ABSTRACT

KIKWAY, ISAACK. Epidemiology and Ecology of Cucurbit Downy Mildew: Temporal Dynamics, Oospore Occurrence and Shifts in Fungicide Sensitivity. (Under the direction of Dr. Peter S. Ojiambo).

Cucurbit downy mildew caused by the biotrophic oomycete *Pseudoperonospora cubensis* is economically the most important disease the Cucurbitaceae family. In the continental United States, the pathogen is largely thought to overwinter in regions located below 30-degree latitude in south Florida and Texas around Gulf of Mexico, where cucurbits are grown year-round. While disease development is well characterized at the landscape level, there is limited information on disease dynamics at the field level. Temporal dynamics of cucurbit downy mildew epidemics were characterized in North Carolina and South Carolina to understand how management strategies influence disease epidemics. Standardized area under disease progress curve, final disease severity and weighted mean absolute rates of disease progress were significantly affected by fungicide treatment and host type (P < 0.0001), with significantly lower values in fungicide treated plots than in untreated control plots. Due to lack of cultivars with disease resistance, fungicides are still the most effective method for disease management. However, the pathogen has a high risk for developing resistance to fungicides and there is need for evaluating programs that slow development of fungicide resistance. Within a season, directional shifts in fungicide sensitivity profiles of *P. cubensis* populations was observed following alternation of chlorothalonil with either cymoxanil, fluopicolide or propamocarb in field experiments in North Carolina and South Carolina. Kruskal-Wallis test showed the mean disease severity increased significantly among early- and late-season isolates where chlorothalonil was alternated with fluopicolide (P = 0.0010) but not with cymoxanil (P = 0.2378) or propamocarb (P = 0.5604). Based on a combined data, a directional shift in fungicide sensitivity distribution were significant for fluopicolide (χ^2 = 6.64; P

= 0.0100) but not propamocarb (χ^2 = 0.11; P = 0.7372). Early- and late-season *P. cubensis* isolates sampled from the cymoxanil treated plots were all resistant, and no significant (χ^2 = 0.06; P = 0.8087) shifts in their fungicide sensitivity profile during a growing season. Finally, while oospores have been observed under laboratory conditions, their occurrence and survival during the winter period under field conditions has not been reported in the United States. In a survey of infected leaves collected in North Carolina and South Carolina, about 5% of 1,658 naturally infected cucumber and cantaloupe leaves sampled during the study had oospores. In addition, absolute (slope = −0.27; P < 0.0001) and standardized (slope = −0.39; P < 0.0001) oospore viability declined linearly over a six-month period following exposure during the winter season. With other variables held constant, the decline in oospore viability was significantly affected by soil temperature (b = −0.03 to −0.05; P < 0.0001) and number of rainy days (b = 21.6 to 40.46; P < 0.05). About 20% of the oospores exposed to outdoor conditions at the end the study period were putatively viable and deemed potentially infective. These results provide information on the potential role of oospores in the epidemiology of cucurbit downy mildew in the southeastern United States.

Epidemiology and Ecology of Cucurbit Downy Mildew: Temporal Dynamics, Oospore Occurrence and Shifts in Fungicide Sensitivity

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BIOGRAPHY

Kikway Isaack was born in the County of Uasin-Gishu, Kenya, and had the opportunity to live with diverse communities while growing up. From a humble background where peasant farming was the main economic activity Isaack completed his primary education from Lainguse primary in 2003 and proceeded for Secondary education in Cengalo Secondary School where he finished his Kenya Certificate of Secondary Education (KCSE) in the year 2007. From Cengalo Secondary school, Isaack joined University of Eldoret to pursue his Bachelor of Science degree in Horticulture Science and Management. He graduated in December of 2013 with a Bachelor of Science in Horticulture Science and Management. After mentorship from Dr. Sitienei on availability of research opportunities in United States based universities, he realized his passion for research in field and laboratory work. Isaack joined the Department of Plant Pathology at Louisiana State University under the guidance of Dr. Melanie Lewis Ivey in spring 2015 for MS program in Plant Pathology. In spring 2017, Isaack graduated with a Master of Science in Plant Pathology and immediately proceeded to start his PhD program in fall 2017 at North Carolina State University under advisement of Dr. Peter Ojiambo, in the Department of Entomology and Plant pathology. During his PhD program, Isaack's research focused on the epidemiology, biology and management of cucurbit downy mildew caused by the oomycete *Pseudoperonospora cubensis*.

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CHAPTER 1

Literature Review

1.0. Cucurbitaceae family

The family of Cucurbitaceae commonly known as the gourd family are large dicotyledonous plants distributed around tropical and subtropical regions (Ajuru and Nmom, 2017). It is of high economic value to man as major source of raw or cooked food (McCreight, 2016). Cucurbitaceae family comprise of about 120 genera and more than 800 species (Ajuru and Nmom, 2017; Schaffer and Paris, 2003). The Cucurbitaceae family has vast socio-economic importance such as food, medicinal value, source of biodiesel, and complimentary dietary for poultry and remedies for livestock (Ajuru and Nmom, 2017). Members of the family are herbaceous vines with tendrils for climbing and can grow annually or perennially (Schaffer and Paris, 2003). This family is comprised of world's most valuable crops including cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), squash (*Cucurbita spp*.), zucchini (*Cucurbita pepo*) and pumpkin (*Cucurbita moschata)* (Kousik et al., 2003; Schaffer and Paris, 2003).

Morphologically, cucurbits are characterized by extensive root system, hollow angled, hairy, and prickly stems. With leaf axil bearing a flower bud and tendril, cucurbit leaf is large simple deeply lobed spirally and arranged along petioles. Cucurbits are monoecious with unisexual flowers born in the leaf axil (Kousik et al., 2003). Cucurbit fruits are large fleshy with hard outer covering with seeds attachment in the ovary wall are in parietal placentation. Cucurbits are subjects to many disease and insect pests that affects growth and yield. Powdery mildew, gummy stem blight, cucurbit downy mildew and many other diseases affects cucurbits globally. While disease control is not always successful, it can be managed using cultural practices, chemical protectants or eradicants, and host resistance (Jahn et al., 2002; McCreight, 2016; Zitter et al., 1996). Cucurbits are produced globally in over 80 countries and, in the United States, cucurbits among other fresh vegetables are of high economic value and are grown in the eastern portion of United States, and California. In 2018 alone, 333,000 acres were harvested in the United States with a value of $1.38 billion (USDA-NASS, 2019).

1.1. Cucurbit downy mildew

Cucurbit downy mildew (CDM) is a foliar disease caused by an oomycete pathogen *Pseudoperonospora cubensis*. It is an important foliar disease of cucurbits and has devastating impacts on cucurbit production, causing significant yield losses in the United States, Europe, China, Israel, and in other parts of the world when poorly managed (Lebeda and Cohen, 2010; Thomas, 1996). The disease has been reported in wide geographical environments ranging from temperate, semi-arid, and tropical open fields, tunnels or under greenhouse production system (Savory et al., 2011). Cucurbit downy mildew affects foliar portions of the plant causing significant losses in fruit quality and yield among members of Cucurbitaceae family that include Cucumber (*Cucumis sativa*), cantaloupe (*Cucumis melo*), watermelon (*Citrullus lanatus,*), pumpkin (*Cucurbita maxima*) and acorn and summer squash (*Cucurbita pepo*, *Cucurbita moschata*) (Savory et al., 2011; Palti and Cohen, 1980). *Pseudoperonospora cubensis* pathogen shows differences in virulence and pathogenicity depending on cucurbit host type with cucumber being the most susceptible as compared to other cucurbits. Additionally, evidence of existence of different physiological races or pathotypes has been described in other parts of the globe (Cohen et al., 2003; Lebeda and Gadasova, 2002; Sarris et al., 2009; Savory et al., 2011).

1.2. Pathogen Taxonomy

Pseudoperonospora cubensis is classified into Kingdom Straminipila (chromista), Phylum oomycota, Class oomycetes, Order peronosporales, Family Peronosporaceae, Genus *Pseudoperonospora* and Species *Pseudoperonospora cubensis* (Savory et al., 2011; Thomas, 1996). *Pseudoperonospora cubensis* belong to genus *Pseudoperonospora* together with *Pseudoperonospora humuli*, *Pseudoperonospora cannabina*, *Pseudoperonospora celtidis*, *Pseudoperonospora urticae* and *Pseudoperonospora cassiae* (Choi et al., 2005; Savory et al., 2011). Systemic classification of *P. cubensis* is based on the thalli morphology and phylogenetic studies. Morphological characters in taxonomic classification include conidiosporangiophore morphology, conidiosporangium shape and color, haustoria types and the ability to discharge zoospores (Savory et al., 2011; Voglmayr et al., 2004). Unlike species of Peronospora with sporangia that germinate directly via germ tube, *Pseudoperonospora* species possess a special kind of germination where sporangia germinate via cytoplasmic cleavage to produce motile zoospores (Palti and Cohen, 1980; Rostovzev, 1903).

1.3. Morphology

Pseudoperonospora cubensis is composed of hyaline, coenocytic, intercellular mycelium that develop on host’s mesophyll cells and palisade tissues. Within the host tissues is a specialized clevate-branched intercellular haustoria within the mesophyll (Lange et al., 1989b). Haustoria are specialized structures for nutrient absorption and delivery of effector proteins used by pathogens in suppressing host defense response during pathogen host interaction (Savory et al., 2010; Hahn and Mendgen, 2001; Whisson et al., 2007). Sporangiophores holding reproductive structures emerge from host stomata on the lower side of infected leaves and are hyaline, dichotomously branched bearing lemon shaped, grey-purple single sporangia on their tips (Choi et al., 2005; Palti, 1975). Gray to purple sporangia on the underside of infected cucurbit leaves are classic sign of CDM disease in the field. Sporangium which is attached to specialized hyphal structure called sporangiophore is a vegetative asexual structure that holds zoospores they develop. *Pseudoperonospora species* sporangia germinate via cytoplasmic cleavage to produce 2 to 15 (Palti and Cohen, 1980) biflagellate zoospores. The two flagella (i.e., tinsel and whiplash) aid zoospores swim towards open stomata where they encyst before penetration (Iwata, 1949). Whereas Oomycetes such as *Schelospora sorghi*, *Plasmopara halstedii*, and *Pseudoperonospora humuli* are homothallic (Gent et al., 2017; Kitner et al., 2021), species such as *Phytopthora infestans* (potato and tomato), *Plasmopara viticola* (grape), and *Bremia lactucae* (lettuce) are heterothallic. Unlike homothallic species in which male antheridium fertilizes female oogonium produced by the same mycelium (Kitner et al., 2021), heterothallic species reproduce sexually when mycelia of two opposite mating types grow together to form thick -walled oospores.

Most oomycetes produce thick-walled sexual structures adapted to withstand extreme weather conditions in the soil for up to 10 months (Zang et al., 2012). Oospores act as over-seasoning and survival structures. They can survive in soil, plant debris, fruits, and seeds of infected host. At the onset of growing season oospores may act as primary initial inoculum in regions where host plants cannot survive harsh winter condition. Studies conducted under laboratory conditions in Israel and in the United States have shown that *Pseudoperonospora* *cubensis* is heterothallic. Co-inoculation of paired mixed sporangia of field isolates of A1 and A2 opposite mating types within the populations in the United States on detached cucumber or cantaloupe leaves and incubated at 15 or 21^0C resulted in production of hyaline to golden yellow, spherical thick-walled oospores within 6-11 days in the mesophyll (Thomas et al., 2017). Oospore production is dependent on the co-inoculation sporangia isolate pair, the host inoculated, and temperature. Occurrence of oospores in nature is rare and has been reported in open fields in Russia (Rostowzew, 1903), Japan (Hiura and Kawada, 1933), in North china (Chen, 1959) and under greenhouse conditions in China (Zhang et al., 2012) and Italy (D'Ercole, 1975). However, no reports on oospores of *P. cubensis* occurrence in nature have been reported in the United States and efforts to determine oospore occurrence in nature is an ongoing process.

1.4. Life and disease cycle

1.4.1 Asexual reproduction

Pseudoperonospora cubensis reproduces primarily asexually where dichotomously branched sporangiophore emerge from stomata openings bearing lemon shaped sporangia on sterigmata (Savory et al., 2011). Asexual spores are the main infective units of *P. cubensis* (Lebeda and Cohen 2010). Zoosporangium or conidiosporangium are light grey to deep purple in color, ovoid, and lemon shaped are infective and responsible for initiation and disease spread. Due to high reproductive capacity and repetitive disease cycles within a season, *P. cubensis* is regarded as polycyclic pathogen and consequently known for polycyclic epidemics (Kranz, 2003). *Pseudoperonospora. Cubensis* is an obligate biotrophic parasite (Savory et al., 2011). In the United States, growth and survival of *P. cubensis* depend on the presence of cucurbit hosts in the regions with mild winters where production of cucurbits occurs whole year-round such as South Florida, Texas, Gulf of Mexico, and greenhouse production (Holmes et al., 2015; Nusbaum, 1944; Ojiambo and Holmes, 2011; Thomas, 1996). Regions above 30-degree latitude experiencing freezing winters receives sporangia aerially dispersed over long distance via air currents. These melanized sporangia act as source of primary inoculum that initiate primary cycle of disease in the growing season. Secondary disease cycles are initiated by locally produced sporangia dispersed by rain splash, wind, or anthropogenic activities (Lebeda and Cohen, 2010). The pathogen overwinters in regions that receive mild winter conditions without lethal frost. During spring when cucurbit host are present in northern latitudes, long distance aerial blown sporangia land on host leaf surfaces where it undergoes indirect germination in presence of water (rain or dew) (Lebeda and Cohen, 2010). Under favorable conditions such as moisture (irrigation water, rain, or dew) and optimum temperature, airborne zoosporangia undergo cytoplasmic cleavage to release 5 to15 biflagellate zoospores that chemotactically swim towards host stomata where they encyst (Cohen, 1981). Germ-tube emerge from cystospore and appressorium penetrate host tissue via stomata aperture (Cohen and Lebeda, 2010). Infection hyphae grow into host intercellular space and colonize mesophyll tissue. The pathogen establishes web like structures called haustoria for uptake of nutrients and release of effector proteins to suppress host defense response. Depending on environmental conditions, inoculum load and host genotype (resistance/susceptibility), incubation period from penetration, and colonization until visible symptoms appear may take between 4-12 days (Lebeda and Cohen, 2011). *P. cubensis* asexually reproduce and branched sporangiophore emerge from stomata bearing lemon shaped sporangia at the tips within 5-7 days post infection. These sporangia are easily dislodged from sporangiophore by air or rain splash and act as either primary or secondary inoculum locally or wind blown over long distance (Cohen et al., 2015). Polycyclic nature of *P. cubensis* as a result of asexual reproduction is attributed for regional and temporal dynamics on the diversity and epidemics of *P. cubensis.*

1.4.2 Sexual reproduction and overwintering

Oomycetes reproduce sexually through the production of thick-walled sexual, survival structures that can overwinter between seasons as oospores in soil or on plant debris when a living host is absent. Sexual reproduction in *P. cubensis* is rare. However, sexual reproduction in *Perenosporaceae* results in formation of oospores under special conditions (Michelmore, 1981). Few reports of sexual reproduction in *P. cubensis* have been reported under laboratory conditions (Thomas et al., 2017; Cohen and Rubin, 2012). Like other oomycetes sexual reproduction in *P. cubensis* results in formation of thick-walled oospore structures. A few studies in Asia and Europe have shown the possibility of *P. cubensis* to overwinter in the soil or plant debris during winter (Zhang et al., 2012). Sexual reproduction of *P. cubensis* have been successful under laboratory condition in Israel (Cohen and Rubin, 2012) and in the United States (Thomas et al., 2017). Documented sexual reproduction in *P. cubensis* occurs mainly late in the season when the disease is present and infected tissues are necrotic (Bedlan, 1989; Lebeda, 1990). However, no reports on formation of oospores under natural field conditions have been reported in the United States. Being an obligate biotrophic parasite, *P. cubensis* is known to survive only on the living host (Savory et al., 2011). In the United States *P. cubensis* pathogen mycelia overwinter in the regions that receive mild winter temperatures with whole year-round cucurbit production such as southern Florida, Texas, and Gulf of Mexico.

Existence of opposite compatible mating types (A1 and A2) in the United States has been reported (Thomas et al., 2017). Under laboratory-controlled conditions A1 and A2 mating types sexually reproduce oospores which are thick walled, spherical, brown structures (Cohen et al., 2012; Thomas et al., 2017). Occurrence of oospores of *P. cubensis* in green house chambers was reported in Austria (Bedlan, 1989) and in China (Zhang et al., 2012). Additionally, reports on the occurrence of overwintering oospores in nature has been reported in other parts of the world such as in Bulgaria (Elenkov, 1967), Japan (Hiura and Kawada, 1933), India (Bains and Jhooty, 1976), Italy (D'Ercole, 1975), Israel (Cohen et al., 2003) and Russia (Rostowzew, 1903). Features of oospores as reported by Hiura and Kawada (1933) showed presence of obovoid to ellipsoid or irregular pyriform measuring about 28-56 × 24-44 µ. The male part involved the antheridia that are clevate to globose and measure 14-22 × 10-16 µ. Production of oospores by invitro co-inoculation of A1 and A2 opposite mating types on detached leaves under laboratory conditions has been reported in the United States (Thomas et al., 2017). Reports on the existence of oospores under greenhouse chambers were spherical, hyaline to pale yellow structures on leaves attached to the plant on necrotic lesions. Bedlan (1989) detected oospores in older leaves of cucumber in a green house. Studies in china reported occurrence of oospores towards the end of the season on greenhouse grown cucumbers (Zhang et al., 2012). Whereas oospores of *P. cubensis* reported in the US were produced under laboratory conditions, to date there are no reports of oospore occurrence in nature. The existence of A1 and A2 mating types in nature and consequently formation of oospores under laboratory conditions in the United States, suggest that oospores may occur in continental United States under natural environmental conditions, and this may influence the annual epidemics and management programs of cucurbit downy mildew.

1.5. Symptoms and Signs

Pseudoperonospora cubensis is an obligate parasite that needs living cucurbit plant tissue to grow, survive and reproduce (Savory et al., 2011). Under favorable environmental conditions and in presence of susceptible cucurbit host, the pathogen causes cucurbit downy mildew disease and the reaction results in salient symptoms upon infection. Symptoms are generally characterized by development of water-soaked lesions that turn chlorotic and later necrotic on the adaxial leaf surface (Savory et al., 2011). However, symptoms morphology in terms of lesion size, shape and development may vary based on cucurbit host species (Savory et al., 2011). During prolonged leaf wetness due to rainfall, dew or irrigation cucumber symptoms may start as water-soaked lesions on the upper side or underside of an infected leaf that gradually increase forming angular chlorotic lesion that are restricted by leaf veins on the foliage (Savory et al., 2011). In other cucurbit species such as cantaloupe, watermelon and squash, leafspots may start as pale green that enlarge and turn brown. On the underside of the infected leaves, lesions bear grey to dark sporangia that are lemon shaped and attached on to sporangiophores emerging from stomates. As the disease progresses, the lesions tend to coalesce into large necrotic areas (Thomas, 1996). The “wildfire like” disease progress may lead to a rapid plant death in a few days. Due to defoliation, cucurbit downy mildew infection leads to reduced canopy and consequently limiting photosynthetic area hence leading to halted fruit development, stunted growth, and increased sun exposure (Savory et al., 2011; Keinath et al., 2007). Sun scald affects quality fruit due misshapen fruit and sun-scalded fruits and rots and eventually reduced yields (Keinath et al., 2007). In summary, without effective intervention *P. cubensis* greatly reduce cucurbits crop yield and quality of marketable fruits.

1.6. Ecology and Epidemiology

1.6.1. Overwintering, survival, and spring infection

Oomycetes such as *Bremia lactucae*, *Sclerospora sorghi* and *Phytophthora infestans* overwinter in plant debris in soil, and seeds as thick-walled oospores (Savory et al., 2011). Oospores have occasionally been reported from parts of Asia and Europe such as in Japan (Hiura and Kawada, 1933), China (Zhang et al., 2012; Chen, 1959), India (Bains and Jhooty, 1976), Italy (D'Ercole, 1975), and in Russia (Rostowzew 1903). However, quest for oospores occurrence in many countries including the United States has not been successful, and the role of *P. cubensis* oospores formation and their role as primary inoculum is unknown. While oospores of *P. cubensis* can be generated under laboratory conditions (Thomas et al., 2017; Cohen and Rubin, 2011) little is known on their existence in nature and their implications on annual cucurbit downy mildew epidemics. With oospores reports on existence in nature in parts of the world and successful formation in the laboratory, oospores need to be considered as potential local primary inoculum that carry disease over winter and initiate disease during spring in the United States. However, the occurrence in nature, germination and infection by oospores of *P. cubensis* has not been established.

Being an obligate biotroph, *P. cubensis* may survive on overwintering cucurbit host under glass or plastic cover in temperate and semi-arid regions. These protected winter crops in regions that experience freezing winter provide a 'green-bridge' effect and thus are highly suspected as sources of downy mildew inoculum for field-grown crops grown in spring early and summer in regions that receive freezing winter. Additionally, *P. cubensis* may survive during winter on wild hosts, cucurbits in greenhouses and in open production fields in southern regions such as Southern Florida, Texas, and Mexico (Thomas et al., 2017; Savory et al. 2011, Wallace et al., 2015). These regions are below 30-degree latitude and experience subtropical climate that allows for year-round production of cucurbits plants. Sporangia are the primary infective units of *P. cubensis*. Long distance aerial dispersal of sporangia via wind currents from southern warm regions to northern latitudes act as source of inoculum (Holmes et al., 2004; Savory et al., 2011). During the season, local transport of secondary inoculum is dispersed via wind, rain splash or physical transfer by anthropogenic activities within a field initiate polycyclic epidemics (Thomas, 1996). In the spring and summer months, the pathogen typically spreads over long distance from its overwintering locations in the southern regions to northern regions in the United States. Disease occurrence has been found to follow regular planting dates probably due to south to north aerial dispersal of sporangia (Ojiambo et al., 2015). With the primary inoculum that cause devastating epidemics arising from long distance aerial dispersal of sporangia, presence of locally produced inoculum in form of oospores under natural environmental condition has not been documented in the continental United States.

The lifespan of a sporangium is about 48 h after detachment from sporangiophore (Savory et al., 2011; Cohen and Rotem, 1971a). Upon deposition on host surface in presence of water or dew, sporangia germinate indirectly via production of multiple biflagellate zoospores. The zoospores swims within moisture into the leaf stomata where they encyst. Thereafter, germ tubes forms appressorium which penetrate leaves cuticle through stomata and subsequently colonize leaf tissues by forming haustoria. Under favorable relative humidity (RH 90-100%) (Sun et al., 2017), temperature 5–30°C (Cohen, 1977b; Cohen, 1981) and minimal wetting period of about 2 h, the infection, colonization, and parasitism of the susceptible host (Cohen, 1976) cells is relatively faster and sporangiophore bearing lemon shaped sporangia may emerge from stomata on the underside of the leaves within 5 to 7 days.

Asexual reproduction of zoosporangia can occur at temperatures from 5 to 30°C, with optimum production at 15 to 20°C (Cohen et al., 1971).

1.7. Heterothallism, pathotypes and host specialization and sexual reproduction

Pseudoperonospora cubensis is a highly diverse pathogen and several pathotypes have been documented in the United States, Israel, Czech Republic, and Kazakhstan (Lebada et al., 2013; Rsaliyev et al., 2018; Thomas et al., 2017). Studies conducted in Israel, China and in the United States showed the existence of A1 and A2 opposite mating types in nature (Cohen and Rubin, 2012). Additionally, population biology studies conducted in the United States found the existence of clade I and clade II in the United States (Runge et al., 2011; Thomas et al., 2017). Globally, A2 mating type has been reported in Angola, China, Russia, Kenya, Israel, Ukraine and Vietnam (Cohen et al., 2013; Cohen et al., 2015). Phylogenic studies have grouped *P. cubensis* into clade I and clade II with heterothallic mating types A1 in clade II and A2 grouped in clade I (Thomas et al., 2017; Wallace et al., 2020). Thomas et al. (2017) in their study on the *P. cubensis* host specialization found that A1 mating types is associated with cucumber while A2 mating type were found associated with squash. Discovery of A2 mating type in 2010 in Asia was a breakthrough for studies involving sexual reproduction and possible formation of oospores by *P. cubensis* pathogen (Cohen et al., 2012). Co-inoculation of sporangia from A1 and A2 mating types on detached leaves under controlled laboratory studies in Israel and in the United States resulted in formation of spherical thick-walled oospores structures (Cohen et al., 2012; Thomas et al., 2017). Oospores produced under controlled laboratory conditions in Israel were able to infect and cause disease and produce sporangia within 7-20 days (Cohen, 2012). However, in the United States oospores produced under lab conditions did not cause disease on detached leaf bioassay (Thomas et al., 2017). The discovery of mating types and subsequent formation of oospores under lab conditions suggest probable formation of oospores under natural environmental conditions. Additionally, A1 and A2 mating type in nature in the United States may suggest possibility of oospore occurrence in nature and therefore, more studies are needed to ascertain their occurrence and potential role in initiation of cucurbit downy mildew disease epidemics.

1.8. Plant host resistance and disease resurgence

Development of host resistant cucumber cultivars carrying recessive *dm-1* resistant gene in 1960s provided effective management of cucurbit downy mildew with minimal fungicide application for about 40 years (Holmes et al. 2006). However, in 2004, *P. cubensis* cryptic strains of the A1 mating type overcame *dm-1* resistance gene and since then host resistance alone has ceased being an effective management strategy. To date, chemical application has been the most reliable CDM management program (Cohen et al., 2015). Back in 2004 and 2005, cucurbit downy mildew disease was characterized by severe disease epidemics that resulted in crop failure in most parts of eastern United States in 2004 and 2005. *P. cubensis* is considered a “high risk pathogens” with high evolutionary potential resulting in geographical and seasonal genetic diversity that can over host resistance. Development of durable host resistance in susceptible host has been unsuccessful. High evolutionary potential of *P. cubensis* result in multiple races of pathogen hence multiple genes for resistance to enhance quantitative resistance would need to be identified and bred into commercial cucurbits cultivars.

The resurgence of cucurbit downy mildew in the United States in 2004 was described as a unique phenomenon characterized by devastating epidemics, rapid spread, failure of fungicide control programs, complete crop loss in several regions, and abandoned cucumber fields prior harvest (Holmes et al., 2015). Prior to severe epidemics experienced in the United States in 2004 and 2005, cucumber host resistance management was the most reliable disease management tool. While cucurbit downy mildew incidence was not absent in the United States, management on cucumber crop did not require fungicide application and other cucurbit host such as squashes survived with minimal application of fungicide (Holmes et al., 2015). Breeding of cucumber cultivars in 1960s resulted in durable host resistance *dm-1* gene from Plant introduction (PI) accession 197087 that was effective for about four decades (Barnes and Epps, 1954). However, severe disease epidemics reported in 2004 and 2005 were due to the resurgence of the disease due to the breakdown *dm-1* gene conferring host resistance in cucumber. Epidemics in 2004 and 2005 were characterized by high disease levels, failure of disease management methods and abandonment of cucurbit fields before harvest and total crop loss. The shift within the pathogen populations reported in Israel (Cohen et al., 2003) and Europe around this period resulted in discovery of aggressive pathotypes that lead to increased CDM epidemics. Emergence and spread of new pathotypes or new cryptic species of pathogen (A1 mating type) and new genetic recombinant due to possible sexual reproduction are possible causes of *P. cubensis* resurgence in United States and the rest of the world (Runge et al., 2011).

1.9. Temporal progress of cucurbit downy mildew during the growing season

Cucurbit downy mildew epidemics in the United States occurs annually for early, mid- and late-season crop. The interaction of a susceptible host, viable infective units of a pathogen and conducive environmental factors determines onset and the extent of the epidemic within a season in different geographic locations. Factors that influence cucurbit downy mildew progress and dynamics of epidemics are the leaf wetness essential for infection, rate of foliage growth and physiological age of the host, amount of primary inoculum available and rate at which lesions necrotize (Cohen and Palti, 1980). While presence of host and inoculum are important for disease to initiate, epidemics cannot occur without leaf wetness. Increased amount of moisture plays important role in cucurbit downy mildew progress. While dew provides leaf wetness at night, rainfall during daytime extends wetness period at higher temperatures. Additionally, rainfall not only penetrates to lower leaves, but also aids in dispersal of inoculum when conditions for infection are favorable. Like rain, irrigation system provides leaf wetness. However, effect of irrigation system is greater in that it alters microclimate in favor of disease development. According to Cohen and Palti (1980), while sprinkler may aid in dispersal, furrow system, flooding, or trickling irrigation system, promotes the general growth of cucurbits, causes foliage to be denser, and rows to close at an earlier stage, thus altering the microclimate. In addition to rain, anthropogenic activities such as irrigation, dew at night and guttation also play an important role in leaf wetness.

The effect of light on dynamics of *P. cubensis* development is highly complex as it affects duration of leaf wetness during darkness or light as well as sporulation of *P. cubensis.* Optimum temperature for *P. cubensis* development is between (26 to 30°C). However, *P. cubensis* development has been reported during high temperatures of up to 48°C (Bains and Jhooty, 1976). In the scenario where the disease developed at higher temperature, rainfall was found to play important role. It was reported that temperature reductions were affected by occasional rainfall, and the resultant long wet periods, were considered to maintain the disease even under these extreme temperature conditions (Cohen and Palti, 1980). Understanding how the dynamics of host, pathogen inoculum and the environment affects the disease inception and progress are helpful in management of cucurbit downy mildew.

Comparison of epidemics in a pathosystem is useful in determining an effective disease control strategy, such as effectiveness of cultivar resistance or fungicide efficacy. Comparative epidemiology not only defines the differences or similarity among diseases but is also used to derive models and principles that can be applied in epidemiology studies (Kranz, 1974). Quantitative and experimental comparative epidemiology can either be described horizontally or vertically. Horizontal comparison of epidemics compares crop facets such leaf vs root disease, diseases in open vs closed environments. Vertical comparison on the other hand compares elements of disease such as primary inoculum, spore formation, spore release, spore dispersal, landing, infection, incubation, latent period, infectious period (Kranz 1974). Disease progress curves represent a graph of an epidemic. It shows how the pathogen, host, environment and anthropogenic factors interact at a field or landscape level. The estimate of rate parameter, and standardized rate parameter ρ (rho) are important components used to statistically compare temporal and spatial dynamics of disease epidemics (Campbell and Madden, 1990). Disease progress curves being an interaction between the host plant, pathogen and environment can be fitted to monomolecular, exponential, logistic and Gompertz epidemiological growth models (Madden et al., 2007). Best performing growth models for comparison of epidemics can be selected based on fit statistics such as mean squared error (MSE) and coefficient of determination (R^2) as measures of model performance. When comparing epidemics with similar growth model such as monomolecular, exponential, logistic or Gompertz, estimate of the rate parameter derived using the same model cannot be used to comparison of epidemics directly. Rather, the standardized rate parameter ρ (rho) can be used to compare dynamics of epidemics when epidemics are described by different disease progress models. For instance, when disease epidemics is described by several models within Richards family such as monomolecular, logistic and Gompertz, the weighted mean absolute rate of disease increase ρ (rho) is applicable. The final disease severity (y_0), area under disease progress curve (AUDPC) and yield loss are also important temporal and spatial components in comparison of epidemics.

1.10. Chemical control and fungicides resistance

The three primary sources of inoculum for cucurbit downy mildew in regions that receive cold winter include (a) oospores, which has been found in nature in Asia, and Europe and whose role in infection is unknown; (b) protected crops in greenhouses or glass houses that carry inoculum throughout the cooler season; and (c) long distance wind-borne inoculum blown in from warmer regions. Depending on the impending source of inoculum from the overwintering source, timing of the start of fungicidal treatment is important for effective management of cucurbit downy mildew. Timing of chemical application should take the following into consideration: source, proximity and size of inoculum; source of leaf wetness and duration of wetness periods; age of plants; weather; irrigation regime; and type of fungicide (protectant or systemic). Understanding of the source of primary inoculum may be the most important aspect of estimating the time of early outbreaks of downy mildew and of timing preventive control measures. However, a coordinated attempt to predict specific dates for spring outbreaks has not been possible to work out schemes of timing for the beginning of fungicide applications by meteorological indicators, and disease monitoring through Cucurbit

Downy Mildew ipmPIPE (Integrated Pest Management Pest Information Platform for Extension and Education) platform has been helpful to growers by giving near real time information on when it is appropriate to begin intensive application of fungicides (Ojiambo et al., 2011).

According to Cohen and Palti (1980), the appearance of the first symptoms of the disease in production field, may influence the type and timing of fungicide applications. Further, timing and application of fungicides is subject to the following considerations: (a) expected absolute rate of downy mildew development, as influenced by prevailing weather and proximity of sources of inoculum: (b) rate of foliage development; (c) irrigation type and intervals; and (d) type of fungicide chosen be it protectant or systemic (Cohen and Palti, 1980). Protectant fungicides also known as multisite inhibitors are basically non-systemic, preventative fungicides that form a protectant barrier at the surface of plant. Timing in application of these fungicides is important for their efficacy. They technically inhibit pathogen germination, growth and development prior infection and penetration into host tissue. Copper formulations, chloroisophphthalonitriles (chlorothalonil), dithiocarbamates (mancozeb), phthalimides (folpet) and are non-systemic multisite inhibitors used against downy mildew. When inoculum and weather conditions favor rapid disease development on susceptible cucurbits, application of any protectant fungicide require maximum coverage of foliage. Resistance to multisite inhibitors rarely develop. However, in the control of downy mildew, all protectant fungicides suffer from one important drawback in that they do not provide protection to newly formed young tissues. Additionally, when vegetative growth is vigorous, protectants have to be applied at very short intervals. Since the risk for resistance is lower in protectant fungicides, they are included in spray program that reduces the aggressiveness, pressure, and the frequency of single site fungicide within a season. These fungicides can be applied singly, in alternation with single site or tank mixed to improve efficacy of single site and slow down resistance evolution in pathogen population.

Up until the 1960s fungicide control in agricultural production system were mainly protectant or multisite inhibitors. However, after 1960 fungicides with specific modes of action characterized by selective, systemic, and curative nature were introduced (Edgington et al., 1980; Morton and Staub, 2008). In late 1960s and 1970s, several systemic fungicides introduced included oxathiins, 2-aminopyrimidines, benzimidazoles, morpholines, dicarboximides, phenylamides, phosphorothiolates, phenylamides, alkyl-phosphonates were introduced (Thind, 2009). These specific fungicides were not only specific on the target pathogen specific pathway, but also could penetrate and protect host at the growing regions and were effective at much lower dose rates than the earlier surface protectants (Thind, 2009). Unlike contact fungicides which only act as protectants, systemic fungicide can penetrate host tissues and act on pathogens at the initial infection site or away from point of entry. Introduction of single site-specific systemic fungicides improved *P. cubensis* management in that timing of application against cucurbit downy mildew is much simpler (Cohen and Palti, 1980). Site-specific chemical classes available in the market include Quinone outside inhibitors (QoIs), phenylamides (PAs), carboxylic acid amides (CAAs), cyano-acetamide oximes (CO), benzamides, phosphonates, dinitroanilines carbamates and plant defense inducers such as benzothiadiazoles (Gisi and Sierotzki, 2008). Quinone outside inhibitors are inhibitors of mitochondrial respiration and by binding to the Qo site it inhibits the electron transport at cytochrome b (complex III). Quinone outside inhibitors also known as strobilurins, include azoxystrobin, famoxadone, and fenamidone. The second major group is phenylamides

(PAs) fungicides such as mefenoxam (metalaxyl-M), metalaxyl and benalaxyl. Phenylamides inhibit ribosomal RNA synthesis and specifically RNA polymerization. Carboxylic acid amides (CAAs) are the third major group including dimethomorph, iprovalicarb, benthiavalicarb, mandipropamid. Carboxylic acid amides potential targets are phospholipid biosynthesis and cell wall deposition. Cyano-acetamide oximes such as cymoxanil is widely used in mixtures with multisite inhibitors. However, the biochemical mode of action in cymoxanil is still unknown. While specific biochemical mode of action is not well understood, carbamate chemical class (propamocarb) is reported to inhibit the permeability of cell membranes (Gisi and Sierotzki, 2008). Benzamides such as fluopicolide have both preventative and curative actions on oomycete pathogens. It has a novel mode of action works by decolonization of spectrin-like proteins (Thomas et al., 2018; Toquin et al., 2007). Other groups include phosphonates (mainly fosetyl-Al), dinitroanilines (fluazinam), carbamates (propamocarb) and plant defense inducers such as the benzothiadiazoles (BTH; acibenzolar-S-methyl/Bion) (Gisi and Sierotzki, 2008).

Currently cymoxanil (FRAC code 27), ethaboxam (FRAC code 22), fluopicolide (FRAC code 43), propamocarb (FRAC code 28), and oxathiapiprolin (FRAC code 49), are some of the sites-specific fungicides for managing the disease following infection (Cohen et al., 2015; D'Arcangelo et al., 2021; Salas et al., 2019). While few reports on reduced efficacy of oxathiapiprolin and ethaboxam are known (Dutta 2021), reduced sensitivity to cymoxanil, fluopicolide, and propamocarb have been reported in different states and within seasons in the United States (Keinath 2016; Thomas et al., 2018).

Qualitative resistance to fungicides is monogenic that involve single major gene. In this case modification of a single gene results in insensitive or sensitive phenotypes and associated to major or complete loss of disease control. Unlike qualitative resistance, quantitative resistance is polygenic and involve interaction of several genes. In contrast to qualitative resistance, polygenic resistance result from modification of several interacting genes. These modifications may lead to a range of sensitivity phenotypes such as sensitive, moderately sensitive, and completely insensitive. Directional selection for fungicide resistance can be mainly attributed to genetic diversity of pathogen and fungicide application pressure during disease management process.

1.11. Management of resistance to fungicides

Among oomycetes, *P. cubensis* is considered high risk pathogen with high evolutionary potential. In chemical protection, fungicides not only affect growth rates of pathogen populations but also the life cycle for a sensitive population (van den Bosch et al., 2015). In fungicide application programs, insensitive population are not affected and therefore have a conducive environment that increases growth rates compared to that of sensitive strains. Resistance therefore occurs when frequency in phenotype population size of resistant strains increases relatively faster than sensitive (van den Bosch et al., 2015). Resistant phenotype increasing frequency is dependent on fungicide exposure time (Staub and Sozzi, 1983; Dekker, 1986; van den Bosch et al., 2015). Fungicide application controls pathogen life cycle parameters by; (i) reducing reproduction efficiency and the proportion of pathogen spore inoculum for infection, (ii) increasing the latent period, which is the time from infection to spore production, and (iii) reducing the infectious period by reducing the rate of sporulation (van den Bosch et al., 2015; Hobbelen et al. 2011a, b). Unlike multi-site inhibitors, single-site fungicides are associated with high risk of selection and evolution of resistant populations in agricultural practice. Thus, effective disease management method is characterized by facets such as effective disease control, reduced selection for resistant population, increased fungicide effective life, decreased selection of resistant individuals in a population and keep yield losses below an economic threshold. The per capita rate of increase of both sensitive and resistant strains and time span over which selection process take place comprise three strategies that can guide development of fungicide application programs that minimizes the selection for fungicide resistance. Effective fungicide resistance management strategies can be achieved when; per capita rate of increase of both sensitive and resistant strains in a population are reduced; per capita rate of increase of the resistant strain is reduced relative to that of sensitive strain ; and when time span over which selection takes place is reduced (Ishii and Hollomon, 2015; van den Bosch et al., 2015). Features of a successful and effective management tactics for fungicide resistance management, includes the following: (i) reliable disease monitoring and forecasting, (ii) the management of application dose and rates of application, (iii) the management of the number and frequency of sprays, (iv) the use of fungicide mixtures, (v) the use of fungicide alternation and (vi) the avoidance of curative use of fungicides (Gisi and Sierotzki, 2008).

Managing application dose is an important tactic for fungicide resistance management. Appropriate dose reduction that cannot negatively impact fungicide efficacy is considered management resistance tactic (Paveley et al., 2001). Increasing fungicide dose than the recommended not only increase the cost of production but also increases selection pressure for insensitive pathogen population. However, reducing the dose of a fungicide may compromise effective disease control and consequently reduced yield and quality (van den Bosch et al., 2011, 2014a, b). In terms of selective pressure, the higher the number of fungicide application the greater the selective pressure in favor of resistant pathogen population. Thus, reducing the number of applications reduce the selection due to reduced exposure of fungicide on insensitive population (van den Bosch et al., 2014a). Mixing fungicides of different modes of action has been found useful resistance management strategy. Fungicide mixtures include adding a mixing partner to an at-risk fungicide, adding a mixing partner as well as reducing the dose of the at-risk fungicide and mixing two at-risk fungicides. Fungicide alternation involves a program where; (i) a different mode of action is added in between the sprays of at-risk fungicide and (ii), replacement of at-risk fungicide with sprays of a fungicide with a different mode of action (van den Bosch et al., 2015). In cases where cross-resistance to a specific fungicide in not present, selection for resistance is reduced due to reduced exposure of fungicide.

1.12. Rationale and objectives

In the United States, cultivation of cucurbits is undertaken in regions where the environment is conducive for production such as in the eastern United States and California. However, foliar disease caused by pathogens such as oomycetes affect production of cucurbits are also favored in such an environment. Between 1940s to the 1960s studies on long distance transport of *P. cubensis* was initiated and during this period, breeding resulted in the release of cultivars with high level of resistance to cucurbit downy mildew disease (Barnes, 1948, 1966; Holmes et al., 2015). Resistant cultivars were important management tool and were effective cucurbit downy mildew control with minimal chemical control for about four decades. The resurgence of cucurbit downy mildew in 2004 changed the dynamics of cucurbit downy mildew drastically as characterized by annual frequent disease epidemics, increased disease pressure and cucurbit downy mildew specific fungicide inefficacy. Studies on epidemics of cucurbit downy mildew at land scape level such as spatio-temporal progression, sources of inoculum, dispersal and infection dynamics, and prediction of disease outbreaks has been studied in the United States (Ojiambo et al., 2015). However, temporal dynamics of cucurbit downy mildew disease at field level as affected by environment, host and pathogen has not been characterized. For example, to effectively implement an effective management strategy, one requires a thorough understanding of how the environment, host type, and pathogen affect disease dynamics at field production level. To increase crop yield, maximize the efficacy of fungicides, and reduce cost incurred in cucurbit downy mildew management, there is need to understand how host type and fungicide application affect disease dynamics such as initiation of disease, rate of disease increase and final disease severity at the end of the season. To fully understand spatio-temporal dynamics of cucurbit downy mildew at field level in the United States it is of substance to, i) characterize the temporal dynamics of cucurbit downy mildew epidemics during the growing season, ii) determine the impact of alternating a multi-site with a site-specific fungicide and cucurbit host type on epidemic progress and final disease severity, and iii) examine if fungicide treatments influenced the distribution of the A1 and A2 mating types in cucurbit fields.

Rich population diversity of *P. cubensis* is due to high reproduction capacity, mutation, migration, gene flow and recombination as a result of sexual reproduction. These factors not only lead to shifts in population structure within season, but also dynamics in sensitivity profile to site-specific systemic fungicides. Additionally, rich diversity in *P. cubensis* influence spatial and temporal dynamics of cucurbit downy mildew epidemics. Changes in population structure of *P. cubensis* has been reported globally including in Asia, Europe and in the United States.

In the United States shifts in population structure may have emerged in 2004 when new races overcame cucumber host resistance (Holmes et al., 2006, 2015). Resurgence of cucurbit downy mildew led to differential host reaction among cucurbit hosts with cucumber cultivars being the most devastated. Currently, while cucurbit hosts such as pumpkin, butternuts squash and watermelon are tolerant, cucumber in contrast is highly susceptible to cucurbit downy mildew. Chemical control of cucurbit downy mildew is currently the most reliable management strategy. However, rampant cases of fungicides inefficacy derails efforts of disease management. Implementation of effective spray programs is recommended not only to reduce disease severity and improve yield, but also to minimize the development of fungicide resistance in pathogen populations. Chlorothalonil and Mancozeb (FRAC codes M 05 and M 03, respectively) are multi-site fungicides recommended to protect the crop prior to infection. Currently Cymoxanil (FRAC code 27), ethaboxam (FRAC code 22), fluopicolide (FRAC code 43), propamocarb (FRAC code 28), and oxathiapiprolin (FRAC code 49), are some of the sites-specific fungicides for managing the disease following infection (Cohen et al., 2015; D'Arcangelo et al., 2021; Salas et al., 2019). Due disease pressure and frequent use of these fungicides, reports of inefficacy have been documented for cymoxanil, fluopicolide, propamocarb and oxathiapiprolin (Keinath, 2016; Thomas et al., 2018). Efficacy of site-specific fungicides was reported to diminish immediately after their release in different states and within seasons in the United States (Adams et al., 2014; Hausbeck and Linderman, 2014; Vallad et al., 2016). To curb losses associated with fungicide failure, understanding population structure of *P. cubensis* pathogen early in the season and within the season will elucidate shifts associated with selection due to frequent application of site-specific site fungicides. Reducing fungicide exposure time may reduce selection pressure and hence slow resistance development for pathogen isolates. One effective strategy used to slow build-up of fungicide resistant isolates in pathogen population within a growing season is to alternate and tank mix different modes of action (van den Bosch et al., 2015). For instance, applying single sites fungicides in alternation with other single sites and tank mixing with multisite fungicides are best for both management of cucurbit downy mildew and subsequently reduce the emergence and build-up of fungicide resistant *P. cubensis* isolates (D'Arcangelo et al., 2021; van den Bosch et al., 2015). Alternation of multisite (chlorothalonil) and site-specific (azoxystrobin) or alternation of site specific in cucurbit powdery mildew, *Podpsphaera xanthii* act as evidence of success in the above-mentioned strategies for delaying selection for fungicide resistance (McGrath, 2001). However, there are no studies so far undertaken to determine how the strategy influences fungicide efficacy against cucurbit downy mildew. This study, therefore, aims at elucidating shifts in sensitivity structure of *P. cubensis* population within season by quantifying sensitivity structure early in the season and at the end of the season following alternation of multisite and site-specific fungicides and subsequently provide fundamental information for resistance management studies. Such information is useful in informing growers on the choice of different fungicide classes during the growing season.

The occurrence of oospores of *P. cubensis* outside laboratory has been reported in parts of Asia and Europe (Bedlan, 1989; Cohen et al., 2003; Cohen et al., 2015; Zhang et al., 2012). However, there are no reports on the occurrence of oospores in under field conditions in the United States. The role of *P. cubensis* oospores in nature depends on the occurrence, survival, and the potential to remain viable during the freezing winter until the onset of the growing season in regions above 30-degree latitude. Studies on the potential of oospores to initiate disease has been reported in China and Israel but none has been observed in the United States.

In the United States, outbreaks of cucurbit downy mildew diseases in the northern states have largely thought to be due to spatio-temporal progress of the epidemic from overwintering sources in southern Florida. However, about 5% of the disease reports has been found inconsistent with northward progression of epidemic and could be due to local inoculum in production field in greenhouses, wild hosts or oospores (Cohen et al., 2015; Ojiambo et al., 2011; Wallace et al., 2015). There are several factors that suggest that oospores of *P. cubensis* occur in nature and potentially play a role in initiation of local epidemics. First, the presence of A1 and A2 mating type and the potential of sexual reproduction that has been proofed under laboratory condition. Secondly, results of coalescent analysis of the pathogen population indicated genetic mixing between lineages I and II of *P. cubensis* in United States (Thomas et al., 2017c). This genetic mixing is only possible if oospores are indeed able to germinate and cause infection under field conditions. Therefore, this study was undertaken to determine the occurrence of oospores in nature and their potential role in disease initiation and on the epidemiology of cucurbit downy mildew disease in the United States.

Given the above considerations, the overall goal of this dissertation was therefore, to understand the temporal dynamics of cucurbit downy mildew as affected by environment, host type, and chemical control and investigate the ecology of the disease as it relates to pathogen survival during the overwintering season and the potential role of oospores in the epidemiology of the disease in the United States. The specific objectives of this research are as follows:

1. Characterize the temporal dynamics and severity of cucurbit downy mildew epidemics as affected by chemical control and cucurbit host type (Chapter 2)
2. Establish the temporal shifts in sensitivity of *Pseudoperonospora cubensis* population in response to cucurbit downy mildew fungicides (Chapter 3)

3. Determine the field occurrence and overwintering of oospores of *Pseudoperonospora cubensis* in the Southeastern United States (Chapter 4)

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CHAPTER 2

Temporal Dynamics and Severity of Cucurbit Downy Mildew Epidemics as Affected by Chemical Control and Cucurbit Host Type

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Abstract

Cucurbit downy mildew caused by the oomycete *Pseudoperonospora cubensis* is an important disease that affects members of Cucurbitaceae family globally. However, temporal dynamics of the disease have not been characterized at the field scale to understand how control strategies influence disease epidemics. Disease severity was assessed visually on cucumber and summer squash treated with weekly alternation of chlorothalonil with either cymoxanil, fluopicolide or propamocarb, during the 2018 spring season and 2019 and 2020 fall seasons in North Carolina, and the 2018 and 2020 fall seasons in South Carolina. Disease onset was observed around mid-June during the spring season and early September during the fall season, followed by a rapid increase in severity until mid-July in the spring season and late September or mid-October in the fall season, typical of polycyclic epidemics. The Gompertz, logistic and monomolecular growth models were fitted to disease severity using linear regression and parameter estimates used to compare the effects of fungicide treatment and cucurbit host type on disease progress. The Gompertz and logistic models were more appropriate than the monomolecular model in describing temporal dynamics of cucurbit downy mildew, with the Gompertz model providing the best description for 34 of the 44 epidemics examined. Fungicide treatment and host type significantly ($P < 0.0001$) affected standardized area under disease progress curve (sAUDPC), final disease severity (Final DS) and weighted mean absolute rates of disease progress (ρ), with these variables, in most cases, being significantly ($P < 0.05$) lower in fungicide treated plots than in untreated control plots. Except in a few cases, sAUDPC, Final DS and ρ were lower in cases where chlorothalonil was alternated with fluopicolide or propamocarb than in cases where chlorothalonil alternated with cymoxanil or when chlorothalonil was applied alone. These results characterized the temporal progress of cucurbit downy mildew and provided an

improved understanding of the dynamics of the disease at the field level. Parameters of disease progress obtained from this study could serve as inputs in simulation studies to assess the efficacy of fungicide alternation in managing fungicide resistance in this pathosystem.

Keywords: cucumber, disease progress, epidemiology, fungicide, oomycete, summer squash

Introduction

In the United States, cucurbits are produced primarily in the eastern part of the country and in California, where the environment is conducive for the cultivation of the crop. Cucumber (*Cucumis sativus*), squash (*Cucurbita pepo*), cantaloupe (*Cucumis melo*), pumpkin (*Cucurbita maxima*) and watermelon (*Citrullus lanatus*), are some of the economically important cucurbits grown globally. In 2019, cucurbits were planted on about 156,100 ha in the United States with the crop being valued at $1.6 billion (Anonymous 2020). Several foliar diseases are known to affect production of cucurbits and of these diseases, cucurbit downy mildew caused by the oomycete *Pseudoperonospora cubensis* is considered one of the most economically important globally (Lebeda and Cohen 2011; Ojiambo et al. 2015). Since *P. cubensis* is an obligate pathogen and requires its host to reproduce and survive, epidemics in the eastern United States are largely thought to be initiated annually by sporangia that are dispersed aerially from overwintering sources in southern Florida and Texas along the Gulf of Mexico, where mild winters allow for year-round production of cucurbits (Ojiambo and Holmes 2011). Warm weather with temperatures ranging from 18 to 25°C and humid conditions that prevail during the growing season in this region favor disease development (Palti and Cohen 1980), with temperature being the predominant factor that determines the rate of disease development (Arauz et al. 2010; Neufeld and Ojiambo 2012). Severe epidemics lead to rapid necrosis of leaf tissues and defoliation and subsequent reduction in fruit yield and quality (Salcedo et al. 2020; Savory et al. 2011). In the absence of effective disease control measures, severe disease epidemics can also result in complete crop losses (Holmes et al. 2015).

Cucurbit downy mildew epidemics have been well characterized at the landscape level in the Continental United States (Ojiambo et al. 2015). Such studies have been fundamental in understanding the spatio-temporal progress and the corresponding infection windows of the disease in eastern United States (Ojiambo and Holmes 2011), risk of disease outbreak across the region (Ojiambo and Kang 2013) and predicting the epidemic wave and testing hypotheses on alternative sources of initial inoculum (Ojiambo et al. 2017; Ojwang et al. 2021). However, studies on characterizing temporal dynamics of the disease at the field level are still lacking. These studies are useful in quantifying how the environment, host and pathogen affect disease dynamics to establish how the effects of control strategies, for example fungicides, affect disease epidemics and identify factors that influence the magnitude of the rate of disease increase and final disease severity (Campbell and Madden 1990; Madden et al. 2007). For example, while cucurbit downy mildew is known to progress rapidly under conducive weather conditions, rates at which epidemics progress have not been established for different cucurbit host types or how fungicides could influence rates of epidemic progress. In this regard, quantifying temporal dynamics of epidemic is especially important where chemical control is the main method for disease control and the affected pathogens have a high risk of developing resistance to these chemistries. Further, quantifying the temporal dynamics of cucurbit downy mildew is useful in generating parameters for mechanistic models that link disease risk models to models that predict fungicide efficacy based on physical mode of action (Caffi and Rossi 2018; Gent et al. 2015), and for simulation models for testing the usefulness of fungicide resistance management strategies (Hobbelen et al. 2013).

Fungicides, host resistance and cultural practices that reduce prolonged leaf wetness were typically the main components in the integrated management of cucurbit downy mildew (Holmes et al. 2006). However, the global resurgence of the disease in the past 20 years that has been driven primarily by changes in the virulence structure of the pathogen, has resulted in an increase in the frequency of highly virulent strains (Cohen et al. 2015; Lebeda and Urban 2007). This resurgence has been linked to the breakdown of resistance gene in cucumber and increase in the frequency of fungicide resistance in cucurbit production in the United States (Thomas et al. 2017a; Thomas et al. 2018). Currently, growers rely heavily on fungicides for managing cucurbit downy mildew because cucurbit cultivars with commercially acceptable levels of resistance are not available in most cases. In the presence of disease, an aggressive spray program where fungicides are applied every 5 to 7 days in cucumber and every 7 to 10 days for other cucurbits, is usually recommended. When cucurbit downy mildew infects the crop early in the season, growers can apply up to 11 sprays in an effort to control the disease (Ojiambo et al. 2010). The presence of A1 and A2 mating types within the pathogen population has raised the possibility of sexual reproduction in *P. cubensis*, a process that can also increase the ability of the pathogen to develop resistance to fungicides routinely used to control cucurbit downy mildew (Ojiambo et al. 2015). Sexual recombination provides *P. cubensis* a greater advantage in the evolutionary 'arms race', given the pathogen's wide host range and the sole reliance on fungicides to manage the disease. Sexual reproduction also results in the production of oospores, and F1 progeny isolates from oospores show altered sensitivity to fungicides

(Cohen and Rubin 2012). Recombinant F1 progenies may also have enhanced virulence towards resistant host genotypes, as reported for other oomycetes (Klarfeld et al. 2009). Greater diversity and persistence of novel pathogen genotypes may further hasten breakdown of host resistance and reduced fungicide efficacy, resulting in difficulties in controlling the disease. There is thus an increasing need to evaluate current fungicide control programs recommended to reduce disease severity and/or delay the emergence of fungicide resistance. When coupled with analysis of temporal dynamics of disease epidemics, such studies provide insights in how to apply chemical controls effectively beyond what can be obtained from routine testing of fungicide products.

The Fungicide Resistance Action Committee (FRAC) considers *P. cubensis* to be a high-risk pathogen for developing resistance to fungicides (FRAC 2021). Currently, fungicides available to control downy mildew effectively are limited due to the high propensity of *P. cubensis* to develop resistance to fungicides. Chlorothalonil and mancozeb (FRAC codes M 05 and M 03, respectively) are multi-site fungicides recommended to protect the crop prior to infection. Multi-site fungicides do not provide adequate protection from yield losses when they are applied season-long and growers must apply other fungicides after downy mildew appears (Adams et al. 2015; Colucci et al. 2007). Cymoxanil (FRAC code 27), ethaboxam (FRAC code 22), fluopicolide (FRAC code 43), propamocarb (FRAC code 28), and oxathiapiprolin (FRAC code 49), are some of the sites-specific fungicides for managing the disease following infection (Cohen et al. 2015; D'Arcangelo et al. 2021; Salas et al. 2019). While these site-specific fungicides were effective when first introduced to the market, isolates of *P. cubensis* with reduced sensitivity to cymoxanil, fluopicolide, and propamocarb have been reported in the United States (Keinath 2016; Thomas et al. 2018). Similarly, reduced efficacy of these site-specific fungicides has been reported in different states and within seasons in the United States (Adams et al. 2014; Hausbeck and Linderman 2014; Vallad et al. 2016).

Since the rate of loss of effective fungicides threatens to exceed that rate of introduction of new products (van den Bosch et al. 2014), fungicide resistance management is key for maintaining or prolonging the efficacy of the few fungicides that are still effective against cucurbit downy mildew. Alternations of single-site systemic and multi-site fungicides and mixtures of single-site and multi-site fungicides are some of the tactics proposed for improving fungicide efficacy and delaying buildup of resistant pathogen populations (Hobbelen et al. 2013; Skylakakis 1981; van den Bosch et al. 2014). These tactics are particularly important for cucurbit downy mildew where growers continue to intensively use the few remaining effective fungicides, which further increases the risk of fungicide resistance development in *P. cubensis* (Zhu et al. 2008). Simulation models shows alteration of site-specific, and non-cross-resistant fungicides delays buildup of pathogen genotypes insensitive to high-risk site-specific fungicides (van den Bosch et al. 2014). However, systematic studies have not been conducted to establish the effectiveness and consistency of this strategy in improving fungicide efficacy and reducing cucurbit downy mildew severity. Further, the impact of different cucurbit host types on fungicide efficacy when implementing this strategy has not been established. This is particularly important given the nature of host specialization in this pathosystem where recent and more virulent pathotypes of *P. cubensis* with the A1 mating type are frequently associated with cucumber than other cucurbit host types (Thomas et al. 2017b). Most of the isolates found to be resistant to fluopicolide or propamocarb in the United States have been of the A1 mating type (Thomas et al. 2018). Based on the above considerations, this study was conducted with the following specific objectives: i) characterize the temporal dynamics of cucurbit downy mildew epidemics during the growing season, ii) determine the impact of alternating a multi-site with a site-specific fungicide and cucurbit host type on epidemic progress and final disease severity, and iii) examine if fungicide treatments influenced the distribution of the A1 and A2 mating types in cucurbit fields.

Materials and Methods

Field sites, plant material and planting. Field experiments were conducted at two research locations during the 2018, 2019 and 2020 growing seasons in North Carolina and South Carolina. Experiments in North Carolina were conducted at the Upper Coastal Research Station located in Rocky Mount in Edgecombe County, during the summer season (April to July) in 2018 and fall season (September to November) in 2019 and 2020. Experiments in South Carolina were conducted at the Clemson University Coastal Research and Education Center in Charleston, Charleston County, during the fall season (August to November) in 2018 and 2020. In North Carolina plots were made of raised beds (0.15 m high by 0.60 m wide) covered with plastic mulch and fitted with drip irrigation along the center of the beds. Plots measured 3 m long by 0.6 m width and were separated by 2.5 m fallow area on each end in 2018. In 2019 and 2020 plots measured 6 m long by 0.6 m with a 3 m fallow area on each end. In South Carolina, plots were set up as in North Carolina except that each plot included three rows that were 3.3 m long and 0.9 m wide.

Two cucurbit host types, cucumber (cv. 'Straight 8') and summer squash (cv. 'Spineless Perfection'), were used in all experiments at both locations except at Charleston where cucumber cultivar 'Speedway' was used in the experiments in 2020. In general, cucumber is more susceptible to cucurbit downy mildew than summer squash. Plots were seeded manually by placing 3-5 seeds per hill at a spacing of 0.6 m within the row. In North Carolina, plots were planted on 9 May 2018, 14 August 2019, and 19 August 19 2020. When necessary, plots were reseeded 1 week after initial seeding to replace non-germinated seeds. After 2 to 3 weeks, plots were subsequently thinned to one plant per hill to allow for five plants per plot in 2018 and 10 plants per plot in 2019 and 2020 seasons. In South Carolina, seedlings of the two host types were first raised in a greenhouse before transplanting on 3 September 2018 and 18 August 2020. A total of 15 plants were transplanted per plot in both 2018 and 2020 growing seasons.

Disease initiation and fungicide treatments. Cucurbit downy mildew was initiated in all experimental plots in growing seasons by naturally occurring, windblown sporangia from neighboring infected cucurbit fields (in North Carolina) or sentinel plots (in South Carolina) (Keinath 2016). One multi-site fungicide (chlorothalonil) and three site-specific fungicides (cymoxanil, fluopicolide and propamocarb) were used in this study (Table 2.1). Alternating fungicides with different FRAC codes was the resistance management strategy that was evaluated to assess its impact on disease severity. Specifically, chlorothalonil (FRAC code M 05) was applied in alternation with either cymoxanil (FRAC code 27), fluopicolide (FRAC code 43) or propamocarb (FRAC 28) on a weekly basis after disease symptoms developed in the field. Prior to the start of this fungicide application regime, two sprays of chlorothalonil, a protectant fungicide, were applied in treatment plots in North Carolina but only in 2018, at a 14-day interval until disease was observed, while the protectant fungicide was not applied in plots prior to the initiation of the fungicide application regime in South Carolina. No fungicides were applied in the untreated control plots at both locations. In North Carolina, disease symptoms were first observed on 15 June 2018, 3 September 2019 and 8 September 2020, while onset in South Carolina occurred approximately on 19 September 2018 and 25 August 2020.

In North Carolina, fungicides were applied using a CO_2-pressurized backpack sprayer calibrated to emit 203.9 liter/ha at 137.9 kPa via a boom with four nozzles spaced 45.7 cm apart. In South Carolina, fungicides were applied with a tractor-mounted hydraulic sprayer calibrated to emit 655 liter/ha at 689 kPa via a boom with four nozzles spaced 45 cm apart. Five treatments were evaluated: i) chlorothalonil alone (i.e., chlorothalonil treatment), ii) weekly alternation of chlorothalonil with cymoxanil (i.e., cymoxanil treatment), iii) weekly alternation of chlorothalonil with fluopicolide (i.e., fluopicolide treatment), iv) weekly alternation of chlorothalonil with propamocarb (i.e., propamocarb treatment) and v) untreated control. Three applications of systemic fungicides were applied to plots in North Carolina, these applications were on 21 June, 5 July, and 17 July in 2018; on 3 September, 17 September, and 1 October in 2019; and on 11 September, 23 September and 6 October in 2020. Plots in South Carolina received a maximum of four applications of systemic fungicides, and these applications were done on 24 September, 10 October, 20 October and 5 November in 2018 and on 28 August, 10 September, 24 September and 8 October in 2020.

Disease assessment. Plants were monitored regularly for growth and once plants reached ‘tip-over’ or about 30 days old, 5 plants were randomly selected and tagged in each plot at all locations and growing seasons except in North Carolina in 2018 were all plants were selected for tagging. The sixth, seventh and eighth leaf of every plant was tagged using zip ties to allow for consistent disease assessment throughout the experiment. Disease severity on all the tagged leaves was assessed by visually estimating the percentage of leaf area with chlorotic and necrotic symptoms. Mean leaf area infected (%) across all the tagged leaves represented estimated disease severity for the plot. In North Carolina, disease severity was recorded on five assessment dates in each growing season: June 25, July 2, 12, 18 and 24 in 2018; September 11, 19, 24, October 1 and 10 in 2019; and September 23, October 1, 6, 15 and 22 in 2020. In South Carolina, disease severity was recorded on the same 10 plants per plot on six assessment dates in 2018 (September 13, October 1, 9, 16, 23 and 29) on both hosts. In 2020, disease severity on cucumber was recorded on seven assessment dates (September 2, 9, 15, 21, October 5, 12 and 19), while severity on squash was recorded on six assessment dates (August 29, September 2, 9, 21, October 5 and 13).

Sampling of isolates and mating type determination. Isolates were collected at two-time points; i) from untreated control plots at the onset of disease symptoms and ii) from respective fungicide treated plots, one week after the last application of the systemic fungicide. The mating type of the sampled isolates was determined as described by Thomas et al. (2017c) for a total of 68 isolates collected from untreated plots (n = 9) and fungicide-treated plots of cucumber (n = 48) and squash (n = 20) across the entire study. Briefly, isolates were co-inoculated with a known A1 and A2 tester strains in equal volumes (1:1) with a spore concentration of 2×10^4 sporangia/ml on detached leaves of cucumber cultivar Straight 8. Detached leaves were inoculated with 10 μl of the spore mixture suspension and were placed on moist paper towels in clear acrylic boxes and incubated in a growth chamber at 21°C under a 12 h/12 h light and darkness cycle. About 7 to 10 days after inoculation, leaf discs (11-mm in diameter) were cut from incubated leaves and clarified in ethyl alcohol-acetic acid solution (3:1 v/v). Clarified leaves were washed in deionized water and then examined under a compound microscope (100×) for the presence of oospores. An isolate was designated as A1 or A2 if oospores were observed when co-inoculated with its opposite mating type tester strain.

Data analyses, experimental design, and statistical analyses. To assess the effect of fungicide treatment and host type on disease severity, different response variables were calculated. First, area under disease progress curve was calculated from disease assessment data as follows: $\mathrm{AUDPC} = \sum_{i=1}^{n} \left[\left(y_i + y_{i+1} \right) / 2 \right] \left(t_{i+1} - t_i \right)$, where y_i = diseased leaf area (%) estimated on the *i*th disease assessment date, t_i = time (days) from disease first to the *i*th disease assessment date and n = total number of disease assessments during the epidemic. Since the duration of disease assessments was not similar at the test sites, a standardized area under disease progress curve (sAUDPC) was calculated to allow for direct comparison of treatment effects: $\mathrm{sAUDPC} = \mathrm{AUDPC} / t_n$, where t_n = time (days) from first to the final disease assessment date. Secondly, final disease severity (Final DS, %) was recorded for each treatment effect and Final DS in the untreated control was used to establish the disease pressure (DP = Final DS/100) in each season.

To quantify the temporal progress of cucurbit downy mildew epidemics, individual disease severity values recorded over time for each fungicide treatment were fit to monomolecular, logistic and Gompertz models to estimate apparent rates of disease increase (r) (Campbell and Madden 1990). Severity values were fitted to the models through simple linear regression using PROC REG and models were evaluated for their fit to disease data and the most appropriate model was selected based on coefficients of determination (R^2), mean squared error (MSE) and plots of residual versus observed data. Since r values from different models cannot be compared directly, the weighted mean absolute rate of disease increase parameter, rho (ρ) was calculated to facilitate direct comparison of rates from different models: $\rho = rK / (2m + 2)$, where K = upper asymptote (here assumed to be 1), m is the shape parameter and m = 0, 1 and 2 for the monomolecular, Gompertz and logistic model, respectively (Campbell and Madden 1990).

Experiments were laid out in a split-plot design with host type as the whole-plot treatment and fungicide as the split-plot treatment. Treatments were replicated three times in North Carolina and four and three times in 2018 and 2020, respectively, in South Carolina. The effect of fungicide treatment and host type on sAUDPC, Final DS and ρ were analyzed using PROC GLIMMIX. The model fitted to the data can be expressed as follows: $y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + b_k + d_{ik} + w_{ijk} + e_{ijkl}$, where y is the response variable (sAUDPC, Final DS or ρ), μ is the constant (intercept), α is the host type treatment effect, β is fungicide treatment effect, $(\alpha\beta)_{ij}$ is the host × fungicide interaction effect, b_k is the block effect, d_{ik} is the whole-plot error, s_{ijk} is the sub-plot error and e_{ijkl} is the residual error. In this statistical model, host type and fungicide treatment were treated as fixed effects, while blocks were treated as a random effect. The statistical model was implemented separate for each year after preliminary analyses showed differences between years at both experimental sites. Pairwise comparison of treatment effects was conducted using the Tukey's test at α = 0.05. Contingency tables and the number of *P. cubensis* isolates tested for mating type were used to examine the association between the frequency of A1 and A2 mating types and i) cucurbit host type and ii) fungicide treatments. Statistical differences in the distribution of A1 and A2 mating type isolates within these two categories were tested based on Fisher's exact test using PROC FREQ. All statistical analyses were implemented in SAS version 9.4 (SAS Institute, Cary, NC).

Results

Weather variables during experimental periods. Mean temperatures during the spring season in 2018 at Rocky Mount, North Carolina varied from 21.9 to 29.8°C with the lowest mean temperature being recorded in early July, while the highest temperature in late June (Fig 2.1). Generally, rainfall increased during the season with daily amounts ranging from 0.15 to 45.47 mm. Mean temperatures during the fall season were higher in 2019 (mean = 22.1°C) than in 2020 (mean 18.9°C) (Fig. 2.1). Rainfall amounts during the fall seasons were higher in 2020 (total = 295.13 mm) than in 2019 (total = 195.58 mm). In addition, while rainfall generally decreased during fall 2020, rainfall in 2019 was mainly present early and late in the season with a period of no precipitation in between mid-September to mid-October (Fig. 2.1). At Charleston, South Carolina, mean temperatures generally decreased during the growing season in fall seasons in 2018 and 2020, with temperatures being slightly higher in 2018 (mean = 24.6°C) than in 2020 (mean = 23.8°C) (Fig. 2.1). However, precipitation at this site was lower in 2018 (total = 102.1 mm) than in 2020 (total = 282.7 mm).

Disease pressure and disease severity. Both sAUDPC and FDS were significantly affected by growing season at Rocky Mount (P = 0.0001) and Charleston (P < 0.0006) and thus results at each experimental location are presented by growing season. Cucurbit downy mildew developed on cucumber and summer squash in all growing years at Rocky Mount, North Carolina. Across all growing seasons, disease pressure at this site was high and similar for both cucumber and summer squash except in 2018, when the disease pressure was higher on cucumber (DP = 100%) than on summer squash (DP = 70%) (Table 2.2). Disease severity expressed as sAUDPC was marginally significantly (P = 0.049) different between the two hosts. In addition, there was a significant (P < 0.0001) host × year interaction for sAUDPC and Final DS recorded at both sites. For example, sAUDPC was higher for cucumber than summer squash in 2018 and 2020 but lower in 2019 (Table 2.2). At Charleston, South Carolina, disease pressure was substantially higher on cucumber than on summer squash in both years but especially in 2020 where DP was 89.7% in cucumber compared to 56.5 % in summer squash (Table 2.3). Disease severity followed a similar trend being comparable for both hosts in 2018 but about 50% higher in cucumber (sAUDPC = 38.6) than in summer squash (sAUDPC = 18.1) in 2020.

Effect of fungicide treatments on disease severity and final severity. Fungicide treatments significantly affected sAUDPC (P = 0.0001) and Final DS (P = 0.0002) at Rocky Mount, North Carolina. Similar effects of fungicide treatment on sAUDPC (P = 0.0001) and Final DS (P = 0.0001) were also observed at Charleston, South Carolina.

At Rocky Mount, North Carolina, sAUDPC for cucumber was significantly lower in treated than untreated plots in all years except in 2019, where only values for fluopicolide and propamocarb treatments were significantly lower than for the untreated control (Table 2.2). Among fungicide treated plots, sAUDPC was consistently low in cucumber plots that received the fluopicolide treatment except in 2018 where sAUDPC was not significantly different among fungicide-treated plots. Similarly, Final DS was significantly lower in plots that received either the fluopicolide or propamocarb treatment than all other fungicide treatments except in 2019 where although values were numerically lowest for the fluopicolide and propamocarb treatments, no significant differences in Final DS were observed between all treatments (Table 2.2). A similar pattern in the effects of fungicide treatment on sAUDPC and Final DS was also observed on summer squash. For example, sAUDPC was consistently low in plots that received the fluopicolide treatment except in 2018, when sAUDPC was not significantly different among fungicide treatments. Final DS on summer squash was significantly different among treatments across all years except in 2020. Although Final DS was lowest in propamocarb-treated plots in 2018, it was not significantly different from disease severity values in other treatments except the untreated control (Table 2.2). In 6 of 12 cases examined, sAUDPC or Final DS in plots treated with chlorothalonil alone were not different from values for the untreated control plots.

At Charleston, South Carolina, sAUDPC on cucumber was lowest in fluopicolide or propamocarb treated plots in both 2018 and 2020, although differences among fungicide-treated plots were more pronounced in 2020 than in 2018 between fungicide treatments. For example, sAUDPC was significantly lower in plots that received the fluopicolide and propamocarb treatment in 2020. However, these two treatments were not significantly ($P >$ 0.05) different from all other treatments in 2018 except the untreated control (Table 2.3). A similar trend in treatment effects was observed for Final DS on cucumber where the lowest Final DS was observed in fluopicolide treated plots in both years (Table 3), with more pronounced differences between treatments in 2020 than in 2018. Similar results on sAUDPC and Final DS were observed on summer squash where fluopicolide treated plots had the lowest values with treatment differences following a similar pattern as observed for cucumber in both years (Table 2.3). In most cases (7 of 8 cases), sAUDPC or Final DS in plots treated with chlorothalonil alone were not different from values for the untreated control plots except for summer squash in 2018.

Temporal progress of disease epidemics. The increase of disease severity over time was characteristic of a sigmoid curve with disease severity being generally low at the start of the seasons at Rocky Mount, North Carolina (Fig. 2.2), with less distinct differences between fungicide treatments during this lag phase of the epidemic. Disease severity increased exponentially thereafter with more distinct differences in fungicide treatments during this period before a gradual decrease in the disease development towards the end of growing seasons. The impact of fungicide treatments on disease development on cucumber was more evident throughout the season in 2018 and 2020 but with more distinct differences during the exponential phase in 2019, while the impact of fungicide treatments on summer squash were generally more evident during the exponential phase of the epidemic in all growing seasons (Fig. 2.2). In general, disease severity was lower in plots with fluopicolide or propamocarb treatments than other treatments on both host except on cucumber in 2018 and summer squash in 2020. However, final disease severity was always lower in fluopicolide or propamocarb treated plots than in plots that received other fungicide treatments regardless of the host type (Fig. 2.2).

Similarly, temporal increase in disease at Charleston on cucumber and summer squash was low during the start of the season with no differences in fungicide treatments. Disease increased exponentially thereafter during which very distinct differences in fungicide treatments were observed until the gradual decrease in disease development at the end of the season. Although actual levels of disease severity varied in both years, disease development was consistently lower in plots that received either the fluopicolide or the propamocarb treatment than other treatments, irrespective of host type (Fig. 2.3).

Model parameters of epidemic increase and impact of fungicide treatments. The growth models evaluated fit the disease severity data recorded over time reasonably well, but the model that best described the increase in disease severity differed among fungicide treatments and host type. Models selected to describe epidemic progress were characterized by lower mean squared differences between observed and predicted values (MSE) and higher R^2 (data not shown). Model parameters and statistics presented for disease severity recorded at Rocky Mount (Table 2.4) and Charleston (Table 2.5) are for the selected model that best fit data for each fungicide treatment. At both sites, the logistic and Gompertz model fit the data very well (R^2 = 0.91 to 0.99), while the monomolecular model was not a good fit in all epidemics examined. Further, the Gompertz model provided the best description of disease in 34 of 44 (77%) of all the epidemics examined (Tables 2.4 and 2.5).

The impact of fungicide treatment on disease progress was manifested primarily by the reductions in the weighted mean absolute rate of disease increase (i.e., ρ) at both locations. At Rocky Mount, values of ρ were significantly higher in the untreated plots compared to treated plots of cucumber in all seasons. The only exception to this trend was in 2019 where ρ for untreated was not different from that for the chlorothalonil treatment and in 2020 where the rates of increase where significantly higher in plots that received the fluopicolide treatment (ρ = 0.026), although this rate was not significantly different from that in the untreated plots (ρ = 0.024) (Table 2.4). A similar effect was observed on summer squash at this location, where ρ was always higher in the untreated plots than in treated plots except in 2019 where rates of disease increase in propamocarb treated plots (ρ = 0.086) were significantly higher than in the untreated control (ρ = 0.070). Among fungicide treatments at Rocky Mount where rates of disease progress were lower than for untreated control, values of ρ was lowest in either fluopicolide or propamocarb treated plots for both cucumber and squash. Based on the untreated control, disease progressed slightly faster on cucumber (mean ρ = 0.058) than on summer squash (mean ρ = 0.050). In all epidemics examined, predicted initial disease severity,

y_0, was consistently low and very close to zero with little discernable differences between fungicide treatments (Table 2.4).

The impact on fungicide treatment on disease progress at Charleston also followed a similar pattern as observed in Rocky Mount with ρ were significantly lower in fungicide treated than in untreated plots in both cucumber and summer squash (Table 2.5), although the extent of the differences in infection rates varied during the season. For example, ρ in the untreated plot was significantly higher than rates in either the propamocarb or fluopicolide plots of cucumber in 2018, while ρ was significantly higher in untreated than for all fungicide treated plots in summer squash in 2020. As was the case at Rocky Mount, values of ρ were lowest in either fluopicolide or propamocarb treated plots for both cucumber and squash among the plots that received fungicide treatments except for summer squash in 2020 where ρ was lowest in chlorothalonil treated plots. Based on the untreated control, disease progressed slightly faster7 on cucumber (mean ρ = 0.020) than on summer squash (mean ρ = 0.018). As was the case at Rocky Mount, y_0 was low and very close to zero with no discernable differences between fungicide treatments (Table 2.5).

Mating type and association with host type and fungicide treatment. Of the 68 isolates tested for mating type, 48 (71%) isolates were of the A1 mating type, while 20 (29%) isolates were of the A2 mating type. There was significant association of mating types with host type (χ^2 = 44.4; P = 0.0001), where most of the A1 and A2 isolates were associated with cucumber and summer squash, respectively. For example, of the 48 A1 isolates, 46 (96%) isolates were from cucumber, while 2 (4%) isolates were from summer squash. Of the 20 A2 isolates, 18 (90%) were from summer squash, while 2 (10%), were from cucumber. However, there was no significant (P > 0.05) association between mating type and fungicide treatment in cucumber (χ^2 = 0.98; P = 0.254) or summer squash (χ^2 = 3.33; P = 0.095), with mating type distribution in fungicide treated plots being influenced primarily by the cucurbit host type (Fig. 2.4).

Discussion

Chemical control remains the most effective method for controlling cucurbit downy mildew due to the lack of commercial cucurbit cultivars with adequate levels of disease resistance. Effective deployment of fungicides requires a thorough understanding of how epidemics develop and how these products affect disease epidemics under different environmental conditions. In this study, temporal dynamics of cucurbit downy mildew were characterized to quantify the effects of fungicide treatments on disease epidemics and how these fungicide treatments together with cucurbit host types affected the magnitude of the rate of disease increase and final disease severity. Disease severity increased logistically, as is typical of polycyclic epidemics, with more effective fungicide treatments expressing lower sAUDPC values, final disease severity and lower rates of disease progress than less effective fungicide treatments. While disease severity and the rate of epidemic progress was marginally higher on cucumber than on summer squash, fungicide treatments involving alternation of chlorothalonil and either propamocarb or fluopicolide were more effective than treatments involving alternation of chlorothalonil with cymoxanil or repeated applications of chlorothalonil during the growing season in North Carolina and South Carolina.

In this study, while cucurbit downy mildew developed in all experimental sites, disease pressure varied among seasons and experimental locations. For example, disease pressure during the fall season was higher in 2019 than 2020 at Rocky Mount in North Carolina.

Similarly, disease pressure during the fall season was higher in 2020 than in 2018 at Charleston, South Carolina. Temperature, relative humidity, and moisture are important weather factors that influence the development and extent of cucurbit downy mildew on cucurbit host types. Temperatures between 15 and 25°C have been reported to be optimum for disease development, with temperatures >28°C limiting disease development (Cohen and Rotem 1969; Yang et al. 2007). Further, moisture is primarily required to initiate the disease epidemics, while temperature determines the rate of disease development (Arauz et al. 2010). Thus, differences in disease pressure observed in the study could partly be explained by the prevailing weather factors. For example, warmer temperatures at Rocky Mount may have contributed to high disease pressure in 2019 (mean = 22.1°C) than in 2018 (mean = 18.9°C) that was relatively cooler. Further, while precipitation was higher in 2018 than in 2019, precipitation was available at the start of the epidemic in both years. Since moisture is required to initiate disease and temperature determines the rate of disease progress, the warmer temperatures and moisture availability at the onset of epidemic could partly explain why disease pressure was higher in 2019 than in 2020 at Rocky Mount in North Carolina. This interplay between temperature and moisture availability in determining the extent of disease severity has been reported in both cucumber and squash (Arauz et al. 2010; Neufeld and Ojiambo 2012). Disease pressure at Charleston, South Carolina was also higher in 2020 than in 2018, where temperatures in 2020 were low and within the conducive range (mean = 23.4°C) except at the start of the epidemic, while 2018 was relatively very warm (24.6°C) for most of the season. Further, while precipitation at Charleston was spread out in both seasons, 2018 was relatively drier than 2020. Differences in disease pressure were also evident among host types with disease being slightly higher on cucumber than on summer squash. Generally, cucumber is more susceptible to cucurbit downy mildew than squash (Neufeld and Ojiambo 2012). In addition, following the resurgence of the disease in the United States, cucumber is now typically infected by pathotypes 1 and 3 of *P. cubensis* that are more aggressive than pathotypes 4, 5 and 6 that usually infect squash (Thomas et al. 2017a,b; Wallace et al. 2020).

Cucurbit downy mildew is known to progress rapidly in the field especially when weather conditions are conducive for disease development (Homes et al. 2015). However, the temporal dynamics of the disease in a field during a growing season have not been characterized prior to the present study. Such information is useful for quantifying the effects of control strategies on disease epidemics or identifying factors affecting the magnitude of the rate of disease progress and final disease severity (Madden et al. 2007). In this study, cucurbit downy mildew epidemics were best described by either the Gompertz or logistic growth models. The fit of these two models is consistent with a polycyclic-type disease where the primary inoculum is present within or outside the field, and production and spread of secondary inoculum during the season and secondary infection of healthy tissues drive epidemics. In the Continental United States, primary inoculum for cucurbit downy mildew (i.e., sporangia), is thought to originate from overwintering sites in southern Florida and along the Gulf of Mexico where cucurbits are grown year-round (Ojiambo et al. 2015). These sporangia can be dispersed over a long distance to initiate disease in more northern states (Ojiambo and Holmes 2011; Neufeld et al. 2018). Further, the Gompertz model best described most of the epidemics that developed in this study. This model provides a good description of epidemics for which most of the area under the disease progress curve is to the right of the inflection point (Campbell and Madden 1990; Madden et al. 2007). This positive skew in the disease progress curve is due to a very rapid development of the disease soon after disease onset. This rapid development of cucurbit downy mildew was evident for several epidemics at both sites especially in untreated plots for which the lag phase was relative short. The Gompertz model has often been found to provide a more realistic description of observed epidemic data than the logistic model (Berger 1981).

The impact of fungicide treatment on the progress of cucurbit downy mildew epidemics at Rocky Mount and Charleston was expressed as lower values of sAUDPC and ρ. For example, values of ρ for cucumber in 2018 at Rocky Mount were about 2-4 times as lower for plots that received either the cymoxanil, fluopicolide or propamocarb treatment compared to the untreated control plots. The corresponding values of ρ for summer squash were 1-2 times lower in fungicide treated plots than in the untreated plots. Similarly, values of sAUDPC for cucumber in 2018 at Rocky Mount were about 2 times lower for all fungicide treated plots than for the untreated plots. The corresponding values for summer squash were 2 to 3 times lower in fungicide treated plots than untreated control plots. Epidemiologically, ρ reflects the daily arrival of inoculum at disease-free host tissue, and since fungicides protect the host or reduce the production of secondary inoculum, the effect of fungicides on disease epidemics is to reduce values of ρ. Both sAUDPC and ρ reflected the impact of fungicide treatment on disease development, but the separation of treatment effects varied between these two measures of disease. For example, while there were no significant differences based on sAUDPC between untreated and treated plots except plots that received the fluopicolide treatment for summer squash in North Carolina in 2019, ρ indicated significant differences between untreated plots and fluopicolide, propamocarb and chlorothalonil treatments. Similar observations on sAUDPC and ρ in separating treatment effects have also been reported for potato late blight (Fry 1978). Previous studies have considered sAUDPC a better index of fungicide efficacy than ρ for several reasons. First, sAUDPC uses all available data, it does not obscure variations in rate of disease development due to transformations and minor differences in disease severity early in the season that have limited impact on sAUDPC (Shaner and Finney 1977). Further, sAUDPC incorporates disease onset and the rate of epidemic development (Fry 1978). Unlike sAUDPC and ρ, values of y_0 were close to zero with no differences among fungicide treatments. This observation could be explained by the fact that chlorothalonil was applied to all plots until disease symptoms were observed in the field, which ensured disease to be low and uniform at the start of the epidemic prior to the initiation of fungicide treatments.

Three systemic fungicides, cymoxanil, fluopicolide and propamocarb, were tested in alternation with chlorothalonil, a protectant fungicide. These fungicides are some of the currently recommended products for downy mildew control and were selected primarily based on documented reports of reduced sensitivity of *P. cubensis* to these chemistries (Goldenhar and Hausbeck 2019; Keinath 2016; Thomas et al. 2018). Thus, while evaluation of fungicide efficacy was not the main goal of this study, we can make inferences on the efficacy of these products based on how they influenced disease development and final disease intensity. With a few exceptions, alternation of chlorothalonil with either fluopicolide or propamocarb resulted in lower sAUDPC, final disease severity and rates on disease progress compared to either untreated control or where chlorothalonil was in alternation with cymoxanil or was applied weekly during the season. In a recent study, propamocarb was among the most effective fungicides in suppressing cucurbit downy mildew (D' Arcangelo et al. 2021). However, we were unable to directly compare our results with those in the study by D' Arcangelo et al. (2021), where fluopicolide and propamocarb were evaluated as a single treatment application rather than in alternation with chlorothalonil. Following reports of reduced sensitivity of *P.*

cubensis to fluopicolide in the United States, it was recommended that growers not to use the product for a few years with the expectation that the pathogen population could revert to a largely sensitive population (Thomas et al. 2018). While, fungicide sensitivity assays were not conducted in this study, alternation of fluopicolide with chlorothalonil was still useful in the management of cucurbit downy mildew. Alternation may be especially critical since single treatments of fluopicolide were found to be less effective in disease control (D' Arcangelo et al. 2021).

Fungicide resistance is one of the most important challenges that threaten the use of fungicides in the control of plant diseases. The downy mildew pathogen *P. cubensis*, is considered a high-risk pathogen and has developed resistance to important fungicide groups (Cohen et al. 2015). Recent field studies have also shown reduced sensitivity of *P. cubensis* to the recently introduced chemistry, oxathiapiprolin (Dutta 2021). Alternation of fungicides is one of the strategies recommended to manage fungicide resistance (Hobbelen et al. 2013; Skylakakis 1981; van den Bosch et al. 2014). This strategy is thought to act by reducing the time of exposure of the pathogen to the fungicide. In an effort to evaluate this strategy, we examined if any associations existed between mating type distribution and fungicide treatment. As in other studies (Thomas et al. 2018; Urban and Lebeda 2007), we observed no significant association between A1 and A2 mating types and fungicides treatments. Most strains of *P. cubensis* that are insensitive and sensitive to propamocarb or fluopicolide are of the A1 and A2 mating type, respectively (Thomas et al. 2017b). However, mating types may not be a good indicator of the actual frequency of isolates that are resistant and sensitive to respective products, since fungicide resistance management strategies are aimed at preventing or delaying emergence of resistant strains in a sensitive pathogen population (Hobbelen et al. 2013).

Simulation models have been found to be useful in testing the usefulness of fungicide management strategies especially where conducting field experiments is difficult (Fry 1988). For example, initial disease severity and rate of increase in disease have been used as input variables in a mathematical model to predict selection for fungicide resistance in foliar pathogens of cereals (Hobbelen et al. 2013). Apparent infection rates following inoculation of plants with susceptible and resistant strains have also been used as input variables in a mathematical model to study the effects of different fungicide doses on the population dynamics of resistant fungi (Vendite and Ghini 1999). Thus, some of the parameters generated in this study that quantified the temporal dynamics of cucurbit downy mildew, could be used as inputs in simulation models for subsequent studies on fungicide resistance management within this pathosystem. This study represents the first characterization of the temporal dynamics of cucurbit downy mildew and provides useful model parameters that can be used in simulation studies designed to assess the usefulness of fungicide alternations in management of fungicide resistance in *P. cubensis*.

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Table 2.1. Description of fungicide products used to evaluate the efficacy of application programs in controlling cucurbit downy mildew in North Carolina and South Carolina from 2018 to 2020

Product trade name	Active ingredient	Mode of action	Formulation (rate/ha)	Group name[x]	FRAC code[y]
Bravo Weather Stik	Chlorothalonil 54%	Protectant	6SC (2.34 L)	Multi-site inhibitor	M05
Curzate	Cymoxanil 60%	Systemic	60DF (0.35 kg)	Cyanoacetamide-oxime	27
Presidio	Fluopicolide 39.5%	Systemic	4SC (0.29 L)	Benzamide	43
Previcur Flex	Propamocarb HCl 66.5%	Systemic	6F (0.40 L)	Carbamate	28

[x] Benzamide (pyridinylmethyl-benzamide).

[y] FRAC code = Fungicide Resistance Action Committee mode-of-action classification.

Table 2.2. Efficacy of fungicide spray programs with a protectant and systemic products on the severity of cucurbit downy mildew in cucumber and summer squash in North Carolina from 2018 to 2020.

		2018[b,c]		2019[b,c]		2020[b,c]	
Host type	**Fungicide treatment[a]**	**sAUDPC**	**Final DS (%)**	**sAUDPC**	**Final DS (%)**	**sAUDPC**	**Final DS (%)**
Cucumber	Chlor + Cymox	36.9 b	77.7 bc	47.8 ab	97.6 a	35.4 b	64.3 b
	Chlor + Fluop	37.9 b	77.8 bc	33.7 c	89.7 a	9.9 c	30.4 c
	Chlor + Propam	30.2 b	59.4 c	41.4 bc	89.6 a	29.3 b	55.9 b
	Chlor + Chlor	29.5 b	92.6 ab	49.7 ab	98.2 a	33.7 b	65.3 b
	Untreated	66.4 a	100.0 a	58.7 a	100.0 a	52.8 a	90.9 a
Summer Squash	Chlor + Cymox	12.1 ab	50.8 ab	60.8 ab	99.9 a	30.2 ab	82.3 a
	Chlor + Fluop	8.8 b	51.9 ab	52.9 b	96.9 b	25.8 b	76.0 a
	Chlor + Propam	6.1 b	29.7 b	59.8 ab	100.0 a	26.9 b	77.7 a
	Chlor + Chlor	7.4 b	35.6 b	63.2 a	99.9 a	24.9 b	75.6 a
	Untreated	21.9 a	70.0 a	68.4 a	100.0 a	41.7 a	97.1 a

[a] Fungicide treatment denotes weekly alternation of a multi-site (chlorothalonil) fungicide with a multi-site (Chlorothalonil) or a single-site specific fungicide (cymoxanil, fluopicolide or propamocarb) during the growing season; Chlor = chlorothalonil, Cymox = cymoxanil, Fluop = fluopicolide and Propam = propamocarb.

[b] sAUDPC = standardized area under disease progress curve based on percent leaf area infected, while Final DS = final disease severity (%) recorded on the last disease assessment date.

[c] Within columns, least squares means followed by the same letter are not significantly different at $\alpha = 0.05$ based on Tukey's test.

Table 2.3. Efficacy of fungicide spray programs with a protectant and systemic products on the severity of cucurbit downy mildew in cucumber and summer squash in South Carolina in 2018 and 2020.

		2018 [b,c]		2020 [b,c]	
Host type	**Fungicide treatment[a]**	**sAUDPC**	**Final DS (%)**	**sAUDPC**	**Final DS (%)**
Cucumber	Chlor + Cymox	14.1 ab	44.3 ab	29.8 b	84.3 ab
	Chlor + Fluop	6.3 b	27.2 b	16.2 c	60.7 c
	Chlor + Propam	9.6 b	30.8 b	18.2 c	66.3 b
	Chlor + Chlor	10.8 ab	40.2 ab	36.2 ab	85.7 ab
	Untreated	21.6 a	61.0 a	38.6 a	89.7 a
Summer Squash	Chlor + Cymox	12.4 b	29.5 b	12.2 b	38.9 bc
	Chlor + Fluop	7.6 b	18.4 b	6.4 c	24.5 c
	Chlor + Propam	9.5 b	22.4 b	9.9 bc	34.1 c
	Chlor + Chlor	12.2 b	33.3 ab	14.2 ab	51.8 ab
	Untreated	22.4 a	57.5 a	18.1 a	56.5 a

[a] Fungicide treatment denotes weekly alternation of a multi-site (chlorothalonil) fungicide with a multi-site (Chlorothalonil) or a single-site specific fungicide (cymoxanil, fluopicolide or propamocarb) during the growing season; Chlor = chlorothalonil, Cymox = cymoxanil, Fluop = fluopicolide and Propam = propamocarb.

[b] sAUDPC = standardized area under disease progress curve based on percent leaf area infected, while Final DS = final disease severity (%) recorded on the last disease assessment date.

[c] Within columns, least squares means followed by the same letter are not significantly different at $\alpha = 0.05$ based on Tukey's test.

Table 2.4. Parameter estimates for models selected to describe the temporal progress of cucurbit downy mildew on cucumber and summer squash in treatment plots at Rocky Mount, North Carolina from 2018 to 2020.

				Parameter estimate and statistics[c,d]						
Host	**Year**	**Treatment[a]**	**Model[b]**	y_0	**Rate (*r*)**	**SE(*r*)**	**Rho (ρ)**	**MSE**	R^2 **(%)**	***P*-value**
Cucumber	2018	Chlor + Cymox	Gompertz	1.63E-05	0.125	0.0098	0.032 c	0.00295	98.30	0.0009
		Chlor + Fluop	Gompertz	4.66E-05	0.120	0.0099	0.032 c	0.00531	96.92	0.0023
		Chlor + Propam	Gompertz	3.76E-03	0.088	0.0113	0.047 b	0.00571	94.53	0.0055
		Chlor + Chlor	Logistic	9.48E-04	0.353	0.0223	0.070 a	0.00339	98.54	0.0008
		Untreated	Logistic	5.42E-04	0.406	0.0434	0.071 a	0.00002	99.99	0.0001
	2019	Chlor + Cymox	Gompertz	3.13E-129	0.214	0.0157	0.052 b	0.00398	98.40	0.0009
		Chlor + Fluop	Gompertz	1.49E-28	0.154	0.0132	0.042 b	0.00097	99.49	0.0002
		Chlor + Propam	Gompertz	1.77E-31	0.159	0.0112	0.052 b	0.00193	99.05	0.0004
		Chlor + Chlor	Gompertz	2.48E-111	0.209	0.0199	0.054 b	0.00414	98.28	0.0010
		Untreated	Logistic	6.14E-06	0.450	0.0249	0.080 a	0.00545	98.11	0.0011
	2020	Chlor + Cymox	Gompertz	8.86E-04	0.069	0.0690	0.018 ab	0.00275	96.74	0.0025
		Chlor + Fluop	Gompertz	2.06E-02	0.127	0.0131	0.026 a	0.00020	98.89	0.0005
		Chlor + Propam	Gompertz	1.08E-02	0.053	0.0016	0.013 b	0.00012	99.77	0.0001
		Chlor + Chlor	Gompertz	3.52E-02	0.115	0.0095	0.019 ab	0.00232	97.24	0.0020
		Untreated	Gompertz	1.70E-02	0.145	0.0096	0.024 a	0.00141	98.97	0.0004
Squash	2018	Chlor + Cymox	Gompertz	1.25E-03	0.094	0.0108	0.024 bc	0.00058	98.85	0.0005
		Chlor + Fluop	Gompertz	1.84E-03	0.089	0.0485	0.015 c	0.00009	99.86	0.0001
		Chlor + Propam	Gompertz	8.99E-03	0.074	0.0096	0.018 bc	0.00014	99.07	0.0004
		Chlor + Chlor	Gompertz	7.79E-03	0.076	0.0347	0.013 c	0.00074	98.55	0.0007
		Untreated	Gompertz	1.87E-04	0.110	0.0044	0.030 a	0.00084	99.25	0.0003
	2019	Chlor + Cymox	Logistic	9.93E-06	0.433	0.0221	0.073 bc	0.00182	99.35	0.0002
		Chlor + Fluop	Gompertz	1.24E-59	0.188	0.0039	0.048 d	0.00052	99.77	0.0001
		Chlor + Propam	Logistic	1.76E-06	0.497	0.0576	0.086 a	0.00343	98.85	0.0005
		Chlor + Chlor	Gompertz	0.00E+00	0.318	0.0206	0.081 ab	0.00511	97.96	0.0012

	Untreated	Logistic	1.77E-05	0.415	0.0435	0.070 c	0.00209	99.18	0.0003
2020	Chlor + Cymox	Gompertz	1.76E-13	0.119	0.0078	0.032 a	0.00239	98.49	0.0008
	Chlor + Fluop	Logistic	4.62E-04	0.265	0.0473	0.049 b	0.00169	99.05	0.0004
	Chlor + Propam	Gompertz	1.31E-13	0.119	0.0066	0.030 a	0.00186	98.73	0.0006
	Chlor + Chlor	Logistic	8.87E-05	0.322	0.0775	0.054 b	0.00477	97.64	0.0016
	Untreated	Gompertz	6.63E-34	0.178	0.0234	0.052 b	0.00505	97.89	0.0013

[a] Fungicide treatment denotes weekly alternation of a multi-site (chlorothalonil) fungicide with a multi-site (Chlorothalonil) or a single-site specific fungicide (cymoxanil, fluopicolide or propamocarb) during the growing season; Chlor = chlorothalonil, Cymox = cymoxanil, Fluop = fluopicolide and Propam = propamocarb.

[b] Denotes the model that best described the temporal progress of cucurbit downy mildew during the growing season.

[c] MSE = estimated mean squared error (i.e., average squared differences between predicted and observed disease severity; *r* = rate parameter for the model that best described disease progress; SE(*r*) = standard error of *r*; ρ = weighted mean absolute rate of disease progress; R^2 = percentage of variability in disease severity explained by the model; $\boldsymbol{y_0}$ **=** initial disease severity (predicted), for which intercept values based on linearized logit or Gompit functions were divided by 10 to facilitate calculations of $\boldsymbol{y_0}$.

[d] Within columns for ρ, values followed by the same letter are not significantly different based on Tukey's mean separation test (α = 0.05).

Table 2.5. Parameter estimates for models selected to describe the temporal progress of cucurbit downy mildew on cucumber and summer squash in treatment plots at Charleston, South Carolina in 2018 and 2020.

				Parameter estimate and statistics[c,d]						
Host	**Year**	**Treatment[a]**	**Model[b]**	y_0	**Rate (*r*)**	**SE(*r*)**	**Rho (ρ)**	**MSE**	R^2 **(%)**	***P*-value**
Cucumber	2018	Chlor + Cymox	Gompertz	2.28E-02	0.045	0.0063	0.012 ab	0.00084	97.52	0.0002
		Chlor + Fluop	Gompertz	5.74E-02	0.033	0.0133	0.006 c	0.00022	94.94	0.0010
		Chlor + Propam	Gompertz	5.42E-02	0.035	0.0104	0.009 b	0.00056	96.06	0.0006
		Chlor + Chlor	Gompertz	2.74E-02	0.042	0.0053	0.011 ab	0.00094	96.14	0.0006
		Untreated	Gompertz	2.66E-03	0.062	0.0099	0.016 a	0.00323	95.79	0.0007
	2020	Chlor + Cymox	Gompertz	3.81E-05	0.085	0.0087	0.023 b	0.00069	99.54	0.0001
		Chlor + Fluop	Gompertz	1.96E-03	0.064	0.0044	0.017 a	0.00099	98.50	0.0001
		Chlor + Propam	Gompertz	2.09E-03	0.064	0.0071	0.017 a	0.00095	98.74	0.0001
		Chlor + Chlor	Gompertz	4.66E-05	0.085	0.0050	0.021 b	0.00121	99.28	0.0001
		Untreated	Gompertz	1.04E-05	0.091	0.0045	0.024 b	0.00064	99.64	0.0001
Squash	2018	Chlor + Cymox	Gompertz	6.78E-02	0.033	0.0036	0.010 bc	0.00129	92.82	0.0020
		Chlor + Fluop	Gompertz	1.47E-01	0.020	0.0048	0.006 c	0.00043	90.54	0.0035
		Chlor + Propam	Gompertz	7.53E-02	0.031	0.0029	0.008 bc	0.00096	91.96	0.0025
		Chlor + Chlor	Logistic	7.94E-02	0.079	0.0052	0.013 ab	0.0004	98.06	0.0001
		Untreated	Gompertz	1.73E-02	0.048	0.0048	0.017 a	0.00176	97.02	0.0003
		Chlor + Cymox	Gompertz	5.74E-02	0.036	0.0089	0.009 c	0.00155	93.65	0.0015
	2020	Chlor + Fluop	Logistic	6.66E-02	0.087	0.0271	0.015 b	0.00016	97.06	0.0003
		Chlor + Propam	Logistic	7.38E-02	0.085	0.0248	0.014 b	0.00059	95.47	0.0008
		Chlor + Chlor	Logistic	5.79E-02	0.096	0.0217	0.016 b	0.00078	97.79	0.0002
		Untreated	Logistic	3.99E-02	0.112	0.0154	0.019 a	0.00110	98.26	0.0001

[a] Fungicide treatment denotes weekly alternation of a multi-site (chlorothalonil) fungicide with a multi-site (Chlorothalonil) or a single-site specific fungicide (cymoxanil, fluopicolide or propamocarb) during the growing season; Chlor = chlorothalonil, Cymox = cymoxanil, Fluop = fluopicolide and Propam = propamocarb.

[b] Denotes the model that best described the temporal progress of cucurbit downy mildew during the growing season.

[c] MSE = estimated mean squared error (i.e., average squared differences between predicted and observed disease severity); r = rate parameter for the model that best described disease progress; SE(r) = standard error of r; ρ = weighted mean absolute rate of disease progress; R^2 = percentage of variability in disease severity explained by the model; $\boldsymbol{y_0}$ = initial disease severity (predicted), for which intercept values based on linearized logit or Gompit functions were divided by 10 to facilitate calculations of $\boldsymbol{y_0}$.

[d] Within columns for ρ, values followed by the same letter are not significantly different based on Tukey's mean separation test (α = 0.05).

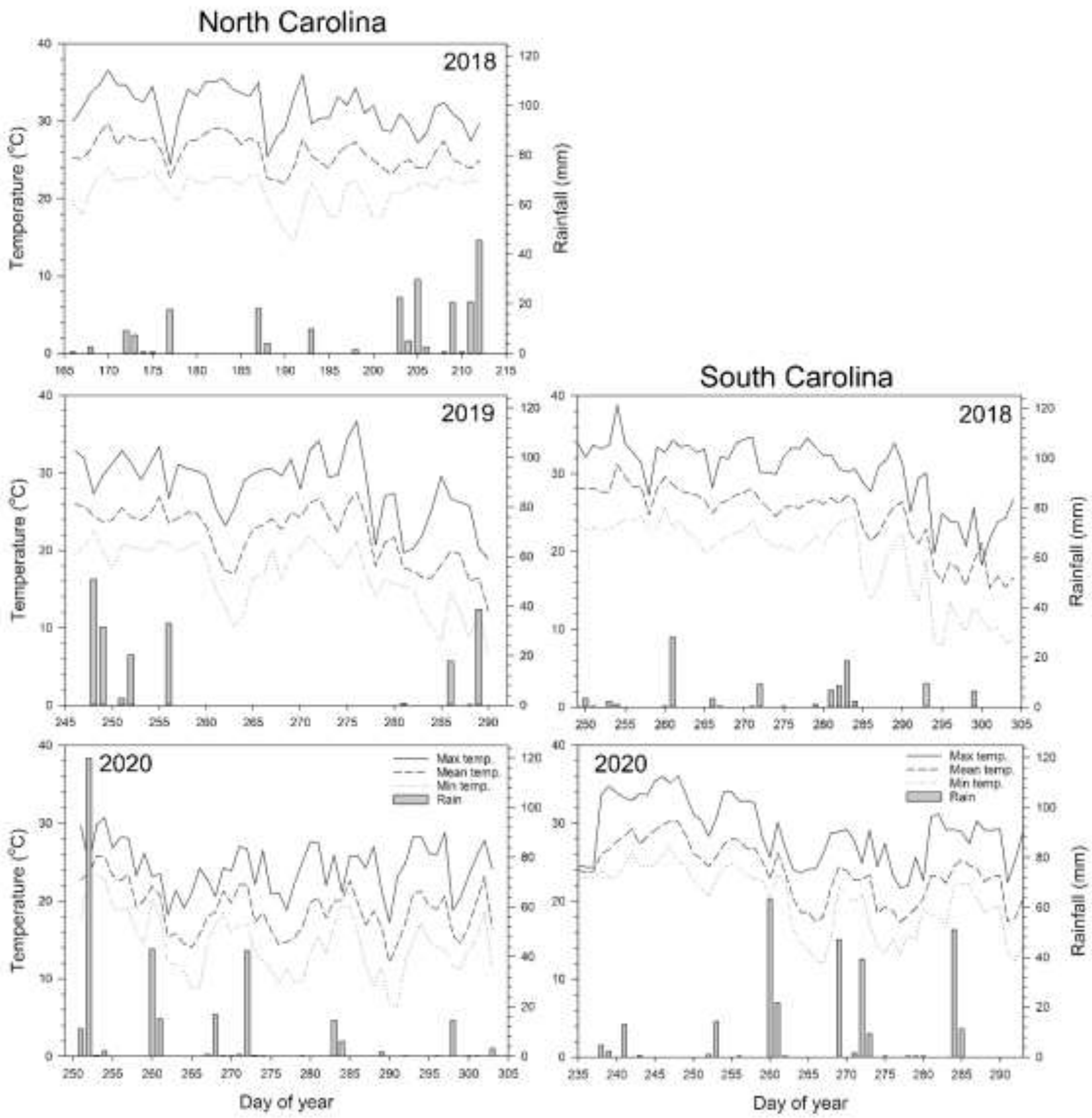

Figure 2.1. Temperature and daily rainfall amount recorded during the experimental period at Rocky Mount, North Carolina (2018 to 2020) and Charleston, South Carolina (2018 and 2020) in experiments conducted to assess the efficacy of fungicide spray programs on severity of cucurbit downy mildew. In North Carolina, experiments in 2018 were conducted in spring, while those in 2019 and 2020 were conducted in fall. Experiments in South Carolina were conducted in fall in both 2018 and 2020

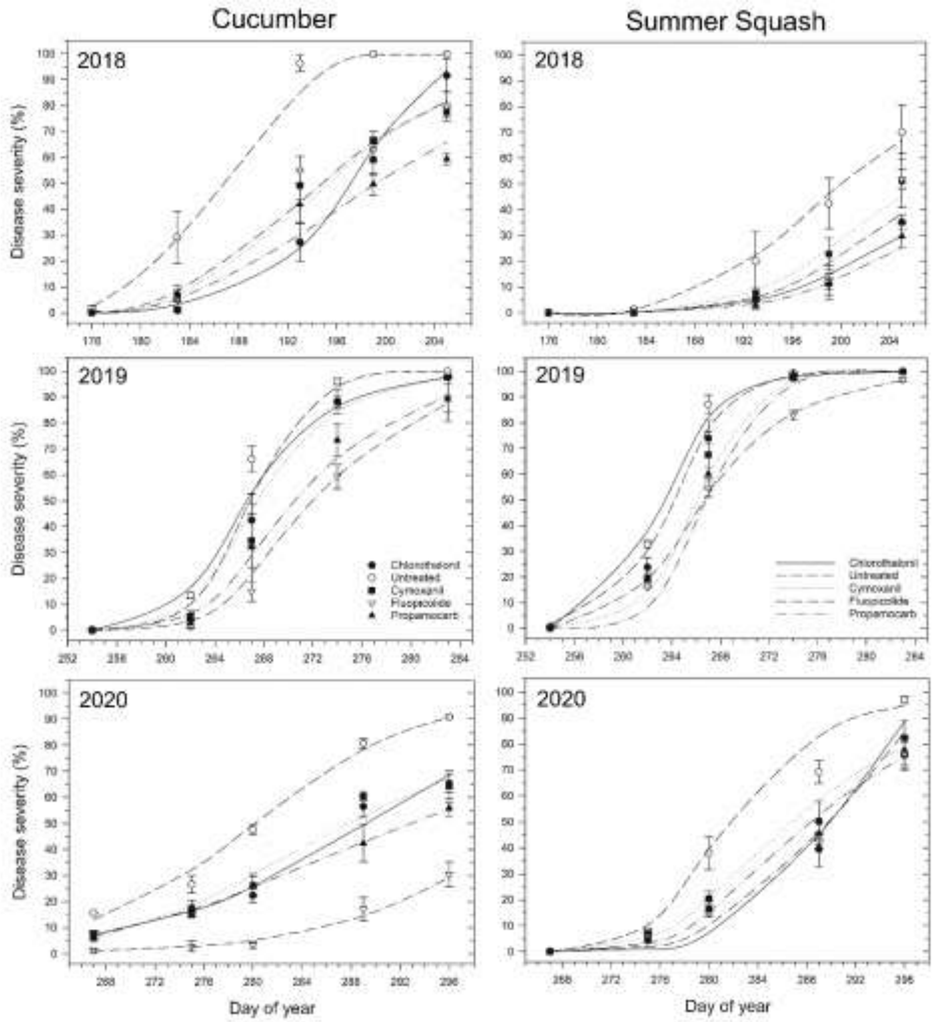

Figure 2.2. Temporal progress of cucurbit downy mildew on cucumber and squash in fungicide treated plots and untreated plots during the 2018 to 2020 growing seasons at Rocky Mount, North Carolina. Experiments were conducted during the spring (late June to late July) in 2018 and during the fall season (mid-September to late October) in 2019 and 2020. Data points represent mean values, the vertical bars are standard errors of the mean observed disease severity values, and the curves represented predicted values obtained from fitting either a Gompertz or logistic growth model to observed disease severity values. Within the legends, cymoxanil, fluopicolide and propamocarb denotes plots treated with chlorothalonil and the respective systemic fungicide.

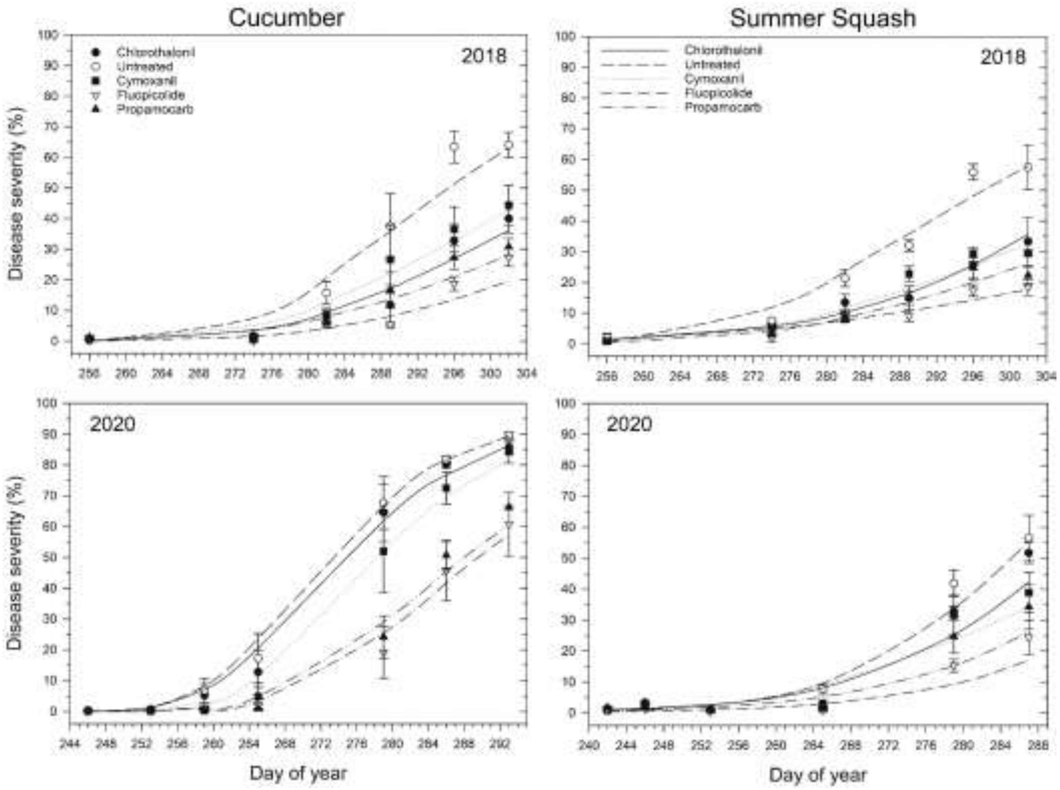

Figure 2.3. Temporal progress of cucurbit downy mildew on cucumber and summer squash in fungicide treated plots and untreated plots in 2018 and 2020 at Charleston, South Carolina. In both years, experiments were conducted during the fall season (early September to late October). Data points represent mean values, the vertical bars are standard errors of the mean observed disease severity values, and the curves represented predicted values obtained from fitting either a Gompertz or logistic growth model to observed disease severity values. Within the legends, cymoxanil, fluopicolide and propamocarb denotes plots treated with chlorothalonil and the respective systemic fungicide.

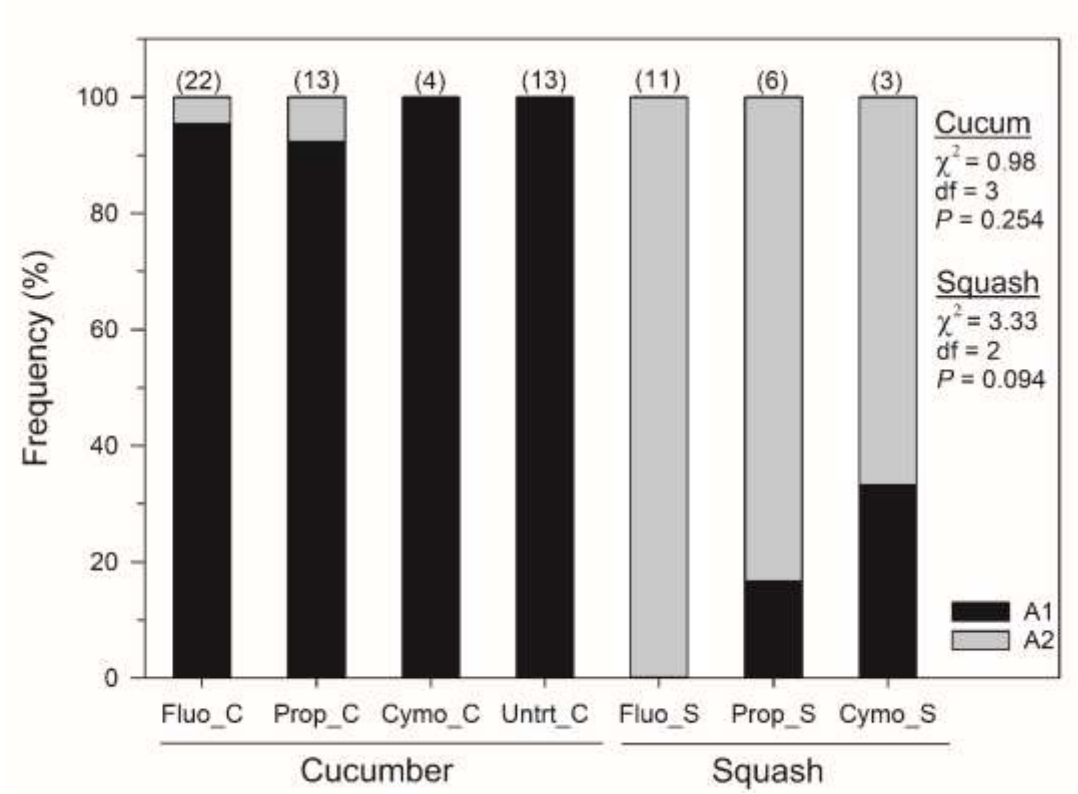

Figure 2.4. Frequency distribution of A1 and A2 mating types of *Pseudoperonospora cubensis* isolates based on isolates collected from fungicide treated plots (fluopicolide, propamocarb, cymoxanil and untreated control) and isolates collected from plots of cucurbit host types (i.e., cucumber and summer squash). For fungicide treatment, 'Cymo', 'Fluo', 'Prop' and 'Untrt' refer to Cymoxanil, Fluopicolide, Propamocarb and Untreated, respectively, while for host type, 'C' and 'S' denote cucumber and summer squash, respectively. Differences in the frequency distribution of the number of A1 and A2 isolates among fungicide treatments by host is based on the Fisher's exact test. Values in parenthesis are numbers of isolates tested for each fungicide treatment and host type.

CHAPTER 3

Within-season Shift in Fungicide Sensitivity Profiles of *Pseudoperonospora cubensis* Populations in Response to Chemical Control

To be submitted to *Plant Disease*

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Abstract

Cucurbit downy mildew, caused by *Pseudoperonospora cubensis*, is an important disease affecting cucurbits worldwide. Chemical control is the most effective method for disease control but *P. cubensis* has a high risk for developing resistance to fungicides. Alternating fungicides with different modes of action is recommended to avoid an increase of resistant subpopulations. Thus, this study was conducted to establish shifts in the sensitivity profiles of *P. cubensis* isolates during the growing season wherein chlorothalonil was applied in alternation with either cymoxanil, fluopicolide or propamocarb in field experiments conducted from 2018 to 2020 at Rocky Mount, North Carolina and in 2018 and 2020 at Charleston, South Carolina. The sensitivity of isolates sampled early (i.e., baseline isolates) or late in the season, to these single-site fungicides was determined using the detached leaf disc bioassay where isolates were classified as sensitive, or resistance based on the mean relative disease severity values. Based on the Kruskal-Wallis test, mean RS increased significantly among early- and late-season isolates where chlorothalonil was alternated with fluopicolide (P = 0.0010) but not with cymoxanil (P = 0.2378) or propamocarb (P = 0.5604). Although there was a directional selection towards resistance for isolates sampled from plots that were treated with fluopicolide or propamocarb alternated with chlorothalonil during a growing season, a significant shift in fungicide sensitivity distribution based on combined data were observed for fluopicolide ($\chi^2 = 6.64$; P = 0.0100) but not propamocarb ($\chi^2 = 0.11$; P = 0.7372). Early- and late-season *P. cubensis* isolates sampled from the cymoxanil treated plots were all resistant to this fungicide and there were no significant shifts in their fungicide sensitivity profile during a growing season ($\chi^2 = 0.06$; P = 0.8087). These results indicate that a shift towards reduced sensitivity in *P. cubensis* can occur during a growing season and the efficacy of fluopicolide and propamocarb is likely to decrease as the frequency of the less sensitive subpopulations increases

during the production season. The resultant effect on disease severity and selection of an insensitive subpopulation may accelerate the development of resistance to propamocarb in the southeastern United States.

Keywords: cucurbits, fungicide resistance, cucurbit downy mildew, oomycete, resistance selection

Introduction

Cucurbit downy mildew (CDM) caused by the oomycete *Pseudoperonospora cubensis* is considered one of the most economically important disease of cucurbits in field and greenhouse production around the world (Lebeda and Cohen 2011; Holmes et al. 2015; Ojiambo et al. 2015). The disease affects important cucurbit crops, including cucumber, melon, squash, and watermelon (Holmes et al. 2015; Lebeda 1992). *Pseudoperonospora cubensis* is a polycyclic pathogen that can undergo numerous infection cycles during the growing season under favorable weather conditions (Kikway et al. 2021). In the United States, plant breeding efforts from 1940s to 1960s resulted in the development of CDM resistant cucumber cultivars (Barnes 1948; Barnes 1966). Indeed, effective host resistance in cucumber derived from the cultivar Chinese Long Plant Introduction 197087 and the associated *dm-1* resistance gene in cucumber was so effective that fungicides were not required for CDM management (Barnes and Epps 1954; Sitterly 1972). Until 2004, cucumber did not require intensive chemical protection due to effective host resistance (Holmes et al. 2015; Thomas et al. 2018). However, a resurgence of the disease characterized by rapid disease spread and fungicide inefficacy resulted in severe to complete crop losses across the east coast and mid-west in 2004 and 2005 (Holmes et al. 2006; Holmes et al. 2015). This resurgence of CDM was subsequently associated with breakdown of the *dm-1* resistance gene and rendered host resistance in cucumber cultivars ineffective (Criswell et al. 2010). While, the *dm-1* gene still exhibits moderate resistance to new A1 strains of *P. cubensis*, fungicides are required to protect cucumber yield and quality (Cohen et al. 2015; Wang et al. 2019).

During the last several decades, systemic fungicides have been developed and widely used to control CDM (Brent 2012). The development of these systemic fungicides was considered as a hallmark in chemical crop protection since these fungicides could protect newly formed tissues and are able to suppress pathogen growth after infection. However, these fungicides are single-site inhibitors and pathogens like *P. cubensis* with high evolutionary potential (McDonald and Linde 2002) were able to develop resistance due to selection pressure from frequent application and render these chemistries ineffective following their introduction to the market. As such, the Fungicide Resistance Action Committee (FRAC) considers *P. cubensis* to be a high-risk pathogen for developing resistance to fungicides (FRAC 2021). Examples of the single-site systemic fungicides for managing CDM following infection include cymoxanil (FRAC code 27), ethaboxam (FRAC code 22), fluopicolide (FRAC code 43), propamocarb (FRAC code 28), and oxathiapiprolin (FRAC code 49) (Cohen et al. 2015; D'Arcangelo et al. 2021; Salas et al. 2019). Unlike single-site fungicides, multi-site fungicides are recommended to protect the crop prior to infection. Chlorothalonil and mancozeb (FRAC codes M 05 and M 03, respectively) are example of multi-site fungicides used to control CDM. Multi-site fungicides do not provide adequate protection from yield losses when they are applied season-long, and growers must apply other fungicides after downy mildew appears in the field (Adams et al. 2015; Colucci et al. 2007). Typically, single-site fungicides are used in concert with multi-sites fungicides for effective disease control and to prolong the efficacy of single-site fungicides (Massi et al. 2021).

The emergence of fungicide resistance has become a serious problem in several pathosystems since the introduction of single-site fungicides in the 1970s. Temporal shifts in fungicide resistance within populations of *P. cubensis* have been well documented in several studies (e.g., Lebeda et al. 2019). For example, propamocarb was highly effective, while metalaxyl was ineffective against pathogen populations in Czech Republic from 2001 to 2011 (Pavelková et al. 2014). Similarly, the efficacy of cymoxanil, fluopicolide and propamocarb monitored from 2012 to 2014 showed that cymoxanil was completely inffective, while fluopicolide and propamocarb were highly effective during this period in the Czech Republic (Lebeda et al. 2017). In the United States, a quantitative synthesis of fungicide effiacy data showed that floupicolide and propamocarb were the most effective for disease control between 2000 and 2008, while demethylation inhibitors were the least effective (Ojiambo et al. 2010). A subsequent study showed a shift towards increased resistance to fluopicolide, while propamocarb was still largely effective (Thomas et al. 2018). More recently, field studies have shown reduced sensitivity of *P. cubensis* populations in the United States to the recently introduced chemistry, oxathiapiprolin (Dutta 2021). Most of the studies on the temporal shifts in fungicide resistance within populations of *P. cubensis* have been conducted over growing seasons (e.g., Lebeda et al. 2017; Pavelková et al. 2014). Temporal shifts in fungicide resistance can also occur within a growing season for polycyclic plant pathogens (Cosseboom et al. 2019; Grünwald et al. 2006; Matasci et al. 2008; Odilbekov et al. 2019). However, no studies have been conducted to understand the temporal shift, distribution and frequency of resistant isolates of *P. cubensis* within a growing season. Such studies have practical implications since late season application of fungicides in combination with shifts in fungicide resistance could accelerate resistance development (Köller et al. 1995). Similarly, a late season sampling of isolates for monitoring the status of fungicide resistance could overestimate resistance levels since shifts caused by a fungicide could be superimposed on natural seasonal shifts in fungicide senstivity. In addition, fungicides groups applied early in the season may need to be different from those applied late in the season to reduce emergence of fungicide resistance and improve disease control.

Resistance to single-site fungicides within pathogen populations is considered a major threat to modern agriculture since it can potentially lead to reduction in disease control in the field. Thus, appropriate disease control strategies have to be implemented to reduce the emergence of resistance by reducing the selection pressure associated with fungicide use. In general, to avoid an increase of the resistant subpopulation, anti-resistant strategies prescribe application of single-site fungicides/classes in alternation and/or in mixture with fungicide partners with a different mode of action to limit the number of sprays within a season (Hobbelen et al. 2013; Skylakakis 1981; van den Bosch et al. 2014). These anti-resistance strategies are especially important for cucurbit downy mildew where growers continue to intensively use the few remaining effective single-site fungicides, a practice that further increases the risk of fungicide resistance evolution in *P. cubensis* (Cohen et al. 2015). Simulation models shows alteration of site-specific and non-cross-resistant fungicides delays buildup of pathogen genotypes insensitive to high-risk site-specific fungicides (van den Bosch et al. 2014). However, systematic studies have not been conducted to determine how this strategy influences the distribution and frequency of resistant phenotypes of *P. cubensis* in cucurbit fields within a growing season. In some studies, repeated use of particular fungicide classes has not always been associated with a decrease in sensitivity (Gang et al. 2015; Thygesen et al. 2009). This observation creates a complex scenario that is usually addressed at theoretical level using simulation models to establish a relationship between fungicide spray programs and the possible emergence of fungicide resistance in the field (Castle and Gilligan 2012; van den

Bosch et al. 2014). Based on the above considerations, this study was conducted with the following specific objectives: i) determine the shift in the sensitivity distribution (i.e., selection) within populations of *P. cubensis* within a growing season, and ii) establish the impact of alternating a multi-site with a site-specific fungicide on the shift in selection within the populations of *P. cubensis* within a growing season. Our approach was to compare sensitivity distributions of isolates from unsprayed population early in the season to sensitivity profiles of isolates sampled late in the season after exposure to alternation of a multi-site fungicide with specific single-site fungicides within a growing season.

Materials and Methods

Field trials and planting materials. Field experiments were conducted at the Upper Coastal Research Station in Rocky Mount, North Carolina, during the summer season in 2018, and the fall season in 2019 and 2020. Field experiments were also conducted at the Coastal Research and Education Center in Charleston, South Carolina, during the fall season in 2018 and 2020. Plots at Rocky Mount were raised beds (0.15 m high by 0.60 m wide) covered with plastic mulch and fitted with drip irrigation along the center of the beds and they measured 3-6 m long by 0.6 m width and were separated by 2.5-3.0 m fallow area on each end. Plots at Charleston were also set up as above except that each plot included three rows that were 3.3 m long and 0.9 m wide.

Cucumber (cv. ‘Straight 8’) and summer squash (cv. ‘Spineless Perfection’) were used in all experiments except at Charleston in 2020 where the cucumber cv. ‘Speedway’ was used instead of ‘Straight 8’. At Rocky Mount, planting was done manually by placing 3-5 seeds per hill at a spacing of 0.6 m per row on 9 May 2018, 14 August 2019, and 19 August 2020. Plots were reseeded 1 week after initial seeding when necessary to replace non-germinated seeds. Thinning was done after 2-3 weeks, to allow for 1 plant per hill and 5 plants per plot in 2018 and 10 plants per plot in 2019 and 2020. At Charleston, seedlings were first raised in a greenhouse and transplanted on 3 September 2018 and 18 August 2020 for a total of 15 plants per plot in each growing season.

Fungicide treatments and application. Disease epidemics in all field plots were initiated from naturally occurring sporangia from either neighboring infected fields or sentinel plots at Rocky Mount and at Charleston (Keinath 2016). One multi-site fungicide (chlorothalonil) and three site-specific fungicides (cymoxanil, fluopicolide and propamocarb) were used in this study (Table 3.1), with alternation of fungicides with different FRAC codes was the resistance management strategy that was evaluated to assess its impact on shifts in fungicide sensitivity profiles. At both locations, chlorothalonil (FRAC code M 05) was applied weekly in alternation with either cymoxanil (FRAC code 27), fluopicolide (FRAC code 43) or propamocarb (FRAC 28) following the appearance of disease symptoms in the plots. Disease symptoms were observed on 15 June 2018, 3 September 2019, and 8 September 2020 at Rocky Mount and on 19 September 2018 and 25 August 2020 at Charleston. Prior to the start of this fungicide application regime, two sprays of chlorothalonil (a contact fungicide), were applied in treatment plots at Rocky Mount (but only in 2018), at a 14-day interval until disease was observed, while the contact fungicide was not applied in plots prior to the initiation of the fungicide application regime at Charleston. No fungicides were applied in the untreated control plots at both locations.

At Rocky Mount, fungicides were applied in the plots using a CO_2-pressurized backpack sprayer calibrated to emit ~204 liter/ha at 138 kPa via a boom spaced with four nozzles spaced at ~46 cm apart. Fungicides were applied at Charleston with tractor-mounted hydraulic sprayer calibrated to emit 655 liter/ha at 689 kPa via a boom with four nozzles spaced 45 cm apart. Three fungicide treatments namely, i) weekly alternation of chlorothalonil with cymoxanil (i.e., cymoxanil treatment), ii) weekly alternation of chlorothalonil with fluopicolide (i.e., fluopicolide treatment), iii) weekly alternation of chlorothalonil with propamocarb (i.e., propamocarb treatment), were evaluated to assess their impact on shifts in fungicide sensitivity profiles of *P. cubensis* isolates. At Rocky Mount, three applications of single-site fungicides were applied to plots, and these were on 21 June, 5 July, and 17 July in 2018, on 3 September, 17 September, and 01 October in 2019 and on 11 September, 23 September, and 06 October 6 in 2020. At Charleston, a maximum of four applications of single-site fungicides were applied to plots on 24 September, 10 October, 20 October, and 05 November in 2018 and on 28 August, 10 September, 24 September, and 08 October in 2020.

Isolate sampling and collection. Isolates of *P. cubensis* were collected from experimental plots at Rocky Mount and Charleston at two-time points; early season (i.e., baseline isolates collected from untreated plots or sentinel plots not exposed to any fungicide application) and late season (i.e., isolates collected from respective treatment plots 1 weeks after the last application of a site-specific fungicide at the end of the season). At both time points, infected leaf samples were arbitrarily sampled from the field at both locations. A total of 54 isolates (11 in 2018, 14 in 2019 and 29 in 2020) were collected during the early season sampling period (Table 3.2). Late season isolates were collected from cymoxanil, fluopicolide, or propamocarb treated plots for a total of 28, 53, and 43 isolates, respectively, across the entire study (Table 3.2). Specifically, leaf samples for early season isolates were collected on 13 June 2018, 03 September 2019, and 09 September 2020, while samples for late season isolates were collected on 24 August 2018, 08 October 2019, and 13 October 2020 at Rocky Mount. At Charleston, leaf samples for early season isolates were collected on 17 September 2018, 24 June 2020, and 25 August 2020, while infected leaf samples late season isolates were collected on 12 November 2018, 23 July 2020, and October 2020. All infected leaf samples collected at Charleston were shipped overnight to NC State University immediately after collection from the field. All sampled leaves were subsequently stored at -20°C until processing.

Isolate propagation and maintenance. Single lesion isolates were obtained from the sampled plant leaves as described by Thomas et al. (2017). Briefly, infected leaves were placed with the abaxial side on moist paper towels in clear acrylic boxes and incubated in a growth chamber at 21°C/18°C under 12/12 h light/dark cycle. Following sporulation, a single lesion was cut out using a cork borer and used to inoculate respective host plants to multiply sporangia of each isolate. When discrete single lesions became visible, plants were bagged and kept under dark conditions for 24 h to stimulate sporangia production. Individual lesions were then cut out and used to inoculate respective host plants to propagate sporangia of each isolate. All the isolates were maintained in the laboratory by propagating them on their respective host plants using detached leaves until they were ready for use in leaf disc fungicide sensitivity bioassays. To propagate isolates, the abaxial side of the first or second leaves from host plants were placed on a moist paper towel in clear acrylic boxes and the adaxial side was inoculated with about 2×10^4 sporangia/ml a sporangial suspension. Inoculated leaves were then incubated in a growth chamber at 21/18°C day/night with a 12/12-h light/day cycle.

Fungicide sensitivity bioassay. To establish the sensitivity profiles of *P. cubensis* isolates collected early and late season in the growing season the effect of cymoxanil, fluopicolide and propamocarb on disease severity were assessed using a leaf disc bioassay (Urban and Lebeda 2007) as described by Thomas et al. (2018). Discriminatory doses were used to differentiate sensitive from resistant isolates and the discriminatory dose used for cymoxanil, fluopicolide and propamocarb was 486 mg a.i./liter (Lebeda and Sedláková 2014), 10 mg a.i./liter (Thomas et al. 2018) and 468 mg a.i./liter (Thomas et al. 2018), respectively (Table 3.3). Briefly, leaf discs (12 mm in diameter) were cut from the first, second, or third leaves of 3-to-4-week-old cucumber or summer squash plants and placed adaxial side down into wells (one disc per well) of a 24-well plate filled with 1 ml of 0.5% water agar. The isolates collected from cucumber or summer squash were inoculated onto their respective cucurbit host in the leaf disc bioassay. Each fungicide treatment was replicated six times using leaf discs from the same leaf. The assay was repeated once, and a new leaf was used for each run of the assay. To each leaf disc was treated by pipetting 10 µl of each fungicide in an about 1-µl droplets across the surface of the leaf disc to ensure uniform fungicide application. These droplets were then spread evenly using sterile paintbrush dipped in the respective fungicide at its discriminatory doses. After 24 h, fungicide treated leaf discs were inoculated with a sporangial suspension at a concentration of 1×10^4 sporangial/ml. Untreated leaf discs were used as a control in each well plate during all experiments. Treated and non-treated control leaf discs were then incubated in a growth chamber at 21/18°C under 12/12h light/dark cycle for 5 days.

Five 5 days post inoculation, disease severity on each fungicide-treated and non-treated leaf disc was visually assessed and recorded. Relative mean severity (RS) was calculated as disease severity on treated (DS_T) leaf disc relative to disease severity on non-treated control (DS_C) for each isolate ($RS= [DS_T/DS_C] \times 100$). Isolates were classified as either resistant or sensitive to cymoxanil, fluopicolide or propamocarb, based on RS values. Isolates with a RS <35% were classified as sensitive, while isolates with RS ≥35% were classified as resistant (Urban and Lebeda 2007) to either cymoxanil, fluopicolide or propamocarb.

Data analyses. Disease severity data recorded from two experimental runs were combined after analysis of homogeneity of variance assumption for equality among runs was determined using GLIMMIX procedure in SAS. The homogeneity test for equality of variance among runs was not significant ($P > 0.05$) for all the assays and data from experimental runs were combined for analysis. An analysis of variance was used to determine differences in RS values between early and late season isolates. Further, the distributions of mean RS for early- and late-season isolates exposed to each single-site fungicide for the combined data were compared using the Kruskal-Wallis test (Stokes et al. 1995) performed using PROC NPAR1WAY, a non-parametric test of the null hypothesis that the distribution of a response is the same in multiple groups. The association between sampling time (i.e., early, or late season) and sensitivity of isolates to either cymoxanil, fluopicolide or propamocarb was examined using contingency tables. Statistical differences in the frequency distribution of resistant and sensitive isolates early and late in the season (i.e., shift in sensitivity) was examined based on Fisher's exact test using PROC FREQ in SAS to determine significant shifts in fungicide sensitivity. All statistical analyses were conducted using the SAS software version 9.4 (SAS Institute, Cary, NC).

Results

Relative disease severity in fungicide sensitivity assays. Relative disease severity (RS) was to assess the response of sampled *P. cubensis* isolates to the evaluated single-site fungicides. Based on RS values, the sensitivity of isolates collected early and late in the season varied between years and single-site fungicides (Table 3.3). For example, mean RS for early-season isolates treated with cymoxanil was significantly ($P < 0.05$) higher in 2019 (mean = 84.1%) than in 2018 (mean = 72.9%) and 2020 (mean = 66.8%). Similarly, mean RS for late-season isolates was significantly (P < 0.05) higher in 2019 (mean = 80.2%) than in 2018 (mean = 58.2%) and 2020 (mean = 68.1%). Further, while mean RS across years was higher for early-season isolates (mean = 72.6%), it was not significantly (P > 0.05) different from that of late-season isolates (mean 67.4%).

Relative severity for isolates treated with fluopicolide were significantly (P < 0.05) lower than those treated with cymoxanil. Further, patterns in the differences in mean RS for isolates between years and sampling period for fluopicolide were different from those observed for cymoxanil. For example, while the mean RS for early-season isolates treated with fluopicolide was higher in 2018 (mean = 32.4%), it was not significantly (P > 0.05) different from that in 2019 (mean = 16.6%) and 2020 (mean = 15.2%). Similarly, mean RS for late-season isolates was significantly (P < 0.05) higher in 2019 (mean = 52.8%) than in 2020 (mean = 31.5%) but not 2018 (mean = 39.2%). Further, the mean RS across years was significantly (P < 0.05) higher for late-season isolates (mean = 39.1%) than for early-season isolates (mean = 19.0%) (Table 3.3).

As was the case with fluopicolide, RS values for isolates from plots treated with propamocarb were also significantly (P < 0.05) lower than those treated with cymoxanil (Table 3.3). Further, mean RS for early-season isolates treated with propamocarb was significantly higher in 2019 (mean = 34.6%) than in 2018 (mean = 12.1%) and 2019 (mean = 16.5%), while mean RS for late-season isolates was significantly (P < 0.05) higher in 2019 (mean = 33.4%) than in 2018 (mean = 9.1%) but not 2020 (mean = 23.8%). Across years, the mean RS was not significantly (P > 0.05) higher for late-season isolates (mean = 22.1%) than for early-season isolates (mean = 20.4%) (Table 3.3). The mean RS for isolates collected early in the season from the cymoxanil treated showed that these isolates were resistant and they remained resistant for collected later in the season. However, isolates collected early in the season that were exposed to fluopicolide and propamocarb were sensitive to these two fungicides. Later in the season however, populations exposed to fluopicolide developed resistance based on RS values Based on the Kruskal-Wallis test, the distributions of mean RS values among the early-season and late-season isolates were significantly different for isolates exposed to fluopicolide (χ^2 = 10.82; P = 0.0010) but not those exposed to cymoxanil (χ^2 = 1.39; P = 0.2378) or propamocarb (χ^2 = 0.34; P = 0.5604) (Fig. 3.1).

Fungicide sensitivity profiles and shifts in sensitivity. As described by Urbana and Lebeda (2007), a relative severity value of 35% was used as the threshold to classify the sensitivity of *P. cubensis* isolates to the single-site fungicides. Based on this threshold, sensitivity profiles for isolates differed among test fungicides, between and within sampling periods (Table 3.3). For example, all isolates sampled early and late in the season were found to be resistant to cymoxanil across the entire study period. However, while all isolates sampled early in the season were sensitive to both fluopicolide and propamocarb, differences in sensitivity profiles were observed among the late-season isolates across the study period. For example, while early-season isolates were sensitive to fluopicolide in 2018 to 2020, late-season isolates were resistant to fluopicolide in 2018 and 2019 but remained sensitive in 2020 (Table 3.3). Similarly, while early-season isolates were sensitive to propamocarb in 2018 to 2020, late-season isolates were resistant to propamocarb in 2019 but remained sensitive in 2018 and 2020.

Shifts in sensitivity profiles of *P. cubensis* isolates after exposure within a season as detected based on Fisher's exact test varied depending on the single-site fungicide evaluated and the growing season. For example, sensitivity profiles of isolates exposed to fluopicolide shifted from a sensitive population early in the season to an insensitive population late in the season. However, frequency distributions significantly shifted towards insensitivity in 2019 (χ^2 = 4.49; P = 0.0341) and 2020 (χ^2 = 3.81; P = 0.0509) but not in 2018 (χ^2 = 0.04; P = 0.8506) as reflected by the mean RS for each population (Fig. 3.2), indicating a directional selection towards resistance in 2019 and 2020. Based on the combined data across the three years, frequency distribution showed a significant ($\chi^2 = 6.64$; $P = 0.0100$) shift towards insensitivity and directional selection towards resistance for *P. cubensis* isolates exposed to fluopicolide within a growing season (Fig. 3.2).

Based on the mean RS, there was indication of sensitivity profiles of isolates exposed to propamocarb shifting from a sensitive population early in the season to an insensitive population late in the season especially in 2019 and 2020. However, there was no significant shift in the frequency distributions towards insensitivity in 2019 ($\chi^2 = 0.004$; $P = 0.9512$), 2020 ($\chi^2 = 0.934$; $P = 0.3339$) and for the combined data ($\chi^2 = 0.113$; $P = 0.7372$), with the population remaining largely sensitive to propamocarb throughout the growing season (Fig. 3.3). Unlike for fluopicolide and propamocarb, isolates sampled early and late in the season were both insensitive to cymoxanil and there was no directional shift in sensitivity during the growing season in all years (Fig. 3.4), although there was a decrease in the mean RS especially in 2018 ($\chi^2 = 0.1523$; $P = 0.2172$). Similarly, while there was a decrease in the mean RS for the combined data across all years, there was no significant ($\chi^2 = 0.059$; $P = 0.8087$) shift in the frequency distributions towards sensitivity during the growing season (Fig. 3.4).

Discussion

Fungicides are currently the most effective method to control CDM given that most commercial cucurbit cultivars, with the exception of a few new cucumber cultivars, do not confer adequate disease resistance. Thus, preservation of the efficacy of available single-site fungicides through the deployment of resistance management strategies is of primary importance in an effort to avoid loss of disease control due to fungicide resistance. In this study, we examined the impact of a resistance management strategy of alternation of single-site with multi-site fungicide on temporal shift in sensitivity of *P. cubensis* isolates within a growing season, to establish the effect of this spray program on the pathogen population under field conditions. We observed significant directional selection towards insensitivity within a growing season for isolates sampled from plots treated with weekly alternation of fluopicolide with chlorothalonil. Further, while there was directional shift towards insensitivity for isolates sampled from plots treated with weekly alternation of propamocarb with chlorothalonil, shifts in sensitivity profiles were not significant based on established sensitivity thresholds. The observed temporal shift in the sensitivity suggests that as the frequency of the insensitive isolates of *P. cubensis* increases during the growing season, the efficacy of fluopicolide and possibly propamocarb is likely to decrease during the production season. Consequently, selection of a resistant sub-population is expected to accelerate the emergence of fungicide resistance. This work represents the first documentation of the selection for fungicide resistance within a growing season in populations of *P. cubensis* and has important implications for fungicide resistance management within this pathosystem.

This study builds on our previous work on the effectiveness of the resistance management strategy of alternation single-site and multi-site fungicides in improving fungicide efficacy and reducing cucurbit downy mildew severity (Kikway et al. 2021). In that study, we observed that CDM severity and rates of disease progress were lower when chlorothalonil was applied in alternation with fluopicolide or propamocarb than when chlorothalonil was alternated with cymoxanil or when chlorothalonil was applied alone. Here, we extended the study by Kikway et al. (2021) to determine how this resistance management strategy influenced the selection for fungicide resistance in populations of *P. cubensis* within a production season. While the Fungicide Resistance Action Committee considers *P. cubensis* to be a high-risk pathogen for developing resistance to fungicides (FRAC 2021), information on the effect of resistance management strategies on the sensitivity profiles of the pathogen populations is still lacking. In this study, we observed a significant shift towards insensitive during the season in plots where fluopicolide was applied in alternation with chlorothalonil. Directional selection towards insensitivity within a growing season in response to single-site fungicides has also been reported in *Phytophthora infestans* (Grunwald et al. 2006), *Plasmopara viticola* (Matasci 2007), *Alternaria solani* (Odilbekov et al. 2019) and *Venturia inaequalis* (Köller et al. 1995). The within-season shift in the sensitivity profiles of *P. cubensis* was most likely due to fluopicolide application and associated selection pressure. This observation was not unexpected given previous reports of insensitivity of the pathogen population (Thomas et al. 2018) and reduced field efficacy of fluopicolide (Adams et al. 2015; Gugino and Grove 2017) in controlling CDM in the United States. In other pathosystem, within-season shifts towards fungicide insensitivity has also been attributed to selection by association without direction selection pressure (Hu et al. 2016), or due to a changing environment or early-season pathogen populations gradually being replaced by late-season populations (Köller et al. 1995). However, it is unlikely that the latter selection processes may not have played a role in this study given that we only observed directional selection for resistance for isolates exposed to fluopicolide but not those exposed to propamocarb during the production season.

A systematic review and synthesis of fungicide efficacy reports found that propamocarb was of the most effective fungicides against *P. cubensis* since its introduction for use in the United States in 2004 (Ojiambo et al. 2010). Although there were reports of reduced efficacy of propamocarb in Pennsylvania (Gugino and Grove 2016) and South Carolina (Keinath 2016) in 2015, a subsequent study found that most of the isolates (~75%) sampled in the eastern United were sensitive to propamocarb (Thomas et al. 2018). In the present study, we did not observe a directional selection towards resistance among isolates exposed to propamocarb during the growing season. Further, while the sensitivity profiles of isolates sampled late in the season were sensitive, the mean RS for these isolates was close to the 35% RS threshold for classifying isolates as resistant (Urban and Lebeda 2007). Thus, while we did not detect selection for resistance within the growing, resistance to propamocarb could still evolve during a production season. Often, evolutionary forces such as migration, mutation and random migration are not expected to be detected within-season in small field plots and sample size of isolates used in this study unless they are very strong (Grünwald et al. 2006; Milgroom et al. 1989). Comparable to durability of host resistance, durability of a fungicide can meaningfully be evaluated over a long time and in large areas (Odile et al. 2010). Given that *P. cubensis* is considered to be a high-risk pathogen for developing resistance to fungicides, regular resistance monitoring programs may be needed to establish the prevailing degree and extent of isolates that are resistant to propamocarb within pathogen populations in the eastern United States. Unlike, for fluopicolide and propamocarb, both early- and late-season sampled isolates of *P. cubensis* were resistant to cymoxanil. This observation is consistent with the higher levels of disease severity in cases where cymoxanil was alternated with chlorothalonil compared to where fluopicolide or propamocarb were alternated with chlorothalonil (Kikway et al. 2021). While a systematic review indicated that cymoxanil alternated with chlorothalonil was effective against CDM in the United States (Ojiambo et al. 2010), reports of reduced efficacy of cymoxanil against CDM were reported in bioassay experiments in South Carolina (Keinath 2016) and in field studies in Michigan (Goldenhar and Hausbeck 2019). There also reports on the emergence of cymoxanil resistant populations in other oomycetes in other parts of the world (Gisi and Sierotzki, 2008; Grünwald et al. 2006). However, a recent report from China indicated that cymoxanil was highly effective against *P. cubensis* with the majority of isolates tested (~77%) being sensitive (Yanju et al. 2019). The study by Yanju et al. (2019) did not specify their discriminatory dose to assess sensitivity of *P. cubensis* to cymoxanil and thus, we are unable to meaningfully compare our results with their findings, although differences in the pathogen populations could be a contributing factor in our observed results.

The shift in the sensitivity profile of *P. cubensis* towards isolates that are less sensitive to fluopicolide and propamocarb during the course of a growing season has some practical implications. First, application of fluopicolide or propamocarb may accelerate development of fungicide resistance by imposing selection for resistance. In other pathosystems, there has been reports of naturally reduced sensitivity in the latter part of the growing season for example in the use of DMI to control *Venturia inaequalis* in peach orchards (Braun and McRae 1992). While this phenomenon has not been reported in the control of *P. cubensis* in cucurbits, naturally shifting populations impose an additional interactive selection force that could further accelerate resistance development (Köller et al. 1995). Regardless of the source of the imposed selection pressure, the outcome is reduced disease control towards the end of the season and increased opportunities for sexual reproduction and local overwintering (Kikway et al. 2022). The latter further emphasizes the need for effective disease control towards the end of the growing season. As such, single-site fungicides with a different mode of action from that of fluopicolide or propamocarb, may need to be used late in the season. Secondly, given that shifts caused by fungicide application could be superimposed with natural seasonal shifts in resistance, a late-season sampling of *P. cubensis* isolates to monitor the status of fluopicolide or propamocarb resistance in cucurbit fields may overestimate levels of resistance. Thus, sampling of isolates to assess fungicide sensitivity may need to be conducted before any natural shifts in the pathogen population occur in the field.

Oxathiapiprolin is a newly introduced single-site fungicide for use to control cucurbit downy mildew. While recent field studies have shown reduced sensitivity of *P. cubensis* to this newly introduced chemistry (Dutta 2021), there are no reports on the sensitivity profiles of *P. cubensis* in response to oxathiapiprolin. It would be interesting to establish how alternation of oxathiapiprolin with chlorothalonil would influence selection of resistant isolates of *P. cubensis* during the growing season.

In summary, we observed that application of fluopicolide or propamocarb in alternation with chlorothalonil affects directional shift in the sensitivity profiles of *P. cubensis* during the growing season. Fungicide resistance management is critical to preserve and prolong the efficacy of single-site fungicides. Thus, control strategies against the pathogen will need to be adjusted to decrease the selection pressure for decreased sensitivity to fluopicolide. During the growing season, fungicides should also be applied according to environmental conditions influencing the risk of disease rather than calendar-based spray programs to reduce the number of spray applications per season (Neufeld et al. 2017). Other fungicide resistance strategies such as mixing fungicides with different modes of actions should also be evaluated systematically to assess their effectiveness in managing resistance in *P. cubensis*. Even in the presence of fungicide resistance strategies, selection of resistant isolates of *P. cubensis* cannot be completely avoided. Thus, resistance must be carefully monitored and managed not only at the field level but also at a regional level, to avoid the selection and spread of resistant strains. Further, rapid detection of location-specific resistance profiles could allow growers to identify changes needed in their spray programs and tailor sprays sequences to field-specific needs (Schnabel et al. 2015). The latter has been demonstrated to be useful in monitoring fungicide resistance in *Botrytis cinerea* causing gray mold in strawberries.

Acknowledgments

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Table 3.1. Description of fungicide products used to assess shifts in sensitivity in populations of *Pseudoperonospora cubensis* during the growing season at Rocky Mount, North Carolina and Charleston, South Carolina.

Product trade name	Active ingredient	Mode of action	Formulation (Rate/ha)	Group name[y]	FRAC code[z]
Bravo Weather Stik	Chlorothalonil 54%	Contact	6SC (2.34 L)	Multi-site inhibitor	M05
Curzate	Cymoxanil 60%	Systemic	60DF (0.35 kg)	Cyanoacetamide-oxime	27
Presidio	Fluopicolide 39.5%	Systemic	4SC (0.29 L)	Benzamide	43
Previcur Flex	Propamocarb HCl 66.5%	Systemic	6F (0.40 L)	Carbamate	28

[y] Benzamide (pyridinylmethyl-benzamide).

[z] FRAC code = Fungicide Resistance Action Committee mode-of-action classification.

Table 3.2. Number of isolates of *Pseudoperonospora cubensis* sampled to assess shifts in fungicide resistance during the growing season.

Fungicide program [a]	Sampling period [b]	Treatment group [c]	Number of isolates (n)			
			2018	2019	2020	Total [d]
Untreated	Early season	Non-exposed (baseline)	11	14	29	54
Chlorothalonil + Cymoxanil	Late season	Cymoxanil exposed	13	9	6	28
Chlorothalonil + Fluopicolide	Late season	Fluopicolide exposed	16	13	24	53
Chlorothalonil + Propamocarb	Late season	Propamocarb exposed	13	12	18	43

[a] Fungicide treatment denotes weekly alternation of a multi-site (chlorothalonil) fungicide with a multi-site (chlorothalonil) or a single-site specific fungicide (cymoxanil, fluopicolide or propamocarb) during the growing season.
[b] Early season represents time at onset of disease symptoms, while late season represents time after the last application of the respective systemic fungicide.
[c] Non-exposed isolates represent isolates collected early in the season; exposed isolates represent isolates collected late in the season after the last application of the respective systemic fungicide.
[d] Number of isolates for combined for each isolate group.

Table 3.3. Mean relative disease severity of early and late season isolates of *Pseudoperonospora cubensis* on detached leaf discs of cucumber treated with systemic fungicides at their respective discriminatory doses.

			Early season [a]		Late season [a]	
Treatment group	Year	Discriminatory dose (mg a.i./l) [b]	Mean relative severity (%) ± S.E.	Sensitivity [c]	Mean relative severity (%) ± S.E.	Sensitivity [c]
Cymoxanil exposed	2018	480	72.9 ± 6.5	Resistant	58.2 ± 7.3	Resistant
	2019	480	84.1 ± 3.9	Resistant	80.2 ± 4.4	Resistant
	2020	480	66.8 ± 4.0	Resistant	68.1 ± 4.5	Resistant
Fluopicolide exposed	2018	10	32.4 ± 11.7	Sensitive	39.2 ± 9.7	Resistant
	2019	10	16.6 ± 5.9	Sensitive	52.8 ± 9.7	Resistant
	2020	10	15.2 ± 5.2	Sensitive	31.5 ± 6.9	Sensitive
Propamocarb exposed	2018	468	12.1 ± 2.4	Sensitive	9.1 ± 2.5	Sensitive
	2019	468	34.6 ± 8.1	Sensitive	33.4 ± 8.9	Sensitive
	2020	468	16.5 ± 3.0	Sensitive	23.8 ± 4.6	Sensitive

[a] Early season represents time at onset of disease symptoms, while late season represents time after the last application of the respective systemic fungicide.
[b] Discriminatory dose is for the systemic fungicide that corresponds to the treatment group for sampled isolates.
[c] Isolates were classified as either sensitive (i.e., mean relative disease severity <35%) or resistant (i.e., mean relative disease severity ≥35%) as described by Urban and Lebeda (2007); S.E. denotes the standard error.

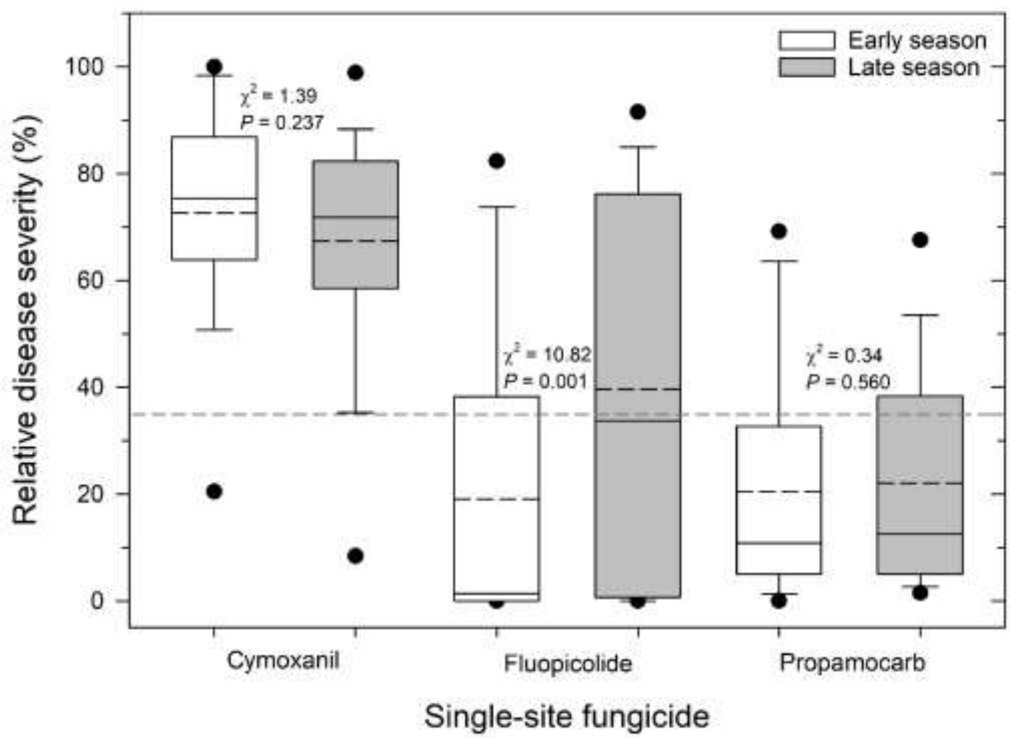

Figure 3.1. Box-whisker plots showing the distribution of mean relative disease severity for isolates of *Pseudoperonospora cubensis* collected early in the season (baseline) or late in the season (exposed), before and after expose, respectively, of the pathogen population to fungicides in field experiments conducted in North Carolina and South Carolina from 2018 to 2020. Fungicide sensitivity bioassays were conducted using a leaf disc assay at the corresponding discriminatory dose for each single-site fungicide: cymoxanil (480 mg a.i./liter), fluopicolide (10 mg a.i./liter) and propamocarb (468 mg a.i./liter). The number of isolates assayed for each fungicide is presented in Table 1. The dashed horizontal line denotes the relative disease severity (RS) threshold used to classify an isolate as either sensitive (RS < 35%) or resistant (RS ≥ 35%). The boxes represent the interquartile range, with the whiskers indicating the 5- and 95-percentiles. Solid and dashed lines within the boxes represent the median and mean RS, respectively. The chi-square values are for the Kruskal-Wallis test for differences in the distribution of mean RS between early- and late-season isolates.

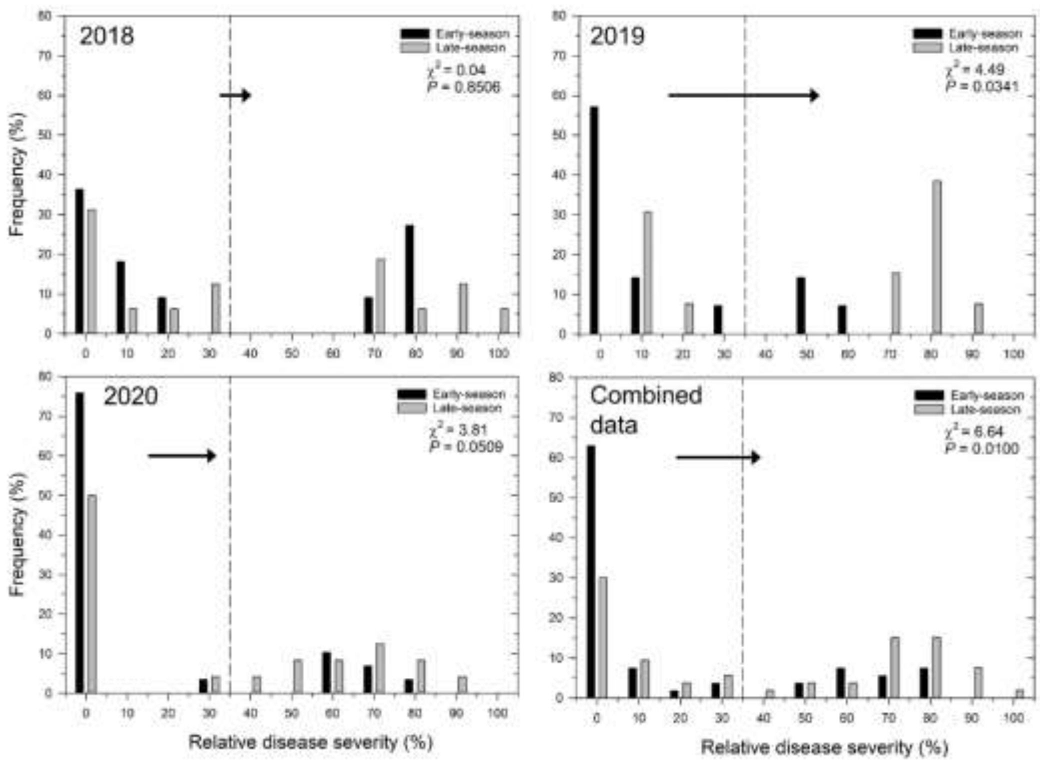

Figure 3.2. Frequency distribution of relative disease severity on cucumber leaf discs inoculated with early-season (baseline) or late-season (exposed) isolates of *Pseudoperonospora cubensis* before and after exposure, respectively, of pathogen population to fluopicolide in field experiments conducted to assess shift in fungicide during the growing season in North Carolina and South Carolina. Data shown is for isolates collected in both locations and screened in 2018 (n = 16), in 2019 (n = 13), in 2020 (n = 24), and isolates collected in all the three years (n = 53). Fluopicolide sensitivity assays were conducted using a discriminatory dose of 10 mg a.i./liter. The dashed vertical line denotes relative disease severity (RS) threshold used to classify an isolate as either sensitive (RS < 35%) or resistant (RS ≥ 35%). The arrows show the direction and approximate magnitude of the shift in mean of relative disease severity before and after exposure of pathogen population to fluopicolide.

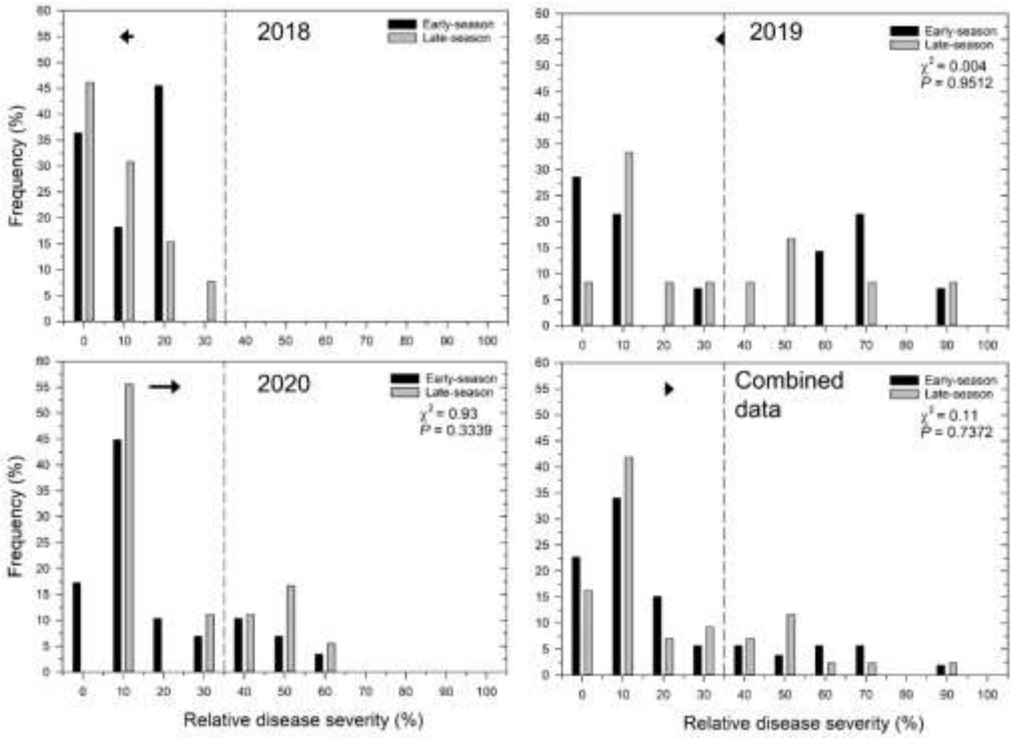

Figure 3.3. Frequency distribution of relative disease severity on cucumber leaf discs inoculated with early-season (baseline) or late-season (exposed) isolates of *Pseudoperonospora cubensis* before and after exposure, respectively, of pathogen population to propamocarb in field experiments conducted to assess shift in fungicide during the growing season in North Carolina and South Carolina. Data shown is for isolates collected in both locations and screened in 2018 (n = 13), in 2019 (n = 12), in 2020 (n = 18), and isolates collected in all the three years (n = 43). Propamocarb sensitivity assays were conducted using a discriminatory dose of 468 mg a.i./liter. The dashed vertical line denotes relative disease severity (RS) threshold used to classify an isolate as either sensitive (RS < 35%) or resistant (RS ≥ 35%). The arrows show the direction and approximate magnitude of the shift in mean of relative disease severity before and after exposure of pathogen population to fluopicolide.

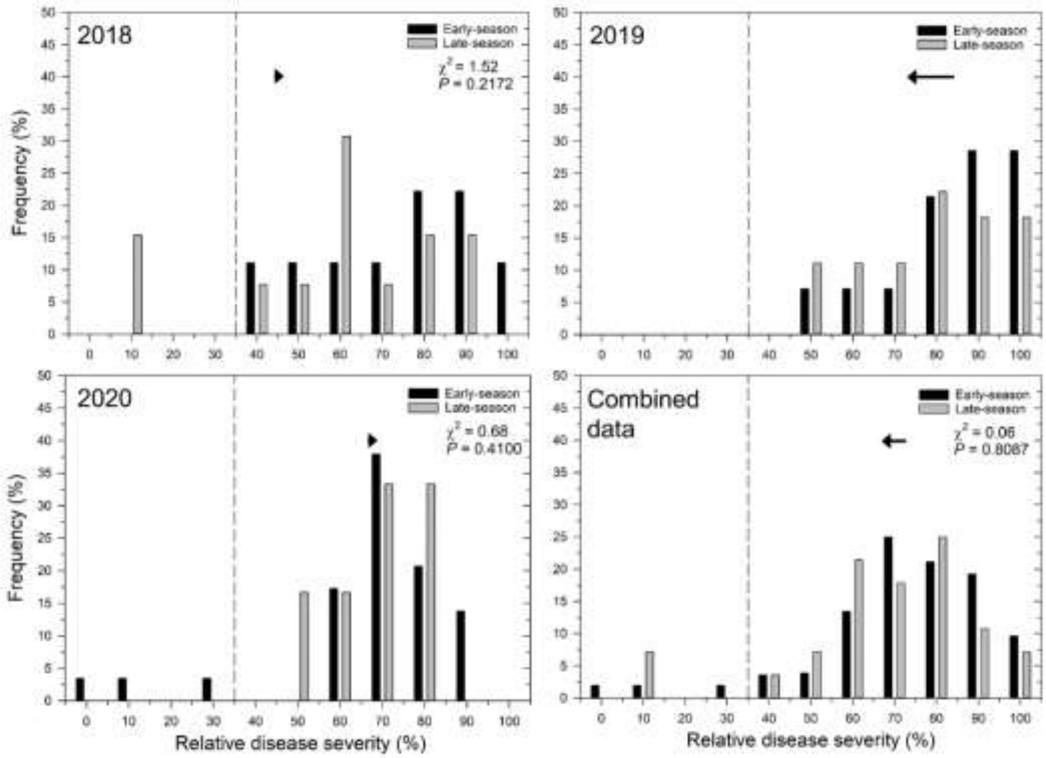

Figure 3.4. Frequency distribution of relative disease severity on cucumber leaf discs inoculated with early-season (baseline) or late-season (exposed) isolates of *Pseudoperonospora cubensis* before and after exposure, respectively, of pathogen population to cymoxanil in field experiments conducted to assess shift in fungicide during the growing season in North Carolina and South Carolina. Data shown is for isolates collected in both locations and screened in 2018 (n = 13), in 2019 (n = 9), in 2020 (n = 6), and isolates collected in all the three years (n = 28). Cymoxanil sensitivity assays were conducted using a discriminatory dose of 480 mg a.i./liter. The dashed vertical line denotes relative disease severity (RS) threshold used to classify an isolate as either sensitive (RS < 35%) or resistant (RS ≥ 35%). The arrows show the direction and approximate magnitude of the shift in mean of relative disease severity before and after exposure of pathogen population to cymoxanil.

CHAPTER 4

Field Occurrence and Overwintering of Oospores of *Pseudoperonospora cubensis* in the Southeastern United States

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ABSTRACT

In the United States, the cucurbit downy mildew pathogen, *Pseudoperonospora cubensis*, has been shown to form oospores under laboratory conditions, but there are no reports on the formation of oospores in naturally infected cucurbit plants in the field. This study investigated the occurrence of oospores in naturally infected leaves from cucurbit fields in North Carolina and South Carolina from 2018 to 2020. Oospore viability and survival was also determined outdoors during the winter in North Carolina during this study period using soil containing leaves infested with oospores. About 5% of 1,658 naturally infected cucumber and cantaloupe leaves sampled during the study had oospores, with a mean density of 585 oospores per cm^2 of infected leaf tissue. Absolute oospore viability, as assessed using the plasmolysis method, declined linearly (slope = −0.27; $P <$ 0.0001) over the six-month exposure period from 67.8% in November to 19.3% in May. Other variables being equal, the decrease in oospore viability was significantly affected by soil temperature ($b = -0.03$ to -0.05; $P < 0.0001$) and number of rainy days ($b = 21.6$ to 40.46; $P <$ 0.05), while the effects of soil moisture on oospore viability were less clear. About 20% of the oospores exposed to outdoor conditions at the end the study period were putatively viable and deemed potentially infective. However, these putatively viable oospores failed to germinate or initiate disease when inoculated onto cucumber or cantaloupe leaves. These results indicate that oospores might require some unrecognized stimuli or physiological factors to initiate germination and infection. Nonetheless, viability of oospores at the end of the winter season suggests that once exposed to the right conditions that stimulate germination, these oospores could potentially serve as a primary inoculum source in the southeastern United States where winter temperatures are cold enough to kill cucurbits plants.

Keywords: cucurbits, dormancy, germination, oospore formation, oospore viability, pathogen survival

INTRODUCTION

Plant pathogenic oomycetes cause devastating diseases in several important crops and significantly affect agricultural production globally. Oomycetes that cause downy mildews in important agricultural crops are found in the genera *Albugo*, *Bremia*, *Hyaloperenospora*, *Peronospora*, *Phytophthora*, *Plasmopara*, *Pseudoperenospora* and *Pythium* (Kamoun 2009). In plant pathogens, sexual reproduction and the formation of resting structures is a mechanism that generates genetic diversity within the pathogen population and enables pathogens to survive during unfavorable environmental conditions (McDonald and Linde 2002). Oospores are the sexual or resting spores in oomycetes. They are typically spherical, thick-walled, and brown to hyaline in color. These structures act as a means of pathogen survival in soil or plant debris during the offseason when no host plant tissue is available for infection (Cohen et al. 1997; Hanson and Shattock 1998; Zhang et al. 2006). Homothallic species of plant pathogenic oomycetes such as *Plasmopara halstedii* and *Pseudoperenospora humuli* form oospores when an antheridium fertilizes an oogonium of the same mycelium (Gent et al. 2017). However, heterothallic plant pathogenic oomycetes, such as *Phytophthora infestans*, *Bremia lactucae*, *Plasmopara viticola* and *Pseudoperenospora cubensis*, undergo sexual reproduction when mycelia of two compatible opposite mating types, A1 and A2, are in close proximity and grow together, resulting in the formation of oospores (Cohen and Rubin 2012; Cohen et al. 2016; Michelmore and Ingram 1981). Thus, for biotrophic oomycetes such as *P. cubensis* that require living host tissue to complete their life cycle, oospores could play a significant role in disease epidemiology by serving as a primary source of inoculum.

Pseudoperonospora cubensis is the causal agent of cucurbit downy mildew, a disease that affects all commercially important cucurbits such as cucumber, watermelon, cantaloupe, squash, and pumpkin (Salcedo et al. 2020; Savory et al. 2011). There has been a resurgence of the disease globally during the past two decades that has been accompanied by significant crop losses (Cohen et al. 2015; Holmes et al. 2015; Ojiambo et al. 2015). In the United States, the resurgence of cucurbit downy mildew occurred in 2004 and was characterized by the breakdown of *dm1*, a single recessive gene in cucumber that had been stable for 40 years (Holmes et al. 2015). Subsequent research showed that disease resurgence was most likely due to the introduction of new pathogen strains and specifically pathotypes 1 and 3 of the A1 mating type from east Asia that were not previously present in the United States (Runge et al. 2011; Thomas et al. 2017a). Additional studies revealed that both A1 and A2 mating types were present in the United States and oospore formation was demonstrated under laboratory conditions (Thomas et al. 2017b). Sexual reproduction within the pathogen population was subsequently demonstrated based on genomic analyses of *P. cubensis* populations in the United States (Thomas et al. 2017c). The presence of both mating types indicates that the pathogen has the ability to form oospores in the field, but no studies have been conducted to establish their presence in cucurbit fields in the United States. This is particularly important since *P. cubensis* is primarily thought to overwinter in areas located below 30-degree latitude in south Florida and Texas around the Gulf of Mexico, where cucurbits are grown during winter resulting in year-round production of cucurbits in the continental United States (Nusbaum 1944; Ojiambo and Holmes 2011). These winter production locations are assumed to be the sources of initial primary inoculum (wind-dispersed sporangia) for disease epidemics that occur in cucurbit fields in more northern states that experience cold winters. The presence of oospores in northern states could enable an earlier disease onset than would be expected if disease onset relied on aerial transport of sporangia from overwintering sources in south Florida or along the Gulf of Mexico. Under favorable conditions, early disease onset will necessitate initiation of fungicide applications much earlier and thus increase the number of applications needed to control the disease and the cost of production during the growing season.

Formation of oospores in *P. cubensis* is considered rare, and reports of oospore formation under crop production conditions have previously been limited to Asia and Europe (Cohen et al. 2015). For example, Bedlan (1989) detected oospores in older necrotic cucumber leaves in a greenhouse in Austria. Formation of *P. cubensis* oospores in greenhouse-sampled cucumber leaves has also been reported in China (Chen et al. 1959; Zhang et al. 2006; Zhang et al. 2012). Oospore formation has been reported under natural conditions in Israel (Cohen et al. 2003), India (Bains et al. 1977), Japan (Hiura and Kawada 1933) and Russia (Rostowzew 1903). Hiura and Kawada (1933) observed oospores in vigorously growing leaves (presumably asymptomatic) and in young chlorotic or partially necrotic lesions in Japan. Similarly, D'Ercole (1975) found oospores in the field in Italy late in the growing season in senescing leaves on the soil surface. Zhang et al. (2012) found oospores on plant debris and older leaves in a greenhouse towards the end of the crop season. In the United States, oospores have only been observed under laboratory conditions through crossing of isolates that belong to compatible mating types (Thomas et al. 2017b). Although formation of oospores of *P. cubensis* in temperate and tropical regions has been documented in the literature, reports on the occurrence of oospore in cucurbit fields are still very rare (Cohen et al. 2015). As such, the role of oospores in the initiation of disease epidemics and the overall epidemiology of cucurbit downy mildew is still largely unknown (Cohen et al. 2015; Savory et al. 2011). The potential role oospores could play in initiating disease epidemics has been confined to a single study conducted in China, where oospores present in soil collected from infected cucumber in a greenhouse were found to infect and cause disease on new cucumber seedlings under greenhouse conditions (Zhang et al. 2012).

The study by Zhang et al. (2012) suggested that oospores of *P. cubensis* could overwinter in plant debris and serve as an initial source of inoculum. In the United States, about 5% of the reported disease cases were inconsistent with northward progress of the epidemic wave from overwintering sources in southern Florida (Cohen et al. 2015). We postulate that the existence of undocumented local inoculum sources such as year-round greenhouses, perennial hosts and oospores of *P. cubensis*, or unreported disease cases could explain the observed inconsistency in disease occurrence and progression (Cohen et al. 2015; Ojiambo and Holmes 2011; Wallace et al. 2015). While, oospore formation has been demonstrated under controlled conditions in the United States (Thomas et al. 2017b), their occurrence under field conditions and potential role in cucurbit downy mildew epidemics has not been determined. Thus, the potential impact of oospores on disease initiation and on the epidemiology of the disease in the United States was the impetus to undertake the present study. The specific objectives of this study were to (i) determine the occurrence of *P. cubensis* oospores under field conditions in the southeastern United States, (ii) establish the temporal dynamics of the viability and survival of oospores of *P. cubensis* in the field during the winter season, and iii) identify weather factors associated with the dynamics of oospores survival and viability during the overwintering season. Information on potential weather factors associated with oospore viability could be useful in developing models to predict germination of oospores and the risk of disease initiation from oosporic inoculum at the start of the spring season. Preliminary findings on certain aspects of this work have been published elsewhere (Kikway et al. 2020).

MATERIALS AND METHODS

Monitoring of oospore formation in cucurbit fields. Experiments to establish whether oospores can be produced in cucurbit fields in the southeastern United States were conducted at the Upper Coastal Plain Research Station at Rocky Mount, North Carolina, and at the Coastal Research and Education Center in Charleston, South Carolina. All experiments were conducted during the fall season in 2018, 2019, 2020. Cucumber is the preferred host for isolates of the A1 mating type, while cantaloupe is the preferred host for isolates belonging to both A1 and A2 mating types (Thomas et al. 2017b). Thus, to increase the probability of oospore formation in the field, cucumber (cv. Straight 8) rows were inter-planted with cantaloupe (cv. Hales Best Jumbo) rows at an interval of 0.6 m for field experiments conducted in North Carolina. These experimental plots were composed of 4 to 6 rows that were 30 m in length and 0.6 m in between rows and planted on 13 August 2018, 16 August 2019, and 10 August 2020. In addition to experimental plots, oospore formation was also monitored in sentinel plots at Rocky Mount, North Carolina. Sentinel plots measured about 61 m × 15 m and were planted with eight different cucurbit hosts (10 plants per host and 0.6 m within rows): cucumber (*Cucumis sativus*, cv. Straight 8 or Silver Slicer), cantaloupe (*Cucumis melo* cv. Hales Best Jumbo or Halona), acorn squash (*Cucurbita pepo* ssp. *ovifera*, cv. Table Ace), pumpkin (*Cucurbita maxima*, cv. Big Max), butternut squash (*Cucurbita moschata*, cv. Waltham) and watermelon (*Citrullus lanatus*, cv. Micky Lee). Sentinel plots were part of the cucurbit downy mildew monitoring network within the region (Ojiambo et al. 2011). Sentinel plots at Rocky Mount were also planted on 13 August 2018, 16 August 2019, and 10 August 2020. At Charleston, South Carolina, formation of oospores was monitored only in sentinel plots (as described above) that were also part of the disease monitoring network. Sentinel plots at this site were transplanted on 27 August 2018, 12 August 2019, and 19 August 2020. No fungicides were applied to experimental plots at Rocky Mount or sentinel plots at both sites.

Symptomatic leaves were randomly sampled from cucumber and cantaloupe plants infected with downy mildew during the months of October and November during the study period. In nature, oospores of *P. cubensis* have been found primarily on senescent leaf tissues towards the end of the growing season, a period that is thought to coincide with onset of the overwinter phase of the pathogen (D`Ercole 1975; Zhang et al. 2006). Briefly, partially necrotic leaves were sampled randomly from upper, middle, and lower parts of cucumber and cantaloupe plants. Leaf samples were collected on 25 October 2018, 31 October 2019, and 02 November 2020, at Rocky Mount, while leaves were collected on 05 November 2018, 17 October 2019, and 14 October 2020, at Charleston (Table 4.1). Samples collected at Rocky Mount were enclosed in sealed plastic bags and transported to the Plant Pathology laboratory at North Carolina State University (NCSU) in Raleigh, while those collected at Charleston were shipped overnight to the Plant Pathology laboratory at NCSU immediately after collection from the field. All sampled leaves were subsequently stored at -20°C until processing. Disease severity in fields where symptomatic leaves were sampled from cucumber and cantaloupe plants ranged from 3% (in sentinel plots) to 95% (in experimental plots) across the study.

Leaf samples and tissue processing for presence of oospores. A total of 1,000 leaf samples were collected from experimental and sentinel plots at Rocky Mount, North Carolina during the study period. These samples were composed of 550 cucumber leaves and 450 cantaloupe leaves. Similarly, 658 leaf samples composed of 340 cucumber leaves and 248 cantaloupe leaves, were collected from sentinel plots at Charleston, South Carolina, during the study (Table 4.1).

Sampled leaves were examined for the presence of oospores in tissues as described elsewhere (Frinking et al. 1987; Thomas et al. 2017b). Briefly, leaf discs measuring 12-mm in diameter were excised from necrotic parts of the leaf and clarified in a 3:1 (v/v) mixture of ethanol (96%) and glacial acetic acid (approximately 100%) at room temperature. Leaf discs were suspended in the clarification mixture until they were completely fixed and then washed in three changes of distilled water. Clarified discs were placed on a glass slide and examined under a light microscope at ×100 magnification for the presence of oospores. Oospores were counted in an area field of view of approximately 2.5 mm^2 for each leaf disc. About 10 leaf discs were examined for each of the 1,658 leaves sampled in this study. In each collection year, the number of leaves with oospores were used to calculate the frequency of oospore occurrence at each sampling site. For leaves found to contain oospores, the number of oospores per leaf area was estimated based on the area field of view on the leaf disc using a compound microscope. Finally, oospore diameter (μm) and other morphological attributes were recorded microscopically (n = 333 and 326 oospores, for leaf samples collected in North Carolina and South Carolina, respectively) using a calibrated lens, and oospore images were captured, and their diameter measured using the Olympus Cellsens Imaging Software (Olympus America Inc., Center Valley, PA).

Dynamics of oospore viability and survival during winter season. Experiments to establish the temporal dynamics of oospore survival and viability and weather factors that influence oospore survival under field conditions were conducted at Rocky Mount, North Carolina from 2018 to 2021. These experiments were conducted in a series of five steps: i) oospore production in the laboratory, ii) oospore extraction and enumeration from inoculated leaves, iii) exposure of oospores to outdoor conditions in the field during the winter season, iv) assessment of oospore viability following exposure outdoors, and v) measurement and recording of weather factors during the oospore exposure period. Each of these five steps was followed in each year and is described in detail below.

Production of oospores under laboratory conditions. Oospores were produced under controlled laboratory conditions by crossing a compatible A1 strain (A11) with an A2 strain (D3) of *P. cubensis* as described by Thomas et al. (2017b). Strain A11 was originally isolated from cucumber in North Carolina, while D3 was originally isolated from butternut squash in South Carolina and both strains had been stored at -80°C. Briefly, sporangia of each strain were produced by propagating isolates on their respective original hosts using a detached leaf assay. After 5 to 7 days, sporangial suspensions (2×10^4 spores/ml) were mixed in equal proportion (1:1 [vol/vol]) and spray inoculated on the abaxial side of detached first to third true leaves of cantaloupe (cv. Hales Best Jumbo) or cucumber (cv. Straight 8). Inoculated leaves were placed with the adaxial side on moist paper towels in clear acrylic boxes and incubated in a growth chamber at 21°C under a 12/12 h light/dark cycle. About 7 to 10 days post-inoculation, the presence and the number of oospores in leaf tissues was determined as described above.

Extraction and enumeration of oospores from inoculated leaves. Oospores were extracted from inoculated leaves as described elsewhere (Flier et al. 2001; van der Gaag and Frinking 1996) but with some modifications. Briefly, oospore-infested leaves were air dried on a laboratory bench at room temperature until extraction of oospores. Drying was conducted to differentially kill vegetative structures such as mycelia and sporangia. Dried leaves were placed in a blender containing 140 ml distilled water and 140 ml ice and macerated for 5 min. The resultant suspension and material were passed through a 65-µm sieve, and the contents were captured on a 20-25-µm nylon sieve. The suspension was then collected by centrifugation at 10,000 rpm for 5 min and re-suspended in 0.5 ml deionized water. Oospores were then collected on a 25-µm sieve, and after rinsing with deionized water, they were stored in sterile distilled water at 4°C for up to 4 months. To estimate oospores in dried leaves, leaf discs were observed for oospore presence and the number of oospores per leaf area (cm^2) estimated as described above. Here, leaf area was estimated separately by calculating the area of about 50 leaves of cucumber and cantaloupe plants raised in a phytotron. The number of leaves in 1 g of leaf material was subsequently estimated and multiplied by the number of oospores per leaf to estimate the number of oospores per gram of dried infested leaf material.

Exposure of oospores outdoors during winter season. Oospores in infested leaf tissues were mixed in soil and exposed to outdoor weather conditions at Rocky Mount during the winter season from November 2018, 2019 and 2020 to May 2019, 2020, and 2021, respectively (henceforth referred to as 2019, 2020 and 2021 winter season, respectively) as described by Gent et al. (2017). Briefly, PVC pipes measuring 5 cm in diameter and 60 cm in length were first filled with 600 cm^3 of a 2:1 mixture of dried soil and perlite. The soil used was a loamy sand (Vann et al. 2013) and was collected from a fallow field at NCSU Upper Coastal Research Station at Rocky Mount, where the previous crop was cotton. The soil-perlite mixture was then infested with 1 g of air-dried infested leaf material containing approximately 16,000 oospores of *P. cubensis*. The PVC pipe containing the oospore infested soil-perlite mixture was sealed on both ends using 20-µm nylon mesh fitted with 5-cm PVC coupler. Twenty sealed PVC pipes were placed outdoors (vertically) in a field at the research station on 29 November 2018, 26 November 2019, and 27 November 2020.

Following exposure to outdoor winter conditions, three PVC tubes were removed from the field every month from January to May during the experimental period to assess survival and viability of oospores. The PVC tubes were collected on 13 February, 15 March, 12 April, and 31

May 2019; 24 January, 28 February, 27 March, 30 April and 29 May 2020; and on 29 January, 26 February, 31 March, 30 April and 31 May in 2021. Oospores were isolated and extracted from the entire 600 cm^3 of soil mixture by sieving and centrifugation as described by Parker (2007). Extracted oospores were suspended in distilled water, and their viability assessed as described below.

Assessment of viability of oospores. Viability of oospores was determined using the plasmolysis method as described by Thomas et al (2017b). While the plasmolysis test has been reported in other oomycetes to correlate with oospore viability as measured by infectivity (Pitts and Shattock 1994), there is no data that demonstrates conclusively that this test predicts viability or infectivity in *Pseudoperonospora*. Briefly, 100 µl of an oospore suspension was centrifuged for 1 min at 10,000 rpm, and the supernatant discarded. The resultant pellet was resuspended with 100 µm of 4M NaCl solution (i.e., osmoticum) in a centrifuge tube and the mixture incubated at room temperature for 20 to 45 min. About 100 to 500 oospores per biological replicate (three replicates total) at each assessment time, were microscopically observed for plasmolysis and subsequently classified as either viable (i.e., plasmolyzed with a contracted cytoplasm) or non-viable (i.e., non-plasmolyzed with a non-contracted cytoplasm) (Jiang and Erwin 1990). In each year, oospore viability was assessed prior to their exposure to outdoor conditions (i.e., baseline viability) and monthly following exposure in PVC tubes in the field (i.e., absolute viability). Baseline viability was assessed on 14 November 2018 and 2019 and 15 November 2020. For each biological replicate, absolute or baseline viability (%) was calculated as: (number of viable oospores/total number of oospores assessed) × 100.

Germination and infectivity of oospores. Germination and infectivity of oospores was tested as described by Rubin and Cohen (2006) using oospores exposed outdoors during the 2019 and 2020 winter seasons and processed as described above. Briefly, 5-ml homogenized oospore suspension was pipetted into a 9-cm petri dish and exposed to two cycles of drying and wetting (12 h per cycle at 26°C). Dry homogenates were re-suspended in 3-ml sterile distilled water and 20 µl of the final homogenate inoculated on the abaxial side of cucumber (cv. Straight 8) and cantaloupe (cv. Hales Best Jumbo) leaves. Positive controls were inoculated with 10 µl of sporangia. Inoculated leaves were placed in clear acrylic boxes and incubated in a growth chamber at 21°C under a 12/12 h light/dark cycle for 21 days. Germination and infectivity of oospores was also tested using the Perlite baiting method (Rubin and Cohen 2006). Briefly, 2 g of Perlite (no. 4; Agrikal, Kibbutz Habonim, Israel) was added to each petri dish containing 20 ml of the oospore suspension. After thorough mixing of perlite and oospore homogenate, 2 to 3 leaves of 3 wk old cucumber (cv. Straight 8) plants were placed on the surface of the mixture in each petri dish with the abaxial side facing down. Petri dishes were covered with lids and incubated at 20ºC under a 12/12 h light/dark cycle for 4 weeks for symptom development.

Weather variables. Records of air temperature, soil temperature, amount of precipitation, soil moisture, and number of rainy days were obtained from the State Climate Office of North Carolina weather station (CRONOS/ECONet) located at the Upper Coastal Research Station in Rocky Mount. For both air and soil temperature, cumulative degree days (CDD) above a base temperature were used to express the change in oospore survival in response to accumulated thermal time units (Lovell et al. 2004): $\mathrm{CDD} = \int_0^d (Temp(u) - Tbase)H(Temp(u) - Tbase)du$, where CDD is the cumulative degree days from the first day up to day *d* of oospore exposure in the field, $Temp(u) = (Tmax + Tmin)/2$ and the Heaviside function, $H(Temp(u) - Tbase)$, is either 0 (*Temp*

< *Tbase*) or 1 (*Temp* ≥ *Tbase*) ensuring that daily accumulation of degree days occurs only when *Temp*(*u*) exceeds the base temperature $(Tbase)$. The temperature below which oospores of *P. cubensis* are not biologically active is not known, and thus $Tbase$ was assumed to be 0°C, since this temperature has been reported to strongly limit viability and germination of oospores in other oomycete pathogens (Pittis and Shattock 1994).

Data analysis. Baseline viability prior to exposure outdoors varied between years. Thus, to facilitate comparison across years, standardized viability was calculated by dividing mean absolute viability (V_T) at each assessment period following exposure with the corresponding mean baseline oospore viability, V_0, prior to exposure to outdoor conditions.

The SAS procedure MIXED was used to determine if a common slope could be fitted to absolute and standardized oospore viability over time for combined data. Based on the fixed effects results of this analysis, year did not significantly affect either absolute (P = 0.1031) or standardized (P = 0.7622) viability. Thus, viability data combined across all years were fitted to a linear model of the form $y = b_0 + b_1 x$ using PROC MIXED with year as a random variable, where y = oospore viability (%), and b_0 and b_1 are the parameters to be estimated.

To identify potential weather factors that could influence viability, standardized oospore viability data were first analyzed using the PROC CORR procedure of SAS to assess whether significant (α = 0.05) linear correlations existed between standardized oospore viability and weather variables: cumulative degree days based on air temperature (CDD_{AT}) or soil temperature (CDD_{ST}), number of rainy days (i.e., days with at least 2.54 mm of rain), rainfall amount and soil moisture (i.e., moisture content (%) measured at 0.2 m below the soil surface). Following preliminary analysis, repeated measures analysis of covariance (ANCOVA) with changing covariates (Verbyla 1988), was performed using the PROC MIXED procedure in SAS to determine the effects of year on standardized oospore viability using CDD_{ST}, number of rainy days, and soil moisture as changing covariates. All the statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

Weather conditions during outdoor overwintering experiments. Temporal trends in air temperature were generally similar during the experimental years although absolute values varied between years (Fig. 4.1). For example, air temperatures starting in November typically decreased reaching lowest values in January (mean = 5.6°C, 8.6°C and 4.8°C in the 2019, 2020 and 2021, respectively) before increasing steadily thereafter until May (mean = 23.6°C, 18.3°C and 19.9°C in the 2019, 2020 and 2021, respectively). However, the decrease and subsequent increase in temperature was more pronounced in 2019 and 2021 than in 2020, partly reflecting the January mean temperatures. Soil temperatures followed a similar trend as that of air temperatures except that absolute values were slightly higher than those of air temperatures (Fig. 4.1). The corresponding cumulative degree-days based on air temperature (CDD_{AT}) ranged from about 3 to 2222, 10 to 2288 and 14 to 1994 degree-days in the 2019, 2020 and 2021 winter season, respectively. Cumulative degree-days based on soil temperature (CDD_{ST}) were slightly higher than those of CDD_{AT} and ranged from 6 to 2410, 10 to 2495 and 15 to 2262 degree-days in the 2019, 2020, and 2021 winter season, respectively.

Incidence of naturally infected field samples with oospores. The frequency of naturally infected leaves with oospores was low with a mean frequency of 4.6% across the entire study and varied slightly between seasons, locations, and hosts. For example, the frequency of leaves with oospores ranged from 2.0% to 9.0% with a mean of 4.95% for samples collected in North Carolina

(Table 4.1). The frequency of leaf samples with oospores within sampling seasons in North Carolina also was dependent on the cucurbit host. For example, incidence of leaves with oospores in cucumber was lowest in 2019 (4.5%) and highest in 2020 (9.0%), while in cantaloupe, the frequency of samples with oospores was lowest in 2019 (2.0%) and highest in 2018 (4.5%). Finally, although the frequency of leaves with oospores in North Carolina was twice as high in cucumber (mean = 6.50%) than in cantaloupe (mean = 3.40%), there was no significant difference in mean oospore frequency between these two hosts (Table 4.1). The frequency of leaves with oospores for samples collected in South Carolina was lower than that of samples in collected in North Carolina and ranged from 1.0% to 6.3% with a mean of 3.9%. However, there was no significant difference in mean oospore frequency between the two states. As was the case with leaves collected in North Carolina, the frequency of leaves with oospores in South Carolina was slightly higher for cucumber (mean = 4.1%) than for cantaloupe samples (mean = 3.8%) but with no significant difference in the frequency between the two hosts (Table 4.1).

Morphology, density, and size of oospores formed in field samples. Oospores that formed in leaf tissues were round or spherical in shape and hyaline to golden yellow or light brown in color (Fig. 4.2a-c). Oospore walls were composed of two thick layers (Fig. 2d) and an outer smooth surface with an intact cytoplasm (Fig. 4.2b-d). The diameter of oospores was variable and ranged between 12.5-65.0 µm in cucumber leaves and 10.9-59.4 µm in cantaloupe leaves collected in North Carolina (Table 4.2). Diameter of oospores in leaf samples collected from South Carolina ranged between 15.6-62.5 µm in cucumber leaves and 9.4-62.5 µm in cantaloupe leaves. Further, oospores formed in cantaloupe leaves (mean = 33.9 µm) were significantly larger than those formed in cucumber leaves (mean = 22.5 µm) for samples collected in North Carolina. However, oospores formed in cucumber leaves (mean = 28.9 μm) were significantly larger than those formed in cantaloupe leaves (mean = 22.5 μm) for samples collected in South Carolina (Table 4.2).

The density of oospores presents in the sampled leaves varied between sampling date, host type and sampling location (Table 4.2). Oospore density varied from 269 to 748 oospores/cm^2 in North Carolina, with the density being slightly higher in cucumber leaves (mean = 548 oospores/cm^2) than in cantaloupe leaves (mean = 498 oospores/cm^2) but with no significant difference between the two hosts. Samples collected in South Carolina showed an opposite trend, with the density of oospores being higher in cantaloupe leaves (mean = 898 oospores/cm^2) than in cucumber leaves (493 oospores/cm^2) but with no significant difference between the two hosts. Across the study, the density of oospores was higher in leaf samples collected in South Carolina (mean = 684 oospores/cm^2) than in samples collected in North Carolina (mean = 532 oospores/cm^2) but there was no significant difference between the two states (Table 4.2).

Oospore viability and dynamics during winter. Detachment of cytoplasm from the oospore wall during exposure to the osmoticum was used to distinguish non-viable oospores from viable oospores. The cytoplasm of non-viable oospores, i.e., non-plasmolyzed oospores, did not contract and remained intact as these oospores had lost the differential permeability of their cellular membrane (Fig. 4.3 a,b). In contrast, the cytoplasm of the viable plasmolyzed oospores was contracted to form a central ball-like structure, due to the loss of cell membrane integrity due to the osmoticum (Fig. 4.3 c, d).

Based on combined data, absolute oospore viability decreased linearly with time ($y = 64.2 - 0.27x$; P < 0.0001) during the winter season ranging from 67.8% at the start of the experiments in November to 19.3% at the end of the experiments in May (Table 4.3) (Fig. 4.4). Similarly, standardized viability decreased steadily with time ($y = 95.4 - 0.39x$; P < 0.0001), ranging from 100% in November to 28.5% in May. Discernable differences in the viability between winter seasons were observed for both absolute and standardized viability values (Fig. 4.4). Absolute viability values at the start of the experiments in November were significantly (α = 0.05) higher in 2019 (mean = 73%) than in 2020 (mean = 63%) and 2021 (mean = 65%) winter seasons. Absolute viability about 3 months after exposure (i.e., February) was significantly (α = 0.05) higher in 2021 (mean = 49.4%) than 2019 (24.5%) and 2020 (mean = 38.5%) winter seasons. However, no differences in absolute viability were observed between years about 4 months after initial exposure (i.e., March) or at the end of the season, about 6 months after initial exposure (i.e., May) (Fig. 4.4). Differences in standardized viability between years followed a similar trend as that of absolute viability but with more pronounced differences in viability values about 3 months after initial exposure (Fig. 4).

Effects of weather variables on oospore viability during winter. Oospore viability was significantly negatively correlated with CDD_{AT} ($0.78 < |r| < 0.90$, $P < 0.0006$) and CDD_{ST} ($0.78 < |r| < 0.89$; $P < 0.0006$) in all three study periods (Table 4.4). However, oospore viability was not significantly correlated with the other weather variables except the number of rainy days in 2020 ($r = -0.64$, $P < 0.0043$) and 2021 ($r = 0.62$, $P < 0.0131$) and soil moisture in 2020 ($r = -0.75$, $P < 0.0003$) (Table 4.4).

Fixed-effects results of ANCOVA on the effects of experimental year on oospore viability with weather variables showed that year ($P = 0.0174$), CDD_{AT} ($P < 0.0001$), CDD_{ST} ($P < 0.0001$), rainfall amount ($P = 0.0421$), number of rainy days ($P < 0.0001$) and soil moisture ($P < 0.0206$) significantly affected oospore viability. Further, when ANCOVA was conducted on oospore viability separately for each year, CDD_{ST} was significantly ($P < 0.0001$) associated with reduced oospore viability in all experimental winter seasons, with a unit increase in thermal units in soil reducing viability by a factor of approximately 0.04 (Table 5). Similarly, the number of rainy days significantly ($P < 0.00101$) affected viability in all the three years, with reduction in the number of rainy days reducing the viability oospores during the winter season. However, the effects of soil moisture on viability were significant in 2019 ($P = 0.0001$) and 2021 ($P = 0.0421$) but not in 2020 ($P = 0.0835$) winter season (Table 4.4).

Germination and infectivity of oospores. Oospores exposed to outdoor conditions until the end of May did not germinate following conditioning in the field. Similarly, cucumber and cantaloupe leaves inoculated with oospore homogenate following conditioning did not develop lesions 3 weeks post-inoculation. Subsequently, inoculated leaves started to disintegrate and daily observations for any possible infection or sporulation on these leaves were discontinued. Both cucumber and cantaloupe leaves inoculated with sporangia (positive control) developed disease symptoms 5 days after inoculation.

DISCUSSION

The potential of sexual reproduction and formation of oospores within the pathogen population of *P. cubensis* in the United States has been demonstrated under controlled conditions. However, formation of oospores in cucurbit fields under natural conditions has previously not been reported. The present study examined the formation of oospores in cucurbit fields and their potential to survive as overwintering structures for *P. cubensis* in the southeastern United States in areas above the 30-degree latitude, where winters are cold enough to kill cucurbit plants. Oospores were present in necrotic leaves sampled during the fall in cucurbit fields in North Carolina and South Carolina. However, the frequency of leaves with oospores was low with a mean of 5% across the entire study. In addition, while viability of oospores exposed to winter weather in the field decreased steadily over time, 20% of the oospores were still viable in March to May, which is the start of the planting season for cucurbits in these states. Among weather factors examined, temperature was consistently associated with viability of oospores, with viability declining with an increase in thermal time during the overwintering period. The inability of oospores to germinate following exposure to outdoor overwintering conditions in the field suggests that these sexual structures possibly require some unrecognized stimuli or physiological factors in order for them to germinate and infect cucurbit host tissue. This study represents the first documentation of the occurrence of oospores of *P. cubensis* under natural field conditions and further contributes to our knowledge on the potential role of oospores in the epidemiology of cucurbit downy mildew in continental United States.

In the United States, the quest for oospores of *P. cubensis* in natural field conditions can be traced back to 1889 when Halsted (1889) failed to observe oospores in cucumber leaves. Subsequent studies (Doran 1932; Foster 1920) continued the search for oospores in cucumber leaves, but neither reported formation of oospores. The resurgence of cucurbit downy mildew in 2004 prompted renewed efforts to search for oospores in the United States, but these efforts also were unable to find oospores in nature (Cohen et al. 2015; Holmes et al. 2015). In the first documentation of the A1 mating type of *P. cubensis* in the United States and oospore formation under controlled conditions (Thomas et al. 2017b), it was suggested that sampling of fields where cucumber is planted next to cantaloupe would increase the probability of oospore formation under field conditions due to host preference in *P. cubensis*. In the present study, the frequency of leaves with oospores ranged from 1 to 9%. Previous studies (Bains et al. 1977; Cohen et al. 2003; D'Ercole 1975; Hiura and Kawada 1933) that reported oospore formation under natural conditions did not quantify the frequency of leaves with oospores. For example, the number of oospores in field samples in Israel was very erratic, and no attempt was made to quantify leaves with oospores (Cohen et al. 2003). The frequency of leaves with oospores in a study conducted in China was relatively high ranging from 4 to 61% (Zhang et al. 2012). However, the latter study was conducted under greenhouse conditions, and it is also not clear how host plants were infected. It is possible that the inoculation method used could have influenced the frequency of leaves with oospores. Further, while the study by Zhang et al. (2012) is informative, it is not clear if artificial inoculation captures the heterogeneity in mating type frequency of *P. cubensis* that may influence sexual reproduction and oospore formation under natural field conditions. The high frequency of leaves with oospores in Zhang et al. (2012) is most likely due to the controlled conditions that were optimized in the greenhouse. Thus, we cannot meaningfully compare our results with those of Zhang et al. (2012) due to the differences in the methods that were used in the respective studies.

The range of oospore density (number of oospores per leaf) in naturally infected samples reported in the present study was 269 to 683 oospores/cm^2 for cucumber and 401 to 1,363 oospores/cm^2 for cantaloupe. These numbers are similar to those reported for artificially infected leaves under controlled conditions. For example, the range of oospore density for cucumber and cantaloupe leaves reported by Thomas et al. (2017b) in laboratory study conducted in the United States was 562 to 883 oospores/cm^2 and 281 to 1,004 oospores/cm^2, in cucumber and cantaloupe, respectively. However, oospore density observed in the present study varied across sample locations, years and cucurbit host. For example, there was a 1.5 to 2.5-fold difference in oospore density in cucumber leaves sampled in North Carolina during the study period. This difference was higher in cantaloupe leaves sampled in South Carolina where there was a 2.5 to 3.0-fold difference in oospore densities during the experimental period. This variation in oospore density has also been reported in other studies involving *P. cubensis* (Cohen et al. 2003; Cohen and Rubin

2012; Thomas et al. 2017b) and other oomycetes (Gent et al. 2017). Under controlled conditions, variation in oospore density has been attributed to several factors, including variability in the amount of diseased tissue, host susceptibility, and inoculum concentration (Cohen and Rubin 2012; Gent et al. 2017). For example, *P. cubensis* produces more oospores in cucumber leaves that are primarily chlorotic, while more oospores are produced in cantaloupe leaves that are more necrotic (Cohen and Rubin 2012). In addition, the Straight 8 cucumber cultivar used in this study lacks the *dm1* gene (Wang et al. 2019) and its oospore formation profile may not be typical of newer cucumber hybrids that possess the *dm1* gene.

Inherent fecundity of the A1 and A2 isolates has also been reported to influence oospore production with different combinations of opposite mating types resulting in different oospore densities on the same host type (Cohen and Rubin 2012; Thomas et al. 2017b). While no studies have been conducted to establish how pathogen and host factors could influence oospore density under field conditions, we speculate that inherent fecundity of pathogen strains and differences in environmental factors are likely to play a major role in oospore density. Lineage II (clade 2) of *P. cubensis* that is composed primarily of the A1 mating type strains usually appear in the field earlier in the season, while lineage I (clade 1) that consist mainly of the A2 mating type strains appear later in the fall (Rahman et al. 2021). Thus, it is plausible that dynamics of these strains combined with their inherent fecundity could influence oospore density under field conditions. Similarly, variation in weather conditions influences disease severity, with more optimum conditions resulting in more diseased tissues and hence potential for formation of more oospores in the tissues (Gent et al. 2017). As such, effective disease control especially towards the end of the season has the added benefit of reducing the opportunity for production of oospores and thereby potentially limiting the availability of this potential source of local initial inoculum in areas in the continental

United States that experience cold winters. Reduction of oospore production would also reduce the emergence of new recombinant isolates that could potentially be insensitive to currently effective fungicides or isolates that have enhanced virulence towards resistant host genotypes (Cohen et al. 2015; McDonald and Linde 2000; Ojiambo et al. 2015).

Knowledge of disease epidemiology for pathogens such as *P. cubensis* can be improved significantly by a quantitative assessment of both the asexual and sexual phases of the pathogen (Rossi et al. 2008). While the role of sporangia of *P. cubensis* in the epidemiology of cucurbit downy mildew has received considerable attention (e.g., Arauz et al. 2010; Neufeld and Ojiambo 2012; Neufeld et al. 2018), information on the ecology of oospores and the potential role these overwintering structures could play in disease epidemiology is still lacking. In this study, viability of oospores decreased steadily with time when oospores were exposed to natural field conditions during winter. About 20% of the exposed oospores were still viable at the end of the study period in April and May. This decrease in viability over time is also consistent with reports on *P. cubensis* oospores in outdoor experiments conducted in China (Zhang et al. 2012) and *P. infestans* oospores under natural weather conditions during the winter period (Mayton et al. 2000). In the southeastern United States, cucurbits are usually planted between early March and late May, and thus it is plausible that oospores still viable during this period could be an initial source of primary inoculum. Among the weather variables examined, temperature consistently affected oospore viability wherein viability decreased with increasing thermal time during the experimental period. An increase in temperature has also been reported to steadily reduce viability of oospores of *Phytophthora kernoviae* (Widmer 2011). The effect of other weather variables examined in this study, in particular soil moisture and rainfall amount, on viability of *P. cubensis* oospores was less clear. For oospores of the grape downy mildew pathogen *P. viticola*, rainfall distribution rather than rainfall amount is more important for viability (Vercesi et al. 1999), with restoration of viability when rain resumes (Rouzet and Jacquin 2003). Thus, additional studies are needed to clearly establish the role of these other weather variables on the viability of oospores of *P. cubensis*. The intent in the present study was not to quantify the empirical relationship between oospore viability and weather factors but rather identify potential variables that could be useful in a quantitative assessment of the effects of weather factors on viability and germination of *P. cubensis* oospores in subsequent studies.

In general, oospores of oomycetes are endogenously dormant and thus, their germination is typically dependent on fulfillment of specific conditions that allow them to mature and break dormancy or a stimulus to initiate germination (McMeekin 1960; Oliver and Schweizer 1999; Tommerup 1981). Temperature, light, chemical treatments, oospore age and nutrition are some of the factors that influence oospore germination, with germination being more rapid and higher for oospores of homothallic than heterothallic species (Zentmeyer and Erwin 1970). In the present study, about 20% of the oospores were viable at the end of the study period in May and thus potentially infective. However, these oospores neither germinated nor induced infection using techniques that have previously been successful with *P. cubensis* (Cohen et al. 2011; Cohen and Rubin 2012). Inability of oospores to germinate and cause infection following exposure to overwintering conditions in the field has also been reported for the related species *P. humuli* (Gent et al. 2017) and in other oomycetes (e.g., Kandel et al. 2019). Similarly, Thomas et al. (2017b) failed to induce germination and infection of viable oospores of *P. cubensis* produced under controlled conditions. It is important to note that instances of successful germination and infectivity of oospores of *P. cubensis* or *P. humuli* are very rare (Cohen and Rubin 2012; Gent et al. 2017). For example, where germination has been successful in *P. cubensis*, the frequency of oosporic infection has been less than 0.2% (Cohen and Rubin 2012). Zhang et al. (2012) also reported infection of cucumber seedlings in the greenhouse inoculated by oospore suspension from overwintered outdoor debris although they did not demonstrate oospore germination in their study. Since the methodology applied in this study has previously resulted in successful germination and infectivity of oosporic inoculum, albeit at a low frequency, it is plausible that our inability to obtain oosporic infection was probably due to currently unrecognized physiological factors (Gent et al. 2017; Horner et al. 2019). Thus, it is plausible that these viable oospores could germinate and cause infection once exposed to the right conditions that stimulate germination, and could serve as a local source of inoculum before sporangia typically are blown into a given area from sources further southward. This assumption is supported by results of coalescent analysis of the pathogen population that indicated genetic mixing between lineages I and II of *P. cubensis* in United States (Thomas et al 2017c). This genetic mixing is only possible if oospores are indeed able to germinate and cause infection under field conditions. However, they were unable to germinate and cause symptoms on inoculated host tissue. Further studies on factors that influence germination of *P. cubensis* oospores are still needed to better understand their role as an initial source of primary inoculum for cucurbit downy mildew in areas that experience cold winters in the eastern United States.

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TABLE 4.1. Occurrence of oospores of *Pseudoperonospora cubensis* in leaves of cucumber and cantaloupe naturally infected by cucurbit downy mildew in cucurbit fields in North Carolina and South Carolina from 2018 to 2020

Location, State	Host type	Year	Sampling date	Leaves assayed	Leaves with oospores	Frequency of oospores (%)[a]	95% CL for oospore frequency[b]	
							LCL	UCL
Rocky Mount, NC	Cucumber	2018	25-Oct	150	9	6.0	…	…
		2019	31-Oct	200	9	4.5	…	…
		2020	02-Nov	200	18	9.0	…	…
		2018-2020	…	550	36	6.5	4.4	8.6
	Cantaloupe	2018	25-Oct	150	5	4.6	…	…
		2019	31-Oct	200	4	2.0	…	…
		2020	02-Nov	100	6	3.6	…	…
		2018-2020	…	450	15	3.8	1.9	5.7
	All hosts	2018-2020	…	1000	41	5.1	3.4	6.9
Charleston, SC	Cucumber	2018	05-Nov	100	4	4.0	…	…
		2019	17-Oct	240	10	4.2	…	…
		2018-2019	…	340	14	4.1	4.0	4.2
	Cantaloupe	2018	05-Nov	70	3	4.3	…	…
		2019	17-Oct	200	2	1.0	…	…
		2020	14-Oct	48	3	6.3	…	…
		2018-2020	…	318	8	3.8	1.4	6.3
	All hosts	2018-2020	…	658	22	3.9	2.5	5.4
All locations[c]	All hosts	2018-2020	**…**	**1658**	**73**	**4.6**	**3.4**	**5.8**

[a] Values for data combined over years, hosts and locations are means of frequency of oospores across years, hosts, and locations, respectively.

[b] LCL and UCL are the 95% lower and upper confidence limits (CL) of mean frequency of oospores, respectively.

[c] Refers to the total number of leaves assayed and leaves with oospores across the entire study based on samples collected in North Carolina and South Carolina from 2018 to 2020.

TABLE 4.2. Characteristics of oospores of *Pseudoperonospora cubensis* formed in leaves of cucumber and cantaloupe naturally infected by cucurbit downy mildew in cucurbit fields in North Carolina and South Carolina from 2018 to 2020

				Oospore density (number/cm^2)[a]			Oospore diameter (µm)		
Location, State	Host type	Year	Sampling date	Mean	LCL	UCL	Mean	LCL	UCL
Rocky Mount, NC	Cucumber	2018	25-Oct	269	64	475	28.1	26.2	29.9
		2019	31-Oct	406	319	493	24.0	22.5	25.4
		2020	02-Nov	683	618	748	27.5	26.4	28.5
		2018-2020	…	548	486	609	26.7	25.8	27.5
	Cantaloupe	2018	25-Oct	401	266	535	38.5	35.7	41.3
		2019	31-Oct	459	329	588	20.1	17.4	22.7
		2020	02-Nov	592	449	735	36.6	33.4	39.9
		2018-2020	…	498	412	583	33.9	31.7	36.1
	All hosts	2018-2020	…	532	483	582	29.0	28.0	30.0
Charleston, SC	Cucumber	2018	05-Nov	451	315	588	24.1	22.3	25.8
		2019	17-Oct	562	395	729	33.8	31.4	36.2
		2018-2019	…	493	391	595	28.9	27.3	30.6
	Cantaloupe	2018	05-Nov	547	364	730	42.9	40.5	45.2
		2019	17-Oct	455	276	634	28.8	23.4	34.1
		2020	14-Oct	1363	780	1946	11.1	10.8	11.4
		2018-2020	…	898	601	1196	22.5	19.9	25.0
	All hosts	2018-2020	…	684	530	838	26.0	24.5	27.4
All locations[c]	**All hosts**	**2018-2020**	**…**	**585**	**523**	**647**	**27.5**	**26.6**	**28.4**

[a] Refers to the number of oospores per leaf area of tissue assayed for presence of oospores using 10-mm leaf discs.

[b] LCL and UCL are the 95% lower and upper confidence limits (CL), respectively, of mean oospore density and diameter.

[c] Values are mean oospore density or oospore diameter across the entire study based on values for infected leaf samples collected in North Carolina and South Carolina from 2018 to 2020.

TABLE 4.3. Temporal dynamics of the viability of *Pseudoperonospora cubensis* oospores exposed to overwintering conditions during the winter season in a field at Rocky Mount in North Carolina based on combined data collected in 2019 to 2021

			Viability of oospores over winter [a,b]		
Month	Number of assayed oospores	Number of viable oospores	Absolute Viability (%)	Standardized Viability (%)	S.E.
November	3132	2119	67.8	100.0	0.00
January	1195	453	38.4	58.4	5.12
February	1874	682	44.8	66.7	4.66
March	1666	443	26.7	40.1	2.92
April	984	195	22.7	33.8	3.64
May	1549	291	19.3	28.5	2.06

[a] Viability values for the month of November represent baseline numbers prior to the exposure of oospores in the field during the experimental period. Oospore viability is standardized based on mean initial viability in November to allow direct comparison of results from different experimental years.
[b] Viability of oospores was determined by plasmolysis method involving incubating oospores in a 4M NaCl solution for 45 min at room temperature; S.E. denotes the standard error of the mean standardized viability.

TABLE 4.4. Matrix of Pearson's correlation coefficients for standardized viability of *Pseudoperonospora cubensis* oospores and weather factors recorded during winter seasons from 2019 to 2021 at Rocky Mount, North Carolina

		Variable[x,y]					
Year		V_T/V_0	CDD_{AT}	CDD_{ST}	Rain (mm)	Rainy days	Soil moist (%)
2019	V_T/V_0	…	−0.78 (0.0006)	−0.78 (0.0006)	−0.45 (0.1421)	−0.53 (0.0742)	−0.08 (0.8155)
	CDD_{AT}		…	0.99 (0.0001)	0.89 (0.0001)	0.81 (0.0015)	−0.50 (0.0972)
	CDD_{ST}			…	0.89 (0.0001)	0.81 (0.0015)	−0.49 (0.1006)
	Rain (mm)				…	0.65 (0.0205)	−0.84 (0.0006)
	Rainy days					…	−0.30 (0.3409)
	Soil moist (%)						…
2020	V_T/V_0	…	−0.89 (0.0001)	−0.88 (0.0001)	−0.47 (0.0508)	−0.64 (0.0043)	−0.75 (0.0003)
	CDD_{AT}		…	0.99 (0.0001)	0.54 (0.0216)	0.85 (0.0001)	0.89 (0.0001)
	CDD_{ST}			…	0.53 (0.0245)	0.85 (0.0001)	0.89 (0.0001)
	Rain (mm)				…	0.78 (0.0001)	0.74 (0.0005)
	Rainy days					…	0.95 (0.0001)
	Soil moist (%)						…
2021	V_T/V_0	…	−0.86 (0.0001)	−0.86 (0.0001)	0.00 (0.9918)	0.62 (0.0131)	0.34 (0.2048)
	CDD_{AT}		…	0.99 (0.0001)	0.15 (0.5971)	−0.42 (0.1199)	−0.55 (0.0346)
	CDD_{ST}			…	0.17 (0.5460)	−0.42 (0.1169)	−0.52 (0.0455)
	Rain (mm)				…	0.67 (0.0060)	0.52 (0.0447)
	Rainy days					…	0.43 (0.1056)
	Soil moist (%)						…

[x] V_T/V_0 is the standardized oospore viability, where V_0 is the baseline viability of oospores prior to exposure in the field, while V_T is the absolute oospore viability following exposure during the winter period; CDD_{AT} is the cumulative degree days based on air temperature, CDD_{ST} cumulative degree days based on soil temperature; Rainy days is the number of rain days and Soil moist (%) is the moisture content in the soil measured at 0.2 m below the soil surface.

[y] Values in parenthesis are *P*-values for the strength of association between a pair of variables.

TABLE 4.5. Analysis of fixed effects in the analysis of covariance for the effect of experimental year on standardized viability of oospores of *Pseudoperonospora cubensis* with soil temperature, soil moisture and days with rain as covariates at Rocky Mount, North Carolina

	2019				2020				2021			
Effect[a]	Estimate	S.E.[b]	*t*-value	$P > t$	Estimate	S.E.[b]	*t*-value	$P > t$	Estimate	S.E.[b]	*t*-value	$P > t$
Intercept	806.19	54.026	14.92	0.0045	384.81	154.30	2.49	0.1301	141.88	27.905	5.08	0.0366
CDD_{ST}	−0.05	0.003	−18.44	0.0001	−0.03	0.005	−5.85	0.0001	−0.04	0.006	−7.77	0.0001
RDays	27.18	4.263	6.37	0.0007	40.46	13.262	3.05	0.0101	21.60	5.863	3.69	0.0050
SMoist	−2352.92	175.12	−13.44	0.0001	−1071.27	567.540	−1.89	0.0835	−240.53	101.580	−2.37	0.0421

[a] CDD_{ST} is cumulative degree days based on soil temperature, RDays is the number of rain days and SMoist is the soil moisture content (%) measured at 0.2 m below the soil surface.
[b] S.E. is the standard error.

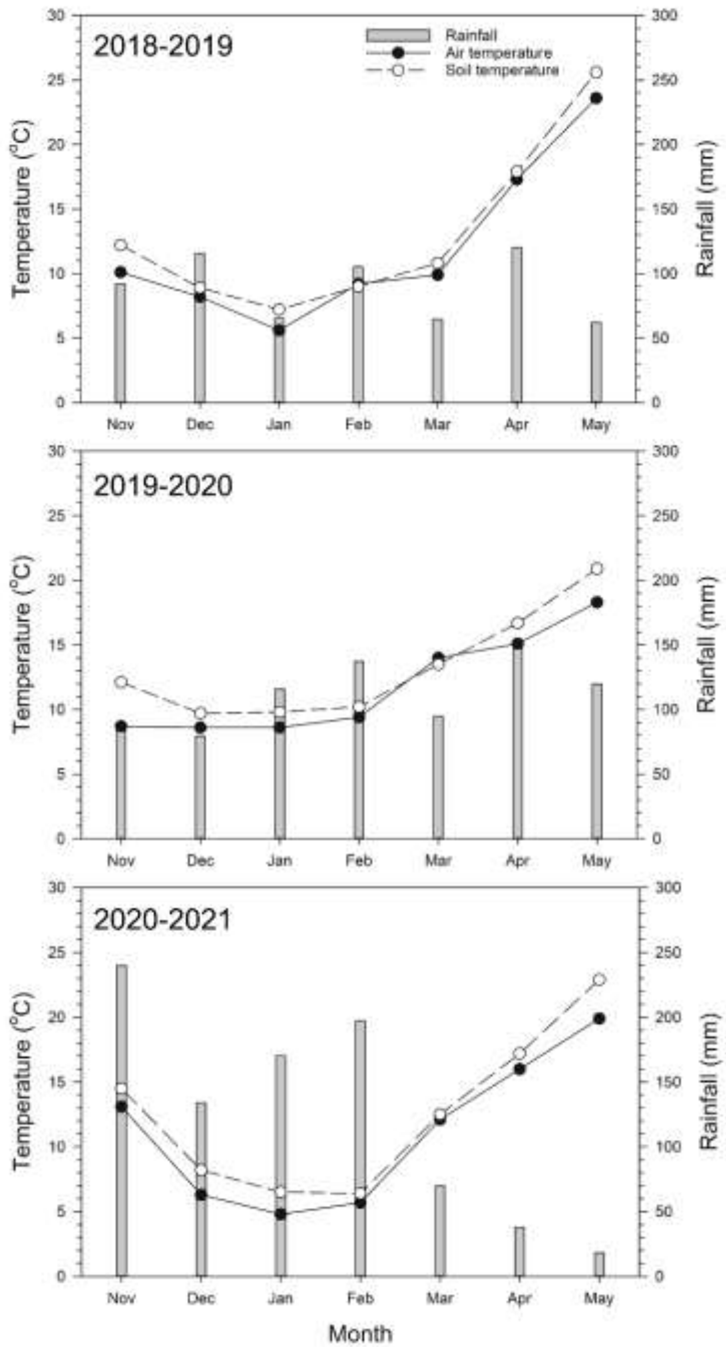

Figure 4.1. Weather conditions recorded at Rocky Mount, North Carolina, during the experimental period for the study to establish the dynamics of the viability of oospores of *Pseudoperonospora cubensis* under field conducted during the winter season of 2018/19, 201/20 and 2020/21. Temperature values are monthly means, while precipitation values are totals recorded during the month.

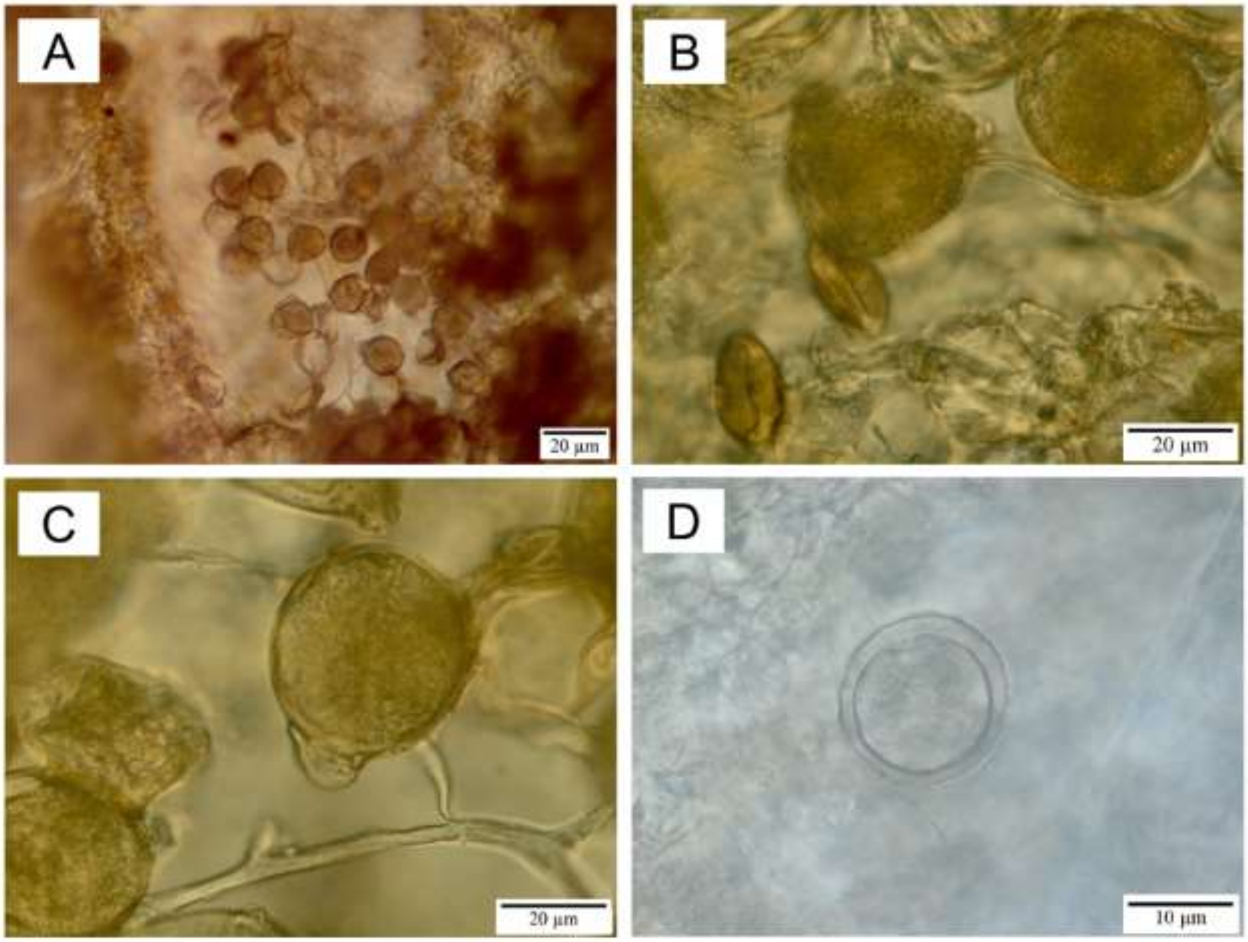

Figure 4.2. Oospores of *Pseudoperonospora cubensis* within leaf tissues of naturally infected cucumber cultivar Straight 8. Oospores embedded in clarified leaf tissue (**A**), a close-up of an intact oospore in plant tissue (**B**, **C**), and characteristic spherical shape of an oospore composed of thick cell walls with two layers (**D**). Within Fig. 2B are two sporangia of P. cubensis displayed at the same magnification.

Figure 4.3. Viability of oospores of *Pseudoperonospora cubensis* as determined using the plasmolysis method involving incubating oospores in a 4M NaCl solution for 45 min at room temperature. Untreated oospore (**A**) treated oospore but not viable shown by an unplasmolyzed oospore (**B**), and treated and viable oospores showing plasmolysis (**C**, **D**). Plasmolysis (i.e., contraction of the cytoplasm) indicates a viable oospore, while unplasmolyzed (i.e., intact cytoplasm) denotes a non-viable oospore.

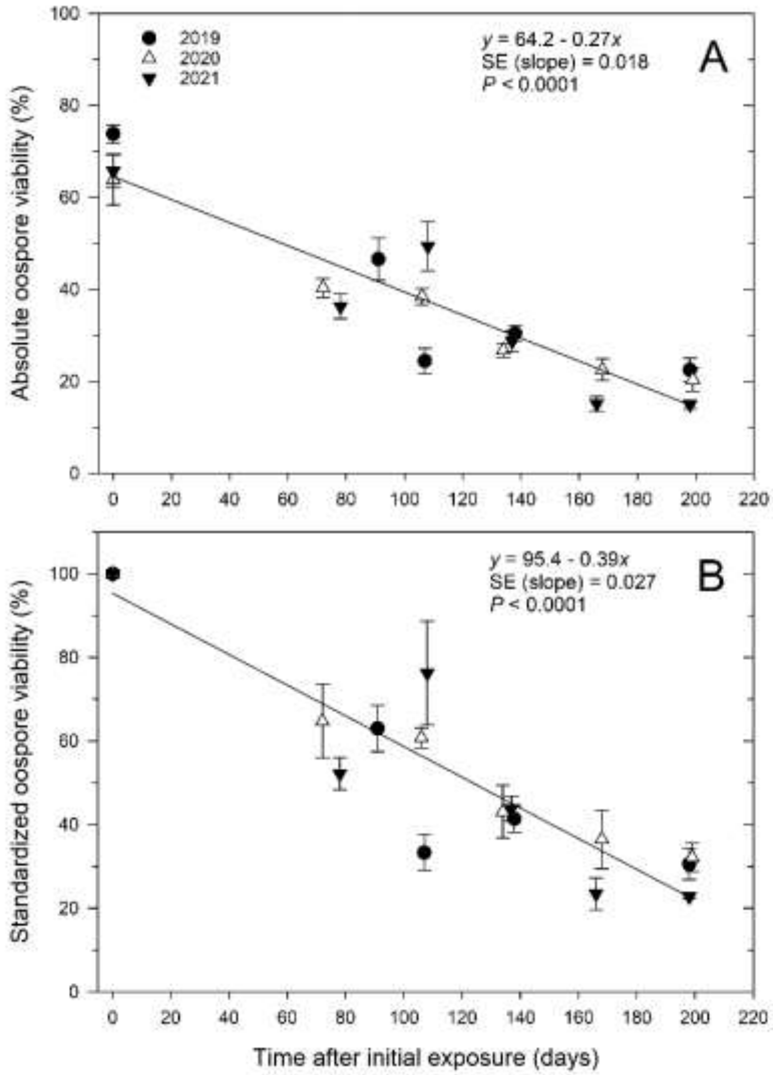

Figure 4.4. Dynamics of the viability of *Pseudoperonospora cubensis* oospores based on samples exposed in the field at Rocky Mount, North Carolina during the winter season in 2019, 2020 and 2021. **A**) Absolute oospore viability and **B**) standardized oospore viability over winter. Standardized viability is based on the mean baseline viability of oospores in November to allow for direct comparison of results from different experimental years. Each data point is a mean of three subsamples in each year and vertical bars represent the standard error of mean absolute or standardized viability. The line on each graph is the predicted oospore viability generated from fitting viability data (y) over time (x) to a linear function of the form: $y = b_0 + b_1 x$, where b_0 and b_1 are the parameters to be estimated.

CHAPTER 5

Conclusions

Pseudoperonospora cubensis a causal agent for cucurbit downy mildew is a diverse, high-risk pathogen responsible for severe epidemics in cucurbits globally. Breeding of resistant cucumber cultivars in the past 40 years had resulted in resistant cultivars with disease management relying mostly on host resistant and adequately managed by minimal chemical application. However, resurgence of cucurbit downy mildew in 2004 in the United States changed management strategy and host resistance alone was no longer effective in disease control. Since its resurgence, chemical control remains the most effective method for controlling cucurbit downy mildew due to the lack of commercial cucurbit cultivars with adequate levels of disease resistance. Widespread fungicide resistance has been a bottle neck in CDM management. In the United States, diversity of *P. cubensis* and long-distance aerial spread of inoculum are responsible for onset of annual epidemics in regions that receive harsh winters. It is unknown whether local inoculum in form of oospores can overwinter in this region. The research in this dissertation aimed at elucidating three main objectives; i) characterize the temporal dynamics and severity of cucurbit downy mildew epidemics as affected by chemical control and cucurbit host type; (ii) establish temporal shifts in sensitivity of *P. cubensis* populations in response to cucurbit downy mildew fungicides; and (iii) determine the occurrence and overwintering of oospores of *P. cubensis* under field conditions in the Southeastern United States.

Work contained in this dissertation characterized the temporal dynamics and severity of CDM at the field scale in order to understand how chemical control strategies together with host types influence disease epidemics. Chemical control strategies involving alternation of protectant fungicide with systemic fungicide on two different cucurbit host type were used to characterize disease severity and determine parameters of disease progress on temporal scale and eventually, assess shifts in sensitivity profiles involving chemical control strategies. There were differences in disease pressure among host types with disease intensity being slightly higher on cucumber than on summer squash. Chemical control strategy involved the alternation of three systemic fungicides, cymoxanil, fluopicolide and propamocarb, with chlorothalonil, a protectant fungicide. Polycyclic-type disease caused by *P. cubensis* was confirmed in this research study where cucurbit downy mildew epidemics were best described by logistic or Gompertz model. Thus, the extent of the epidemics was dependent on primary inoculum originating within or outside the field as well as secondary inoculum generated during the season. Fungicide treatment and host type significantly affected cucurbit downy mildew disease dynamics resulting in lower values of standardized area under disease progress curve (sAUDPC), final disease severity, estimate of rate of disease increase. Weighted absolute rates of disease progress ρ were significantly lower in fungicide treated plots than in untreated control plots. In this dissertation, results show alternation of chlorothalonil with fluopicolide or propamocarb was consistently effective and reduced disease pressure, and resulted in lower values of sAUDPC, final disease severity and ρ was observed in most cases. These results, thus, provide understanding on how disease management strategies involving host type with different level of resistance and alternation of protectant fungicides with systemic fungicides affect disease dynamics. Additionally, parameter estimates such as apparent infection rates can be used together with initial and final disease severity values as input variables in mathematical model simulation on development of management strategies as well as prediction of effective fungicide doses and selection factor for fungicide resistance.

Chemical protection is the most effective management tool. However, application of fungicides can result in temporal shift in Pathogen sensitivity profiles. In this study, within season assessment of shifts in sensitivity profiles of *P. cubensis* to fluopicolide, propamocarb and cymoxanil following weekly alternation with chlorothalonil was evaluated. Baseline sensitivity of *P. cubensis* isolates were determined early in the season, and at the end of the season shift was determined after fungicide application. In this study, three reactions namely resistant, sensitive and shift to higher resistance for cymoxanil, propamocarb and fluopicolide respectively were observed after weekly alternation with chlorothalonil. These results therefore suggest that shift towards reduced sensitivity in *P. cubensis* can occur within a growing season and these may influence the efficacy of single site fungicides such as fluopicolide, and propamocarb and cymoxanil. While control strategies against the pathogen such as mixing of fungicide and alternation may reduce selection pressure, environmental factors that influence disease pressure needs to be considered in order to reduce frequency and intensity of chemical application.

Sexual reproduction in oomycetes results in formation of thick-walled oospore structures that can overwinter on plant debris or in the soil. In the United States, the cucurbit downy mildew pathogen, *P. cubensis*, has been shown to be dimorphic in nature. Additionally, it has been demonstrated that *P. cubensis* potentially forms oospores under controlled conditions in the laboratory. However, there were no reports on the formation of oospores in naturally infected cucurbit plants under field conditions. In this dissertation, occurrence of oospores in cucurbit fields in southeastern United States and their potential to survive and remain viable during winter in areas that experience harsh winter was examined. This research study involved screening of cucurbit downy mildew infected leaves collected for cucurbit fields for the presence of oospores at the end of the growing season. At the end of the growing season oospores were present in necrotic leaves collected from cucurbit fields in North Carolina and South Carolina. These regions are both located above 30-degree latitude where they receive annual harsh winter and cucurbit cost cannot survive, and oospores could be overwintering structures. While the frequency of oospores occurrence in the entire study was very low at about 5%, temporal survival and viability of field deployed oospores suggest that oospores formed in nature could be of important in local initiation of epidemics as primary inoculum. This research study describes the first report on the occurrence of oospores of *P. cubensis* in nature under cucurbit fields in the United States. In obligate parasites such as *P. cubensis* where host cannot survive harsh winter conditions, oospores may be part of pathogen life cycle. Oospores can act as sources of primary local inoculum in regions above 30-degree latitude. In this study, oospores of *P. cubensis* were found to be present in infected leaves in cucurbit fields under natural conditions in the southeastern United States. Further, oospores were also found to survive and remained viable during winter until late spring or early summer during the onset of planting season. While physical and biological factors required for oospore germination are still unknown, further studies are still needed to elucidate the role of *P. cubensis* oospores as an initial source of primary inoculum and their overall role in dynamics of cucurbit downy mildew epidemics not only in the United States, but also other cucurbit producing regions around the world.