collected from *C. sativus*, while 17 of the samples in the Southern region were collected from hosts other than *C. sativus* (Thomas et al. 2017).

Phylogenetic and population genetic analyses has subdivided *P. cubensis* into two major genetic groups, clade 1 and clade 2 (Runge et al. 2011; Kitner et al. 2015; Wallace et al. 2020). Separation of *P. cubensis* clades into two species, with clade 1 referred to as *P. cubensis sensu stricto* and clade 2 as *P. cubensis* cryptic species, has been suggested but researchers have yet to make the definitive statement that the two clades are separate species (Runge et al. 2011; Wallace et al. 2020). Runge et al. 2011, showed that clade 1 originated in North America, while the more virulent clade 2 was indigenous to East Asia and was possibly introduced to Europe and the U.S., causing the epidemics on *C. sativus.* Kitner et al. 2015 discovered an association with clade 1 isolates on hosts other than *C. sativus*, while clade 2 isolates were associated with *C. sativus*. Similar to the 2004 epidemic in the U.S. but with different hosts,

2009 in the Czech Republic was the first year that *P. cubensis* caused epidemics on *Cucurbita* spp. and *C. lanatus* (Kitner et al. 2015). A study with 385 samples from North Carolina identified two distinct genetic clades of *P. cubensis* (Wallace et al. 2020). An association of the two different genetic clades was observed according to cucurbit host: *P. cubensis* clade 1 was primarily collected from *Cucurbita* spp. and *Citrullus* sp. (87.4 and 88.9%, respectively) and *P. cubensis* clade 2 was primarily collected from *Cucumis* spp. (88.4%) (Wallace et al. 2020). At a species level, *C. melo* was host to a mixture of *P. cubensis* clade 1 and clade 2 isolates, along with *C. maxima* and *Cucurbita foetidissima* (buffalo gourd), suggesting host-clade association is in fact at the species level not the genus level (Wallace et al. 2020).

Phylogenetic lineages of *P. cubensis* show a close relationship with *Pseudoperonospora humuli* (hop downy mildew pathogen) (Kitner et al. 2015; Runge et al. 2011). Considered sister species, *P. humuli* perhaps gave rise to *P. cubensis* after a host jump from hops to cucurbits (Runge et al. 2011). Similarity in morphology and internal transcribed spacer (ITS) sequence between *P. humuli* and *P. cubensis* led Choi et al. 2005 to reduce *P. humuli* to a synonym of *P. cubensis*. This was later reversed and *P. humuli* remains a separate species with a narrower host range than *P. cubensis*, but cross infectivity of *P. humuli* on *C. sativus* and *P. cubensis* on *Humulus lupulus* (hop) has been reported (Crandall et al. 2018; Runge et al. 2011; Runge and Thines 2012). Molecular tools and diagnostic markers were developed to separate *P. humuli* from *P. cubensis* (Rahman et al. 2020; Summers et al. 2015; Wallace and Quesada-Ocampo 2017; Withers et al. 2016). Putative single copy genes c2555.2e1 and c2555.3e7 were found solely in *P. cubensis* and the

polymorphism of c2555.3e7 among different cucurbit hosts was subsequently used to create a clade qPCR assay (Rahman et al. 2020). The importance of separating *P. cubensis* from *P. humuli*, one of which readily produces oospores (*P. humuli*) and the other with no reports of oospores occurring naturally in the U.S. (*P. cubensis*) became apparent in our research (Cohen et al. 2015; Gent et al. 2017; Mitchell et al. 2011).

Oospores are the sexual spores produced by oomycetes. These structures typically have thick outer walls, allowing for extended survival in the absence of hosts (Cohen and Rubin 2012). The production of oospores by *P. cubensis* is rare, with reports mainly from Europe and Asia (Cohen and Rubin 2012; Thomas et al. 2017; Zhang et al. 2012). There are no reports of naturally occurring oospores in the U.S. (Thomas et al. 2017). Thomas et al. 2017 determined that 40% of the *P. cubensis* oospores produced *in vitro* were viable using the plasmolysis method. However, Thomas et al. 2017 was unable to cause infection with the oospores in inoculation experiments, similar to the very low infectivity rate found in Israel (0.2%) but much different than the 27-95% rate of infections found in China (Cohen and Rubin 2012; Zhang et al. 2012). Oospores, therefore, play an unknown role in the epidemiology of *P. cubensis* (Ojiambo et al. 2015; Zhang et al. 2012). Disease forecasting models focus on the airborne spread of *P. cubensis* sporangia, in the absence of oospores (Ojiambo et al. 2015). Genetic variability is another consequence of the sexually produced oospores, with a higher potential to overcome host resistance (McDonald and Linde 2002).

Sexual reproduction resulting in oospores occurs in heterothallic organisms when mycelia from both mating types grow in close proximity. However, oospores

can also be formed from the exposure to hormones from other species, due to the interspecies universality of mating hormones (Tomura et al. 2017). Recombination of *P. cubensis* isolates from *C. pepo, C. moschata, C. lanatus, Momordica charantia,* and *Lagenaria siceraria* was found and could be explained by a homothallic clade 2 and heterothallic clade 1, although further research is needed (Wallace et al. 2020). Morphological descriptions of *P. cubensis* oospores are available but infection and sporulation of *P. cubensis* following oospore inoculation or molecular tools to identify the oospores as *P. cubensis* were needed (Cohen and Rubin 2012; Thomas et al. 2017; Zhang et al. 2012)

Use of *P. cubensis* species-specific markers in a qPCR assay is one option to identify oospores following DNA extraction (Summers et al. 2015; Rahman et al. 2020). Another option is the direct visualization of the oospores with fluorescent *in situ* hybridization (FISH). Fluorescent *in situ* hybridization was recently reported for the first time as a species-specific visualization of a downy mildew pathogen, *Plasmopara obducens*, which causes impatiens downy mildew (Salgado-Salazar et al. 2018). The basic steps of FISH protocols are: (i) fixation and permeabilization of the sample, (ii) hybridization of an organismal-specific probe, (iii) washing away excess probe, and (iv) identification/quantification of the sample by microscopy or flow cytometry (Amann and Fuchs 2008). One way to increase the sensitivity of traditional FISH is with catalyzed reporter deposition (CARD-FISH), which can detect single copy genes by allowing for the introduction of numerous fluorescent molecules at the binding site, unlike conventional probes with a single fluorophore (Amann and Fuchs 2008; Pernthaler et al. 2002). A method to increase the specificity of traditional FISH

is with use of locked nucleic acids (LNA) based FISH probes, which increase both thermal stability and binding efficiency compared to conventional DNA based probes (Thomsen et al. 2005).

The objectives of this research were to (i) establish the clade-host relationship of *P. cubensis* in the Mid-Atlantic region of the U.S., (ii) confirm the first discovery of *P. cubensis* oospores in nature in the U.S., and (iii) determine the infectivity and viability of the oospores.

Materials and Methods

Sample collection and DNA extraction

Seventy *P. cubensis* samples were collected from five states between 2016 and 2019 (Table 4.1). The majority of samples (66 of 70) originated in Maryland, Delaware, and Virginia with the remaining samples from New York and Pennsylvania (Table 4.1). Leaves that showed symptoms of cucurbit downy mildew were collected from commercial fields, research plots, and sentinel plots that were part of the cucurbit downy mildew monitoring and forecasting network. The number of samples originating on the host genera *Cucurbita* and *Cucumis* were equal at 34 each, while two samples were collected from *Citrullus*. Lesions were examined under a Zeiss Discovery V20 dissecting microscope (Carl Zeiss Microscopy, Thornwood, NY) and individual sporulating lesions were excised with a sterile scalpel and placed in microcentrifuge tubes for storage at -80ºC.

Genomic DNA was extracted from leaf lesions using the OmniPrep Genomic DNA Isolation Kit (G-BioSciences, St. Louis, MO) following the manufacturer's

instructions. DNA concentration was determined using the Qubit dsDNA BR Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA).

P. cubensis species-specific qPCR assay

An assay to differentiate *P. cubensis* isolates from *P. humuli* and cucurbit pathogens was developed. The target used in the assay was the single copy diagnostic marker c2555.2e1 described by Withers et al. 2016. The primers and probe were designed using the Beacon Designer Software from Premier Biosoft (Palo Alto, CA), BLASTN searched for specificity, and targeted a 124 bp subset of the c2555.2e1 gene fragment. The 20 µL uniplex qPCR reaction contained 1x LightCycler 480 Probes Master (Roche, San Francisco, CA), 300 nM of the forward primer, 300 nM of the reverse primer, and 175 nM of the LNA probe with the quencher, Iowa Black FQ (IABkFQ), and 5 µL of DNA. The sequences were as follows: forward primer 5'-GCTTGTCGTTGCGTATTCG-3', reverse primer 5'-GCACGTATGGCTACTCTCG-3', and antisense probe 5'-56FAM-TGT+CTA+ACT+CGT+GCTCCAGG-3IABkFQ-3'. The uniplex qPCR assay was performed using the LightCycler 480 (Roche, San Francisco, CA) and LightCycler 480 Software (Roche). The reaction profile was 5 min at 95°C for initial denaturation followed by 45 cycles of 95°C for 10 s, 62°C for 30 s, and 72°C for 1 s.

Sensitivity of the P. cubensis species-specific qPCR assay

The sensitivity and efficiency of the uniplex qPCR reaction was determined using a standard curve based on the cloned c2555.2e1 marker. Using the PCR protocol described in Withers et al. 2016, a reaction targeting c2555.2e1 was

completed. PCR products were analyzed by gel electrophoresis on a 1% agarose gel and cleaned with QIAquick PCR Purification Kit (Qiagen Sciences Inc., Germantown, MD). The gene fragment c2555.2e1 was cloned using the Topo TA Cloning Kit for Subcloning, with Top10F' *E. coli* (Thermo Fisher Scientific). Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen Sciences Inc.) and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific). Ten-fold dilutions of the plasmid were made, ranging from 10 ng to 1 fg of DNA, in triplicate allowing for calculations of Cq, standard deviation, and reaction efficiency. A QIAxcel Advanced Instrument, using QIAxcel ScreenGel Software (Qiagen Sciences Inc.) was used to examine the amplification of DNA in target and non-target organisms.

P. cubensis clade qPCR assay

P. cubensis clade determination was performed as described by Rahman et al. 2020. The 20 µL multiplex qPCR reaction contained 1x PerfeCta Multiplex qPCR ToughMix (QuantaBio, VWR International, LLC, Beverly, MA), 2 mM $MgCl_2$, 750 nM of the universal forward primer, 500 nM each of the clade 1 and clade 2 reverse primers, 100 nM each of the clade 1 and clade 2 LNA probes with the quencher, IABkFQ, and 1 µL of DNA. The sequences were as follows: the universal forward primer 5'- AACGGTGATCCATGCAGCTTTA-3', clade 1 reverse primer 5'- CGCAGTGGTTGGGTGTGT-3', clade 2 reverse primer 5'- CCATCAAGCCAGCAACTTGTT-3', clade 1 LNA probe 5'- HEX-CAG+TAGCA+TAACCCAAG+ACTTCGT-IABkFQ-3', and clade 2 LNA probe 5'-FAM-TGGTCGAGC+ATTGACAAGAGCCTATCC-IABkFQ-3'. The multiplex

qPCR assay was performed using Mic qPCR Cycler (Bio Molecular Systems, Upper Coomera, Queensland, Australia) and Mic qPCR Analysis Software (Bio Molecular Systems). The reaction profile was 5 min at 95°C for initial denaturation followed by 38 cycles of 95°C for 15 s and 65°C for 1 min. Samples were run in triplicate with known standards and non-template controls included in each run.

Oospore extraction

Initial observations of oospores occurred in butternut squash leaves in 2016. Soil and leaf samples were collected and stored at 0°C and -80°C, respectively. Oospores were extracted from soil via a modified protocol in Parker et al. 2007. Briefly, 100 cc of soil was suspended in water and washed through a series of sieves (250 µm, 88 µm, 53 µm, 32 µm, 25 µm). Material on the 32 µm and 25 µm sieves were washed into 50 ml centrifuge tubes and centrifuged at 3,500 rpm for 5 min, two times. The supernatant was removed with a pipette until a volume of 5 ml remained in the tube, to which 35 ml of 70% sucrose solution was added. The tubes were then centrifuged at 3,500 rpm for 5 min and rinsed on a 25 µm sieve. The material was washed back into the 50 ml centrifuge tube and centrifuged two more times at 3,500 rpm. All except 1 ml of supernatant and material was removed with a pipette. The remaining 1 ml was then aerated and transferred to a 1.75 ml microcentrifuge tube before centrifugation at 6,500 rpm for 5 min. Supernatant was removed with a pipette to 250 µL with subsamples examined under a Zeiss Axio Imager.M2 microscope for quantification, photographs, and measurements taken with the Axiocam 506 Mono digital camera and processed with Zen 2 Pro software (Carl Zeiss Microscopy).

Oospore inoculation

Using the protocol in Cohen and Rubin 2012, vegetative structures of *P. cubensis* were killed, but *P. cubensis* oospores were left viable. Oospore suspensions in a 5 ml volume were poured into 9 cm petri dishes and dried for 12 h at 26°C. Following complete drying 5 ml was added back to the petri dishes and they were exposed again for 12 h at 26°C. The oospore samples were then resuspended in the petri dish and transferred to microcentrifuge tubes for concentration and inoculation. The oospore homogenate was then inoculated in 10-15 spots, in a volume of 20 µL or 25 µL each, onto the detached leaves of watermelon, butternut squash, cucumber, or cantaloupe on damp paper towels in square Nunc BioAssay Dishes (Thermo Fisher Scientific). The inoculated leaves were then placed in a growth chamber with 12 h light/dark at 16.5°C or 20°C until leaves degraded.

Fluorescent in situ hybridization probe design

Multiple oligonucleotide probes were designed from the putative single copy markers (c2555.2e1 and c2555.3e7) from Withers et al. 2016. Probe sequences were as follows: c2555.2e1 LNA dual digoxigenin (DIG) labeled probe 5'-DIG-+TA+CG+CAA+CGA+CAA+GCT+CTT+AT-DIG-3' and c2555.3e7 LNA dual DIG labeled probe 5'-DIG-T+TGCT+TGT+TCG+ACA+TGG+ATT+GA-DIG-3'. The c2555.2e1 probe had a melting temperature of 78°C while c2555.3e7 had a melting temperature of 74°C. Both probes were commercially synthesized (Qiagen Sciences Inc.).

Preparation of materials for FISH visualization

Mycelia, sporangiophores, and sporangia were collected under a Zeiss Discovery V20 dissecting microscope (Carl Zeiss Microscopy). A sterile entomological needle was used to collect the *P. cubensis* or non-target material (*P. humuli* or *Peronospora belbahrii*). Oospores were collected and centrifuged at 6,500 rpm for 5 min before the supernatant was removed. The tissue was then fixed in 400 µL of 4% formaldehyde in 1X phosphate-buffered saline (PBS) overnight (12-16 h) at 4°C (Salgado-Salazar et al. 2018). The next day, the solution was removed with a pipette. The tissue was resuspended in 500 µL of 1X PBS and shaken to wash before the solution was removed with a pipette, this step was repeated for a total of two washes. Finally, the tissue was resuspended in 400 µL of a 1:1 solution of 1X PBS and absolute ethanol before storage at -20°C. Before FISH occurred, the tissue was washed in 1X PBS as described above one time. Digestion of the tissue occurred at 37°C in 3.3 µg ml^{-1} proteinase K for 10 minutes, followed by two washes in 1X PBS.

FISH protocol

The dual DIG probe label was targeted with the Tyramide SuperBoost Kit with Alexa Fluor Tyramide, Goat-Anti-Mouse igG and Alexa Fluor 594 (Thermo Fisher Scientific). In later experiments, FISH was conducted without the Tyramide SuperBoost Kit, instead using Goat Anti-DIG Antibody, DyLight 594 (Vector Laboratories, Maravai LifeSciences, San Diego, CA). Both fluorophore probes had the same emission spectrum (excitation = 590 nm, emission = 617 nm [red]). From the fixed samples, 7.5 µL was heat fixed onto glass microscope slides by incubating on a 60°C hotplate for 15 s then a 65 µL capacity Frame Seal (Bio-Rad Laboratories,

Hercules, CA) was placed around the sample. Later experiments used Poly Prep (poly-L-lysine coated) slides (Sigma-Aldrich, St. Louis, MO) or 0.3% agarose to retain more material on the slides during the multiple washing steps necessary in a FISH protocol. The agarose slides were made by adding 100 μL of 0.3% low melting point agarose to the sample on a glass slide and before placement onto a 60°C hotplate until it was dry.

A modified protocol of the Tyramide SuperBoost Kit was used. Briefly, samples were dehydrated in a series of ethanol washes (70%, 85%, and 95%) for one minute each. Then the endogenous peroxidase activity of the sample was quenched by incubation for 30 s at room temperature in 3% hydrogen peroxidase solution. The samples were then rinsed three times in 1X PBS at room temperature in Wheaton Coplin staining jars (Sigma-Aldrich), which were used for all wash steps. Blocking buffer was added (100-150 μL) to the sample and incubated at room temperature for 60 min. The dual DIG labeled probe was added at a concentration of 40 nM to the hybridization buffer (500 μL formamide, 100 μL 20X saline sodium citrate [SSC] pH 7.0, 400 μL of 25% dextran sulfate; prepared fresh on the day of use, 100 μL per slide). The probe was denatured in the hybridization buffer at 90°C for four minutes and then added to the sample and incubated in the dark in a slide hybridization oven (VWR International, LLC) at 53.5°C for 60 min. A series of stringency washes was then performed: 5X SSC for five minutes at 60.5°C, 1X SSC for five minutes at 53.5°C (2X), 0.2X SSC for five minutes at 53.5°C (2X), and 0.2X SSC for five minutes at room temperature. The DIG probes were then labeled by diluting the primary antibody (anti-DIG mouse) 1:1000 in 100 μL of blocking solution and

incubating at room temperature for 60 minutes. The samples were then washed in 1X PBS for 10 minutes, three times. The poly-horse radish peroxidase-conjugated secondary antibody was added (100–150 μL) to the sample and incubated for 60 minutes at room temperature. The samples were again washed in 1X PBS for 10 minutes, three times. A 1X tyramide solution was prepared by adding 1 μL of 100X Tyramide stock solution, 1 μL of 100X H_2O_2 solution (prepared fresh on the day of use), and 100 μL of Reaction Buffer was prepared and added to the sample for 2-10 minutes. The reaction stop reagent was then applied to the sample (100 μL) before the final three washes in 1X PBS for 1 minute each, followed by dehydration in the series of ethanol washes for 1 minute each (70%, 85%, and 95%). ProLong Diamond Antifade Mountant (Thermo Fisher Scientific) was added to each sample and cured at 4°C in the dark overnight.

Another FISH protocol using the anti-DIG DyLight 594 fluorophore was attempted in order to reduce the number of the wash steps and reduce the amount of sample lost in the process. Sample volumes of 7.5 μL were dried at 60°C on a hotplate before dehydration in a series of ethanol washes as previously described. The probe was denatured and diluted in the hybridization solution as described above before incubation in the hybridization oven at 57°C for 60 minutes. Stringency washes were performed as described above followed by the addition of 100 μL of blocking solution (1% BSA, 2% goat serum, 0.1% PBS T [1 ml of Tween20 + 1L of PBS]) and incubation at room temperature for 15 minutes. The anti-DIG DyLight 594 was diluted 1:1000 in the dilutant solution (1% BSA, 1% goat serum, 0.05% PBS T) and incubated for 60 minutes at room temperature. Samples were then washed in

0.1% PBS T for 3 minutes (3X). Finally, samples were dehydrated in series of ethanol washes as previously described before the addition of the anti-fade mountant and subsequent incubation at 4°C in the dark overnight.

Fluorescent imaging

Fluorescence microscopy was performed using a Zeiss Axio Imager.M2 microscope with an HXP120V fluorescent light and filter set 64 HE (Carl Zeiss Microscopy). Images were acquired with an Axiocam 506 Mono digital camera and processed using the Zen 2 Pro Software (Carl Zeiss Microscopy).

Results

Specificity and sensitivity of the P. cubensis species-specific qPCR assay

The newly developed qPCR assay was highly sensitive, with a range of detection between 10 ng and 1 fg (Fig. 4.1A). The efficiency of the assay was 1.914, with an error of 0.0124, and a slope of -3.546 using the plasmid DNA for the standard curve (Fig. 4.1B). The Cq of the lowest concentration in the standard curve (1 fg) was 33.74; this value was established as the threshold for a positive detection of *P. cubensis.*

Species-specificity was examined in the qPCR assay with a panel of non-target species (Table 4.2, Fig. 4.1C). Thirty-nine of the 41 non-target samples were negative at the Cq threshold of 33.74. Two of the samples had a Cq less than 33.74, *Plasmopara viticola* (the oomycete that causes downy mildew of grapes) with a Cq of 32.79 and DNA extracted from the soil growing *Helianthus annuus* (sunflower) with a Cq of 33.73. Analyses of the qPCR products with the QIAxcel ScreenGel software,

showed amplicons similar in size to the 124 bp target, in the non-target samples. The similar size of the bands suggested not primer-dimer formation but contamination somewhere in the qPCR process from reagents, DNA samples, or pipettes. A trial qPCR run with oospore DNA samples all had Cq value greater than 33.74.

P. cubensis clade-host associations

The qPCR clade assay provided further insight into the clade-host relationship of *P. cubensis* (Table 4.1). Only clade 1 was found on *C. moschata*, *C. pepo*, and *C. lanatus* hosts (Fig. 4.2). Of the samples that exhibited fluorescence, clade 1 accounted for 67% of the samples in *C. maxima*, while clade 2 accounted for the remaining 33% (Fig. 4.2). *C. sativus* samples were only associated with *P. cubensis* clade 2. *C. melo* samples were primarily *P. cubensis* clade 2 (71%) (Fig. 4.2). There was no association with the state of sample origin and clade determination. Clade distribution was nearly even in Delaware, Maryland, and Virginia, while New York samples were from both clades and Pennsylvania only had one sample (Fig. 4.3). The sole *Citrullus* sp. sample that exhibited fluorescence was associated with *P. cubensis* clade 1 (Fig. 4.4A). *Cucurbita* spp. clade distribution was 88% clade 1 and 12% clade 2 (Fig. 4.4B). *Cucumis* spp. had an even stronger association with a specific clade, with 94% of samples identified as clade 2 and only 6% as clade 1 (Fig. 4.4C). Of the 70 samples, 59 fluoresced in the qPCR assay and the remaining 11 were examined with gel electrophoresis, along with positive and negative controls. The positive clade 1 control had a significantly larger product size than the clade 2 control. The sizes of the clade qPCR products were similar to those reported in Rahman et al. 2020. Lack of band formation and smearing of the samples that did not fluoresce, suggests an

annealing time that is too long, or likely that the DNA integrity is low due to shearing and nicking that occurred during isolation.

Oospore measurements and observations

Oospores ranged in diameter from 13.83 µm to 51.58 µm, with a cell wall thickness ranging from 0.90 µm to 5.95 µm. The average size of the oospore diameter from fifty-five measured samples was 34.89 ± 1.26 µm. The average thickness of the cell wall was 3.07 ± 0.16 µm.

Oospores were hyaline to amber in color (Fig. 4.5A, B). Oogonia with antheridia attached (Fig. 4.5D), germinated, and non-germinated oospores were all observed in infected tissue or originating from soil (Fig. 4.5A-C). Oospores were most commonly found in butternut squash leaves and soil.

Oospore inoculations

Inoculation experiments took place on three separate occasions. Temperature in the growth chamber was reduced for the third experiment from 20ºC to 16.5ºC, and droplet size increased from 20 µL to 25 µL, similar to the experimental conditions used by Cohen and Rubin 2012 to cause infection via oospore inoculation. Leaves did not degrade quickly in the growth chamber, which allowed for multiple weeks of observations. The first experiment was conducted using detached cucumber leaves, the second using cantaloupe, watermelon, and butternut squash, and the third using cucumber leaves. No sporulating lesions were observed in any of the three experiments on any of the cucurbit leaves (Fig. 4.6).

FISH using Tyramide SuperBoost Kit

Staining appeared to be non-specific and adhere to any tissue on the slide with the Tyramide SuperBoost Kit (Fig. 4.7). Dilutions of primary antibody, secondary antibody, and probe did not resolve the issue. Reducing the incubation time for the tyramide labeling reaction or increasing the incubation time for the blocking step did not reduce the non-specific staining. Increasing the hybridization temperature and stringency wash temperatures were also unsuccessful. The number and time of wash steps were increased and PBS was replaced with different wash buffers including PBS T, 0.1M Tris-HCl (pH 7.5) + 0.15M NaCl + 0.3% Triton X-100, and 0.05M Tris-HCl (pH 7.5) + 0.15M NaCl + 0.05% Tween 20. Altering the wash buffers or times did not solve the issue either but did highlight the loss of material from the slides. Using 0.3% agarose gel to increase the amount of sample that remained on the slide resulted in the trapping of the fluorophore in the agarose.

FISH using DyLight 594 fluorophore and reduced washes

Using the shortened protocol with the anti-DIG DyLight 594 fluorophore, poly-L-lysine slides, and a lower number of washes resulted in more sample material on the slide for analysis under the fluorescent microscope. The problem remained with non-specificity though, and *P. cubensis*, *P. humuli*, and *P. belbahrii* all fluoresced, even though either probe used targeted a single copy gene of *P. cubensis*. Alterations to the proteinase K treatment and replacement of proteinase K with glucanex or cellulase (each at concentrations of 4 and 10 mg ml^{-1}), probe concentration dilution, anti-DIG fluorophore dilution, hybridization temperature

changes, and stringency wash temperature changes all failed in reducing non-specific staining.

Discussion

Cucurbit downy mildew epidemics in the Mid-Atlantic often develop from initial inoculum spread northward from southern states, including North Carolina (Ojiambo et al. 2015). My research aim was to examine if oospores play a role in the epidemiology of *P. cubensis* in the Mid-Atlantic and if the clade-host relationship was evident in regional cucurbit crops. Both research questions have implications for the future of cucurbit downy mildew disease management.

There was a strong clade-host association of *P. cubensis* observed in our sample collection, notably clade 2 primarily infected *C. sativus* and clade 1 primarily infected *C. moschata* and *C. pepo*. These results align with research from North Carolina and the Czech Republic to further support the association of *P. cubensis* clade with certain host species (Kitner et al. 2015; Wallace et al. 2020). The *P. cubensis* samples from Wallace et al. 2020 all originated in North Carolina, while our study expanded the clade-host relationship to the Mid-Atlantic and showed no signs of a clade-geographic relationship. With a clearer view of the clade-host relationship, disease forecasting can be improved with tools like clade-specific spore trapping (Rahman et al. 2020). Since 2004, *P. cubensis* has caused annual epidemics on *C. sativus* in the Mid-Atlantic, but not consistently on other cucurbit hosts, such as *C. lanatus*, *C. moschata*, and *C. pepo* (Everts *personal communication*; Wyenandt et al. 2017). Crop specific disease forecasting based on *P. cubensis* clade could help prevent unnecessary fungicide applications and drive fungicide recommendations.

Choosing lower efficacy fungicides for the less virulent clade 1 is an option for hosts associated with that clade (Wyenandt et al. 2017). This would reduce growers' input costs and could reduce the amount sprayed of the newest and most efficacious fungicides, such as Orondis (active ingredient oxathiapiprolin) as its use is already under strict fungicide resistance guidelines (Jones et al. *under review*). On the other hand, clade 2 disease outbreaks in *C. sativus* would require a rotation of the higher efficacy fungicides to properly manage the disease. Currently, fungicide efficacy trials and subsequent recommendations for cucurbit downy mildew management are not crop specific and perhaps should be based on clade and updated (Goldenhar and Hausbeck 2019; Jones et al. *under review*; Keinath et al. 2019; Rahman et al. 2020; Wallace et al. 2020).

Oospores found in our surveys were comparable in size and description to those produced *in vitro* in Israel and the U.S., with mean diameters of 40.8 ± 0.1 µm and 36.2 ± 0.6 µm, respectively, but larger than those found from natural infections of cucumber in China (Cohen and Rubin 2012; Thomas et al. 2017; Zhang et al. 2012). Over the course of multiple experiments, using *P. cubensis* oospores found in diseased butternut squash leaves and surrounding soil, no infection occurred. This aligns with the work of other researchers who also report low and erratic infection rates of oospores, perhaps due to dormancy. Thomas et al. 2017 was unsuccessful at causing infection with oospores produced *in vitro* and the 0.2% infection rate was very low with oospore inoculum in Cohen and Rubin 2012. An outlier in oospore inoculation research was a study in China, which used cold treated *P. cubensis* oospores in an attempt to mimic overwintering conditions to break dormancy (Zhang

et al. 2012). The highest infection rate of 95% resulted from planting surface sterilized seeds in oospore infected soil (Zhang et al. 2012). The other method of inoculation used by Zhang et al. 2012, with an oospore solution pipetted onto growing cotyledons, was similar to the detached leaf method used by our lab, Cohen and Rubin 2012, and Thomas et al. 2017, but resulted in an infection rate of 27-46% in the Chinese study. The soil used by our lab for the second and third inoculation experiments had been stored at 4°C for more than 12 months, possibly breaking the dormancy in a portion of the oospores, but again did not result in infection (Zhang et al. 2012). The failure to produce infection is not limited to *P. cubensis* oospores, as Gent et al. 2017 was unable to produce infection with oospore inoculum from *P. humuli.* In both pathosystems, infections via oospores have been reported, which could have implications for overwintering, genetic variation, initial infections, and disease forecasting (Bressman and Nichols 1933; Cohen and Rubin 2012; Gent et al. 2017; Ojiambo et al. 2015).

In *P. cubensis* clade and mating type research, clades 1 and 2 and mating types A1 and A2 shared several hosts, for example *C. melo* and *C. maxima* (Cohen et al. 2013; Thomas et al. 2017; Wallace et al. 2020). If *P. cubensis* is heterothallic, as suggested by Cohen and Rubin 2012, oospore research should focus on sampling *C. melo* and other shared hosts (Thomas et al. 2017). Observations from our research found that oospores were most commonly found in *C. moschata*, which was exclusively associated with clade 1 in Wallace et al. 2020 and our own clade research and exclusively with the A2 mating type in Thomas et al. 2017 and 87% of isolates in Cohen et al. 2013. This challenges the mating type-based research and lends support

to the theory from the clade-based research group. It was suggested that clade 2 could be a homothallic species and clade 1 heterothallic, explaining the evidence of recombination found in clade 1 isolates (Wallace et al. 2020). Both lines of research suggest hybridization is possible between clade 1 and clade 2 or mating type A1 and mating type A2 (Cohen et al. 2013; Kitner et al. 2015; Wallace et al. 2020).

P. humuli and *P. cubensis* are closely related sister species with limited cross-infectivity (Crandall et al. 2018; Runge et al. 2011; Runge and Thines 2012). Separation of the two species with molecular tools before clade and mating type separation became the focus of *P. cubensis* research, was a goal of our lab and others (Summers et al. 2015; Withers et al. 2016). My research was based on the molecular markers from Withers et al. 2016 and developed a highly sensitive qPCR assay to differentiate *P. cubensis* from *P. humuli* and other oomycete pathogens. The separation of the two species was successful with the qPCR assay, as no *P. humuli* samples had a Cq value less than the threshold value for positive *P. cubensis* detection. The qPCR assay was also sensitive, with a detection limit as low as 1 fg of *P. cubensis* DNA. The qPCR assay could be useful in identification of low levels of *P. cubensis*, as its detection limit is lower than the assay from Rahman et al. 2020.

Another goal of my research was to directly visualize *P. cubensis* oospores with a species-specific FISH assay. Fluorescent *in situ* hybridization was used as a direct visualization tool in another downy mildew pathogen, *P. obducens*, but had targeted a high copy number target, ITS 2 (Salgado-Salazar et al. 2018). The close relatedness of *P. humuli* and *P. cubensis* limited the possible diagnostic markers and two single copy genes were targeted (Withers et al. 2016). In order to achieve

maximum specificity and sensitivity, LNA probes were used in a CARD-FISH protocol, which is known to be challenging (Amann and Fuchs 2008; Pernthaler et al. 2002; Thomsen et al. 2005). Issues with the lengthy CARD-FISH protocol and the simpler antibody conjugated DyLight 594 protocol were not resolved. Attempts to optimize and troubleshoot following the probe manufacturer and Tyramide SuperBoost Kit manufacturer recommendations were followed but unsuccessful.

In conclusion, the clade-host relationship of cucurbits in the Mid-Atlantic shows that *C. sativus* is preferentially infected by the more virulent clade 2, while clade 1 preferentially infects *C. moschata*, C. *pepo*, and perhaps *C. lanatus.* Fungicide efficacy trials based on clade are needed and fungicide recommendations are likely to change and become more precise for crops other than *C. sativus*. We remain confident that the oospores were *P. cubensis*, as they were directly observed in infected tissue, but no infection resulted from inoculation trials, highlighting the lack of understanding surrounding the conditions for oospore germination and perhaps their limited role in initial infections.

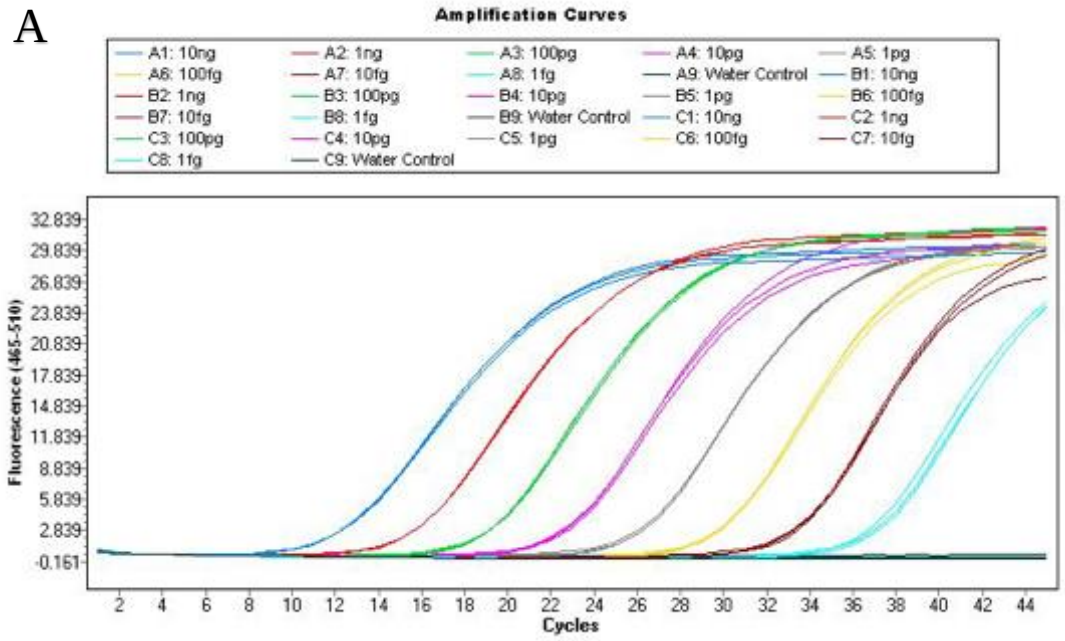
Table 4.1. *Pseudoperonospora cubensis* samples included in the clade qPCR assay.

Sample	Location	Host	Year	Clade Results
1	New York	*Cucurbita moschata*	2019	1
2	New York	*Cucumis melo*	2019	2
3	Virginia	*Cucurbita pepo*	2019	X[a]
4	Virginia	*Cucumis sativus*	2019	2
5	New York	*Cucumis sativus*	2019	2
6	Virginia	*Cucumis sativus*	2019	2
7	Virginia	*Cucurbita moschata*	2019	1
8	Virginia	*Cucumis melo*	2019	2
9	Virginia	*Cucurbita moschata*	2019	1
10	Maryland	*Cucurbita pepo*	2019	X
11	Maryland	*Cucumis sativus*	2019	2
12	Virginia	*Cucurbita maxima*	2019	X
13	Virginia	*Cucurbita maxima*	2019	2
14	Maryland	*Cucurbita foetidissima*	2019	X
15	Maryland	*Cucurbita moschata*	2019	X
16	Maryland	*Cucumis melo*	2019	1
17	Pennsylvania	*Cucumis sativus*	2019	2
18	Delaware	*Cucurbita moschata*	2019	1
19	Delaware	*Cucumis sativus*	2019	X
20	Delaware	*Cucumis melo*	2019	X
21	Delaware	*Cucurbita maxima*	2019	1
22	Delaware	*Cucurbita maxima*	2019	X
23	Delaware	*Cucurbita pepo*	2019	X
24	Delaware	*Cucumis sativus*	2019	2
25	Delaware	*Cucurbita moschata*	2019	1
26	Delaware	*Cucurbita moschata*	2019	1
27	Virginia	*Cucurbita maxima*	2019	1
28	Delaware	*Cucurbita foetidissima*	2019	X
29	Delaware	*Citrullus lanatus*	2019	X
30	Maryland	*Cucumis sativus*	2017	2
31	Maryland	*Cucumis sativus*	2017	2
32	Delaware	*Cucumis sativus*	2017	2
33	Delaware	*Cucumis sativus*	2017	2
34	Delaware	*Cucumis sativus*	2017	2
35	Maryland	*Cucumis melo*	2017	2
36	Delaware	*Cucurbita moschata*	2017	1
37	Delaware	*Cucumis melo*	2017	2
38	Maryland	*Cucumis sativus*	2016	2
39	Maryland	*Cucumis sativus*	2017	2
40	Maryland	*Cucurbita pepo*	2018	1
41	Maryland	*Cucumis melo*	2017	2

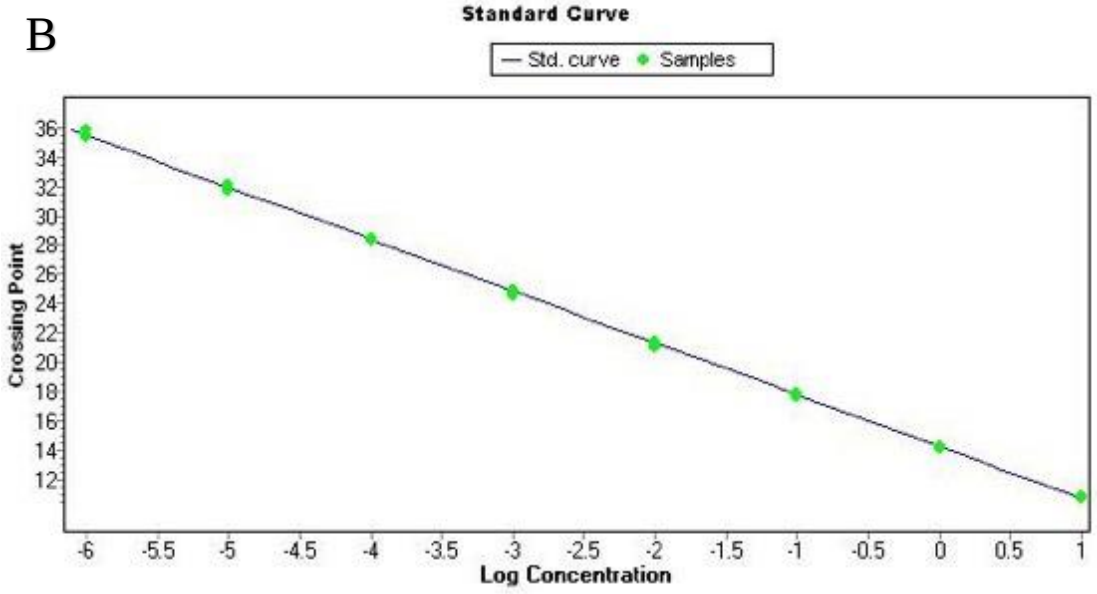
42	Delaware	*Cucurbita maxima*	2017	2
43	Maryland	*Cucurbita moschata*	2017	1
44	Maryland	*Cucurbita moschata*	2017	1
45	Delaware	*Cucurbita pepo*	2017	1
46	Maryland	*Citrullus lanatus*	2017	1
47	Delaware	*Cucumis sativus*	2017	2
48	Delaware	*Cucurbita maxima*	2017	1
49	Maryland	*Cucumis sativus*	2018	2
50	Delaware	*Cucumis sativus*	2018	2
51	Maryland	*Cucurbita pepo*	2018	1
52	Maryland	*Cucurbita maxima*	2018	2
53	Maryland	*Cucumis melo*	2018	1
54	Maryland	*Cucumis sativus*	2018	2
55	Maryland	*Cucurbita maxima*	2018	1
56	Maryland	*Cucumis sativus*	2018	2
57	Maryland	*Cucurbita moschata*	2018	1
58	Maryland	*Cucumis sativus*	2018	2
59	Maryland	*Cucumis sativus*	2018	2
60	Delaware	*Cucumis sativus*	2018	2
61	Maryland	*Cucurbita moschata*	2017	1
62	Maryland	*Cucurbita pepo*	2017	1
63	Virginia	*Cucumis sativus*	2017	2
64	Virginia	*Cucurbita maxima*	2017	1
65	Maryland	*Cucurbita maxima*	2017	1
66	Maryland	*Cucumis sativus*	2016	2
67	Maryland	*Cucumis sativus*	2017	2
68	Maryland	*Cucumis sativus*	2017	2
69	Maryland	*Cucurbita moschata*	2016	1
70	Maryland	*Cucurbita moschata*	2016	1

[a]X = sample did not fluoresce in qPCR assay for clade 1 or clade 2.

A


Amplification Curves
A1: 10ng
A2: 1ng
A3: 100pg
A4: 10pg
A5: 1pg
A6: 100fg
A7: 10fg
A8: 1fg
A9: Water Control
B1: 10ng
B2: 1ng
B3: 100pg
B4: 10pg
B5: 1pg
B6: 100fg
B7: 10fg
B8: 1fg
B9: Water Control
C1: 10ng
C2: 1ng
C3: 100pg
C4: 10pg
C5: 1pg
C6: 100fg
C7: 10fg
C8: 1fg
C9: Water Control
Fluorescence (465-510)
32.839
29.839
26.839
23.839
20.839
17.839
14.839
11.839
8.839
5.839
2.839
-0.161
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycles

B


Standard Curve
Std. curve
Samples
Crossing Point
36
34
32
30
28
26
24
22
20
18
16
14
12
-6 -5.5 -5 -4.5 -4 -3.5 -3 -2.5 -2 -1.5 -1 -0.5 0 0.5 1
Log Concentration

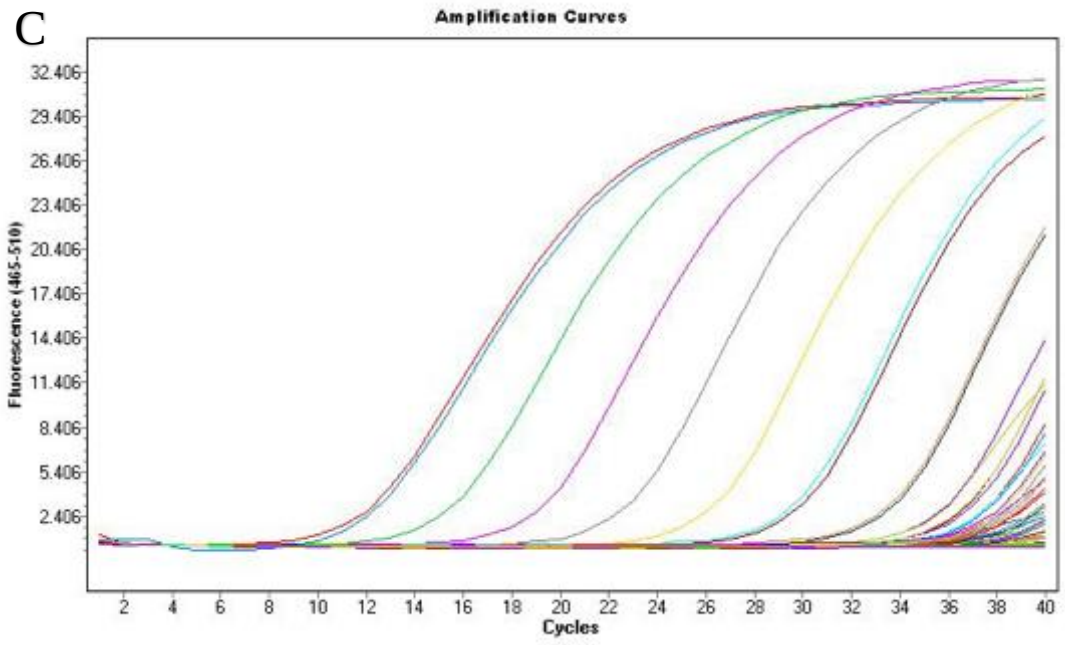


Figure 4.1. A-C: *Pseudoperonospora cubensis* species-specific qPCR uniplex. **A**, plasmid DNA standard curve amplification curves and Cq values, **B,** plasmid DNA standard curve performance, **C,** non-target panel amplification curves.

Table 4.2. List of non-target organisms for the *Pseudoperonospora cubensis* species-specific uniplex qPCR.

Pathogen	Host
Pseudoperonospora humuli	*Humulus lupulus*
Peronospora belbahrii	*Ocimum basilicum*
Plasmopara viticola	*Vitis vinifera*
Plasmopara halstedii	*Helianthus annuus*
Phytophthora capsici	*Citrullus lanatus*
Phytophthora infestans	*Solanum tuberosum*
Pythium spp. 1	Soil
Pythium spp. 2	Soil
Alternaria alternata	*Solanum lycopersicum*
Fusarium spp. 1	*Citrullus lanatus*
Fusarium spp. 2	*Citrullus lanatus*
Phytophthora phaseoli	*Phaseolus vulgaris*
	Cucumis sativus
	Cucurbita pepo

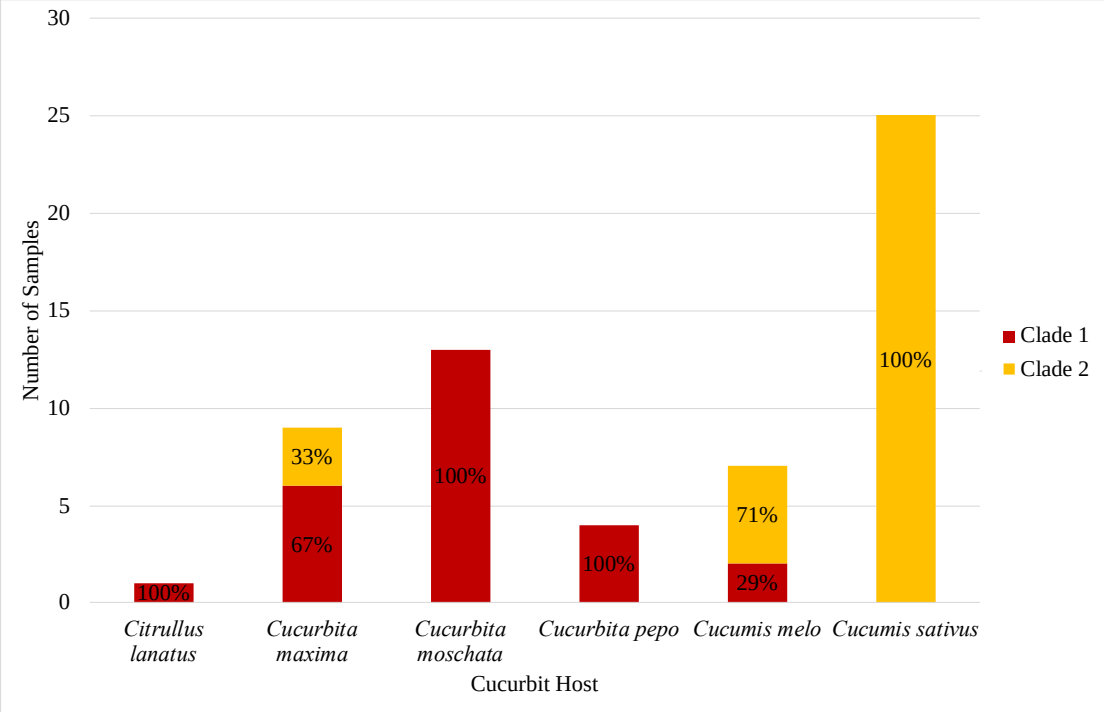


Figure 4.2. *Pseudoperonospora cubensis* clade association with host species.

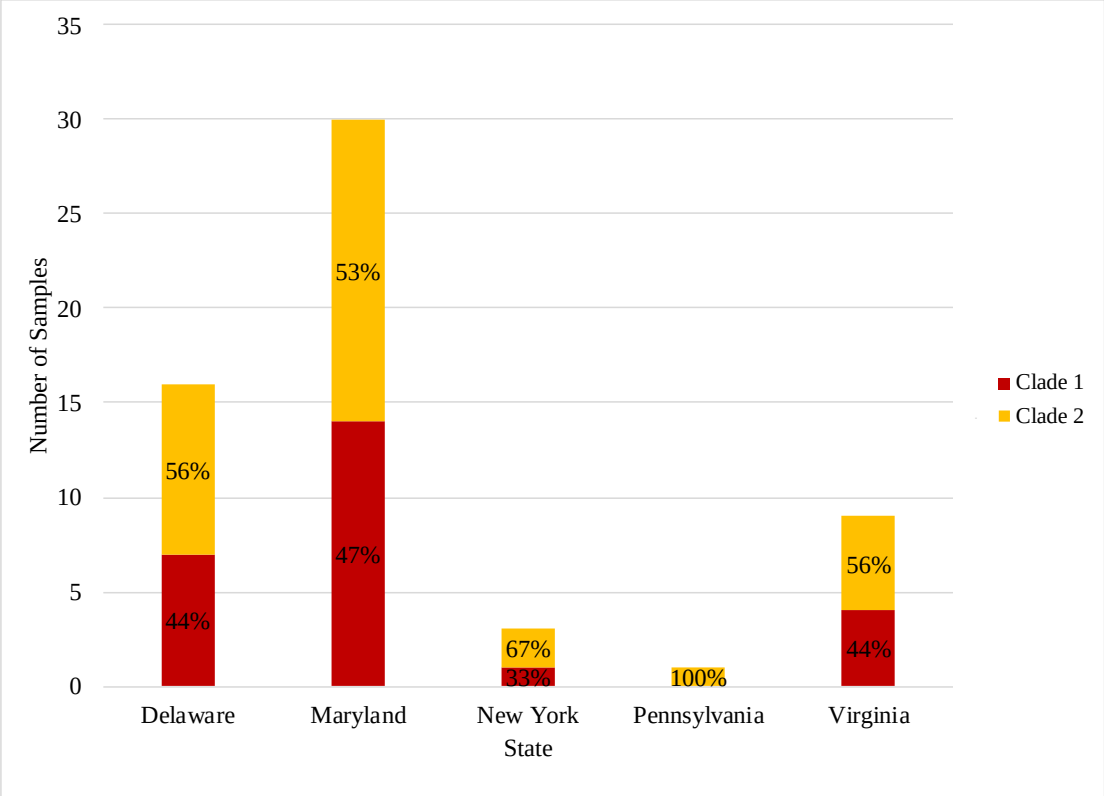
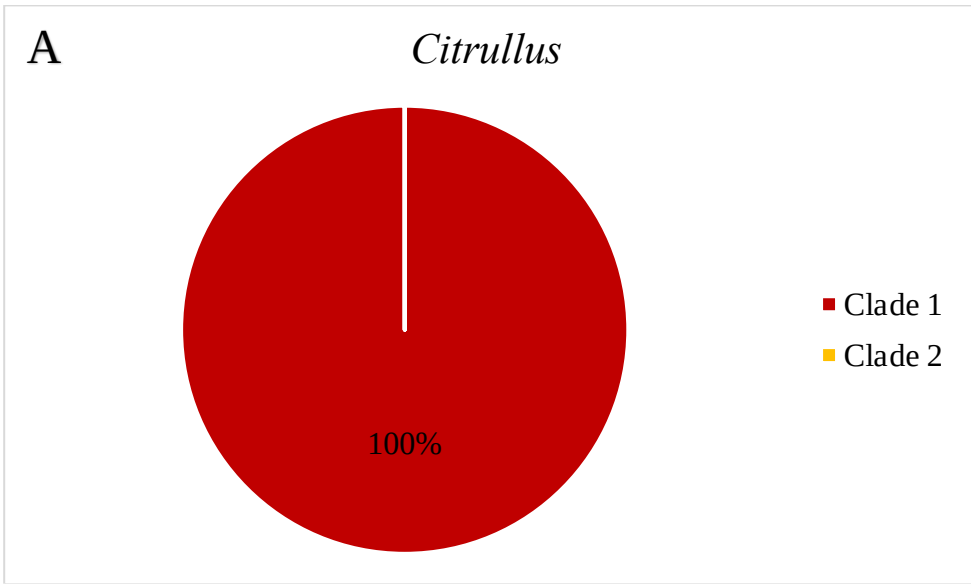
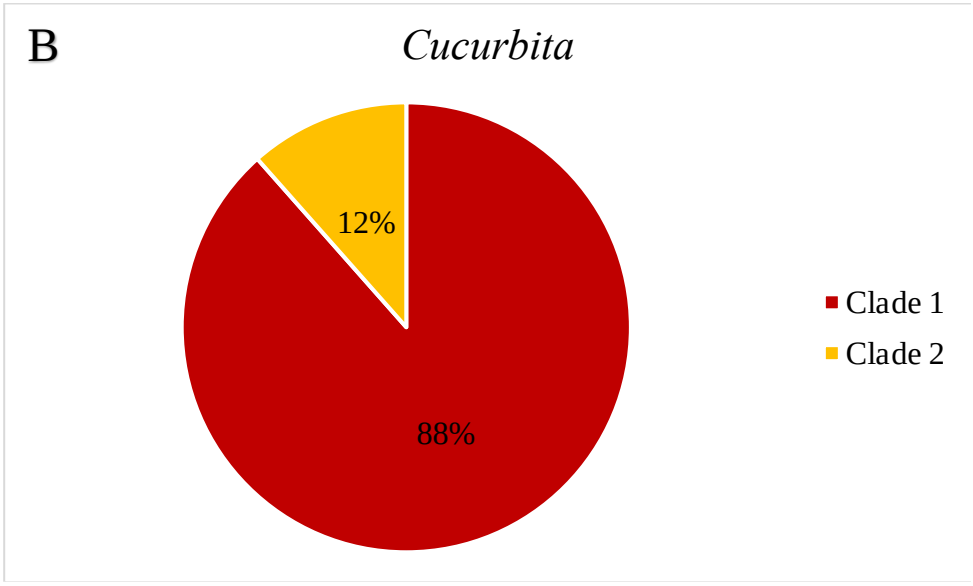


Figure 4.3. *Pseudoperonospora cubensis* clade association with state of origin.


A
Citrullus
100%
Clade 1
Clade 2


B
Cucurbita
12%
88%
Clade 1
Clade 2

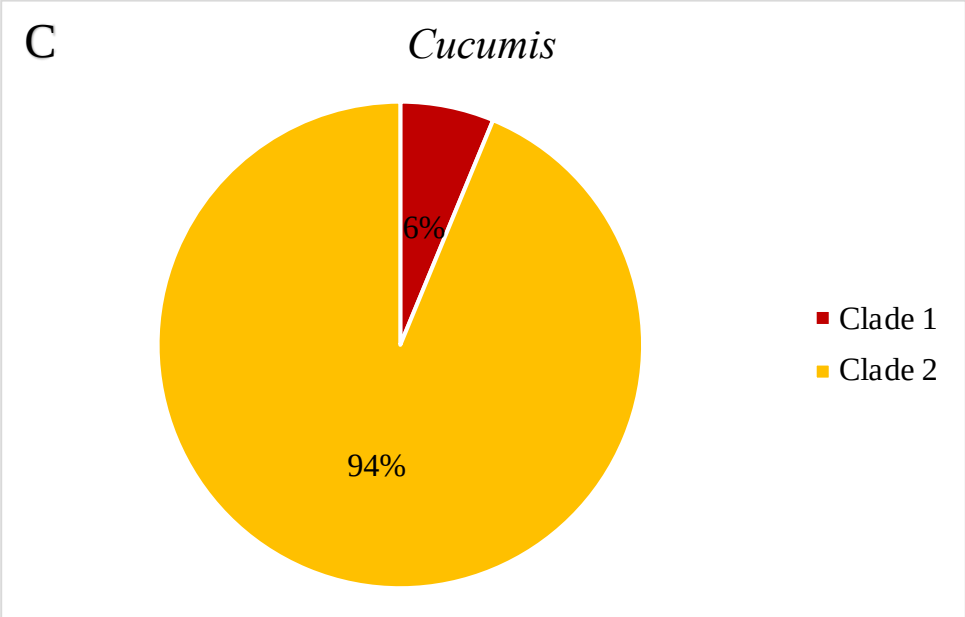


Figure 4.4. A-C. *Pseudoperonospora cubensis* clade association with host genus. **A,** *Citrullus* samples (n=1), **B,** *Cucurbita* samples (n=26), **C,** *Cucumis* samples (n=32).

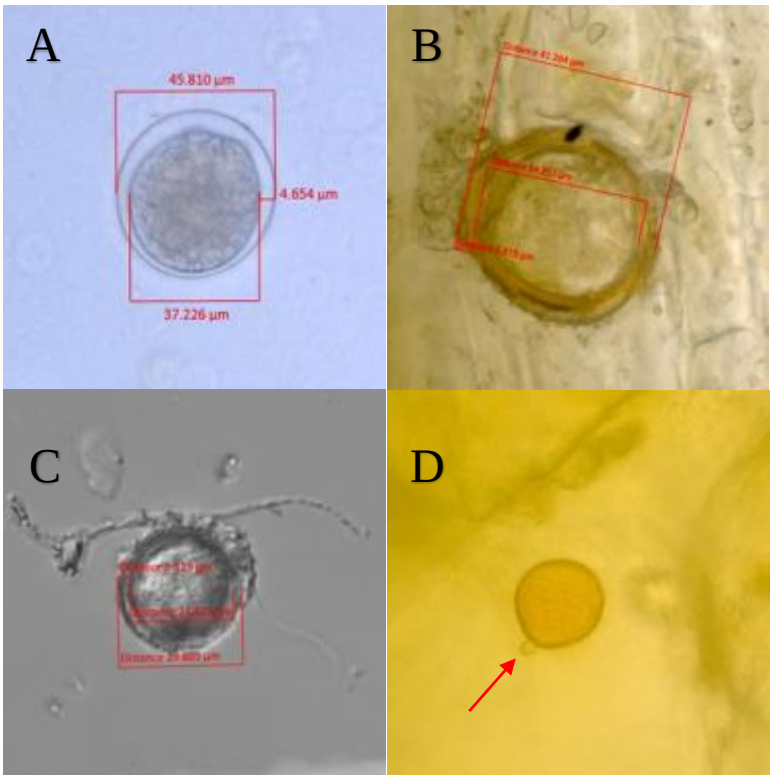


Figure 4.5. A-D: Possible *Pseudoperonospora cubensis* oospores. **A,** oospore extracted from soil, **B,** oospore *in planta*, **C,** germinated oospore, **D,** oogonium with antheridium (arrow).

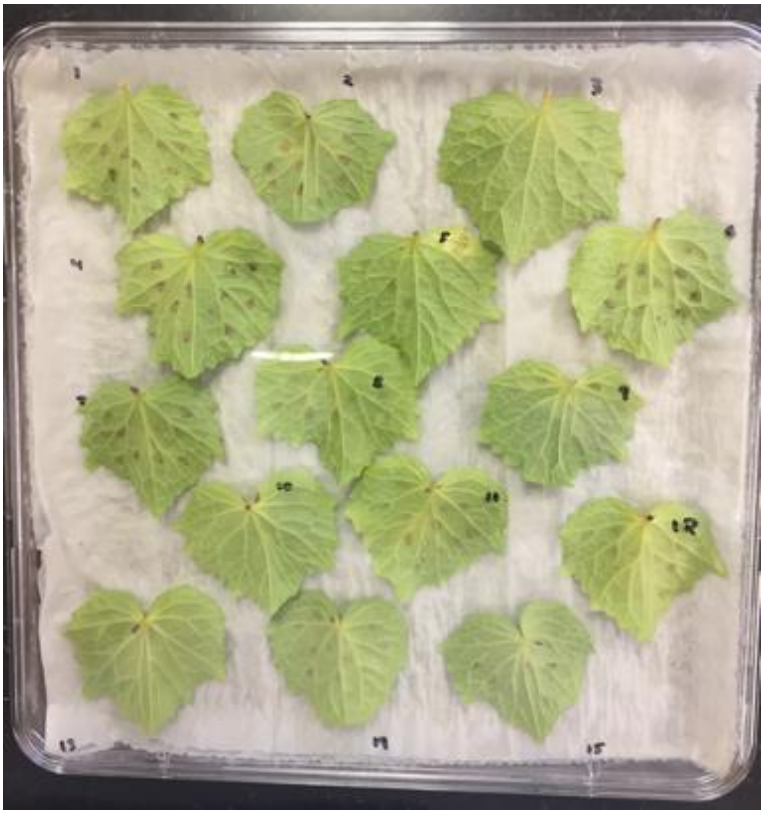


Figure 4.6. Inoculation of *Pseudoperonospora cubensis* oospores onto detached cucumber leaves.

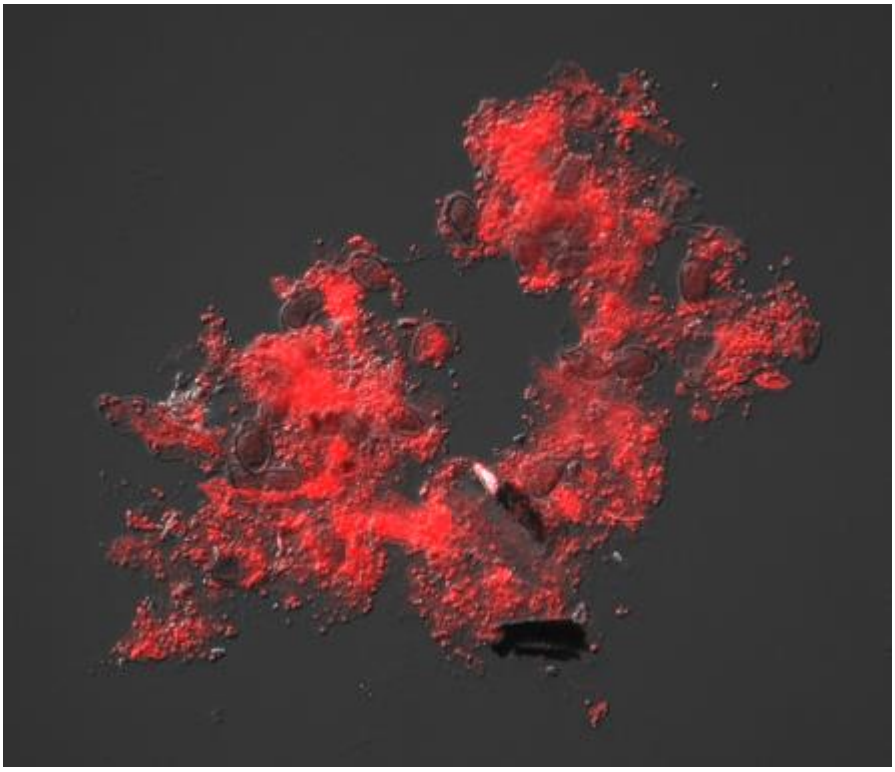


Figure 4.7. Non-specific staining of *Pseudoperonospora cubensis* using fluorescent *in situ* hybridization.

Chapter 5: Conclusions, Limitations, and Future Directions

Cucurbit production in the Mid-Atlantic is challenged annually by conditions that often favor fungal and oomycete growth. Both basic and applied research are needed to answer important questions for cucurbit disease management. The consequences of fungicide applications, the primary tool in managing disease, have become more apparent via impacts on pollinator health, the environment, resistance development, and consumer opinions. Growers need to continually increase the precision of their fungicide applications. Improved decision making can help reduce ineffective applications with proper fungicide timing and selection, based on the targeted disease and efficacy results. In the present work, we provide an alternative to the widely used but scrutinized chlorothalonil, identify currently efficacious fungicides on downy mildew in cucumber, and provide support to help build the relationship between cucurbit downy mildew clade and host, which will play an important role in its management in the future.

Study Highlights

An alternative fungicide spray program was developed in **Chapter 2** that reduced the use of chlorothalonil in the management of gummy stem blight and powdery mildew in cantaloupe, honeydew melon, and watermelon. Chlorothalonil was replaced with two biopesticides rotated as tank mix components with targeted fungicides. One of alternative fungicides, *R. sachalinensis* was more useful as a resistance management component, while the other, polyoxin D, was efficacious on gummy stem blight and anthracnose. The alternative fungicide spray program did not

provide adequate control of anthracnose in watermelon, which can directly threaten yield by infecting fruit. Anthracnose is effectively controlled with chlorothalonil.

Efficacious fungicides were identified in **Chapter 3** for use in management of cucurbit downy mildew. Bioassays were used, which could be completed in as little as four weeks, providing valuable efficacy information in-season to cucurbit growers. This is an important practice, since *P. cubensis* populations are exposed to fungicides as the pathogen spreads northward in the U.S. and could result in fungicide insensitivities in the Mid-Atlantic and Northeast from fungicides used in the South. Multiple fungicides were very effective at managing downy mildew in cucumber across Maryland, Delaware, and at partner universities in Pennsylvania and New York. The most consistently efficacious fungicides were chlorothalonil, zoxamide + chlorothalonil, oxathiapiprolin, and cyazofamid. Whereas, azoxystrobin, mandipropamid, fluopicolide, dimethomorph, and cymoxanil were all ineffective in multiple bioassays.

A clear clade-host relationship of *P. cubensis* and cucurbit crops was found in our sample collection, in **Chapter 4**. Clade 1 was found in summer and winter squash samples and watermelon, while clade 2 was found in cucumber samples. Cantaloupe and pumpkin were hosts of both clades. No relationship between clade and geography was found as cucurbit host species appeared to be the most important factor.

Possible *P. cubensis* oospores were observed in *P. cubensis* infected butternut squash leaves in **Chapter 4**, the first sighting of naturally-occurring oospores in the U.S. Attempts were made to cause infection with inoculation experiments of the oospores but were unsuccessful. Molecular tools to identify the oospores also failed,

either because of a lack of specificity or sensitivity to confirm the structures as *P. cubensis*.

Limitations

This research project had its challenges but each one created the opportunity for learning new methods or asking new questions. In **Chapter 4**, the molecular approaches involved a large amount of optimization and troubleshooting. Fluorescent *in situ* hybridization was especially challenging and a considerable amount of time was spent on each optimization step, as the entire protocol lasted between 4 and 8 hours. Eventually, the non-specific staining with the fluorophore led to the efforts of oospore inoculation. In order to test Koch's Postulates oospores extracted from infected tissue and soil were inoculated onto healthy, detached cucurbit leaves but were unsuccessful at causing infection. The infection rates of oospores in *P. cubensis* and the closely related *P. humuli*, were extremely low in previous research (Gent et al. 2017; Cohen and Rubin 2012; Thomas et al. 2017). The failure to cause infection could be due to a lack of the proper stimuli for germination, flawed methods of oospore extraction/inoculation, or infertility of the oospores.

Subsequently, our focus shifted to using qPCR to identify the oospores as *P. cubensis* in **Chapter 4**. Using a diagnostic marker from published research, our lab designed and optimized a qPCR protocol to separate *P. cubensis* from *P. humuli* and other cucurbit pathogens. Simultaneously, another lab developed and published a qPCR assay, focusing on the polymorphisms within *P. cubensis* which was species-specific, as well as identified clade. This created a situation where our qPCR assay became obsolete as research shifted towards the more precise *P. cubensis* clades. *P.*

cubensis is a widely studied organism and competition can inadvertently occur between labs. The transition was made to examine the clade-host relationship in our sample collection and coordinate with other universities to acquire additional samples (Cornell University, The Pennsylvania State University, and Virginia Polytechnic Institute and State University). The clade-host relationship also was its own limitation as only two samples were collected from watermelon over the four years of sample collection, highlighting the lack of downy mildew on watermelon in the region in recent years.

More information could have been gleaned from the fungicide bioassays in **Chapter 3** if the clade-host component of the project was known at the time. The bioassays were conducted on cucumber and therefore presumably clade 2 *P. cubensis.* In hindsight, sample collection from the bioassays to identify clade would have allowed for some of the first fungicide recommendations based on clade, likely where the future of fungicide recommendations for cucurbit downy mildew is headed. One bioassay was conducted on butternut squash as opposed to cucumber and the results showed high efficacy in all but two fungicides (Supplementary Table 1). Fungicide bioassays performed on each clade host would have provided valuable information on fungicide efficacy, as well.

Finally, when including fungicides as components of spray programs it can be hard to determine the value of one fungicide. In **Chapter 2**, chlorothalonil, polyoxin D, and *R. sachalinensis* were all included as individual treatments but the remaining four targeted fungicides (five active ingredients) were not. In order to include all fungicides, the trial would have become too large, but such information would have

been valuable in order to see the individual impacts of fungicides on the three diseases.

Broader Impacts and Future Directions

The research presented here on chlorothalonil does not take economics into consideration but does provide alternatives to the fungicide in the case of a forced reduction of its use, or a ban, which occurred in Europe. Further research into the detrimental synergistic effects of fungicides on pollinators needs to be completed to guide future use of the fungicides. Changes could be made to application rates and timings to avoid pollinators. Chlorothalonil is widely used, effective for multiple diseases, and a common fungicide resistance management component. While we identified alternatives for gummy stem blight and powdery mildew in melon, anthracnose was not managed and the multiple other cropping systems it is used in also need alternative spray programs to be developed.

The naturally occurring oospores we observed were possibly *P. cubensis*, but further research to definitively identify them is needed. The role of oospores is assumed to be minor for the epidemics of *P. cubensis* but if infective, oospores could serve as initial inoculum and help explains some of the inconsistencies in the downy mildew forecasting model (Cohen et al. 2015). Sexual recombination could give rise to more aggressive populations of *P. cubensis* as well. Crop rotation could become important if oospores were found to cause disease, but historically and in our own research, the infectivity of *P. cubensis* oospores is limited.

The information supporting *P. cubensis* clade and mating types is limited. Our research into the clade-host relationship aligns with other recent work (Wallace et al.

2020). This is important as new biosurveillance tools are unveiled to identify *P. cubensis* clade and provide the basis for fungicide recommendations (Rahman et al. 2020). Educating growers of the *P. cubensis* clade-host relationship and subsequent fungicide recommendations in the Mid-Atlantic will receive a much wider acceptance with the local research we completed to support the claims. Further research into the relationship between clade and mating type is needed to determine if these theories are competing or aligning, just under separate names. Proper management of cucurbit diseases relies on fungicides, host resistance, cultural practices, and the applied and basic research used by extension to educate growers.

Appendix

Supplementary Table 1. Relative downy mildew severity values for fungicides tested on butternut squash in Maryland.

Fungicide	2018
Azoxystrobin	19.0b
Mandipropamid	0c
Fluopicolide	0.2c
Dimethomorph	0c
Ametoctradin + dimethomorph	0c
Cymoxanil	41.1a
Zoxamide	ND
Zoxamide + chlorothalonil	0c
Chlorothalonil	0.2c
Propamocarb	ND
Fluazinam	0c
Cyazofamid	0c
Ethaboxam	ND
Oxathiapiprolin[z]	0c
Fungicide p value	0.0004

[x]Means followed by the same letter are not significantly different, Student's T LSD, *P*=0.05.

[y]ND = no data.

[z]Plenaris 200FS.

Bibliography

Adams, M. L., Collins, H., and Quesada-Ocampo, L. M. 2020a. Evaluation of cultivars in combination with fungicides for control of downy mildew and yield effects on cucumber, Clinton, NC 2019. Plant Dis. Manage. Rep. 14:V117.

Adams, M. L., Collins, H., and Quesada-Ocampo, L. M. 2020b. Evaluation of fungicides and cultivars for control of downy mildew on cucumber, Kinston, NC 2019. Plant Dis. Manage. Rep. 14:V107.

Adams, M. L., Collins, H., and Quesada-Ocampo, L. M. 2020c. Evaluation of fungicides for control of downy mildew on cucumber, Clayton, NC 2019. Plant Dis. Manage. Rep. 14:V116.

Adams, M. L., D'Arcangelo, K. N., Collins, H., and Quesada-Ocampo, L. M. 2019. Evaluation of fungicides and cultivars for control of downy mildew on cucumber, Kinston 2018. Plant Dis. Manage. Rep. 13:V069.

Adams, M. L., Thornton, A. C., and Quesada-Ocampo, L. M. 2015. Evaluation of fungicides for control of anthracnose and gummy stem blight of watermelon, Sampson County 2014. Plant Dis. Manage. Rep. 9:V082.

Adrees, M., Ali, S., Rizwan, M., Ibrahim, M., Abbas, F., Farid, M., Zia-Ur-Rehman, M., Irshad, M. K., and Bharwana, S. A. 2015. The effect of excess copper on growth and physiology of important food crops: A review. Environ. Sci. Pollut. Res. Int. 22:8148-8162.

Amann, R., and Fuchs, B. M. 2008. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol. 6:339–348.

Atwood, D., and Paisley-Jones, C. 2017. Pesticides Industry Sales and Usage 2008-2012 Estimates. United States Environmental Protection Agency. https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf

Avenot, H. F., Thomas, A., Gitatis, R. D., Langston, D. B., Jr., and Stevenson, K. L. 2012. Molecular characterization of boscalid- and penthiopyrad-resistant isolates of *Didymella bryoniae* and assessment of their sensitivity to fluopyram. Pest Manag. Sci. 68:645-651.

Barickman, T. C., Horgan, T. E., and Wilson, J. C. 2017. Efficacy of fungicide applications and powdery mildew resistance in three pumpkin cultivars. Crop Prot. 101:90–94.

Baysal-Gurel, F., Mera, J. R., and Miller, S. A. 2015. Monitoring *Pseudoperonospora cubensis* sensitivity to fungicides, 2014. Plant Dis. Manage. Rep. 9:V051.

Bost, S. C. 2010. An assessment of sensitivity to fungicides in Tennessee isolates of the cucurbit powdery mildew pathogen, *Podosphaera xanthii*. Phytopathology 100. S16.

Brent, K. J., and Hollomon, D. W. 2007. Fungicide Resistance in Crop Pathogens: How can it be Managed? 2nd Edition. Fungicide Resistance Action Committee https://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf

Bressman, E. N., and Nichols, R.A. 1933. Germination of the oospores of *Pseudoperonospora humuli*. Phytopathol. 23:485–487.

Call, A. D., Wehner, T. C., Holmes, G. J., and Ojiambo, P. S. 2013. Effects of host plant resistance and fungicides on severity of cucumber downy mildew. HortScience 48:53-59.

Choi, Y.-J., Hong, S.-B., and Shin, H.-D. 2005. A re- consideration of *Pseudoperonospora cubensis* and *P. humuli* based on molecular and morphological data. Mycol. Res. 109:841–848.

Chu, G., Wakelin, S. A., Condron, L., and Stewart, A. 2010. Effect of soil copper on the response of soil fungal communities to the addition of plant residues. Pebobiologia 53:353-359.

Cohen, Y., Meron, I., Mor, N., and Zuriel, S. 2003. A new pathotype of *Pseudoperonospora cubensis* causing downy mildew in cucurbits in Israel. Phytoparasitica 31:458–466.

Cohen, Y., and Rubin, A. E. 2012. Mating type and sexual reproduction of *Pseudoperonospora cubensis*, the downy mildew agent of cucurbits. Eur. J. Plant Pathol. 132:577–592.

Cohen, Y., Rubin, A. E., and Galperin, M. 2011. Formation and infectivity of oospores of *Pseudoperonospora cubensis*, the causal agent of downy mildew in cucurbits. Plant Dis. 95:874

Cohen, Y., Rubin, A. E., and Galperin, M. 2013. Host preference of mating type in *Pseudoperonospora cubensis*, the downy mildew causal agent of cucurbits. Plant Dis. 97:292.

Cohen, Y., Van den Langenberg, K. M., Wehner, T. C., Ojiambo, P. S., Hausbeck, M., Quesada-Ocampo, L. M., Lebeda, A., Sierotzki, H., and Gisi, U. 2015. Resurgence of *Pseudoperonospora cubensis*: The causal agent of cucurbit downy mildew. Phytopathol. 105:998–1012.

Colucci, S. J., Thornton, A. C., Adams, M. L., and Holmes, G. J. 2007. Evaluation of fungicides for control of downy mildew of cucumber I, 2006. Plant Dis. Manage. Rep. 1:V122.

Coolong, T., and Seebold, K. 2011. Impact of fungicide program and powdery mildew resistance in three varieties of pumpkin. Horttechnology 21:533–538.

Crandall, S. G., Rahman, A., Quesada-Ocampo, L. M., Martin, F. N., Bilodeau, G. J., and Miles, T. D. 2018. Advances in diagnostics of downy mildews: Lessons learned from other oomycetes and future challenges. Plant Dis. 102:265–275.

Dai, P., Jack, C. J., Mortensen, A. N., Bloomquist, J. R., and Ellis, J. D. 2018. The impacts of chlorothalonil and diflubenzuron on *Apis mellifera* L. larvae reared *in vitro*. Ecotoxicol. Environ. Saf. 164:283–288.

Damicone, J. P., and Pierson, T. J. 2013. Evaluation of fungicides for control of watermelon anthracnose, 2012. Plant Dis. Manage. Rep. 7:V020.

Damicone, J. P., and Pierson, T. J. 2014. Evaluation of fungicides for control of foliar diseases on watermelon, 2013. Plant Dis. Manage. Rep. 8:V185.

EFSA. 2018. Conclusion on the peer review of the pesticide risk assessment of the active substance chlorothalonil. European Food Safety Authority. EFSA Journal 2018 16(1):5126. doi: 10.2903/j.efsa.2018.5126.

EFSA. 2015. Peer review of the pesticide risk assessment of the active substance *Reynoutria sachalinensis* extract. European Food Safety Authority. EFSA Journal 2015 13(9):4221. doi:10.2903/j.efsa.2015.4221.

Egel, D. S., and Hoke, S. 2011. Evaluation of fungicides for the control of anthracnose on watermelon, 2010. Plant Dis. Manage. Rep. 5:V022.

EPA. 1999. Chlorothalonil reregistration eligibility decision (RED). EPA 738-R-99-004. United States Environmental Protection Agency. https://archive.epa.gov/pesticides/reregistration/web/pdf/0097red.pdf

EPA. 2018. Biopesticide Active Ingredients. United States Environmental Protection Agency. https://www.epa.gov/ingredients-used-pesticide-products/biopesticide-active-ingredients

Everts, K. L., Osborne, L., Gevens, A. J., Vasquez, S. J., Gugino, B. K., Ivors, K., and Harmon, C. 2012. Extension plant pathology: Strengthening resources to continue serving the public interest. Phytopathol. 102:652-655.

Everts, K. L., Walter, T. L., Jones, J. G., and Johnson, G. C. 2019. Evaluation of fungicide programs for management of downy mildew of processing cucumber – Trial 2, 2018. Plant Dis. Manage. Rep. 13:V128.

FRAC. 2020. FRAC Code List 2020 - Fungal control agents sorted by cross resistance pattern and mode of action. Fungicide Resistance Action Committee https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2020-finalb16c2b2c512362eb9a1eff00004acf5d.pdf?sfvrsn=54f499a_2

Gent, D. H., Cohen, Y., and Runge, F. 2017. Homothallism in *Pseudoperonospora humuli*. Plant Pathol. 66:1508–1516.

Goldenhar, K. E., and Hausbeck, M. K. 2019. Fungicides for control of downy mildew on pickling cucumber in Michigan. Plant Heal. Prog. 20:165–169.

Guan, W., Zhao, X., Treadwell, D. D., Alligood, M. R., Huber, D. J., and Dufault, N. S. 2013. Specialty melon cultivar evaluation under organic and conventional production in Florida. Horttechnology. 23:905–912.

Gugino, B. K., and Grove, T. L. 2016. Evaluation of selected fungicides and fungicide programs for managing downy mildew of cucumber, 2015. Plant Dis. Manage. Rep. 10:V072.

Hausbeck, M. K., Brisco-McCann, E. I., Goldenhar, K. E., and Cook, A. J. 2017. Evaluation of fungicide programs for control of downy mildew of cucumber, 2016. Plant Dis. Manag. Rep. 11:V063.

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E. G., González-Porto, A. V., Barrios, L., Del Nozal, M. J., Bernal J. L., Jiménez, J. J., Palencia, P. G., and Meana, A. 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ. Microbiol. 10:2659.

Hobbelen, P. H. F., Paveley, N. D., and Van den Bosch, F. 2011. Delaying selection for fungicide insensitivity by mixing fungicides at a low and high risk of resistance development: A modeling analysis. Phytopathology 101: 1224-1233.

Holmes, G. J., Ojiambo, P. S., Hausbeck, M. K., Quesada-Ocampo, L., and Keinath, A. P. 2015. Resurgence of cucurbit downy mildew in the United States: a watershed event for research and extension. Plant Dis. 99: 428-441.

Holmes, G. J., Pollard, D. W., and Darden, J. B. 1998. Evaluation of fungicides for control of downy mildew and powdery mildew of squash, 1997. Fungic. Nematic. Tests 53:239.

Ishii, H., Fraaije, B. A, Sugiyama, T., Noguchi, K., Nishimura, K., Takeda, T., Amano, T., and Hollomon, D. W. 2001. Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology, 91:1166–1171.

Ishii, H., Zhen, F., Hu, M., Li, X., and Schnabel, G. 2016. Efficacy of SDHI fungicides, including benzovindiflupyr, against *Colletotrichum* species. Pest Manag. Sci. 72:1844–1853.

Johnson, R. M., Dahlgren, L., Siegfried, B. D., and Ellis, M. D. 2013. Acaricide, fungicide and drug interactions in honey bees (Apis mellifera). PLoS One 8 (1): e54092.

Jones, J. G., Everts, K. L., McGrath, M. T., and Gugino, B. K. *under review*. Efficacy of fungicides for *Pseudoperonospora cubensis* determined using bioassays over multiple years in the Mid-Atlantic and Northeastern United States. Plant Heal. Prog.

Kakumanu, M. L., Reeves, A. M., Anderson, T. D., Rodrigues, R. R., and Williams, M. A. 2016. Honey bee gut microbiome is altered by in-hive pesticide exposures. Front. Microbiol. 7:1255.

Kalischuk, M., Paret, M. L., Freeman, J. H., Raj, D., Da Silva, S., Eubanks, S., Wiggins, D. J., Lollar, M., Marois, J. J., Mellinger, H. C., and Jnaneshwar, D. 2019. An improved crop scouting technique incorporating unmanned aerial vehicle-assisted multispectral crop imaging into conventional scouting practice for gummy stem blight in watermelon. Plant Dis. 103:1642–1650.

Keinath, A. P. 2017. Anthracnose fruit rot. Pages 88–89 in: Compendium of Cucurbit Diseases and Pests, second ed. A.P. Keinath, W. M. Wintermantel, T. A. Zitter, eds. American Phytopathological Society Press, St. Paul, MN.

Keinath, A. P. 2015a. Baseline Sensitivity of *Didymella bryoniae* to cyprodinil and fludioxonil and field efficacy of these fungicides against isolates resistant to pyraclostrobin and boscalid. Plant Dis. 99:815–822.

Keinath, A. P. 2014a. Differential susceptibility of nine cucurbit species to the foliar blight and crown canker phases of gummy stem blight. Plant Dis. 98:247-254.

Keinath, A. P. 2000. Effect of protectant fungicide application schedules on gummy stem blight epidemics and marketable yield of watermelon. Plant Dis. 84:254-260.

Keinath, A. P. 2015b. Efficacy of fungicides against powdery mildew on watermelon caused by *Podosphaera xanthii*. Crop Prot. 75:70–76.

Keinath, A. P. 2019. Integrated management of downy mildew on slicing cucumber with fungicides and host resistance but not trellising. Plant Dis. 103:2592–2598.

Keinath, A. P. 2018. Minimizing yield and quality losses in watermelon with multi-site and strobilurin fungicides effective against foliar and fruit anthracnose. Crop Prot. 106:72-78.

Keinath, A. P. 2016a. Polyoxin D and other biopesticides reduce gummy stem blight but not anthracnose on melon seedlings. Plant Health Prog. 17:177-181. doi:10.1094 / PHP-RS-16-0025.

Keinath, A. P. 2014b. Reproduction of *Didymella bryoniae* on nine species of cucurbits under field conditions. Plant Dis. 98:1379-1386.

Keinath, A. P. 2009. Sensitivity to azoxystrobin in *Didymella bryoniae* isolates collected before and after field use of strobilurin fungicides. Pest Manag. Sci. 65:1090-1096.

Keinath, A. P. 2016b. Utility of a cucumber plant bioassay to assess fungicide efficacy against *Pseudoperonospora cubensis*. Plant Dis. 100:490–499.

Keinath, A. P., and DuBose, V. B. 2014. Evaluation of fungicides rotated with mancozeb to control anthracnose on watermelon, 2013. Plant Dis. Manage. Rep. 8:V220.

Keinath, A. P., and Duthie, J. A. 1998. Yield and quality reductions in watermelon due to anthracnose, gummy stem blight, and black rot. Pages 77-90 in: Recent Research Developments in Plant Pathology, Vol. 2. Research Signpost, Trivandrum, India.

Keinath, A.P., and Hassell, R.L. 2000. Evaluation of diploid and triploid watermelon cultivars for field resistance to anthracnose and gummy stem blight. Biol. Cult. Tests 14:188.

Keinath, A. P., Holmes, G. J., Everts, K. L., Egel, D. S., and Langston, D. B., Jr. 2007. Evaluation of combinations of chlorothalonil with azoxystrobin, harpin, and disease forecasting for control of downy mildew and gummy stem blight on melon. Crop Prot. 26:83–88.

Keinath, A. P., Miller, S. A., and Smart, C. D. 2019. Response of *Pseudoperonospora cubensis* to preventative fungicide applications varies by state and year. Plant Heal. Prog. 20:142–146.

Keinath, A. P., and Zitter, T. A. 1998. Resistance to benomyl and thiophanate- methyl in *Didymella bryoniae* from South Carolina and New York. Plant Dis. 82:479-484.

Kitner, M., Lebeda, A., Sharma, R., Runge, F., Dvořák, P., Tahir, A., Choi, Y.-J., Sedláková. B., and Thines, M. 2015. Coincidence of virulence shifts and population genetic changes of *Pseudoperonospora cubensis* in the Czech Republic. Plant Pathol. 64:1461–1470.

Konstantinidou-Doltsinis, S., and Schmitt, A. 1998. Impact of treatment with plant extracts from *Reynoutria sachalinensis* (F. Schmidt) Nakai on intensity of powdery mildew severity and yield in cucumber under high disease pressure. Crop Prot. 17:649–656.

Kovach, J., Petzoldt, C., Degni, J., and Tette, J. 1992. A method to measure the environmental impact of pesticides. New York's Food and Life Sciences Bulletin 139:1-8.

Langston, Jr. D. B., Sanders, Jr. F. H. 2013. Evaluation of fungicides for control of

downy mildew on cucumber in Georgia I, 2012. Plant Dis. Manage. Rep. 7:V108.

Latin, R.X., and Egel, D.S. 2001. Melcast: Melon Disease Forecaster. Purdue Univ. Coop. Ext. Bull. BP-64-W.

Lebeda, A., and Cohen, Y. 2011. Cucurbit downy mildew (*Pseudoperonospora cubensis*)-biology, ecology, epidemiology, host-pathogen interaction and control. Eur. J. Plant Pathol. 129:157–192.

Lebeda, A., Křístková, E., Roháčková, J., Sedláková, B., Widrlechner, M. P., and Paris, H. S. 2016. Race-specific response of *Cucurbita* germplasm to *Pseudoperonospora cubensis*. Euphytica, 212:145–156.

Marine, S. C., Newark, M. J., Korir, R. C., and Everts, K. L. 2016. Evaluation of rotational biopesticide programs for disease management in organic cucurbit production. Plant Dis. 100:2226-2233.

Marrone, P. G. 2009. Barriers to adoption of biological control agents and biological pesticides. Pages 163-178 in: Integrated Pest Management: Concepts, Tactics, Strategies and Case Studies, E. B. Radcliffe, W. D. Hutchison, and R. E. Cancelado, eds. Cambridge University Press, Cambridge, MA.

Matheron, M. E., and Porchas, M. 2013. Efficacy of fungicides and rotational programs for management of powdery mildew on cantaloupe. Plant Dis. 97:196–200.

McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annu. Rev. Phytopathol. 40:349–379.

McGrath, M. T. 2009. Diseases of cucurbits and their management. Pages 455-510 in: Diseases of Fruits and Vegetables- Diagnosis and Management- Volume I, S. A. M. H. Naqvi, ed. Springer Science + Business Media, Berlin, Germany.

McGrath, M. T. 2017. First report of resistance to quinoxyfen in *Podosphaera xanthii*, causal agent of cucurbit powdery mildew, in the United States. Plant Heal. Prog. 18:94.

McGrath, M.T. 1996. Successful management of powdery mildew in pumpkin with disease threshold-based fungicide programs. Plant Dis. 80: 910-916.

McGrath, M. T., Menasha, S. R., and Sexton, Z. F. 2018. Evaluation of cucumber cultivars resistant to downy mildew, 2017. Plant Dis. Manage. Rep. 12:V069.

McGrath, M. T., and Sexton, Z. F. 2020. Efficacy of fungicides for managing powdery mildew on pumpkins, 2019. Plant Dis. Manage. Rep. 14:V081.

McGrath, M. T., and Thomas, C. E. 1996. Powdery mildew. Pages 28-30 in: Compendium of Cucurbit Diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.

McGrath, M. T., and Wyenandt, C. A. 2017. First detection of boscalid resistance in *Podosphaera xanthii* in the United States associated with failure to control cucurbit powdery mildew in New York and New Jersey in 2009. Plant Health Prog. 18:93. doi: 10.1094/PHP-03-17-0014-BR.

Miller, S. A., Vargas, A., and Amrhein, J. 2020. Bioassay for sensitivity of *Pseudoperonospora cubensis* to fungicides in OH, 2019. Plant Dis. Manage. Rep. 14:V046.

Mitchell, M. N., Ocamb, C. M., Grünwald, N. J., Mancino, L. E., and Gent, D. H. 2011. Genetic and pathogenic relatedness of *Pseudoperonospora cubensis* and *P. humuli*. Phytopathol. 101:805–818.

National Weather Service. 2020. Salisbury, MD Monthly & Annual Summary. https://www.weather.gov/media/akq/climateRECORDS/SBY_Climate_Records.pdf

New England Winter Squash Pest Management Strategic Plan. 2006. New England Pest Management Network Natalia Clifton, University of Massachusetts. https://ipmdata.ipmcenters.org/documents/pmsps/NE_Winter_Squash_PMSP.pdf

Newark, M., Li, P., Yang, X.-P., Paret, M. L., and Dufault, N. S. 2020. Comparing *Stagonosporopsis* spp. Fungicide Resistance Profiles in Florida and East China Production Systems. Plant Dis. 1995:129–136.

Ojiambo, P. S., Gent, D. H., Quesada-Ocampo, L. M., Hausbeck, M. K., and Holmes, G. J. 2015. Epidemiology and population biology of *Pseudoperonospora cubensis* : A model system for management of downy mildews. Annu. Rev. Phytopathol. 53:223–246.

Ojiambo, P. S., Holmes, G. J., Britton, W., Keever, T., Adams, M. L., Babadoost, M., Bost, S. C., Boyles, R., Brooks, M., Damicone, J., Draper, M. A., Egel, D. S., Everts, K. L., Ferrin, D. M., Gevens, A. J., Gugino, B. K., Hausbeck, M. K., Ingram, D. M., Isakeit, T,… Zhang, S. 2011. Cucurbit downy mildew ipmPIPE: A next generation web-based interactive tool for disease

management and extension outreach. Plant Health Prog. https://doi.org/10.1094/PHP-2011-0411-01-RV

Ojiambo, P. S., Paul, P. A., and Holmes, G. J. 2010. A quantitative review of fungicide efficacy for managing downy mildew in cucurbits. Phytopathology 100:1066-1076.

Olaya, G., Kuhn, P., Hert, A., Holmes, G., and Colucci, S. 2009. Fungicide resistance in cucurbit downy mildew. (Abstr.). Phytopathology 99:S171.

O'Neal, S. T., Reeves, A. M., Fell, R. D., Brewster, C. C., and Anderson, T. D. 2019. Chlorothalonil exposure alters virus susceptibility and markers of immunity, nutrition, and development in honey bees. J. Insect Sci. 19(3): 14; 1-8.

Parker, T. B. 2007. Investigation of hop downy mildew through association mapping and observations of the oospore. [Unpublished doctoral dissertation] Oregon State University.

Pavelková, J., Lebeda, A., and Sedláková, B. 2014. Efficacy of fosetyl-Al, propamocarb, dimethomorph, cymoxanil, metalaxyl and metalaxyl-M in Czech *Pseudoperonospora cubensis* populations during the years 2005 through 2010. Crop Prot. 60:9-19.

Pernthaler A., Pernthaler, J., and Amann, R. 2002. Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. 68:3094-3101.

Pest Management Strategic Plan for Cucumbers (Pickling) In Delaware and Eastern Shore Maryland. 2005. https://ipmdata.ipmcenters.org/documents/pmsps/DEpickle.pdf

Pest Management Strategic Plan for Watermelons in Delaware, Maryland, New Jersey, and North Carolina. 2008. https://ipmdata.ipmcenters.org/documents/pmsps/DE-MD-NJ-NCWatermelonPMSP.pdf

Pettis, J. S., Lichtenberg, E. M., Andree, M., Stitzinger, J., Rose, R., and vanEngelsdorp, D. 2013. Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS One. 8:e70182.

PMRA. 2020. Proposed Re-evaluation Decision PRVD2018-17, Mancozeb and its Associated End-use Products. Health Canada- Pest Management Regulatory Agency. https://www.canada.ca/en/health-canada/services/consumer-product-safety/pesticides-pest-management/public/consultations/proposed-re-evaluation-decisions/2018/mancozeb/document.html

Price, D., Sanders, F. H., Jr., and Langston, D. B., Jr. 2013. Evaluation of fungicide spray programs on anthracnose of watermelon in Georgia, 2012. Plant Dis. Manage. Rep. 7:V134.

Quesada-Ocampo, L. M., Granke, L. L., Olsen, J., Gutting, H. C., Runge, F., Thines, M., Lebeda, A., and Hausbeck, M. K. 2012. The genetic structure of *Pseudoperonospora cubensis* populations. Plant Dis. 96:1459-1470.

Rahman, A., Standish, J. R., D'Arcangelo, K. N., and Quesada-Ocampo, L. M. 2020. Clade-specific biosurveillance of *Pseudoperonospora cubensis* using spore traps for precision disease management of cucurbit downy mildew. Phytopathol.

Rideout, S. L., Waldenmaier, C. M., Custis, J. T., Jr., and Wimer, A. F. 2010. Comparison of organic fungicides for the management of downy mildew in pumpkin, 2009. Plant Dis. Manage. Rep. 4:V032.

Runge, F., Choi, Y.-J., and Thines, M. 2011. Phylogenetic investigations in the genus *Pseudoperonospora* reveal overlooked species and cryptic diversity in the *P. cubensis* species cluster. Eur. J. Plant Pathol. 129:135–146.

Runge, F. and Thines, M. 2012. Reevaluation of host specificity of the closely related species *Pseudoperonospora humuli* and *P. cubensis*. Plant Dis. 96:55-61.

Rur, M., Rämert, B., Hökeberg, M., Vetukuri, R. R., Grenville-Briggs, L., and Liljeroth, E. 2018. Screening of alternative products for integrated pest management of cucurbit powdery mildew in Sweden. Eur. J. Plant Pathol. 150:127–138.

Salas, S. E., Shepherd, C. P., Ngugi, H. K., and Genet, J. L. 2019. Disease control attributes of oxathiapiprolin fungicides for management of cucurbit downy mildew. Plant Dis. 103:2812–2820.

Salgado-Salazar, C., Bauchan, G. R., Wallace, E. C., and Crouch, J. A. 2018. Visualization of the impatiens downy mildew pathogen using fluorescence in situ hybridization (FISH). Plant Methods 14:92.

Savory, E. A., Granke, L. L., Quesada-Ocampo, L. M., Varbanova, M., Hausbeck, M. K., and Day, B. 2011. The cucurbit downy mildew pathogen *Pseudoperonospora cubensis*. Mol. Plant Pathol. 12:217-226.

Sitterly, W. R. 1972. Breeding for disease resistance in cucurbits. Annu. Rev. Phytopathol. 10:471-490

Stewart, J. E., Turner, A. N., and Brewer, M. T. 2015. Evolutionary history and variation in host range of three *Stagonosporopsis* species causing gummy stem blight of cucurbits. Fungal Biol. 119:370-382.

Summers, C. F., Adair, N. L., Gent, D. H., McGrath, M. T., and Smart, C. D. 2015. *Pseudoperonospora cubensis* and *P. humuli* detection using species-specific probes and high definition melt curve analysis. Can. J. Plant Pathol. 37:315-330.

Thomas, A., Carbone, I., Cohen, Y., and Ojiambo, P. S. 2017. Occurrence and distribution of mating types of *Pseudoperonospora cubensis* in the United States. Phytopathol. 107:313–321.

Thomas, A., Langston, D. B., Jr., and Stevenson, K. L. 2012. Baseline sensitivity and cross-resistance to succinate-dehydrogenase-inhibiting and demethylation-inhibiting fungicides in *Didymella bryoniae*. Plant Dis. 96:979-984.

Thomas, A., Neufeld, K. N., Seebold, K. W., Braun, C. A., Schwarz, M. R., and Ojiambo, P. S. 2018. Resistance to fluopicolide and propamocarb and baseline sensitivity to ethaboxam among isolates of *Pseudoperonospora cubensis* from the eastern United States. Plant Dis. 102:1619–1626.

Thomsen, R., Nielsen, P. S., and Jensen, T. H. 2005. Dramatically improved RNA in situ hybridization signals using LNA-modified probes. RNA 11:1745-1748.

Tomura, T., Molli, S. D., Murata, R., and Ojika, M. 2017. Universality of the *Phytophthora* mating hormones and diversity of their production profile. Sci. Rep. 7:5007.

Tucker, M. A., Lopez-Ruiz, F., and Oliver, R. P. 2015. Origin of Fungicide-Resistant Barley Powdery Mildew in Western Australia: Lessons to be Learned. Pages 329-340 in: Fungicide Resistance in Plant Pathogens. H. Ishii and D. W. Hollomon, eds. Springer, Tokyo, Japan.

USDA. 2016. Agriculture in Maryland, Summary for 2015. Maryland Agricultural Statistics. United States Department of Agriculture- National Agricultural Statistics Service. https://www.nass.usda.gov/Statistics_by_State/Maryland/Publications/Annual_Statistical_Bulletin/2017/2017-2018%20MD%20Annual%20Bulletin.pdf

USDA. 2006a. Agricultural statistics 2006- Chapter IV- Statistics of vegetables and melons. United States Department of Agriculture, National Agricultural Statistics Service. https://www.nass.usda.gov/Publications/Ag_Statistics/2006/CHAP04

USDA. 2009. Agricultural statistics 2009- Chapter IV- Statistics of vegetables and melons. United States Department of Agriculture, National Agricultural Statistics Service. http://www.nass.usda.gov/Publications/Ag_Statistics/2009/chp04

USDA. 2017a. Polyoxin D zinc salt. Technical evaluation report. United States Department of Agriculture- Agriculture Marketing Service- National Organic Program. https://www.ams.usda.gov/sites/default/files/media/PolyoxinDZincSaltTRFinal20171212.pdf

USDA. 2006b. United States standards for grades of watermelons. United States Department of Agriculture- Agricultural Marketing Service. https://www.ams.usda.gov/sites/default/files/media/Watermelon_Standard%5B1%5D.pdf

USDA. 2017b. Vegetables, Potatoes, and Melons for Sale 2017 and 2012. United States Department of Agriculture, National Agriculture Statistics Service. https://www.nass.usda.gov/Publications/AgCensus/2017/Full_Report/Volume_1,_Chapter_2_US_State_Level/st99_2_0029_0029.pdf

USDA. 2020. Vegetables 2019 Summary (February 2020). United States Department of Agriculture, National Agriculture Statistics Service. https://www.nass.usda.gov/Publications/Todays_Reports/reports/vegean20.pdf

USDA. 2019. 2018 Vegetable Chemical Use. Pesticide Use, cantaloupe, honeydews, watermelons. United States Department of Agriculture- National Agriculture Statistics Service. https://www.nass.usda.gov/Data_and_Statistics/Pre-Defined_Queries/2018_Vegetables/index.php

Van-Zwieten, L., Merrington, G., and Van-Zwieten, M. 2004. Review of impacts on soil biota caused by copper residues from fungicide application. Proceedings of the 3rd Australian New Zealand Soils Conference. University of Sydney, Sydney, Australia.

Wallace, E. C., D'Arcangelo, K. N., and Quesada-Ocampo, L. M. 2020. Population analyses reveal two host-adapted clades of *Pseudoperonospora cubensis*, the

causal agent of cucurbit downy mildew, on commercial and wild cucurbits. Phytopathol. 110: 1578–1587.

Wallace, E. C., and Quesada-Ocampo, L. M. 2017. Analysis of microsatellites from the transcriptome of downy mildew pathogens and their application for characterization of *Pseudoperonospora* populations. PeerJ, 5:e3266.

Wasilwa, L. A., Correll, J. C., Morelock, T. E., and McNew, R. E. 1993. Reexamination of races of the cucurbit anthracnose pathogen *Colletotrichum orbiculare*. Phytopathol. 83:1190-1198.

Withers, S., Gongora-Castillo, E., Gent, D., Thomas, A., Ojiambo, P. S., and Quesada-Ocampo, L. M. 2016. Using next-generation sequencing to develop molecular diagnostics for *Pseudoperonospora cubensis*, the cucurbit downy mildew pathogen. Phytopathol. 106:1105-1116.

Wyenandt, C. A., Kline, W. L., and Ward, D. L. 2017. Effect of fungicide program on the development of downy mildew in three cucurbit crops in New Jersey. Plant Health Prog. 18:181-185.

Wyenandt, C. A., Ward, D. L., and Maxwell, N. L. 2010. Determining “practical” fungicide resistance development and drift in the control of cucurbit powdery mildew in pumpkin. Plant Health Prog. 11. doi:10.1094/PHP-2010-1122-02-RS.

Zhang, Y.-J., Pu, Z.-J., Qin, Z.-W., Zhou, X.-Y., Liu, D., Dai, L.-T., and Wang, W.-B. 2012. A study on the overwintering of cucumber downy mildew oospores in China. J. Phytopathol. 160:469–474.

Zhang, S., Vallad, G. E., White, T. L., and Huang, C.-H. 2011. Evaluation of microbial products for management of powdery mildew on summer squash and cantaloupe in Florida. Plant Dis. 95:461-468.

Zhu, W., Schmehl, D. R., Mullin, C. A., and Frazier, J. L. 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PLoS One. 9:e77547.

Zitter, T. A. 1992. Gummy stem blight of cucurbits. Online Fact Sheet 732.70. Cornell University. http://vegetablemdonline.ppath.cornell.edu/factsheets/Cucurbit_GSBlight.htm