CHARACTERIZATION, VIRULENCE, EPIDEMIOLOGY, AND MANAGEMENT OF *COLLETOTRICHUM SPP.* CAUSING ANTHRACNOSE IN ONION AND CELERY

By

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ABSTRACT

CHARACTERIZATION, VIRULENCE, EPIDEMIOLOGY, AND MANAGEMENT OF *COLLETOTRICHUM SPP.* CAUSING ANTHRACNOSE IN ONION AND CELERY

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Anthracnose symptoms in onions (*Allium cepa* L.) and celery (*Apium graveolens*) were observed for the first time in Michigan during the 2010 growing season. Disease symptoms were found in 37 onion and 50 celery fields during the period of 2010 through 2012. Based on colony morphology, conidial characteristics, internal transcriber spacer (ITS) and glutamine synthetase (GS) sequences and species-specific molecular markers, the causal agents were identified as *Colletotrichum coccodes* (on onions) and *Colletotrichum acutatum* (on celery). These new diseases were named onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose. A total of 901 *C. coccodes* and 549 *C. acutatum* isolates were recovered from symptomatic onion and celery tissue respectively. A subsample isolates of *C. coccodes* (N=110) and *C. acutatum* (N=113) were included in population genetics studies. Four Inter-Simple Sequence Repeat (ISSR) markers banding patterns differed by *Colletotrichum* spp. isolated from onion and celery, and controls (*C. gloeosporoides* and *C. dematium*). Within the *C. coccodes* isolates the banding patterns were identical, while 4% polymorphism was observed among *C. acutatum* isolates. A total of 2,300 and 2,423 transcripts contained Simple Sequence Repeat (SSR) motif suitable for primer design for *C. coccodes* and *C. acutatum* respectively. Out of the primers tested, 64 and 89% amplified the expected fragment size on *C. coccodes* and *C.*

acutatum DNA, respectively. No polymorphisms were observed in the SSR primers tested on *C. coccodes* isolates, while 12% of the primers tested in *C. acutatum* showed polymorphic loci.

Isolates of *C. coccodes* and *C. acutatum* from onion and celery were tested for their pathogenicity and virulence. When ripe cherry tomato (*Solanum lycopersicum* L.) fruit were inoculated with 824 *C. coccodes* isolates from onion, differences in virulence were identified based on the field where they originated. When 81 *C. acutatum* isolates were used to inoculate 'Green Bay' celery seedlings, differences among isolates were found, but differences were not found among collection year, field, or county. A combination of extended high RH period (≥24h) and high temperatures (≥25°C) resulted in >20% disease severity on 'Infinity' onion seedlings. On celery, leaf curl and petiole lesions occurred at all temperatures (15 - 30°C) and leaf wetness duration (0 - 96h) tested. Incidence was highest when inoculated plants were subjected to an extended period of leaf wetness (96h) or at 30°C. When 16 onion cultivars were evaluated for their susceptibility to *C. coccodes,* disease was significantly lower in 'Hendrix' and higher in 'Highlander' and 'Candy'. Fungicides in the strobilurin, multi-site and demethylation inhibitors groups limited disease in onion and celery. Efficacious fungicides resulted in higher marketable celery yields when compared with the untreated inoculated control. Adequate management of onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose should include crop rotation, pathogen free seed, greenhouse and field sanitation, cultivar selection, and alternation of preventative efficacious fungicides to limit infection, symptom development and yield loses caused by these pathogens in onion and celery crops in Michigan.

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LITERATURE REVIEW

ONIONS AND CELERY PRODUCTION IN MICHIGAN

In 2012, Michigan growers produced 1.2% of the U.S. national production of onions (*Allium cepa* L.), valued at $7,748,000 (15). Onions are produced for storage or for fresh market. Michigan farmers grow mainly storage onions and the state ranks firth nationally in this category after California, Colorado, New York and Idaho (15). The majority of onion production is concentrated in the southwest and south central counties of Michigan, including Newaygo, Allegan, Eaton, Ionia and Ottawa. Onions are directly sown into muck or mineral soil. There are up to 30 storage onion cultivars grown in Michigan including white, red and yellow onions.

Michigan growers produced 6% of the U.S. national production of celery (*Apium graveolens*) during 2012 at a valued of $22,380,000 (15). Celery is grown during late spring, summer and early fall in Michigan as opposed to year-round production in California, Florida, and Texas (164). Most of the celery production in Michigan takes place in the southwest side of Lake Michigan including Allegan, Kent, Muskegon, Newaygo, Ottawa, Oceana and Van Buren Counties. In Michigan, celery is seeded in flats, and seedlings are maintained in greenhouses until transplants are approximately seven weeks old. Celery seedlings are transplanted to muck soil or occasionally into coarse textured mineral soil (295). The most common celery cultivars grown in Michigan are ‘Sabroso’ for processing, ‘Green Bay’ for fresh market and ‘Dutchess’ for both markets.

Both onion and celery are cool season biennial crops (34,73,247). In onions, foliar disease such as purple blotch, Stemphylium leaf blotch, Botrytis leaf blight, and downy mildew (*Alternaria porri* (Ellis) Cif., *Stemphylium vesicarium* (Wallroth) E.G. Simmons, *Botrytis squamosa* J.C. Walker, *Peronospora destructor* (Berk.) Casp. ex Berk

respectively) reduce the photosynthetic area and may result in severe defoliation of the plants, compromising bulb enlargement and quality (92).

During the 2010 growing season in Michigan, extension personnel, scouts, and farmers observed unusual onion foliar and neck lesions. A species of *Colletotrichum* was isolated from these lesions and later confirmed as *Colletotrichum coccodes* (Wallr.) Hughes (239). This was the first report of *C. coccodes* infecting onions anywhere in the world (239). Six different *Colletotrichum* spp. have been reported to cause diseases on onions worldwide (99,247). *Colletotrichum circinans* (Berk.) Voglino, which causes smudge, is an occasional pathogen of onion in Michigan and other onion growing regions in the U.S. (247). *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc has been reported infecting onions world wide causing onion twister or anthracnose (90,112,232,280) but the only report from within the U.S. are from Georgia (211). In addition, *C. capsici* (Syd. & P. Syd.) E.J. Butler & Bisby, *C. chardonianum* Nolla, *C. dematium* (Pers.) Grove and *C. lindemuthianum* (Sacc. & Magnus) Briosi & Cavara were reported infection onions in at least nine different counties in the world (97).

In celery, foliar blights such as bacterial, early and late blight (*Pseudomonas syringae* pv. *apii*, *Cercospora apii* Fresen and *Septoria apiicola* Speg, respectively) can negatively impact yield and quality (164). Foliar blights result in additional trimming during postharvest processing and reduced profit for growers. Fusarium yellows (caused by *Fusarium oxysporum* f. sp. *apii* W.C. Snyder & H.N. Hansen) is characterized by plant stunting, yellowing and withering of petioles and vascular tissue discoloration (37-39). Fusarium yellows was described for the first time in Michigan in 1914 (164), and is prevalent throughout the state. Celery fields with a high incidence of *F. oxysporum* f. sp.

apii in the soil can benefit from crop rotation with onions, if the onion residues are incorporated into the soil (91,164). This rotation helps to reduce *F. oxysporum* f. sp. *apii* propagule numbers in the soil and diminish Fusarium yellows incidence in celery (91). For this reason onion and celery have been rotational crops in Michigan since the 1980s (165).

In September of 2010, celery leaves and petioles showed unusual cupping and twisting, respectively. Growers, scouts and extension personnel reported symptoms in Allegan, Barry, Kent, Ottawa and Van Buren Counties. The casual agent of these symptoms was identified as *Colletotrichum acutatum* J. H. Simmonds (238).

Both *C. coccodes* and *C. acutatum* are known to infect other vegetable and fruit crops (81,98,224,285). The first report of onion as host of *C. coccodes* in the U.S. or any other onion producing areas in the world was in 2010 (239). In contrast, *C. acutatum* was reported in the 1990s infecting celery in Australia (135,290), but never before in U.S. (98); the 2010 growing season was the first time *C. acutatum* was reported infecting celery in the state of Michigan and in the U.S. (238).

THE GENUS *COLLETOTRICHUM*

The genus *Colletotrichum* was first described by Corda in 1803 (267). The mycelial thallus of *Colletotrichum* spp. is hyaline, septate, branched and multinucleate. Mycelium forms a conidiomata know as an acervulus, hence the genera is grouped among acervuli-forming fungi or coelomycetes (268). Some members of the genus produce sclerotia in nature and in culture. Acervuli and sclerotia can be setose (i.e. modified thick wall hyphae) and seen as projections from the acervular body (7). Conidia are thin-walled, hyaline, aseptate, straight or falcate and are produced on conidiophores on the upper layer of conidiomata (i.e. acervular hymenia). The conidia from different species within the genus *Colletotrichum* have a high degree of morphological variability, and some degree of variability within a species or species complex (63).

The teleomorphic state of *Colletotrichum* is the genus *Glomerella* (268). For some *Colletotrichum* spp., the sexual stage has been found in nature (268), or observed only in

culture (122). *Glomerella* spp. belong to the Phylum Ascomycota, class Sordariomycetes [NCBI Tax-Browser (29)], also known as *Pyrenomycetes* family *Phylachoraceae* (268). The genus *Glomerella* forms obpyriform to subglobose perithecium, with short ostiolar neck and periphyses. When perithecia are formed, they are partially or completely immersed in the host tissue with aggregated, scattered or single arrangement. In perithecia, thin-walled, unitunicate asci bear biseriate, one-celled, hyaline ascospores (268).

Classification of fungi as species on the genus *Colletotrichum* is based on morphological and culture characteristics; including conidial shape and size, presence or absence and morphology of setae and, presence of sclerotia and appressoria shape (224,258,268). DNA markers have proven useful diagnostic tools to determine species identity and to study phylogenetic relationships among isolates. The sequences of the internal transcribed spacer (ITS) have been used to differentiate among species (67,85,123,153). The ITS regions are located in between the nuclear ribosomal DNA (rDNA), that is comprised by the 5.8S gene, the small subunit (18S) and large unit (28S). Based on ITS sequence differences, species-specific primers have been developed for rapid identification of some *Colletotrichum* spp. (44,67,106,192,199). However, to discriminate among *Colletotrichum* spp. a multiple gene sequence comparison with the type culture sequences is preferred (65,234).

The biological species concept does not entirely apply to *Colletotrichum,* since sexual reproduction is uncommon in nature for the majority of the species in this genus. A phylogenetic species concept is possible to apply, as more information on the evolution and reproductive pathways of the members of this genus is compiled (53). Among the approximately 38 accepted and studied *Colletotrichum* spp., 10 species are known to infect

economically important hosts: *C. acutatum*, *C. capsici, C. circinans*, *C. coccodes*, *C. dematium, C. fragaria*, *C. graminicola C. gloesporoides, C. lindemuthianum* and *C. orbiculare*. However, *C. acutatum, C. gloesporioides* and *C. orbiculare* are considered to be species complexes in the genus and given the name of species *sensu lato* (70,171,183)

Colletotrichum gloesporioides is described as a highly morphologically variable species, able to cause different symptoms on certain hosts. Molecular tools have been used to differentiate *C. kahawae* as a separate species from *C. gloesporioides* (53,209,271), but at least 13 species are included in *C. gloesporioides sensu lato* complex (70,220).

Colletotrichum spp. are widely distributed fungi, pathogenic to plants (6,283) and occasionally to humans and other animals (55). *Colletrotrichum* spp. infect a broad range of crops including tropical fruits, perennial crops, potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), bean (*Phaseolus vulgaris* L.), coffee (*Coffea arabica* L.), and maize (Zea mays L.) (6,98). However, *Colletotrichum* spp. can be specific to a plant species and multiple hosts can be infected by a single *Colletotrichum* sp. Alternatively, several *Colletotrichum* spp. can be associated with a specific host (103,209,224,283). For instance, *C. gloesporoides, C. fragariae*, *C. dematium* and *C. acutatum* have been reported as pathogens of strawberry (140,257). Citrus stone and pome fruits are susceptible to multiple *Colletotrichum* spp. (30,44).

Several members of the genus *Colletotrichum* cause anthracnose in tropical and temperate hosts around the world (6). Anthracnose is a disease characterized by a limited, sunken and necrotic lesion that often has distinctive zones and margins. Anthracnose symptoms can develop in several plant tissues such as leaves, fruits, flowers and/or stems (224,283).

Colletotrichum spp. life cycle

The primary inocula of *Colletotrichum* spp. are ascospores when the teleomorph (*Glomerella* spp.) is present in nature, and conidia in the anamorph (*Colletotrichum* spp.). Conidia are covered with polysaccharides and glycoproteins, a hydrophilic mucilaginous material that enhances dispersal, ensures distribution of inoculum, protects conidia from desiccation and prevents premature spore germination (21,81,221). In *C. truncatum* (Schweinitz) Andrus & W.D. Moore and *C. lindemathianum* (Saccardo & Magnus) Briosi & Cavaraa spore coat of short fibrils (39,59) and glycoproteins (221,222) were observed in the conidial outer layer. The glycoproteins may also be involved in the conidial attachment to the host surface after landing (21).

Conidia that have landed in the host tissue can produce secondary conidia during early infection events, the epiphytic or biotrophic stages (53,172,174,256), and may occur at the infection site or in culture (21,224,285). This process is known as microcyclic conidiation, an ability that can contribute to inoculum proliferation and survival (104), and can be induced by nutrient depletion or temperature stress (173,256).

After the deposition of a conidium on the plant tissue, cell differentiation occurs, then septum formation is initiated, followed by the development of a germination tube, an appressorium and a penetration peg (21,221). The main role of appressoria in infection is the adhesion to host tissue surface.

Appressorium differentiation occurs after the germ tube expands completely, then the tip swells and a septum forms. Later, the penetration pore forms and cell wall thickening occurs coupled with secretion of mucilaginous materials and melanization of the appressorium. The appressorial wall is comprised of two to three layers of carbohydrates

with melanin deposits (221). Melanin is key for the penetration process (229). Melanin protects appressoria from harmful light radiation, strengthens the appressorial wall and, as in other genera (e.g. *Magnaporthe grisea*), may trap surrounding solutes to create hydrostatic pressure (141).

In some *Colletotrichum* spp. an appressorial cone forms as an extension of the penetration peg; this structure may aid in penetration by adding hydrostatic pressure to the penetration point. Penetration peg growth resumes and grows through the pore. Host cuticle penetration occurs directly or indirectly. Direct mechanisms involve mechanical cuticle penetration and enzymatic cuticle digestion while indirect penetration is accomplished through natural openings or wounds (221).

Colletotrichum spp. can penetrate the tissue and remain restricted to the epidermal layer, and the host is asymptomatic, known as biotrophic interaction or quiescent infection (229,230). In the biotrophic interaction with the host, penetration occurs, appressoria form followed by intracellular fungal mycelial growth, but extensive host colonization does not occur or is delayed (5).

During the infection process, *Colletotrichum* spp. can establish a hemibiotrophic interaction, a subcuticular/intramural necrotrophic interaction, and in some instances a combination of the two (21,221,224). During hemibiotrophic infection, *Colletotrichum* establishes a biotrophic interaction with the host tissue. Primary hyphae develop while host tissue remains asymptomatic, this phase can last from 24 hours to 3 or more days and differs among *Colletotrichum* spp. and its hosts (21). These species will form intracellular hyphae that can be swollen or branched, or can arise from infection vesicles as thin hyphae. Intracellular hyphae grow and colonize adjacent host cells (221,224). This biotrophic phase

is followed by extensive colonization of the host tissue. Cell death occurs gradually, is confined to an infected cell and is considered a benign necrotrophy (221). As secondary hyphae develop and expand along the host tissue, a switch to a destructive necrotrophic phase occurs but no host responses are induced (221). While biotrophic host interaction occurs in newly infected cells, necrotrophy can occur at the opposite end, where infection started.

During subcuticular intramural infection, a short biotrophic phase is followed by mycelial growth in the host cell walls. Secondary hyphae then penetrate the host cells (68,224). Necrotrophy can occur afterwards as *Colletotrichum* develops beneath the cuticle causing host cells to swell and collapse. Despite the brief biotrophic phase, a nonhost defense appears to be activated, intramural spread occurs followed by penetration of the host cells (221).

Colletotrichum acutatum

Conidia of *Colletotrichum acutatum* range from 8.5 to 16.5 x 2.5 to 4 μm in size; conidia are fusiform in shape, and sclerotia and setae are absent. In culture, mycelia become gray to pink, some isolates secrete pigment into the media (i.e. chromogenic), and conidial masses are generally present as salmon-pink droplets (267). Acervuli are embedded in the host tissue and bear conidia that can be dispersed mechanically by rain splash (36,292). Short and long distance dispersal have been observed in small fruit (187) and tree fruit (4), respectively. *Colletotrichum acutatum* has a broad host range including cultivated hosts such as fruit, ornamental plants and vegetables (98). Isolates recovered from one host were able to cause disease on a variety of hosts (104). In tree fruit crops, *C. acutatum* is considered not tissue specific when tested under laboratory conditions (106,224).

Colletotrichum acutatum has the ability to infect actively growing tissues, such as flower blossoms, leaves, and shoot tissue (3,4,224). The most common disease caused by *C. acutatum* is strawberry (*Fragaria* L.) fruit rot, but it can also cause crown and bud rot (75,127,224) and lesions on roots and stolons can occur (104,105).

Inoculum of *C. acutatum* can be present in soil, plant debris and weeds. Eastburn et al. (89) were able to recover *C. acutatum* isolates from strawberry nurseries and fields with history of anthracnose, and from infected strawberry tissues buried in soil, demonstrating that *C. acutatum* can survive in soil associated with tissue for up to nine months (89). *Colletotrichum acutatum* can survive in the soil in association with debris and under dry conditions (109), and can survive on other crops and weeds, in many cases not causing evident symptoms (104). *Colletotrichum acutatum* can survive and colonize different plant species, expanding the potential sources of inoculum. For example, *C. acutatum* can colonize weeds such as *Vicia* spp. and *Conyza* spp. in land adjacent to strawberry fields and these isolates are pathogenic on strawberry (104).

In tomato, pepper (*Capsicum* L.) and eggplant (*Solanum melongena* L.) leaves, *C. acutatum* can survive for up to 3 months living as endophyte (139) or as an epiphyte for at least seven weeks (104). In strawberry, symptomatic or infected asymptomatic transplants from nurseries are an important source of primary inoculum (104).

The host interaction and infection strategies of *C. acutatum* appear to be host tissue dependent. In citrus, infection of leaves is biotrophic, while infection of citrus flowers is necrotrophic (296). *Colletotrichum acutatum* is a necrotroph on strawberry fruit, crown and stolons; a hemiobiotroph on immature blueberry (*Vaccinium* L.) fruit; and in almond (*Prunus dulcis* (Mill.) D.A. Webb) and key lime (*Citrus* ×*aurantiifolia* (Christm.) Swingle

(pro sp.) [medica × sp.]), *C. acutatum* begins as a biotroph followed by necrotrophic infection of leaves and fruits (224,285). Plant hormones play an important role in symptom development in diseases cause by *C. acutatum*. Accumulation of indole-3-acetic acid, *trans* jasmonic acid and ethylene on the host occurred during symptom development of citrus postblossom fruit drop caused by *C. acutatum* (167,179).

Colletotrichum acutatum has been regarded as a species complex by some mycologist (85,123,171). *Colletotrichum acutatum sensu lato* include isolates that grouped as *C. acutatum* and differed from other *Colletotrichum* spp. based on molecular markers (123,152). In phylogenetic studies, it is common to find *C. acutatum* isolates grouped as genetic subgroups based on ITS 1 and ITS 2 sequences (107), and those different groups or clades may represent different phylogenetic species (85).

No specific *C. acutatum* subspecies are fully accepted. However, some authors have proposed the use of ITS 2 sequences to create subspecies delimitation (107). Some *forma specialis* have been described, i.e. *C. acutatum* f. sp. *pineum* pathogenic to pines and *C. acutatum* f. sp. *chromogenum* based on their color on culture (224). Host range is not associated with the phylogenetic species (101). However, in some studies, molecular data grouped isolates according to the host of origin (152,153).

In citrus, *C. acutatum* isolates from different regions did not group by geographic area but remain genetically closely related (224). The genetic diversity of *C. acutatum* isolates from the same host can be limited if the population is clonal. However, if the teleomorph occurs in nature, it can lead to a genetically heterogeneous population (107). Recently, Damm et al. (2012) studied the *C. acutatum* species complex using a polyphasic

gene sequences, and concluded this complex is monophyletic and comprises at least 29 closely related species with morphological variability and a broad host range (71).

Colletotrichum coccodes

Colletotrichum coccodes has been reported to infect 50 different hosts worldwide (98) and 18 within the U.S. and is of primary importance on Solanaceous and Cucurbitaceous hosts (79,81,276). It has also been reported to infect weed species in the families Amaranthaceae, Asteraceae, Brassicaceae, Euphorbiaceae, Poaceae, Malvaceae, Oxidalidae and Polygonaceae (11,231).

Colletotrichum coccodes was reported for the first time in the U.S. in 1891 when an outbreak on tomatoes occurred (243). It incites fruit anthracnose in tomato, and causes black dot in potato (81). Recently, Liu et al. (2011) neotypified and characterized *C. coccodes* (182). Additionally, Liu et al. (2013) studied *C. lindemuthianum*, *C. coccodes*, *C. lycopersici* and *C. nigrum* taxonomic relations and reviewed name designations (181). *Colletotrichum lycopersici* and *C. nigrum* were recognized as synonyms. Isolates of *C. coccodes* and *C. nigrum* were closely related, but each formed a separate clade. The isolates in the *C. coccodes* clade were obtained from potato, tomato and pepper, while isolates in the *C. nigrum* clade were isolated from pepper and tomato (181).

Colletotrichum coccodes has been shown to cause symptoms and produce abundant acervuli and sclerotia on senescent tissues in tomato (83), potato (213) and some weed species (57,231). Sclerotia serve as a source of primary inoculum (243), while *C. coccodes* infected weeds can serve as source of primary or secondary inoculum (231,243).

Colletotrichum coccodes produces cylindrical conidia with obtuse ends, 16 to 24 x 2.5 to 4.5 μm in size, in acervuli on leaves, fruits and roots. Acervuli give origin to

abundant black globose microsclerotia. Acervuli and microsclerotia have setae in culture media and in the host tissue (267). Sclerotia comprise three distinct zones: outer, middle and inner (277). The outer and middle zones are comprised of short tightly packed cells. The outer zone contains empty cells. The inner zone develops into a prosenchyma while the middle zone is characterized by a plectenchyma, responsible for the rigidity of the sclerotia (277). *Colletotrichum coccodes* sclerotia will remain in the soil for up to one year in potato fields (96,276), and survive associated with host debris or alone for up to eight years in tomato fields (82). Location of sclerotia within the soil and their association with host debris influences the survival of most sclerotia-forming fungi (61). After five years in the soil, 90% of the *C. coccodes* sclerotia buried at 10 and 20 cm from the surface were viable, compared to sclerotia in the soil surface which were only 50% viable (82).

Sclerotial germination in *C. coccodes* has been shown to be more sensitive to aeration and light than to temperature (in a range from 10 to 35°C). More conidia were produced on sclerotia under aeration, but mycelial germination was equally observed in aerated or air-deprived sclerotia (243). The mycelial growth, sporulation and conidial germination of *C. coccodes* occurred at a temperature range of 24 to 28°C *in vitro* (155), and optimal mycelial growth from sclerotia was observed at 28°C (80).

In potato, tomato and pepper, disease severity is exacerbated by longer periods of wetness (79,138,151). Ten or more hours of continuous leaf wetness were needed for conidia to infect tomato fruits, and optimal conidial production on fruit lesions occurred at 28°C (79). In the field, severe outbreaks are associated with high rainfall or overhead irrigation (81). Furthermore, some authors regard *C. coccodes* as airborne dispersed, when

rain or irrigation are combined with high wind speeds (276). During wet conditions, conidia are known to survive in soil for up to one year (81,276) and 53% of the conidia will survive buried in the soil for one year and 31 weeks (81).

In tomato, anthracnose is especially important in processing tomatoes where the fruits need to ripen on the vine (52) or in postharvest situations where latent infections in asymptomatic fruits result in symptoms when the fruits reach the retailer. Unmanaged tomato anthracnose can cause up to 90% crop loss (52). In tomatoes, infection occurs with and without wounding the tissue (79). Infection occurs in stems, roots and immature and ripe tomato fruits (81); however, symptoms are observed extensively on ripe fruits. Small sunken to water-soaked lesions become enlarged as the central portion of the lesion darkens. Acervuli form in the center of the lesions and salmon-colored sporulation is observed. Lesions coalesce, and setose sclerotia form on the surface of the fruit, and the fruit rots.

Pathogen structures (mycelia and sclerotia) associated with the seed can be an important source of inoculum. *Colletotrichum coccodes* infected 20 to 40% of the seeds extracted from infected tomato fruits (27). Infected seeds appear darker than healthy seeds, but no microsclerotia were observed on the seed coat (27). Highly infected seeds fail to germinate, while moderately infected seeds deteriorate shortly after germination. Anthracnose symptoms in tomato seedlings were observed when seeds were only slightly colonized by the pathogen. Mycelium of a green fluorescent protein (GFP) transformed *C.coccodes* isolate was observed via epi-fluorescent microscope colonizing the seed coat in 40% of the seeds tested but only 1% of the embryos (27).

Potato black dot receives its name from the small black *C. coccodes* sclerotia in the tubers, stolons, roots and stems. Foliar infections can also occur (24,276). Potato foliar

inoculations in glasshouse studies resulted in leaf lesions and a reduction of root dry weight and tuber yield (24). *Colletotrichum coccodes* colonizes potato vascular tissues and internal stem tissues (276). Wounding has been shown to be important for lesion formation in potato foliage, as number of lesions significantly increased when sandblasting occurred prior to inoculation (151). Internal or external tuber infection is possible. Asymptomatic infected seed serves as early season inoculum (24). Infected potato seed is regarded as the most important source of inoculum and dissemination among potato growing areas (276). In Washington state, *C. coccodes* was reported on 50% of certified potato seed lots (151). In other regions of the world, potato seed from Scotland, the Netherlands, Germany and Northen Ireland exported to Israel had low incidence of *C. coccodes* infection (276).

Black dot in potatoes is associated with sandy soils, poor soil drainage, low nitrogen in the soil, and temperatures greater than 23°C during the season (276). Disease severity, increased fungal colonization and sclerotia formation were observed in plants mantained under 8 hours light and 16 hours darkness (276). Infection by *C. coccodes* in potatoes has shown to be latent in young plants and as plants mature, *C. coccodes* growth and colonization is activated by plant hormones, and the symptoms become evident (213). Inoculation of potato plants with infested dry potato stems in the field resulted in significant yield losses (275). Incidence of *C. coccodes*-affected potatoes increased during storage, when unwashed tubers were stored in temperatures ≥15°C and under humid storage conditions (276).

When *C. coccodes* and *Verticilium dahliae* were present in a potato fields, higher disease incidence was observed in comparison to any of the two pathogens alone, suggesting a synergistic relationship that has been proposed as a disease complex (81,150,276).

Colletotrichum coccodes has also been found in lesions caused by flea beetles or *Alternaria solani* in tomatoes (81). Pepper (*Capsicum annuum* L.) seedlings can be killed by *C. coccodes* infection while mature plants have been shown to be more tolerant to the disease (138).

Management of diseases caused by *Colletotrichum* spp.

Colletotrichum spp cause reduction in quantity and/or quality of the harvested yield when left unmanaged, and disease management may result in high costs for growers (283). Cultural and chemical strategies, as well as host resistance, are employed to control diseases caused by this pathogens (6). Disease-free seed is important to avoid introduction of the pathogen into the field. Cultural practices such as crop rotation with non-susceptible hosts, the use of tolerant or resistant cultivars when available, and sanitation practices such as eliminating crop debris and weeds can help ameliorate diseases caused by *Colletotrichum* spp. (2,228).

Strategies to control anthracnose caused by *C. acutatum* include sanitation and the use of tolerant or resistant cultivars when available (228). However, cultivars that have the desired traits for the market are commonly the most susceptible to anthracnose and breeding efforts are expected to deliver the desired horticultural traits and anthracnose resistance. In tropical fruits such as mango (*Mangifera* L.) and other perennial hosts, tree or bush pruning helps to reduce inocula that have persisted in tissues such as flower stalks, twigs, bud scales and leaf or bark lesions (2,224,228). Sanitation needs to be combined with chemical control and other IPM strategies. In susceptible tropical fruits, protectant fungicides are the main tool to manage anthracnose and should be applied at short intervals particularly during the flowering stage. Protectant residual fungicides are less effective after precipitation when

protection is especially needed (228). Systemic fungicides alternated with protectant fungicides are effective for control of anthracnose and may delay potential pathogen resistance issues, as some resistance in various pathogens to single site fungicides has been identified (2,23,40,225). QoI-fungicides (e.g. azoxytrobin) and DMI-fungicides (e.g. tebuconazole and propiconazole) effectively controlled almond anthracnose, but repeated applications of these fungicides are needed in the spring for effective control (2).

In orchards and citrus groves it is very important to avoid fungicide run off, and increase chemical deposition in the plant tissues is achieved by using smaller droplet size during application (228). Postharvest fungicide sprays are used to protect fruits during storage and transit (147).

Colletotrichum acutatum has been reported as a pepper pathogen in the U.S. (178). In Ohio, alternation of protectant and strobilurin fungicides; or alternation of various protectant fungicides effectively controlled pepper anthracnose caused by *C. actutatum* (178). In Michigan, QoI-fungicides, succinate dehydrogenase (SDHI) and protectant fungicides are recommended to manage anthracnose (*Colletotrichum* spp.) in pepper (32).

Effective management of *C. coccodes* in Solanaceous hosts includes a combination of cultural practices and fungicide programs (81,276). Since sclerotia survive in soil for long periods of time (82), control of *C. coccodes* in potato and tomato include three to four years of crop rotation away from Solanaceous hosts. The pathogen can also survive in association with lettuce (*Lactuca* L.), cabbage (*Brassica oleracea* L.), cress (*Lepidium sativum* L.), white mustard (*Sinapis alba* L.) and chrysanthemums (*Chrysanthemum* spp.) among others, and these hosts are not recommended as rotation crops (82). Dillard et. al (1998) proposed a multi-year rotation with nonhost crops as a preventive measure to avoid

increasing microsclerotia population in the soil; however this practice does not completely eliminate (82). Clean potato tubers and clean tomato seeds (27) are needed to avoid introduction of *C. coccodes* inoculum. Fungicide-treated seeds are recommended to kill or reduce inoculum (276).

Well drained soil; elimination of host weeds that can be a source of inoculum can contribute to management of black dot and tomato anthracnose (82,231). *Colletotrichum coccodes* is able to infect common weeds such as velvet leaf (*Abutilon theophrasti* Medik.) and eastern black nightshade (*Solanum ptychanthum*) (11,291), but 10 different weeds have been reported as hosts for *C. coccodes* (231).

Tomato anthracnose is difficult to control under warm temperatures (22 to 28°C), regular rainfall and extended period of moisture (82). As an alternative, the use of resistant and tolerant cultivars is the most environmentally effective method of control of *C. coccodes* in tomato (214) and also in potato (216). In tomato, variety selection influences the fungicide program to be used, as highly susceptible tomato cultivars need significantly higher rates and frequent (seven day interval) applications (50) to produce a quality crop.

Research has focused on tomato to determine the most effective method and timing of fungicide applications (81,276). In Michigan, fungicide applications are recommended at planting in-furrow for control of potato black dot or as a foliar treatment for tomato anthracnose (32). Foliar fungicide applications may be needed even before symptoms start; curative fungicide applications may not provide enough control of black dot or tomato anthracnose since *C. coccodes* may already be present as a latent infection (213).

After the epidemics of onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose in 2010, these diseases were observed in 2011 and 2012. Michigan

onion and celery growers were concerned with the impact these diseases could have on their overall productivity. Therefore, the objectives of this research were i) to characterize the collection of *Colletotrichum* spp. isolated from onion and celery using conidial morphology, and molecular markers, ii) to investigate the pathogenicity and virulence among isolates collected, iii) to study the environmental conditions conducive to the development and progression of onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose, iv) to investigate sixteen onion cultivars susceptibility to *C. coccodes*, and v) to evaluate the efficacy of selected fungicide active ingredients to limit onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose under field conditions.

CHAPTER I

INVESTIGATING GENETIC AND PHENOTYPIC VARIABILITY OF *COLLETOTRICHUM COCCODES* INFECTING ONION (*ALLIUM CEPA*) IN MICHIGAN

ABSTRACT

Onion leaf and neck anthracnose caused by *Colletotrichum coccodes* is a new disease in Michigan not previously described in the U.S. or other onion producing areas in the world. Sampling of onion fields for *C. coccodes* was conducted in 37 fields in Calhoun, Ionia, Eaton, Kent, Montcalm, Newaygo, and Ottawa Counties from 2010 through 2012. Conidial size corresponded to those reported for *C. coccodes*. Species-specific primers amplified the expected 349 bp fragment size. No polymorphisms were observed among the four Inter Simple Sequence Repeat (ISSR) primers tested, but ISSR markers differentiated the *C. coccodes* isolates from other *Colletotrichum* spp. *Colletotrichum coccodes* isolated from tomato and potato in Michigan shared some loci with onion isolates but differences in loci were found. *C. coccodes* transcriptome derived Simple Sequence Repeat (SSR) marker from Expressed Sequence Tags (ESTs) were generated and 2,300 transcripts contained SSR motifs and suitable regions for primer design. Out of the 34 primer pairs tested, 70% amplified the expected product size and 30% of the primers failed to amplify. Polymorphisms were not observed among *C. coccodes* isolates from onion. Differences in virulence among *C. coccodes* isolates were tested by inoculating ripe cherry tomato fruit in greenhouse experiments. Significant differences in virulence were observed based on the field where the samples were obtained. In contrast, no significant differences were found for collection year, county or among isolates. Based on ISSR and SSR markers, the *C. coccodes* population isolated from onions appears to be clonal. However, differences in isolate virulence by field suggest the local environment has an effect on the aggressiveness of the isolates.

INTRODUCTION

The hectares of onion in the U.S. was 62,706 ha in 2012, and accounted for $944 million in revenue (15). The most important diseases in onion affect the onion foliage, and ultimately decrease bulb size (43,133). A new disease in onion, leaf and neck anthracnose was found across onion producing counties in Michigan in 2010. Symptoms of onion leaf and neck anthracnose include oval shaped lesions that are white to light green in color with a light pink to dark brown center that develop on the leaves and pseudostem. Signs of the pathogen (acervuli, conidia and setae) were present in the center of lesions and the causal agent was identified as *Colletotrichum coccodes* (Wallr.) Hughes.

Several species of *Colletotrichum* have been reported to infect and cause disease in onions including *Colletotrichum capsici*, *C. chardonianum*, *C. circinans C. dematium*, and *C. gloeosporioides* (97,157,248). In Michigan, *C. circinans* is an occasional pathogen that causes symptoms on maturing and stored onion bulbs, a disease known as smudge. *Colletotrichum gloeosporioides* has been reported in the tropics (90,112,232,248,280) and in Georgia, U.S. (211) causing onion twister. Typical twister symptoms include leaf distortion and neck elongation. Onion bulbs can suffer from a soft rot before harvest or in storage and these are associated with *C. gloeosporioides* (112).

Colletotrichum coccodes infects more than 18 hosts in the U.S. and 50 hosts world wide (97) including economically important crops such as potato, tomato and pepper. Tomato and pepper fruit, potato tubers, and also leaves, roots and stems can become infected by *C. coccodes* (50,83,138,202). *Colletotrichum coccodes* causes sunken lesions in ripe tomato fruit that can bear acervuli and conidia when environmental conditions are favorable (79). Black globose microsclerotia are the common sign of the pathogen in potato

roots, stems and tubers (12,276).

The systematics of the genus *Colletotrichum* has been problematic. Species designation has been based on morphological characteristics and host range (53,54) and more recently in polygenic phylogenies (54,70,143). *Colletotrichum coccodes* and *C. circinans* were thought at one time to be the same species (94,191) but are now recognized as distinct (54,94,143). Recently, Lie et. al (2013) designated a *C. coccodes* neotype (182). The distinctive morphological characteristic of *C. coccodes* are straight conidia 16–26 × 2.5–5 μm, with smoothed ends and slightly constricted mid-portion, and abundant black setose sclerotia (143,182). The most recent multi locus gene phylogeny of the genus *Colletotrichum* placed *C. coccodes* on the basis of the destructivum/ spaethianum/ graminicola clade (54).

No sexual stage has been observed for *C. coccodes* in nature or tested *in vitro* (54,81,143,276). The transference of genetic material through anastomosis has been proposed as a source of genetic variability in *C. coccodes* populations from potato (25) and has been documented in other *Colletotrichum* spp. (69,226,237). A *C. coccodes* species-specific primer was designed based on ITS sequences (67). Two sets of primer pairs in nested consecutive reactions were designed and tested to diagnose potato tubers infected with *C. coccodes* and to quantify the amount of pathogen in the soil (67).

Characterization of the genetic diversity within pathogen populations can be helpful in developing comprehensive management strategies. *Colletotrichum coccodes* isolates from potato have been described as genetically diverse (17,25). Isolate genetic variation has been studied by determining vegetative compatibility groups (VCGs) (9,17,25,215,251) and molecular markers (9,136). Six North American VCGs (17), seven European and Israeli

VCGs (215,251) and five Australian VCGs (25) have been identified from *C. coccodes* isolates from potato.

VCG testing is based on the generation of nitrate non utilizing (nit) mutants (64,215). This is a technique that is resource and time consuming and may result in unsuccessful paring, preventing VCG designation of isolates (136,215). Genetic molecular markers provide a reliable and rapid technique to investigate genetic diversity in plant pathogen populations (45,118,233). Molecular makers such as Amplified Fragment Length Polymorphism (AFLP) markers have provided similar information when compared to VCGs in *C. coccodes* (9,136). However, the drawback of using AFLP markers are that they are dominant markers that require multiple steps (enzymatic digestion, ligation and amplification), and need to be visualized in acrylamide gels or coupled with a fluorescent detection system (124).

Microsatellites are codominat PCR-based markers that consist of simple sequence repeats (SSR) sequences. Inter Simple Sequence Repeat (ISSR) use the tandem repeats as a primer, which anneal randomly across the genome and result in banding patterns (35). Microsatellites or SSR markers use primer pairs that flank specific tandem repeats resulting in individual PCR fragments. Genomic Microsatellites are SSR repeats located in non-coding regions while genic microsatellite are SSR located in coding regions of the genome (180,279). Both ISSR and SSR have been used to study populations of plant pathogens (49,124,200,233).

Differences in the virulence of *C. coccodes* isolates have been found in isolates from potatoes when experiments were conducted with potato plantlets dipped in inoculum (17,215), when the soil was infested (251), or when mature green tomatoes were injected

with inoculum (25). Isolate aggressiveness differed among different VCGs and geographical origin of the isolates (17,25). Information on genetic and phenotypic variability of *C. coccodes* isolates from onions can contribute to developing efficacious management strategies that account for the pathogen differences in aggressiveness. Therefore, the objectives of this study were to i) genotype a subsample of *C. coccodes* isolates using genetic and genic markers, and ii) investigate the pathogenicity and virulence among isolates obtained from onions.

MATERIALS AND METHODS

Sample collection, and culture collection

In total, 37 onion fields in seven Michigan counties were sampled from 2010 to 2012 (Appendix A, Table A1). In 2010, 17 fields were sampled in the following counties; Calhoun (n=1), Ionia (n=1), Eaton (n=1), Kent (n=4), Newaygo (n=9) and Ottawa (n=1). In 2011, a total of 15 fields were sampled and included the following counties: Calhoun (n=3), Ionia (n=1), Eaton (n=1), Kent (n=1), Montcalm (n=1) and Newaygo (n=8). In 2012, a total of 5 fields were sampled in Ionia (n=1), Kent (n=1) and Newaygo (n=3) Counties.

Isolations were taken by excising a 5 mm^2 square from the margin of foliar or neck lesions and placing four tissue squares onto 50% strength potato dextrose agar (PDA) amended with rifampicin (30 mg/ml) and ampicillin (100 mg/ml). Plates where incubated at 24 ± 2^{o}C under continuous fluorescent light for 7 to 12 days. Transfers were conducted to obtain pure cultures. Once the 7-day old cultures were pure, single spore isolates were obtained by collecting a 4-mm culture plug with a sterile toothpick and placing it into 500 µl of sterile distilled water. These conidial suspensions were vortexed and conidial concentration was calculated using a hematocytometer (Hausser Scientific, Horsham, PA).

Aliquots of conidial suspensions (1:100 or 1:10 dilution) were spread onto water agar plates with a sterile cell spreader. After plates were incubated for 16 h in the dark, a germinated spore was excised from the media and transferred to 50% strength PDA. Seven-day old cultures were used for two long-term storage techniques; 1) a mycelia plug was added into 700 µl glycerol, mixed using a vortex and kept at -20^{o}C, and 2) conidial suspensions were preserved on cryoconservation media (20% glycerol, 0.04% yeast extract, 0.1% malt extract, 0.04% glucose, 0.02% K_2HPO_4) at -80^{o}C; both techniques were modified from Dhingra and Sinclair (77).

Morphological characterization

Microscopic characteristics were examined for 51 *Colletotrichum* isolates obtained from onion. Monoconidial isolates were grown on 25% strength PDA and incubated at 21 ± 2^{o}C under fluorescent light for seven days. Conidial masses were then scraped from the colonies with a sterile toothpick and mounted on a glass slide with 15 µl of lactophenol solution (100 ml lactophenol and 10 ml glacial acetic acid) and sealed with Cytoseal 60 (Thermo Scientific; Waltham, MA). Permanent slides were then inspected with a light microscope Olympus BX43 (Olympus, Center Valley, PA) and photographs were taken with a Leica Digital camera DC 300 (Leica Microsystems, Wetzlar, Germany). For each isolate, conidial length and width of 10 randomly chosen conidia was measured. The experiment was replicated in time twice.

DNA extraction

Colletotrichum spp. isolates were transferred to 35 ml of 50% strength potato dextrose broth and incubated on a rotary shaker (MaxQ™ 4000 Thermo Scientific;

Waltham, MA) for 7 days at 23 ± 2°C. Mycelial masses were vacuum-filtered and lyophilized overnight in a FreeZone 1 Liter Benchtop Freeze Dry System (Labconco, Kansas city, MO). Dry mycelium was then macerated and 500 µl of extraction buffer (AutoGen, Inc; Holliston, MA) were added. The samples were submitted to the MSU Research Technology Support Facility (East Lansing, MI) for robotically assisted DNA extraction using AutoGen 740 (AutoGen Inc; Holliston, MA). DNA concentration was quantified using NanoDrop ND 1000 spectrophotometer (Thermo scientific; Wilmington, DE) and NanoDrop 2.4.7c software (Thermo scientific; Wilmington, DE). DNA quality and integrity was analyzed by electrophoresis in 1% (wt/vol) agarose gel in 0.5X Tris-borate-EDTA buffer (52) and stained with ethidium bromide (5 µg/ml) for visualization (190). DNA was diluted to 10 ng/µl for PCR amplification.

Species-specific primers

The species-specific marker developed by Cullen et al. (67) to amplify the ITS 1-2 region on nested reactions, was tested on a pilot containing DNA of *C. coccodes* from Michigan onions (*n*=2), potato (*n*=1, courtesy of W. Kirk, MSU), tomato (*n*=2) and *C. dematium* (courtesy of A. Schilder, MSU). Amplification was conducted according to Cullen et al. (67), but using pfu DNA polymerase (Agilent Technologies, Santa Clara, CA).

ISSR markers

Five ISSR primers (GTC5, CAG5, GACAC3, GACA4 and TCC5) were tested on a pilot of six *Colletotrichum* spp. from Michigan that included *C. coccodes* isolated from onions (*n*=2), potato (*n*=1, courtesy of W. Kirk, MSU), tomato (*n*=2) and *C. dematium* (courtesy of A. Schilder, MSU). Once amplification conditions were established, ISSR markers were amplified on 54 isolates including the isolates described above, including *C.*

coccodes from onions (*n*=47) and *C. gloeosporioides* (courtesy of A. Schilder, MSU). PCR reactions were carried out on final volumes of 20 µl and containing 50 ng of genomic DNA, 1 mM of primer, 1X PCR buffer (Promega Corp; Madison, WI), 250 mM of total dNTP and 1.25 units of Go Taq polymerase (Promega Corp; Madison, WI). Markers were amplified on an Eppendorf mastercycler ep systems thermal cycler (Eppendorf, Westbury, NY) using the following program: hot start of 95°C for 1 min; then 30 cycles of 95°C denaturing for 30 seconds; 48°C (GACAC3, GACA4 and TCC5) or 60°C (CGA5 and GTG5) annealing for 30 sec and 72°C extension for 1.5 min. ISSR PCR fragments were separated on 2% agarose gels stained with ethidium bromide and a 100 bp or a 1 Kb DNA ladder (New England biolabs Inc; Ipswich, MA).

RNA extraction, purification and sequencing

Five, seven and fourteen-day-old cultures of *C. coccodes* isolate 38-1-3-1 were used for RNA extraction. Mycelium was scraped from the surface of the cultures growing on PDA using RNase free water and sterile tongue depressors. Mycelia were transferred into 1.7 ml microcentifuge tubes. Tubes were kept in dry ice while retrieving scrapings, and stored at -80°C until lyophilized. Mycelia were lyophilized as described above for DNA extraction. Total RNA was extracted using Triazol (Invitrogen, Life Technologies; Grand Island, NY) following the procedure described by Hallen et al. (131). Total RNA was DNase treated using the turbo DNA-free Kit (Ambion- Applied biosystems, Carlsbad CA). RNA concentration and quality was determined using the Bioanalyzer 2100 (Agilent Technologies, San Diego, CA). The RNA obtained from the different growth stages was mixed into a 1:1:1 proportion and submitted to MSU Research Technology Support Facility

(East Lansing, MI) where RNA-Sequencing (RNA-Seq) libraries were constructed and sequenced using Illumina HiSeq platform (Illumina, San Diego, CA).

Transcriptome data analysis, SSR finding

RNA-Seq reads were analyzed using FastQC (Babrahan Bioinfomatics, Cambridge, UK) and FastX Tool kit (Cold Spring Harbor Laboratory, Cold Spring Harbor NY) for base call quality, trimming of barcodes and primers. Cleaned reads were used to construct a transcriptome assembly using Oases (246). The assembled *C. coccodes* transcriptome was compared with the annotated *C. graminicola* genome (223) and the uniref database (269) using BlastX (10). SSR motifs were identified and primers that flank the SSRs were designed as described in Hamilton et al (132).

SSR marker selection and amplification

A set of 34 SSR markers (Appendix A, Table A4). were tested for amplification in two *C. coccodes* isolates from onion, one from potato and two isolates from tomato. Primer selection was based on gene type, 20 primer sets were selected on genes homologs with *C. graminicola* genes and 14 primers on *C. coccodes* specific genes. SSR primer flanking trinucleotides (n=18), tetranucleotides (n=2), pentanucleotides (n=6), and hexanucleotides (n=8) motifs were tested. PCR reactions were tested as a multiplex (including up to 3 primer pair per reaction) or a single primer pair per reaction. Multiplex reactions used primers with expected PCR product sizes that differed by >50 bp. The PCR reactions were carried out on a 25 µl final volume containing 30 ng of genomic DNA, 0.1 mM of each of the forward and reverse primers, 1X PCR buffer (Promega Corp; Madison, WI), 250 mM of total dNTP and 0.5 units of Go Taq polymerase (Promega Corp; Madison, WI). Markers were amplified on an Eppendorf mastercycler ep systems thermal cycler (Eppendorf,

Westbury, NY) using the following program: hot start of 93^{o}C for 4 min; then 30 cycles of 93^{o}C denaturing for 1 min; 55^{o}C annealing for 1 min and 72^{o}C extension for 2 min; and final extension at 72^{o}C for 5 min. PCR fragments were visualized in 4% agarose and stained with ethidium bromide as previously described for ISSR markers. Once amplification conditions were optimal, SSR primers were amplified on a larger isolate panel through pooling *C. coccodes* isolate DNA by collection field (n=35 onion fields) and collection hosts; tomato (n=3), and potato (n=1 from Michigan courtesy of W. Kirk MSU, n=4 from Idaho, courtesy of P. Wharton, University of Idaho).

Virulence experiments

A total of 827 *C. coccodes* isolates were included in the virulence experiments, including 824 *C. coccodes* isolates obtained from onion, two *C. coccodes* isolates from tomato, and one *C. coccodes* isolate from potato (courtesy of W. Kirk, MSU). To test the virulence of the *C. coccodes* isolates, 'Sun Gold' cherry tomato (Johnny seeds, Fairfield, ME) seeds were planted in 412 cm^{3} cells (72 square cell flats, Hummert International, Earth City, MO) and transplanted into 3.8 liter pots (Hummert International, Earth City, MO). After four weeks, seedlings were transplanted into a field plot (MSU Plant Pathology Farm, East Lansing MI) in raised plant beds 0.76 m apart and following cultural practices including the use of plastic mulch (31,72). Ripe cherry tomato fruit were hand harvested and visually inspected for mechanical damage. Selected tomato fruit were surface disinfested with 1% sodium hypochlorite for 5 min, rinsed three times in sterile water for 2 min and air dried at room temperature. Once tomatoes were dried, tomato fruit were placed into a handmade humid chamber. The humidity chamber consisted of a greenhouse bench

(123 cm wide x 293 cm long) covered and enclosed with clear plastic bags. Damp paper towels were placed within the humid chamber to promote high relative humidity (RH). A WatchDog (Spectrum Technologies, Inc., Plainfield, IL) was placed inside the humid chamber to record temperature and RH during pre-incubation and incubation time. Tomatoes were incubated at 95 ± 4% RH for 16 h prior to inoculation. *Colletotrichum coccodes* conidial suspensions were prepared using distilled water and adjusted to 2.0 x 10^5 conidia/ml. Tomatoes were wounded with a sterile pin and 10 µl of conidial suspension placed on the wound. After inoculation, tomatoes were enclosed in a tent at 84 ± 7% RH for 7 days on the greenhouse bench.

After incubation, pictures of the tomatoes were taken using a black background under natural light (greenhouse). Images were enhanced using the software ImageJ (1) to facilitate lesion segmentation in APS ASSESS 2.0 software (168). To make use of the built in segmentation functionality of ASSESS, image backgrounds were changed from black to blue and the red and green color channels were switched. This process does not modify the size or color of the lesion, but has the effect of making the red tomatoes appear green allowing more efficient image processing using ASSESS 2.0 software. A Macro was programmed to import images, calibrate and segment the tomatoes, while lesion area measurement was done using the digital ruler option (free hand closed curve area).

Experimental design and statistical analysis

Virulence experiments were conducted as a complete randomized design with two tomato fruit (experimental units) per isolate (n=827) and replicated in time on 12, 20 and 28 September 2013. The PROC MIXED in the SAS statistical analysis software version 9.2 (SAS Institute Inc., Cary NC) was used to test mean lesion area differences among county,

field or isolates. Data were tested for normality (q-q plots and the Shapiro Wilk test) and homogeneity of variance (Levene's test) of the residuals. ANOVA was evaluated using type III test for fixed effects output. Significant differences ($P<0.05$) were subject to grouping by Least Significant Differences (LSD).

RESULTS

Onion leaf and neck anthracnose was observed in onion fields in the 2010, 2011 and 2012 growing seasons. Lesions caused by *C. coccodes* were found alone or in combination with purple blotch and/or Stemphylium leaf blotch lesions. Onion leaf and neck anthracnose symptoms included oval lesions located on leaves and neck. Lesions appeared bleached to light green in color with a salmon to brown center. A total of 901 *C. coccodes* isolates were obtained from symptomatic onion plants. A total of 122, 681, and 98 *C. coccodes* isolates were recovered in 2010, 2011, and 2012, respectively (Appendix A, Table A1).

Colletotrichum coccodes colonies were initially observed as pale white mycelia three days after transfer, developing into a light gray to salmon colored colonies five five days after incubation. Colony zones (spots) changed from light gray to dark black as microsclerotia formed in the plates 10 to 14 days after incubation. Hyaline cylindrical conidia with rounded to obtuse ends were observed. Conidia size ranged between 9.9 to 16.7 μm long and 2.7 to 4.54 μm wide, and had a length width ratio ranging from 3.24 to 4.85 (Table 1, Appendix A, Table A2).

The species-specific marker resulted in the expected 349 bp fragment on *C. coccodes* from onion (n=4) and potato (n=1) but not on *C. dematium*. Primers GTC5, CAG5, GACAC3, GACA4 amplified on the six isolate pilot while the primer TCC5 did not

amplify. Banding patterns comprised at least 2 bands with a maximum of 5 bands (Table 2). When markers were amplified on a larger panel of isolates, *C. coccodes* isolates from onion (n=49) showed identical banding pattern for all four ISSR primers tested. *Colletotrichum coccodes* isolated from potato or tomato shared bands with *C. coccodes* from onion but had unique bands on primers CAG5 and GACAC3 (Table 2). *C dematium* and C. *gloeosporioides* resulted in unique banding patterns completely different from *C. coccodes* isolates (Figure 1A-C).

Table 1. Conidial size characteristics of *Colletotrichum coccodes* isolated from onion in 2010. Means pooled by collection county and field in Michigan.

			Conidial dimensions					
	Collection	No.	Length (µm)		Width (µm)		L/W Ratio	
County	Field	Isolates	Mean	SE[y]	Mean	SE	Mean	SE
Ionia	24	3	13.6	0.4	3.4	0.1	4.1	0.1
Kent	14	6	14.4	0.4	3.4	0.1	4.2	0.1
Kent	15	1	13.5	0.9	3.6	0.1	3.8	0.2
Kent	32	1	13.1	0.5	3.7	0.1	3.6	0.2
Kent	34	8	15.9	0.3	3.6	0.1	4.5	0.1
Newaygo	3	8	11.8	0.3	3.0	0.1	4.1	0.1
Newaygo	4	7	10.5	0.5	2.9	0.1	3.7	0.1
Newaygo	5	1	13.3	0.7	3.3	0.1	4.1	0.2
Newaygo	6	2	10.3	0.8	2.8	0.2	3.9	0.2
Newaygo	7	11	10.3	0.3	3.7	0.8	3.6	0.1
Newaygo	26	1	12.2	0.5	3.7	0.1	3.3	0.2
Newaygo	27	1	12.3	0.5	3.1	0.1	4.1	0.2
Newaygo	36	1	13.7	0.6	3.6	0.2	4.1	0.3

[y]SE= Standard error.

A total of 12,335 *C. coccodes* genes were sequenced. SSR were found on 2,455 transcripts and a total of 2,300 SSR had designed primer pairs (Appendix A, Table A3). The most abundant SSR types were trinucleotides, tetranucleotides and dinucleotides found on 1306, 383 and 359 transcripts respectively. Suitable regions for primer design were found in 92 to 94% of the *transcript*s that contained dinucleotides, trinucleotides,

tetranucleotides and pentanucleotides SSR repetitions. In contrast, 40 and 76% of the transcripts containing mononucleotide and hexanucleotide had suitable regions for primer design (Appendix A, Table A3).

A total of 34 *C. coccodes* genic SSR markers were used in this study. Amplification was successful for 22 primers out of the 34. A total of 17 primers resulted in the expected PCR product when amplified in multiplex reactions and five primer pairs resulted in the expected product size when amplified in individual reactions. A total of 12 primers did not amplify in multiplex or single reactions.

Table 2. Number of loci amplified with ISSR on *Colletotrichum coccodes* isolated from onion, potato and tomato in Michigan compared with *C. dematium* and *C. gloeosporioides*.

Colletotrichum spp.	No. loci (bands)				
	C. coccodes			*C. dematium* Cd 123	*C. gloeosporioides* Cg 104
Host / Primer motif	Onion (n=49)	Potato (n=1)	Tomato (n=2)	N.a (n=1)	N.a (n=1)
CAG5	3	1[z]	1[z]		
		3	1	4	5
GACAC3	2	1[z]	2[z]		
		1		2	1
GTG5	5	3[z]	3[z]		1[z]
			1	4	4
GACA4	3	2[z]	2[z]		
				4	3

[z]Same band(s) size compared to *C. coccodes* isolated from onion.
N.a= unknown

No polymorphisms were observed in 22 primer pairs on the 110 *C. coccodes* isolates from onions in this survey. Fifteen primer pairs (CO2096, CO2290, CO2447, CO3076, CO3737, CO4187, CO4230, CO4342, CO4383, COS134, COS143, COS191, COS202, COS240 and COS307) showed identical PCR band sizes among *C. coccodes* isolated from onion, tomato and potato. However, PCR products did not amplify for primers COS210 and

COS300 on *C. coccodes* DNA from Michigan and Idaho potato isolates. Primers CO2983 and CO752 amplify the same band in *C. coccodes* from Michigan (onion, tomato and potato) but did not amplify Idaho isolates.

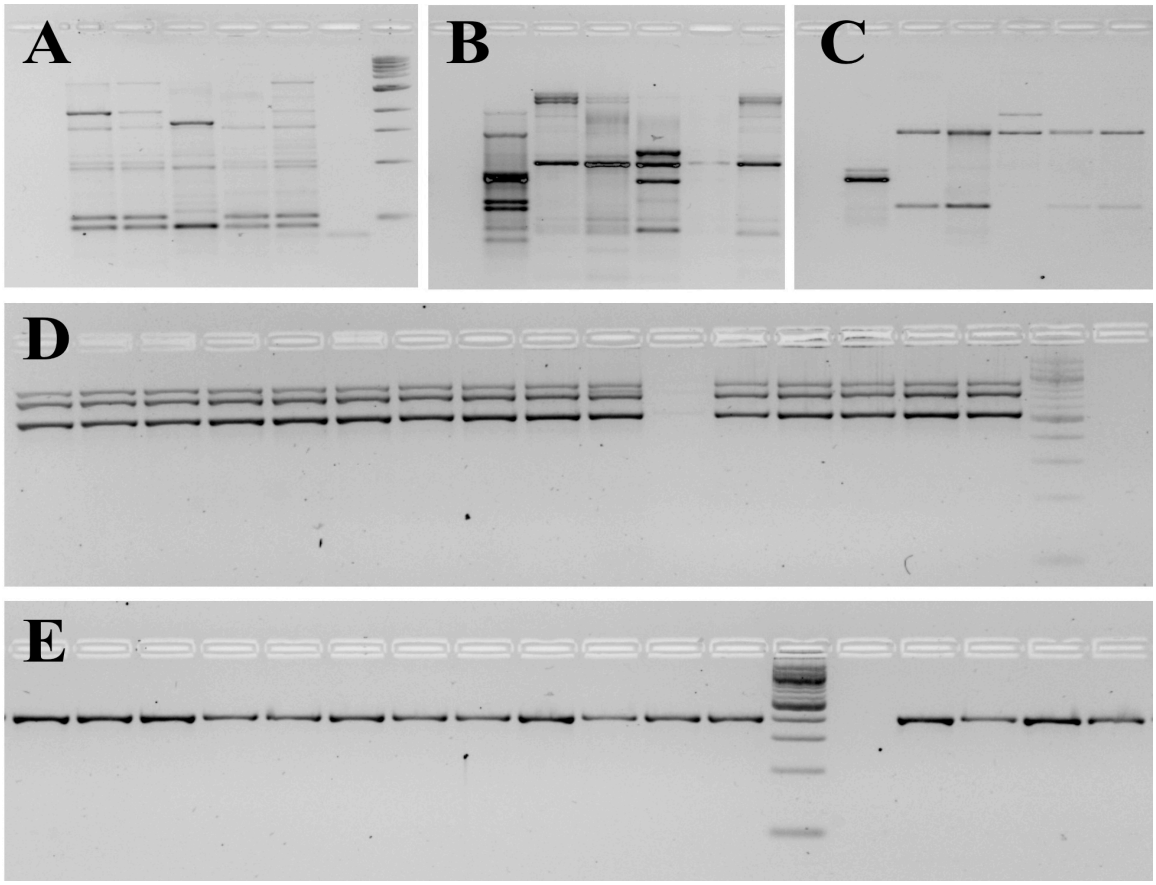

Figure 1. PCR amplification products obtained with A, ISSR marker GTC5 amplified on DNA of isolates Cd123 (*Colletotrichum dematium*), *Colletotrichum coccodes* 7-1-1-3 (onion MI), 40-36 (onion MI), Cco01 (potato MI), TI, (tomato MI), TR (tomato MI) blank and ladder. B, CAG5 blank, Cd123 (*C. dematium*), *Colletotrichum coccodes* 7-1-1-3 (onion MI), 40-36 (onion MI), Cco01 (potato MI), TI, (tomato MI) and TR (tomato MI). C, GACA3 in same isolate distribution asCGA5. D, SSR primers COS240, CO3076, CO4342 in multiplex reaction and E, SSR primers COS210 individual reaction.

Primer CO2530 amplified identical band sizes for *C. coccodes* isolated from tomato and onion, but failed to amplify on isolates from potato (MI and ID). Primers COS309 amplified on onion isolates and ID potato isolates, but failed to amplify on Michigan tomato and potato isolates. Primer COS132 amplified identical band size in onion, tomato, and

potato isolated from ID but failed to amplify on the isolate for MI potato.

All *C. coccodes* isolates tested (n=827) caused lesions on the tomato fruit, including those isolated from onion and potatoes. Lesions on tomato fruit developed as circular to elliptical, sunken and discolored areas. No significant differences in lesion area were observed among *C. coccodes* isolates (P= 0.209). *Colletotrichum coccodes* isolated from onions had similar lesion size compared with the control *C. coccodes* isolated from tomato or potato (Dunnet- Hsu adjustment P= 0.99). No significant differences in lesion area were found among collection year (P=0.72) or among counties (P=0.3932). However, significant differences in lesion area were found among the fields where the isolates were collected (P=0.005, Figure 2).

DISCUSSION

Onion leaf and neck anthracnose was found in several onion producing counties in Michigan over a three-year period. A subsample of *Colletotrichum* spp. isolates associated with these lesions was characterized . Colony appearance of isolates showed little variation, and abundant microsclerotia production in culture. Conidial size and shape was consistent to the *C. coccodes* (Wallr.) Hughes (143) and the neotype (182) descriptions, even though some variation in size was observed. ISSR markers differentiated among *Colletotrichum* spp., for example *C. dematium* and *C. gloeosporioides* had completely different banding patterns compared to *C. coccodes* from onion, potato and tomato, which had similar banding patterns. Few differences in band presence/absence were found among *C. coccodes* isolates by host. It is possible that certain genotypes of *C. coccodes* are associated with specific hosts. Additional ISSR primers and isolates from various hosts could clarify genetic differences among *C. coccodes* isolates from various hosts. However, banding pattern

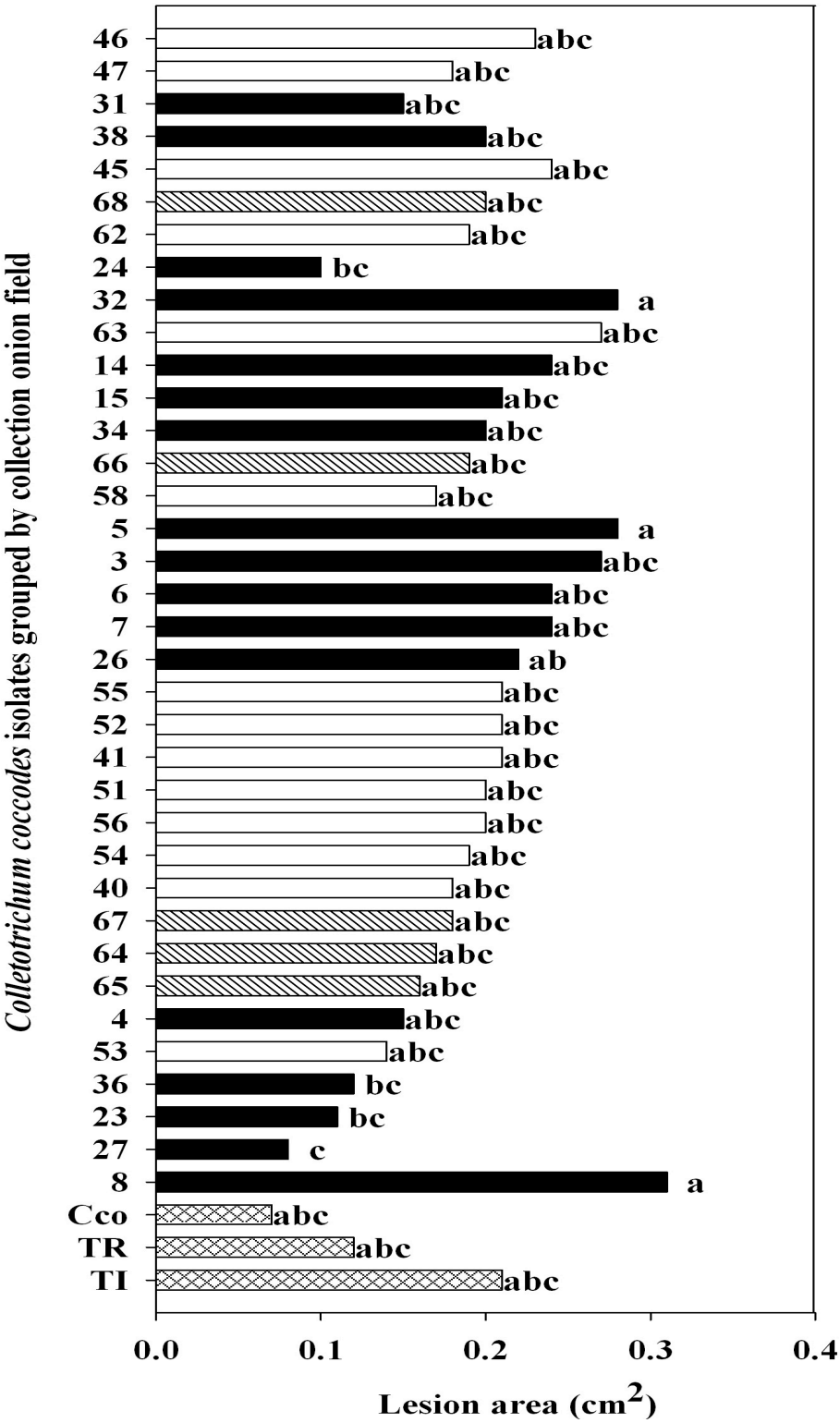

Figure 2. *Colletotrichum coccodes* virulence on 'sun gold' cherry tomatoes inoculated with *C. coccodes* isolates recovered from onion in 2010 (solid bars), 2011 (open bars) and 2012 (hatched bars), tomato and potato (crosshatched bars) in Michigan. Mean lesion size pooled by collection field, and county: C=Calhoun, E=Eaton, I=Ionia, K=Kent, M=Montcalm,

Figure 2. (cont'd). N=Newaygo and O= Ottawa. Bar with common letters are not significantly different, LSD *P*=0.05.

reproducibility and loci scoring was a drawback from this technique in this study and others (265).

Recently Liu et al.(181) investigated the phylogeny/taxonomy of 33 *C. coccodes* isolates from various hosts. As a result, *C. coccodes sensu latu* was divided into two main clades. Isolates from tomato and pepper were present in both clades while isolates from potato were clustered in clade one. The latter clade was designated as *C. coccodes*, while isolates in clade two were designated as *C. nigrum*. This may explain the loci difference between *C. coccodes* from different hosts in these experiments. The relationship of the *C. coccodes* isolates from onion and their appropriate taxonomic designation should be revised based on the most current species separation.

SSR marker finding based on the *C. coccodes* transcriptome resulted in 2,300 SSR primer pairs that can be used in *C. coccodes* population studies with isolates recovered from onions and potentially from other hosts. The major advantage of generating SSR marker from the organisms transcriptome, public EST or genomic sequences, is the potential for lower clustering rates in the genome. Lower clustering rates were observed in linkage maps in sugar cane (62) and grape (249) compared with SSR primers generated from enriched libraries (33,124). Random distribution (independence) of markers across the genome is highly desirable for population genetic studies (45).

The variation of the *C. coccodes* population on genic SSR markers, a technique used in other organisms (125,279) was investigated. Out of the twenty-two SSR markers that yielded the expected PCR products, no polymorphic alleles were observed among the 168

C. coccodes isolates from onion. It is possible that genetic variation among *C. coccodes* isolates from onion exists on a finer scale undetectable in agarose gels. SSR bands could be visualized in acrylamide gels; or primers could be fluorescent labeled and fragments analyzed in a sequencer. The latter will not only detect polymorphism on SSR motif repetition but also Single Nucleotide Polymorphisms (SNP).

Asexual reproduction is thought to be favored in *C. coccodes* since a teleomorph has not been observed in nature or described in laboratory parings (54,81,143,276). The lack of sexual reproduction restricts gene flow, but genotype flow is still possible on asexual pathogens when genetically diverse isolates are introduced into the field. The data support the *C. coccodes* population recovered from onions is clonal. The lack of polymorphism observed in both genetic (ISSR) and genic (SSR) markers, indicate low genotype migration as the underlying lack of genetic diversity.

Genetic complexity of the host is a factor that promotes polymorphism within pathogen population (270). Most of the commercial onion cultivars planted in Michigan and other onion producing areas in the U. S. are F1 hybrids. The lack of genotypic differences of *C. coccodes* population may be related to the narrow gene base of the onion cultivars commercially available (43).

Sources of disease resistance have been found on onion landraces from the center of genetic diversity in eastern Mediterranean countries (43), and open pollinated Alliums with high allelic diversity (43,193). Screening onion genotypes for their response to *C. coccodes* isolates will serve to identify onion genotypes as sources of resistance to include in breeding programs and ultimately develop new onion cultivars with resistance to *C. coccodes*.

Variation in virulence was observed when isolates were grouped by the onion field they were recovered from, which could indicate a local environmental effect. Therefore, local differentiation may be influenced by the microclimate of the field or particular cultural practices (variety selection, weed management, irrigation, fertilization, etc.) utilized for each field (66,116). Studies on *Colletotrichum cereale* and *C. kahawae* (66,253) presented evidence that local adaptation and host specialization play an important role in the structure of emergent *Colletotrichum* populations. Host shift speciation is one of the main routes for emergence of fungal pathogens (116,294), and this hypothesis could be the case in the *C. coccodes* onion pathosystem.

The *C. coccodes* isolates tested caused in similar lesion areas in cherry tomatoes, and no significant differences in virulence were found among the 827 *C. coccodes* isolates. Likewise, no significant differences in virulence were found when comparing among collection years. Even though replications in time were not significantly different, a great portion of the variance remained unexplained in the statistical model. Two factors that could play a role are i) uncovered relationship among county, field and/or year, and ii) variance due to tomato fruit physiological stage. Sampling for *C. coccodes* infecting onions was completed at random per field, county and year. Growers rotate onion fields every year and therefore it was not possible to return to the same field every year and collect true replicates per field and county. Therefore, it was not possible to account for the variance in the statistical model that could explain some of the variability, but instead every factor was investigated separately (isolates, fields, years).

Virulence testing among isolates of *C. coccodes* collected from onion was conducted using tomato fruit and proved to be an effective. Similar techniques were used with isolates

collected from potato plants (25,26). However, the physiological stage of the tomato fruit has an effect on tomato anthracnose lesion development (26,81,155) and this factor could play a role on the variability observed within isolates collected form onion.

No significant differences in lesion area were observed among *C. coccodes* isolated from various hosts (onion, tomato or potato). However, only three isolates of *C. coccodes* from tomato (n=2) and potato (n=1) collected in Michigan were included in these experiments and differences may be uncovered by including isolates of *C. coccodes* from several hosts and regions. For instance, 18 regional VCGs have been identified in *C. coccodes* isolates from potato (17,25,215,251). Limited gene flow between isolates from Australian, European and North American VCGs have been reported (17,25). *Colletotrichum coccodes* infected potato seed could introduce migrants to *C. coccodes* populations among potato growing regions (25).

Michigan growers rarely rotate onions with potatoes, tomatoes or peppers. *Colletotrichum coccodes* is know to be a seed borne pathogen in tomato and potato (27), therefore, the introduction of *C. coccodes* on contaminated onion seed could act as a founder effect. Based on the genetic and genic markers, the population recovered from onion fields in Michigan over a three-year period was clonal. Virulence studies also supported that a clonal population with limited differences in isolate agresiveness was due to a local environmental effect. Once *C. coccodes* has been introduced into a field, this pathogen can prevail in or onassociation with several weed species (231), overwintering in perennial weeds, weed and crop debris, and as microsclerotia in the soil (81,82). Movement of farm machinery among fields may be implicated in spread out of *C. coccodes* microsclerotia.

To summarize, the results support the hypothesis of a local clonal population of *C. coccodes*. Differences in virulence among isolates collected from onion fields were evident, but with overall phenotypic and genetic similarities. A better understanding of the emergence of *C. coccodes* as a pathogen of onion may benefit from additional population studies. Investigating additional genomic and fine genic polymorphism of *C. coccodes* isolated from onion and from various hosts can provide a better understanding of this emerging pathogen and investigate a potential host shift from Solanaceous crops to onions.

CHAPTER II

EFFECT OF INOCULATION METHOD, TEMPERATURE, AND HUMIDITY PERIOD ON FOLIAR INFECTION OF ONION BY *COLLETOTRICHUM COCCODES*.

ABSTRACT

Onion leaf and neck anthracnose caused by *Colletotrichum coccodes* was recently confirmed in Michigan; this pathogen has not previously been reported on onion in the U.S. Greenhouse or growth chamber experiments were conducted to study the effect of inoculation method, temperature, and duration of high (>80%) relative humidity (RH) on foliar infection of onion seedlings. When 'Prince' onion seedlings growing in the greenhouse were inoculated with a conidial suspension of *C. coccodes* (alone or with an abrasive agent), final foliar disease severity was higher (>38%) than methods using 2 or 5g of infested millet seed that was either dried or not (≤24%). Growth chamber studies were conducted to determine the effect of temperature and the duration of high RH on onion leaf and neck anthracnose severity on 'Infinity' onion seedlings when inoculated with a conidial suspension spray. Combinations of temperature (15, 20, 25 or 30°C) and high RH period (0, 12, 24, 48 or 72 h) were factors in a split plot design. Following the designated high RH treatment, severity (symptomatic leaf area %) was evaluated over a four-week period; plants remained at their assigned temperature inside growth chambers with RH of 54 ± 19%. Significant differences and interactions among temperature and high RH period were observed. The combination of high temperature (≥25°C) and extended (≥24h) high RH period resulted in more that 20% of the foliage covered with *C. coccodes* lesions. The combined effect of temperature and high RH period explained 91% of the variance in onion anthracnose severity when modeled with a non-linear Gaussian model. The results suggest that onion leaf and neck anthracnose symptoms are likely to be more severe when the environmental conditions are >15ºC with >12h of high RH.

INTRODUCTION

In 2012, 60,000 ha of onions were planted in the U.S. with a crop value of $944 million; onions produced more than $7.7 million in revenue for Michigan growers (15). Pungent onions suitable for storage following harvest are the mainstay of the Michigan industry; the state ranks fifth nationally after California, Colorado, New York and Idaho (15). *Alternaria porri* (purple blotch), *Stemphylium vesicarium* (Stemphylium leaf blotch), *Botrytis squamosa* (Botrytis leaf blight), and *Peronospora destructor* (downy mildew) are foliar pathogens that commonly occur in Michigan and result in blighting that can reduce yields (133).

Colletotrichum coccodes (Wallr.) Hughes was recently reported as a new pathogen on onion foliage in Michigan and was the first U.S. report (239). *Colletotrichum coccodes* causes elliptical lesions on the onion foliage and pseudostem (neck). Initially, lesions appear sunken and light green to bleached in color with a distinct center. Over time, the center of the lesion may develop a color ranging from salmon to dark brown. Anthracnose lesions may appear to be similar in shape to those caused by IYSV, *A. porri*, and *S. vesicarium* (248), but signs of *C. coccodes* are readily evident viewed through a dissecting microscope or a hand lens (239).

Although *C. coccodes* has been reported to cause disease on 50 different hosts worldwide and 18 hosts within the U.S. (97) it is of primary importance as a pathogen of Solanaceous hosts (81,276). In potato, *C. coccodes* can result in a reduction of tuber size by 16 to 30% (176,212,213). In tomato, *C. coccodes* causes 30 to 70% fruit rot when fungicide sprays are not applied to manage tomato anthracnose (51).

In pepper, more severe foliar symptoms were observed when foliage was inoculated using a *C. coccodes* conidial suspension when compared with using a soil drench (138). Foliar infection of pepper was favored by a temperature of 28°C and a wetness period longer than 24 h (138). Potato foliage inoculated with a *C. coccodes* conidial suspension or a sclerotia suspension resulted in severe foliar symptoms and yield reduction (149,151,202); foliar infection was favored at wetness periods of 24 and 48 h (151).

Colletotrichum coccodes is an important tomato fruit rot pathogen with infection and lesion formation occurring under conditions that include 10 or more hours of continuous humidity and temperatures ranging from 15 to 31°C (79,155); optimal lesion development occurs at 27°C (79,80,155). Byrne et al. studied *C. coccodes* foliar infection in tomato by using a conidial suspension and determined that temperatures of 20 and 25°C in combination with 12 and 16 h of continuous leaf wetness were most favorable (52). Using stepwise regression, Sanogo et al. determined that the numbers of hours without rainfall (within 4-day intervals) predicted 72% of the variation of *C. coccodes* incidence on tomato fruit in the field (244). Combinations of temperature, wetness duration or solar radiation were also significantly correlated with anthracnose incidence (244).

Since *C. coccodes* was first detected in Michigan onion fields in 2010, onion leaf and neck anthracnose has been observed annually in the state. Additional fungicide costs and grower observed yield losses due to this disease have reduced profits for the Michigan onion industry. In order to offer Michigan growers a comprehensive management strategy to limit onion leaf and neck anthracnose, data on the environmental conditions conducive to *C. coccodes* infection and disease progression on onion foliage are needed. The objective of

this study was to test *C. coccodes* inoculation methods using onion seedlings and to determine the effects of temperature and high relative humidity (RH) period on onion leaf and neck anthracnose severity.

MATERIALS AND METHODS

Plant culture and experimental design

Onion seed 'Prince' and 'Infinity' (Bejo, Oceano, CA, and Nunhems, Parma, ID, respectively) were planted into SUREMIX perlite media (Michigan Grower Products, Inc., Galesburg, MI) in 288-cell flats (12 x 24 x 4.5 cm). Onion seedlings were transplanted into 10-cm pots (387 cm^3) when plants were in the three-leaf stage. The seedlings were then grown for 14 days in the greenhouse (25 ± 2^oC, 15 h photoperiod) where they were watered as needed and fertilized weekly with 20-20-20 fertilizer (The Scotts Company, Marysville, OH) and full-strength Hoaglands solution (137).

To investigate inoculation methods 'Prince' onion seedlings were used. The experiment consisted of five inoculation methods with five plants (experimental units) per method. Inoculation methods were tested in a complete randomized design (CRD), replicated in time (8 and 22 February 2011). The inoculation methods included: i) conidial suspension spray, ii) conidial suspension spray mixed with an abrasive agent, iii) conidial suspension applied as a drench, iv) infested millet seed (2 or 5g) subjected to a drying process (dry) and v) infested millet seed (2 or 5g) not subjected to a drying process (wet).

To determine the temperature and duration of high RH optimum for *C. coccodes* infection, growth chambers (Conviron: model CMP3244, Pembina ND) were set at 15, 20, 25 or 30^oC with a 16/8h photoperiod and 95 mE of light intensity. 'Infinity' onion seedlings

were moved into each of the four different growth chambers in a randomized split plot design. In the design, temperature was the whole plot and high RH period was the subplot. The high RH (≥80±15%) periods tested were 0, 12, 24, 48, or 72 h. The experiment had two experimental units (two plants) per temperature and high RH period combination. Growth chamber experiments were conducted 3 times in two years (14 February 2011, 5 April 2011 and 4 March 2012).

Inoculum preparation

Colletotrichum coccodes isolates were obtained from onion foliage in 2010 and used for both experiments. The isolates originated from various counties in Michigan and included: 7-1-1-3 (Newaygo Co.), 8-1-1-1 (Ottawa Co.), 24-1-1-2 (Ionia Co.), 31-1-2-2 (Calhoun Co.), 34-1-6-1 (Kent Co.) and 38-1-3-1 (Eaton Co.). Isolates were transferred from long-term storage onto 50% strength PDA, and maintained at 22 ± 2°C under fluorescent light. To prepare the conidial suspension, seven-day-old cultures of *C. coccodes* were flooded with 1.0 ml of double distilled water with 0.001% Tween and conidia were dislodged with a sterile tongue depressor. The conidial suspension was filtered through three layers of sterile cheesecloth. The concentration was determined using a hemocytometer and the suspension was adjusted to 2.0×10^5 conidia/ml using sterile double distilled water with 0.001% Tween. Equal volumes of conidial suspensions per isolate were mixed and the resulting combination was used to inoculate the onion seedlings. When an abrasive agent was used with a conidial suspension, the 0.001% Tween water was amended with 0.001% (w/v) Concern-diatomaceous earth (Woodstream, Lititz, PA).

The millet seed inoculum was prepared by mixing 100 g of millet seed and 15 ml of water in a glass flask and autoclaving in two consecutive cycles. Eight 0.7 mm plugs of

seven-day-old *C. coccodes* mycelium was used to inoculate millet seed. Two flasks per isolate were infested and incubated at 22 ± 2°C under constant fluorescent lights for 15 days. Following incubation, a portion of the millet was transferred into sterile paper bags and dried for 3 days at 26 ± 2°C, and then spread onto a sterile tray and allowed to dry in a flow hood for 2 days to induce formation of microsclerotia. To create the master *C. coccodes* millet seed inoculum (either dried or not dried), ten grams of infested millet from each isolate were mixed together.

Inoculation and incubation

For both greenhouse and growth chamber experiments, onion seedlings were exposed to a high RH period of 16 h prior to inoculation. Pots containing the seedlings were placed on a 13 cm saucer inside of 7.5-liter clear plastic bags with 350 ml of water (saucer kept plants/pots out of the water); a handmade wire frame placed inside of the bag kept the foliage of the onion plants from direct contact with the surface of the bag.

Greenhouse experiments were conducted to compare inoculation methods. A conidial suspension with or without an abrasive agent was sprayed onto the onion canopy (2 ml/plant) using an airbrush (Badger Air-Brush Co, T63 Vega 2000, Franklin Park, IL). For the drench inoculation, 10 ml of conidial suspension was applied to the base of the onion seedlings. Two or five grams of the *C. coccodes* infested millet seed, dried or not, were placed around the base of the onion seedlings. Control plants were treated with their respective control treatment minus the pathogen. Following inoculation, plants were returned to high (≥80±15%) RH for 72 h. After the 72 h period, bags were opened and plants were removed from the bags and placed on 13 cm saucers where they were watered twice a week by adding water to the saucer to avoid water splash.

Growth chamber experiments were conducted to determine the effect of temperature and high RH periods on onion leaf and neck anthracnose development. Onion seedlings were sprayed with a conidial suspension consisting of a mixture of *C. coccodes* without abrasive agent (as previously described). Inoculated and control seedlings were incubated under high RH for periods of 0, 12, 24, 48, or 72 h at 15, 20, 25, or 30°C. To achieve high RH, plants were incubated in the same high humidity setup (plastic bag + wire frame) as described for the greenhouse experiments but seedlings were placed inside growth chambers at their randomly assigned temperature. Plants assigned to the 0 h high RH period remained unbagged after inoculation. A watchdog (Spectrum Technologies, Inc., Plainfield, IL) was placed inside each growth chamber to record temperature and RH.

Severity rating and isolations

Each leaf was evaluated by assessing area (%) covered with *C. coccodes* lesions, using a scale modified from the leaf blotch of cereals assessment key 1.6.1 (146). The average onion leaf and neck anthracnose severity for each plant was calculated by assessing the percentage area of each leaf covered with lesions, determining the sum of all leaves and dividing by the total number of leaves. Severity ratings were conducted at 4, 7, 14, 21 and 28 days after inoculation (DAI). The area under the disease progress curve (AUDPC) was calculated using the average severity rating per plant. At the end of both experiments, 10% of the plants were randomly selected and isolations were conducted to verify that symptoms were caused by *C. coccodes*. Resulting fungal colonies were confirmed as *C. coccodes* based on their colony appearance and morphology of conidia at 40X magnification (236,267).

Statistical analysis

The PROC MIXED in the SAS statistical analysis software version 9.2 (SAS Institute Inc., Cary NC) was used to test the main effect in both experiments. Logarithmic transformation was conducted to fulfill normal distribution and homogeneity of variance assumptions. ANOVA was evaluated using type III test for fixed effects output. Significant differences ($P<0.05$) were subject to grouping by Least Significant Differences (LSD) per effect (inoculation method for the greenhouse experiments, and temperature and high RH periods for the growth chamber experiments). For the split plot design, the significant interaction was sliced by effect and grouping by LSD was considered significant with an alpha of 0.05. Regression analyses were performed with SIGMAPLOT v. 11 (SYSTAT Software, Chicago IL) to fit models of onion leaf and neck anthracnose severity in relation to the interaction of temperature and high RH period effects. The three-dimensional non-linear regressions models planer, parabolic, Gaussian and Lorentzian were tested. Selection of the best fitting statistical model was based on coefficient of determination (R^2), and mean squared error of residuals (MSE).

RESULTS

Regardless of the inoculation method, all inoculated 'Prince' onion seedlings developed neck and leaf anthracnose symptoms (Figure 3) and control plants remained free of symptoms. The first symptoms were observed 5 days after inoculation (DAI) and consisted of lesions that were oval shaped and approximately 2 mm wide x 3 mm long with a light tan or bleached center (Figure 3). Lesions occurred first on leaves that were inoculated with a conidial spray (with or without an abrasive agent, Figure 3A and B), while lesions on the necks were observed first when inoculated either with the conidial suspension

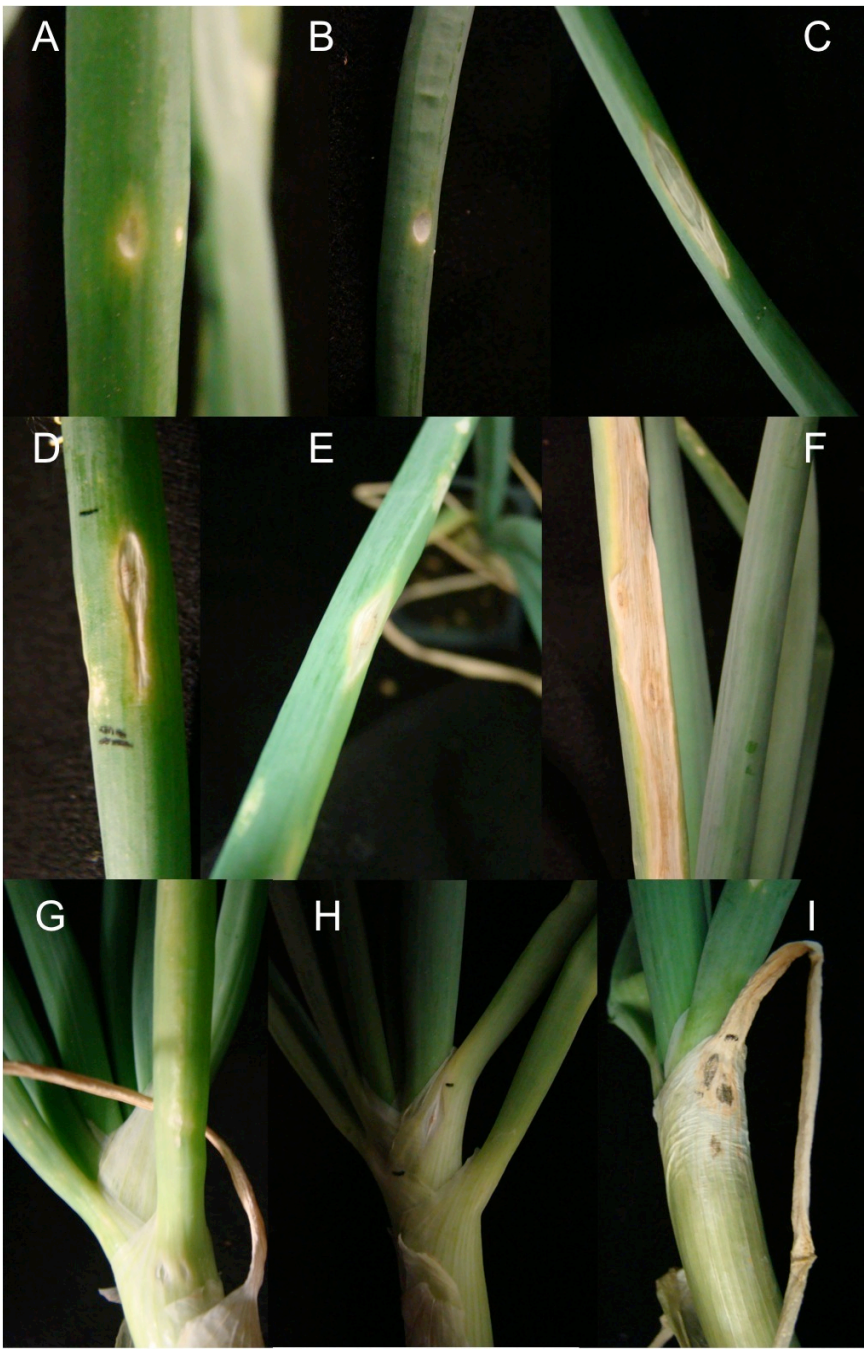

Figure 3. Symptoms of *Colleotrichum coccodes* onion leaf and neck anthracnose on 'Prince' onion seedlings inoculated with isolates 7-1-1-3, 8-1-1-1, 24-1-1-2, 31-1-2-2, 34-1-6-1 and 38-1-3-1. Sunken oval lesions A, 5 days after inoculation (DAI) and B, 7 DAI. Lesion expansion at C, 21 DAI and D, 14 DAI. E, Sporulation on lesion 21 DAI and F, coalescing lesions 28 DAI. Neck lesions G, 7 DAI. H, 21 DAI and I, 28 DAI. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

drench or infested millet (Figure 3G and H). Lesions expanded and coalesced and salmon-colored sporulation was observed 14 to 21 DAI from all inoculation methods (Figure 3C, E and F).

AUDPC data indicated significant differences among inoculation methods (P<0.0001, Table 3). Onion seedlings drenched with a conidial suspension had significantly lower AUDPC values than seedlings inoculated with the conidial spray + abrasive agent but the AUDPC values were not significantly different from that of plants inoculated with a conidial spray alone (Table 3). Inoculation using a conidial spray with or without an abrasive agent did not result in significantly different AUDPC values (Table 3).

Table 3. Effect of inoculation method on the Area Under the Disease Progress curve (AUDPC) and final onion leaf and neck anthracnose severity on 'Prince' onion seedlings inoculated with *Colletotrichum coccodes*.

	Onion leaf and neck anthracnose severity			
Inoculation method	Mean AUDPC		Final (%)	
Conidial spray with abrasive agent	728.2	a[z]	39.3	a
Conidial spray alone	698.1	ab	39.2	a
Drench	514.0	b	30.6	ab
Dry[y] millet (5 g)	146.3	c	23.7	b
Dry millet (2 g)	232.1	c	24.3	b
Wet[x] millet (5 g)	133.7	c	23.3	b
Wet millet (2 g)	172.6	c	19.0	b

[z]Means within a column for inoculation method followed by the same letter are not significantly different (LSD) $\alpha = 0.05$.
[y]Infested millet seed subjected to drying process, 5 or 2 g used for inoculation.
[x]Infested millet seed not subjected to drying process, 5 or 2 g used for inoculation.

Plants inoculated with *C. coccodes* infested millet seed had reduced AUDPC values (Table 3) when compared with the AUDPC value of plants inoculated using a conidial spray or drench inoculation methods (Table 3). Sclerotia were abundant within the millet

subjected to drying process while the wet infested millet seed inoculum consisted of mycelia and conidia only. There were no significant differences among mean AUDPC values for millet amounts (2 or 5 g) or whether the millet inoculum was dried or not (Table 3).

Final disease severity of onion seedlings drenched with a conidial suspension was intermediate (30%) between millet inoculum and conidial suspension inoculum with or without an abrasive agent (39%, Table 3). Final disease severity was lower for onion seedlings inoculated with infested millet regardless of amount or whether the inoculum was dried or not (19 to 24%, Table 3) compared to a conidial suspension applied as a foliar spray (~39% final severity with or without abrasive agent).

On the environmental conditions experiments, initial disease symptoms were observed 4 DAI on'Infinity' onion seedlings inoculated with *C. coccodes* and incubated at high RH for more than 12h at temperatures greater than 15°C and included small sunken oval lesions with no signs of pathogen sporulation. Onion leaf and neck anthracnose severity (leaf area covered with lesions) ranged from 5 to 12% and 15 to 35%, at 4 and 23 DAI respectively. Onion seedlings subjected to 0 h or 12 h of high RH after inoculation had disease severity <5% while seedlings subjected to 72 h of high RH reached 26% disease severity (Figure 4A). Inoculated seedlings at temperatures ≥25°C developed a disease severity level of 22% (Figure 4B).

Significant differences were observed for AUDPC values among high RH periods (P<0.0001) and temperatures (P=00081,Table 4). Disease progression was not significantly different between 24 and 48 h or between 48 and 72 h of high RH, but significant differences between 24 and 72 h were observed (Table 4). Mean AUDPC values for lower

temperatures (15 and 20°C) were significantly different from higher temperatures (25 and 30°C, Table 4).

Table 4. Effect of temperature and relative humidity period on the area under the disease progress curve (AUDPC) for onion leaf and neck anthracnose severity on 'Infinity' onion seedlings inoculated with *Colletotrichum coccodes*.

Effect	Mean AUDPC	
Temperature (°C)		
15	7.5	a[z]
20	68.6	a
25	253.8	b
30	305.8	b
High Relative humidity period (h)		
0	6.8	a
12	11.7	a
24	168.5	b
48	271.3	bc
72	336.3	c

[z]Means within a column for main effect followed by the same letter are not significantly different (LSD) $\alpha = 0.05$.

There was a significant temperature and high RH period interaction for mean AUDPC values ($P<0.0001$, Figure 4). At 15°C, AUDPC values were not significantly different among high RH periods (Figure 4). At 20°C, the AUDPC value for 72h was significantly higher than 0 and 12 h, but similar to 24 and 48 h (Figure 4). AUDPC values indicated that at 12 and 24 h of high RH were not significantly different from 0 or 48h (Figure 4). At 25 and 30°C, periods of 0 and 12 h of high RH had AUDPC values that were significantly lower than those associated with 24, 48 and 72 h of high RH that had similar mean AUDPC values (Figure 4).

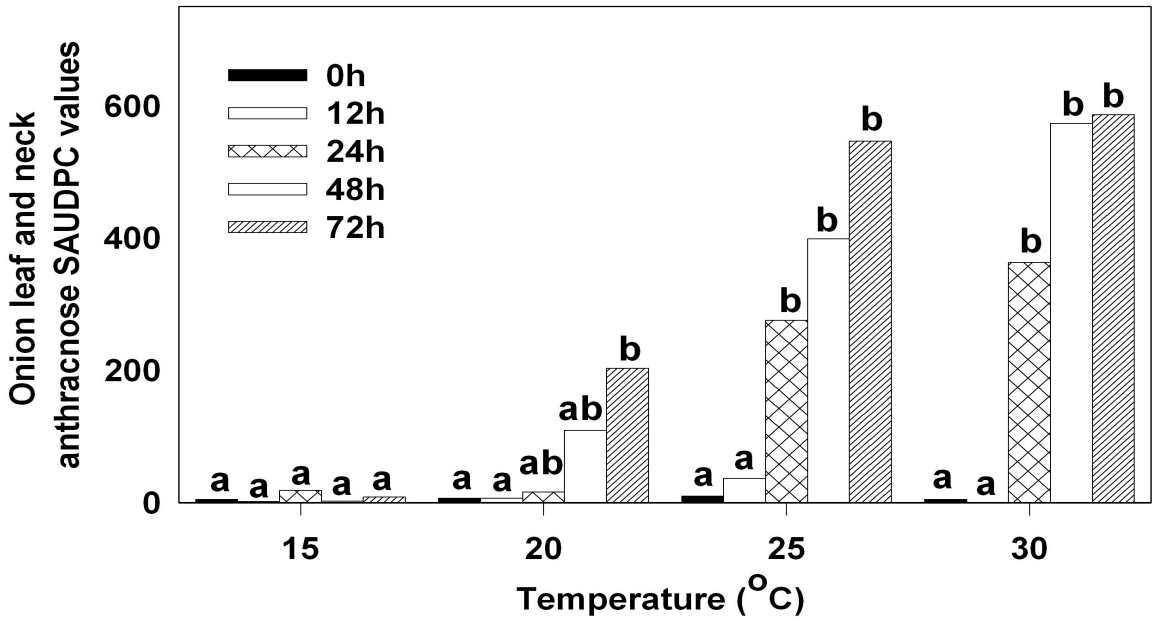

Figure 4. Temperature and relative humidity interaction on AUDPC values of onion leaf and neck anthracnose severity on 'Infinity' onion seedlings inoculated with *Colletotrichum coccodes* sliced by temperature.

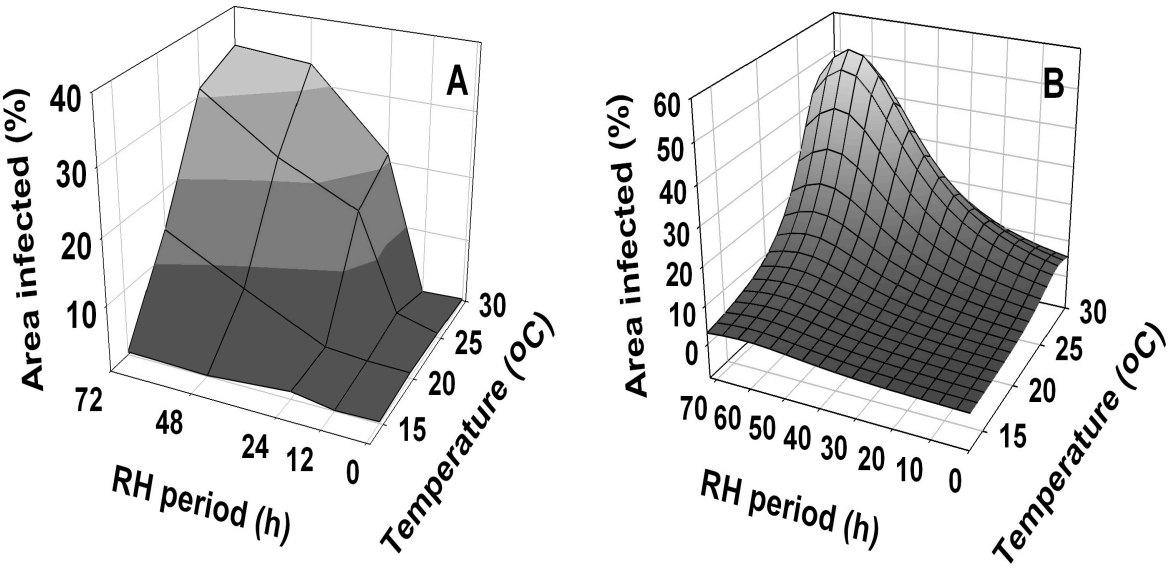

Figure 5. **A**. Observed effect of temperature and relative humidity period interaction on onion leaf and neck anthracnose severity (area infected %). **B**. Predicted response of onion leaf and neck anthracnose severity (area infected %) to the combined effects of temperature and high RH period on 'Infinity' onion seedlings inoculated with *Colletotrichum coccodes*. The predicted values were calculating using Eq. 1.

Disease severity was highest at 30°C at the 48 and 72h high RH followed by 72h of high RH at 25°C (Figure 5A). A Gaussian model was the function that best fit when three dimensional non-linear regression analyses on disease severity raw data (in Figure 5A) were performed (Table 5) (R^2= 0.914, Table 6, Figure 5B):

$$f(t,rh) = a * e^{-\frac{1}{2}\left[\left(\frac{t-t_0}{b}\right)^2+\left(\frac{rh-rh_0}{c}\right)^2\right]} \quad \text{(Eq.1)}$$

Table 5. Statistics of tridimensional regression models tested to fit the data for interaction of temperature and RH period on onion leaf and neck anthracnose severity (% area infected) on 'Infinity' onion seedlings inoculated with *Colletotrichum coccodes* in growth chamber experiments.

Independent/ Dependent variables	Fitted-model (equation)[z]	R^2	Adj. R^2	SEE	*P*-value	Residual MSE
High RH period (RH)	Planer	0.7	0.6	7.1	< 0.001	50.9
Temperature (°C)	Parabolic	0.7	0.6	7.5	< 0.001	55.8
Area infected (%)	Gaussian (Eq 1)	0.9	0.9	3.6	< 0.001	13.3
	Lorentzian	0.8	0.9	4.6	<0.001	21.7

[z]Models were examined for goodness of fit: R^2, SSE, *P* value and MSE.
R^2= coefficient of determination
SEE=standard error of the estimate
MSE=mean squared error of the residuals.

Table 6. Parameter estimates for the Gaussian model (Eq. 1) that described the combined effects of temperature and high RH period on onion leaf and neck anthracnose severity (% area infected).

Dependent variable	Independent variables	Model	Model parameters				
			t_0	rh_0	a	b	c
Area infected (%)	High RH period Temperature	Gaussian	28.6	62.7	38.9	5.4	27.4

For both studies, the plants randomly selected for re-isolation, exhibited lesions that contained acervuli and setae when observed under the dissecting scope (Figure 6). Fungal

colonies had salmon coloration, with production of cylindrical conidia (17 to 22 x 2.8 to 4.0 μm in size) five to seven days after symptomatic tissue was sampled, and black microsclerotia 14 days after isolations were conducted. All colonies matched *C. coccodes* morphological characteristics of the isolates used for inoculation.

DISCUSSION

Michigan onion growers utilize cultural strategies and fungicide sprays to manage fungal foliar diseases each year including purple blotch and Botrytis leaf blight; extensive research has been conducted to reduce the impact of these diseases (56,93,266). With the occurrence of *C. coccodes* as a new onion pathogen, the main environmental conditions conducive to onion leaf and neck anthracnose development were evaluated as an initial step to determine effective management strategies since none existed.

Onion leaf and neck anthracnose symptoms first appeared as white to light green, sunken, oval lesions and are clearly distinguishable from lesions caused by other foliar pathogens. Over time, the tissue in the center of the anthracnose lesion developed a salmon color with *C. coccodes* signs (acervuli, setae, and masses of conidia) evident under a dissecting microscope (≥7.5X). Symptoms caused by *C. coccodes* were restricted to the seedling neck when infested millet was used, while the spray and drench inoculation methods using a conidial suspension resulted in lesions on both the leaves and necks.

In this study, the onion seedlings were at the four to five leaf stage and were susceptible to *C. coccodes*. A previous study utilized seedlings at the two to three leaf stage (239) and they were also susceptible. In this study, conidia, mycelia and/or sclerotia of *C. coccodes* infected onion foliar tissue, but the type of inocula affected final severity and disease progression (AUDPC). When a conidial suspension was applied to onions as

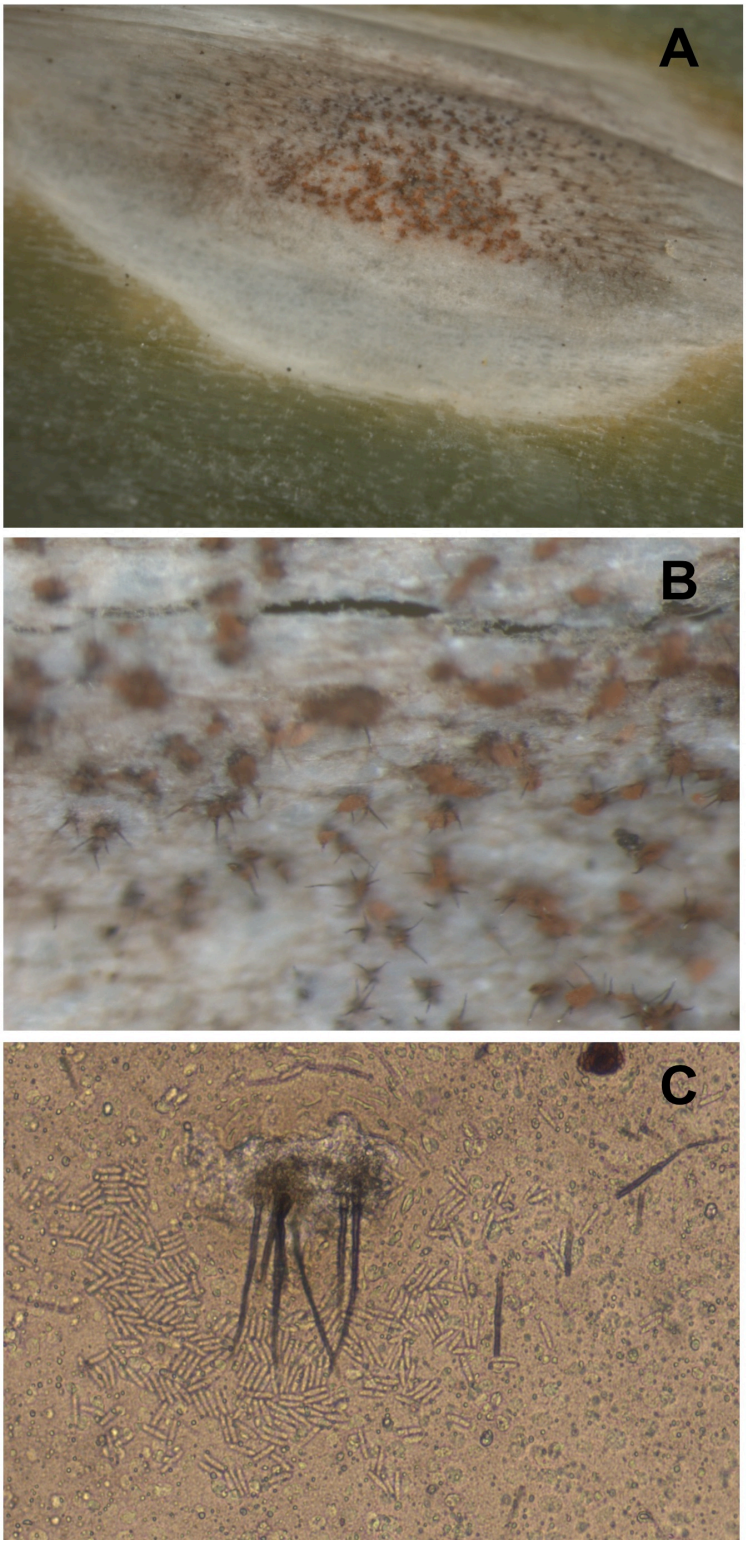

Figure 6. A, Magnified lesion caused by *Colletotrichum coccodes* in 'Prince' onion seedling (7.3 X Magnification). B, *C. coccodes* acervuli and setae (25X). C, setae and conidia (400X).

a foliar spray or drench the resulting disease severity was similar; these methods were also similar in studies with pepper (138). Drench inoculation resulted in damping off of two-leaf pepper seedlings plants but not on older plants (138). The conidial suspension amended with an abrasive agent resulted in similar AUDPC values to that of the conidial suspension alone but differed significantly from the drench. The abrasive agent simulated wounding caused by blowing soil particles. In potato, wounding of the foliage prior to inoculation with *C. coccodes* resulted in higher disease severity (24,149,151,276). In the field, soil particles in combination with wind can cause wounds on the foliage, potentially exacerbating onion leaf and neck anthracnose.

Colletotrichum coccodes is capable of producing sclerotia, a source of primary inoculum, which can remain in the soil (243). When *C. coccodes* infested millet was used as a source of inoculum, regardless of amount or type, the AUDPC values and final disease severity were the lowest compared to other methods of inoculation used in this study. Soilborne inoculum of *C. coccodes* may play a role in causing leaf and neck antracnose of onion as has been demonstrated for potato; soilborne inoculum incites severe black dot disease on potatoes when compared with seedborne inoculum (212). Sclerotia of *C. coccodes* can remain in the soil for up to one year in potato fields (96,276), and survive in association with host debris or alone for up to eight years in tomato fields (82). Experiments comparing *C. coccodes* sclerotia on tomato fruit skin and roots and sclerotia alone, showed that sclerotia alone survive longer compared to the pathogen associated with plant tissue. After five years in the soil, 90% of the *C. coccodes* sclerotia buried at 10 and 20 cm below the surface were viable, compared to sclerotia at the soil surface which were only 50% viable (82). Onions in Michigan are planted primarily on organic soils, and

growers do not rotate with Solanaceous crops, but instead with celery, radishes, field corn or soybeans. Investigation of the role of *C. coccodes* soilborne inoculum in onion fields and the ability of *C. coccodes* to survive on crops (or debris) commonly used in the rotations used by Michigan farmers can guide selection and sequence of crops to rotate with in onion growing counties in the state.

Colletotrichum coccodes may have been originally introduced to onion fields on contaminated seed as documented for tomatoes and potatoes (27,86,175). Onion leaf and neck anthracnose was initially observed in 2010 in six Michigan counties, spanning 17 fields separated by up to approximately 180 km (unpublished data). In Michigan fields where *C. coccodes* was observed in 2010, 2011 or 2012 sclerotia may be overwintering in the soil. Onion roots may have been infected by *C. coccodes* in a manner similar to tomato and potato (82,83,176,213) in that sclerotia associated with onion tissue could have also survived in fields between growing seasons. In Michigan, microsclerotia were observed on the roots of an onion cultivar trial that had been inoculated. When 20 bulbs of each cultivar were harvested and the foliage and root tissue sampled, approximately 2-5% of the onion roots showed signs of *C. coccodes* after isolation from roots were incubated for 14 days (Rodriguez-Salamanca and Hausbeck in press). Machinery carrying soil infested with microsclerotia could aid in the dissemination of the pathogen among fields. Sclerotia can geminate and infect plant tissue (debris or living host), and then produce acervuli and conidia. As for many *Colletotrichum* spp., dispersal in the field occurs mainly via rain splash to nearby plants, therefore the combination of rain and wind may enable splash of conidia within a field (17,244).

The conidial suspension applied as a spray was an effective inoculation technique in onion and therefore can be used for greenhouse and/or field studies. The effect of temperature and RH period on onion leaf and neck anthracnose disease severity was therefore investigated using a conidial suspension spray as a source of inoculum. Onion leaf and neck anthracnose severity was higher at 25 and 30°C when incubated at high RH for 24, 48 or 72h. Optimal mycelial growth, sporulation and conidial germination of *C. coccodes* occurs at a temperature range of 24 to 28°C *in vitro* (155), and optimal lesion development on tomato fruit occurs at a range of 15 to 31°C, being optimal at 26°C (79,80). Lesion development by *C. coccodes* on onion foliage occurred between 15 and 30°C. Infection and lesion development on 'Infinity' onion seedlings required 24 hours or more of high RH in combination with temperatures greater than 25°C were needed to result in significant development of onion leaf and neck anthracnose (disease severity ranging from 22 to 34%).

Severe outbreaks of tomato or pepper anthracnose in the field are associated with high rainfall or overhead irrigation that promote conidial dispersal and provides leaf wetness conducive to *C. coccodes* germination and infection (79,81,244). Studies in potato, tomato and pepper showed that disease severity was exacerbated by relatively long periods of wetness (79,138,151). For example, ten or more hours of continuous leaf wetness were needed for *C. coccodes* conidia to infect tomato fruits (79).

Onion seedlings can be infected by *C. coccodes* and develop symptoms early in the season when the temperature reaches 15°C. In Michigan, onions are direct seeded in late April or May when air temperatures can range from 8 to 19°C (based on 3 year average

Clarskville station, Michigan Automated Weather Network -MAWN). Results showed that optimal anthracnose development could occur in July and August, when average temperatures in Michigan range from 22 to 28°Cwith a maximum of 30°C.

Non linear regression modeling has been employed to describe interaction of weather variables on infection processes in green onion (111), melon (19), blueberry (197) and water yam (129) . In this study, the combined effect of temperature and the high RH period explains 91% of the variance in onion anthracnose severity when it was modeled with a non-linear Gaussian model. Duthie (1997) anticipated between 20 to 30 temperature and wetness period combinations were likely needed for correct parameter estimation on non linear regression modeling (87), and in this study there were 20 combinations of temperature and high RH period and therefore adequate statistical robustness. In order to develop a disease-forecasting model for *C. coccodes* on onion, additional research on the combined effect of temperature wetness duration and interruption of wetness duration and their effect on infection, latent period, sporulation, survival and dispersal, as incorporated in Botcast and Blight-alert for Botrytis blight in onion (56,166,281), is warranted. Incorporation of those parameters to existing disease forecasting systems developed in onion to time fungicide sprays (166,281) can expedite the process. The effect of host susceptibility has been significant and included in forecasting models (51,241) and should be considered in the onion- *C. coccodes* pathosystem.

In summary, the results demonstrated the importance of temperature, high RH duration and their interaction on the severity of onion leaf and neck anthracnose. Further research is warranted to investigate the incorporation of weather variables with the

application and timing of efficacious fungicides that can help limit this emerging disease in Michigan onion fields.

CHAPTER III

EVALUATING HOST RESISTANCE AND FUNGICIDE EFFICACY TO LIMIT *COLLETOTRICHUM COCCODES* CAUSING LEAF AND NECK ANTHRACNOSE IN ONION.

ABSTRACT

Leaf and neck anthracnose incited by *Colletotrichum coccodes* (Wallr.) Hughes is a newly reported foliar disease of onion in Michigan and has been observed in the state from 2010 through 2012. Symptoms include elliptical lesions on the leaves and/or neck that appear bleached with a pale salmon to dark brown center. To develop an effective disease management strategy, field studies were conducted in 2011 and 2012 to test ten fungicides for their efficacy against *C. coccodes* and evaluate sixteen commercial onion cultivars for their susceptibility to the pathogen. The incidence and severity of anthracnose were evaluated weekly following inoculation and the yield determined at the end of the experiment. Significant differences were observed among fungicide treatments but differences between years were also observed. Cabrio (pyraclostrobin), Quadris (azoxystrobin), Priaxor (fluxapyroxad + pyraclostrobin), Inspire (difenoconazole), Bravo (chlorothalonil), and Manzate (mancozeb) significantly limited onion leaf and neck anthracnose compared to the untreated control. Luna Tranquility (fluopyram + pyrimethanil) and Scala (pyrimethanil) were not effective against *C. coccodes*. Onion cultivars differed significantly in disease severity and incidence; differences between years were also observed. 'Hendrix' had the lowest disease severity while 'Highlander' and 'Candy' exhibited severe onion leaf and neck anthracnose symptoms. Choosing less susceptible onion cultivars along with applying fungicides effective against *C. coccodes* may limit crop losses for Michigan growers.

INTRODUCTION

In total, 85 million tons of onions were produced in 2011 on 2 million hectares globally (13). Onion cultivars have been developed to adjust to the diverse seasons where onions are produced (42,43). Cultivars are classified based on the minimum day length needed for bulbing; as short-day (11-12 h), intermediate-day (13-14 h), long-day (13-14 h) and very long-day cultivars (>16 h) (43). Long-day onions suitable for storage are grown in Michigan and are planted primarily in organic soils. In 2012, Michigan onion growers harvested 8,918 ha of onions valued at $7.7 million (15). In the past five years, the Michigan onion industry has experienced reductions in the area planted and harvested (15,16). During the period of 2008 through 2010, 1,618 ha of onions were planted yearly but just 1,214 ha were planted in 2012 (15).

A range of pathogens can compromise onion quality and yield (56,76,247,266) and are an annually threat in Michigan. Foliar diseases including purple blotch (*Alternaria porri*), Stemphylium leaf blotch (*Stemphylium vesicarium*), Botrytis leaf blight (*Botrytis squamosa*), and downy mildew (*Peronospora destructor*) result in leaf blighting and premature leaf senescence that compromise bulb enlargement and quality (56,76,92). Leaf and neck anthracnose caused by *Colletotrichum coccodes* was recently reported as a new pathogen and onions in many Michigan counties were affected from 2010 through 2012 (personal observation). The symptoms of onion leaf and neck anthracnose caused by *C. coccodes* are different from symptoms caused by *C. gloeosporioides* previously reported in the U. S. and the tropics which is referred to as onion twister (90,203,211) or anthracnose (112,130). Michigan onions can have mixed foliar infections of leaf and neck anthracnose, purple blotch, and Stemphylium leaf blotch (personal observation). Onion leaf and neck

anthracnose symptoms include oval lesions on the leaves and neck that differ in appearance from other commonly occurring onion foliar diseases (247). The onion leaf and neck anthracnose lesions range approximately from 0.2 to 0.6 cm wide x 0.4 to 3 cm long and have a characteristic bleached appearance that includes a salmon to brown colored center. The occurrence of this new pathogen contributes to the difficulty of growers in Michigan and other growing regions in maintaining productivity and profitability of producing onions.

In Michigan, onions are direct seeded in late April or early May and harvested in early August to mid September. Temperatures in the onion-growing season in Michigan range from 9 to 30°C. Optimal onion seed germination occurs at 23 to 28°C, and soil moisture is critical to obtain homogenous germination and plant stand. Onions develop and grow from 6 to 32°C, with maximum growth rate occurring from 22 to 28°C (43). Environmental conditions especially conducive to onion leaf and neck anthracnose include a minimum of 12 h at high relative humidity with temperatures higher than 25°C (Rodriguez-Salamanca and Hausbeck in press).

Approximately 40 onion cultivars are available to Michigan growers from various seed suppliers. Some of these cultivars offer resistance to pink root incited by *Setophoma terrestris* (Syn:*Phoma terrestris*) and Fusarium crown rot incited by *Fusarium oxysporum* f. sp. *cepae* but their response to *C. coccodes* has not been evaluated. Michigan growers rely on fungicides to limit foliar diseases on onions including leaf and neck anthracnose and choose from active ingredients representing at least 10 fungicide resistance action code (FRAC) groups (32,40). Fungicides commonly used to manage diseases such as purple blotch and Botrytis leaf blight that occur annually belong to the carboxamides (FRAC group

7), dicarboximides (FRAC code 2), demethylation inhibitors (DMI, FRAC group 3), quinone outside inhibitors (QoI, FRAC group 11), and chloronitriles (FRAC code M5) (134,169,170). Host resistance and/or protective fungicide sprays are commonly used disease management strategies employed to limit disease on onions (208,248) and other crops (121,160,196,262,289). However, since onion leaf and neck anthracnose was only recently reported (239), the objectives of this study were to investigate the response of 16 onion cultivars to *C. coccodes* and to determine the ability of selected fungicides to limit leaf and neck anthracnose under field conditions.

MATERIALS AND METHODS

Field preparation, plant establishment and field maintenance

Cultivar and fungicide trials were direct seeded into mineral soil, Oakville fine sand (14), at the Southwest Michigan Research and Extension Center (SWMREC; Benton Harbor, MI) on 27 May 2011 and 10 May 2012. The field was previously planted to rye and vetch cover crops in 2010 and to onion (*C. coccodes* inoculated plots) in 2011. Prior to bedding, 560 kg/ha of 8-21-29 fertilizer was applied with micronutrients (0.5% Cu, 1% Mn and 0.5% Zn) and side dressed twice with 11 kg/ha of foliar 20-20-20 fertilizer. The target seeding density for both the cultivar and fungicide experiments was 18 plants per 30 cm row/length. For the cultivar trial, 16 commercially available cultivars (Table 7) were planted in a complete randomized block design with four blocks. Each block was 5 m long and consisted of a double row spaced 38 cm apart. 'Infinity' onions were seeded into four blocks 6 m long for the fungicide trial and each block was separated by a gap of 150 cm. A plot consisted of a double row of onions 38 cm apart.

Weed control was achieved by hand weeding and herbicides were applied as needed following current recommendations (32). Insects (thrips and armyworms) were controlled with applications of methomyl (2.3 l/ha). Both the fungicide and cultivar experiments had an overhead mister in place with sprinkler heads delivering 94.6 liter/h, operated 2 days a week for 1 or 2 hours to promote moisture conducive to *C. coccodes* infection and dispersal in the field.

Table 7. Onion cultivars tested for their susceptibility to *Colletotrichum coccodes*, causal agent of onion leaf and neck anthracnose, under field conditions during 2011 and 2012.

Cultivar Name	Bulb Color	Days to Maturity	Seed Company[z]
Bradley	Yellow	118	Bejo
Candy	Yellow	95	Seminis
Hamlet	Yellow	105	Seminis
Hendrix	Yellow	105-110	Nunhems
Highlander	Yellow	85-90	American Takaii
Infinity	Yellow	105-110	Nunhems
Livingston	Yellow	100-110	Solar
Marco	Yellow	100-110	Solar
Milestone	Yellow	110	Siegers
Polo	Yellow	100-110	Solar
Prince	Yellow	105	Bejo, Solar
Pulsar	Yellow	100-105	Nunhems
Redwing	Red	118	Bejo
Stanley	Yellow	100-110	Solar
Talon	Yellow	110	Bejo
Vespucci	Yellow	115	Siegers

[z]American Takaii= American Takii, Inc., Salinas, CA; Bejo= Bejo Seeds, Inc., Oceano, CA; Nunhems=Nunhems USA., Parma, ID; Seminis= Seminis Vegetable Seeds, Inc St. Louis MO; Solar= Solar Seed Inc., Eustis, FL.

Fungicide Treatments

A total of 8 (2011) and 10 (2012) fungicide treatments (Table 8), an untreated inoculated control (2011, 2012), and an untreated non-inoculated

Table 8. Fungicide treatments and their rates applied to 'Infinity' onion plants inoculated with *Colletotrichum coccodes* in the field.

Treatments and Formulation	Active ingredient	Rate, Kg a.i./ha	Labeled	Year	Manufacturer[z]	FRAC Group
Untreated non-inoculated	…	…	…	2012	…	…
Untreated inoculated	…	…	…	2011, 2012	…	…
Quadris 2.08SC	azoxystrobin	0.22	Y	2011, 2012	Syngenta	11
Endura 70WG	boscalid	0.36	Y	2012	BASF	7
Bravo WeatherStik 6SC	chlorothalonil	1.68	Y	2011, 2012	Syngenta	M5
Inspire 5SC	difenoconazole	0.13	N	2011, 2012	Syngenta	3
Luna Tranquility 4.17SC	fluopyram + pyrimethanil	0.58	N	2011, 2012	Bayer	7/9
Priaxor 4.17SC	fluxapyroxad + pyraclostrobin	0.22	N	2012	BASF	7/11
Tilt 2.08EC	propiconazole	0.23	Y	2011, 2012	Syngenta	3
Cabrio 3.3EC	pyraclostrobin	0.17	Y	2011, 2012	BASF	11
Scala 20WG	pyrimethanil	0.79	Y	2011, 2012	Bayer	9
Manzate ProStik 75DF	mancozeb	2.52	Y	2011, 2012	Du pont	M3

[z]BASF = BASF Corp., Research Triangle Park, NC; Bayer = Bayer CropScience LP, Research Triangle Park, NC; DuPont = E. I. du Pont de Nemours and Co., Wilmington, DE; Syngenta = Syngenta Crop Protection, Inc., Greensboro, NC.

control (2012) were replicated four times in a randomized complete block design-RCBD. A total of 8 (2011) and 7 (2012) fungicide sprays, two prior to inoculation and the remaining after inoculation, were applied weekly using a CO_2 backpack sprayer with a three-nozzle boom (R&D Sprayer, Opelousas, LA). The boom was equipped with three XR8003 flat-fan nozzles spaced 48 cm apart, placed 46 cm above the onion tops and calibrated to deliver 473 liter/ha at 344 kPa.

Inoculum preparation, inoculation and disease ratings

Several isolates of *C. coccodes* recovered from onion leaves in 2010 and maintained in long-term storage in the Hausbeck lab culture collection, were used as inoculum. The Michigan *C. coccodes* isolates 7-1-1-3 (Newaygo Co.), 8-1-1-1 (Ottawa Co.), 24-1-1-2 (Ionia Co.), 31-1-2-2 (Calhoun Co.), 34-1-6-1 (Kent Co.) and 38-1-3-1 (Eaton Co.) were obtained from Michigan onion foliage. Isolates were transferred from long-term storage onto 50% strength PDA and incubated at 22 ± 2°C for 7 days. A total of 120 plates (20 plates per isolate) with at least 70 mm diameter fungal growth were blended with approximately 500 ml 0.001% Tween in water and strained through a 19 L paint strainer. This process was repeated until all 120 fungal plates were used. A total of 25 liters of inoculum were made per trial. The conidial suspension was adjusted to 2×10^5 conidia/ml and placed in plastic carboys (Nalgene, Rochester, NY). Inoculum was applied twice during the growing season on 21 Jul and 25 Aug 2011, and 12 Jul and 7 Aug 2012. The conidial suspension was applied using a CO_2 backpack sprayer (R&D Sprayer, Opelousas, LA) with a two-nozzle boom as described avobe but using extended range flat nozzles (XR8002).

Disease assessment and yield measurement

For the cultivar and fungicide trials, the number of plants with leaf and neck lesions (incidence) and severity of disease was evaluated in the inner 4 m of each plot. Disease severity was evaluated using a 0 to 5 disease rating scale based on the percentage of onion foliage (including the necks) covered with lesions using the following scale: 0 (no symptoms), 1 (>0-10%), 2 (>10-25%), 3: (>25- 50%), 4 (>50-75%) and 5 (75-100%). Plots were rated weekly, beginning 14 ± 2 days after inoculation (DAI), until 56 DAI. Onion plots were hand harvested on 5 Oct 2011 and 11 Sep 2012 for the cultivar trials, and on 6 Oct 2011 and 5 Sep 2012 for the fungicide trials. Onion bulbs were separated according to their diameter into three groups: boiler (less than 5 cm), regular (between 5 and 8 cm) or jumbo (more that 8 cm) using a hand wooden grader; bulbs >5 cm in diameter were considered marketable.

Pathogen re-isolation and confirmation

At the end of the trial, five plants per plot and block were sampled by isolating from tissue at the healthy-diseased interface of lesions visible on the leaves. Tissue was plated onto 50% strength PDA amended with 30 ppm rifampicin and 100 ppm ampicillin and maintained at 22 ± 2°C under continuous light. Five days after plating, fungal colonies were observed for the macroscopic appearance and microscopic characteristics corresponding to *C. coccodes* (142,236). Plates were then incubated under the conditions as previously described for nine additional days and then observed for the presence of microsclerotia.

Onion roots from the sampled plants were inspected for the presence of microsclerotia. Roots were surface disinfested with 1% sodium hypochlorite solution for 3

min, rinsed three times with distilled water and allowed to air dry for one hour. Isolations were conducted on 25% strength PDA and cultures were incubated at 22 ± 2°C for 14 day under continuous light.

Statistical analysis

For the cultivar trial, final disease incidence was analyzed using a generalized linear model with a Logit link function and binomial distribution. For the fungicide and cultivar experiments, incidence and severity values were used to calculate the area under the disease progress (AUDPC) curve for the fungicide and cultivar experiments (IAUDPC and SAUDPC for incidence and severity, respectively) and yield were analyzed using the PROC MIXED and PROC GLIMMIX procedure of the SAS statistical analysis software (SAS Institute Inc., Cary NC) using a RCBD model. Data were tested for normality (q-q plots and the Shapiro Wilk test) and homogeneity of variance (Levene's test) of the residuals. The need to include a covariate in the model (ANCOVA) was tested and used for the cultivar trial. Models (ANOVA and ANCOVA) were evaluated using type III effects output. Significant differences were subject to grouping by Least Significant Differences (LSD $P<0.05$ or $P<0.1$).

RESULTS

Leaf and neck anthracnose symptoms were observed 14 and 16 DAI in 2011 and 2012, respectively on the cultivar trial. In general, the lesions were oval in shape with the affected tissue appearing to be bleached or light green (Figure 7). The pathogen sporulation in the center of the lesion initially appeared to be pale pink to salmon in color and then progressed to brown as the lesion aged. An uneven plant stand due to variable germination was observed among cultivars in 2011($P<0.001$) but not in 2012 ($P=0.056$).

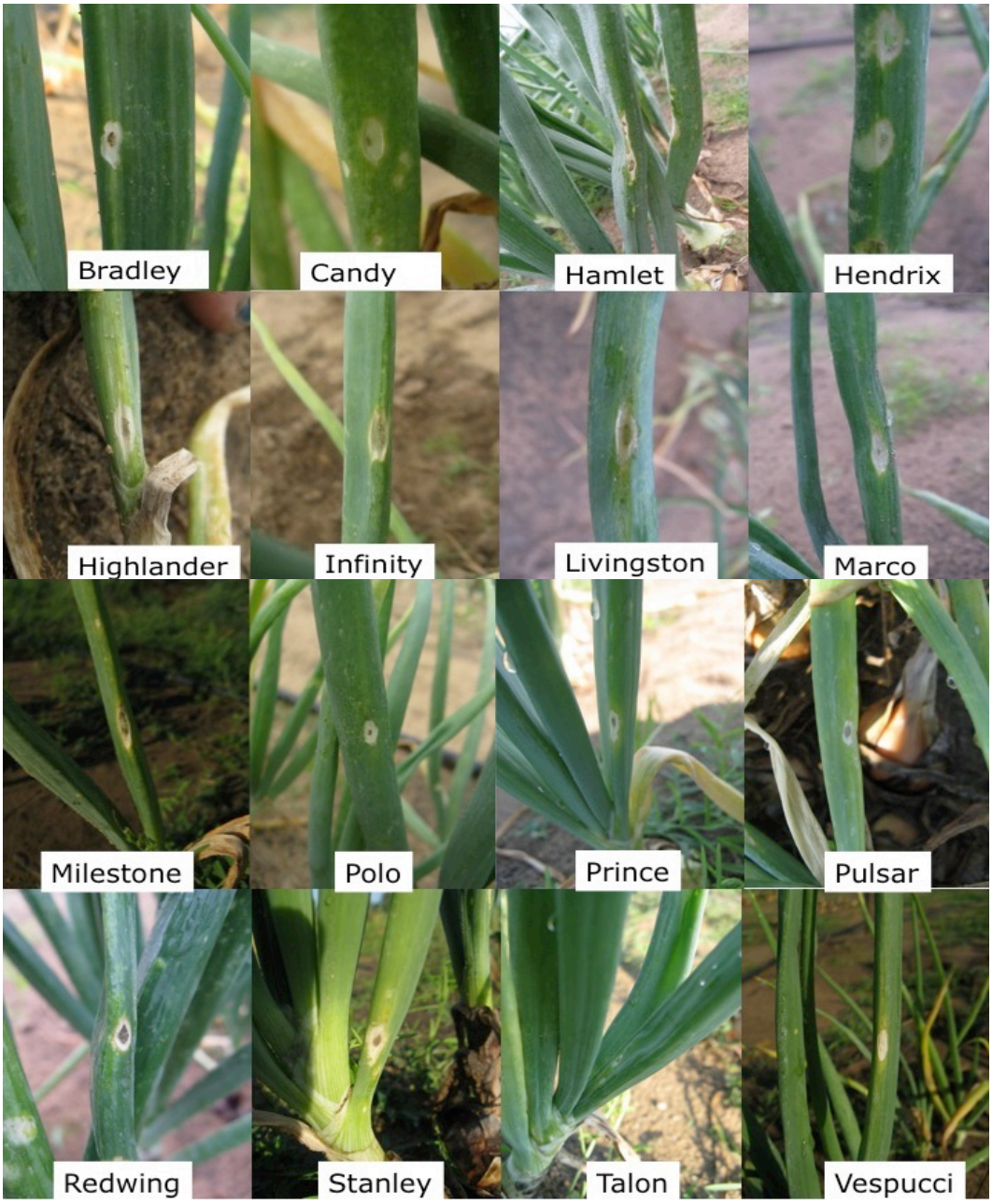

Figure 7. Onion leaf and neck anthracnose symptoms on sixteen onion cultivars inoculated with a mixture of *Colletotrichum coccodes* isolates in the field.

Final onion leaf and neck anthracnose incidence varied between years (P<0.001), cultivars (P<0.001) and a significant interaction between years and cultivars was observed (*P*=0.0025). Cultivars Hamlet (P=0.03), Highlander (*P*<0.001), Pulsar (*P*=0.017), Redwing

(*P*=0.014), and Stanley (*P*=0.006) had significantly higher final disease incidence in 2011 compared with 2012 (Table 9).

Table 9. Incidence of leaf and neck anthracnose (*Colletotrichum coccodes*) on onion cultivars during 2011 and 2012.

	Final Incidence (%)[z]						
Cultivar	Combined years		Differences by year[y]	2011 49 DAI		2012 47 DAI	
Hendrix	35.3	f[x]	ns	30.4	f	41	d
Infinity	48.0	e	ns	50.2	fg	43.8	bd
Polo	48.8	de	ns	53.9	cg	43.6	cd
Vespucci	48.9	de	ns	45.8	gh	50.8	ad
Talon	49.1	de	ns	57.2	cg	44.3	d
Hamlet	52.1	cde	**	69.8	be	36.6	d
Pulsar	55.0	bcde	**	66.1	bf	43.5	bd
Marco	55.2	bcde	ns	51.8	ef	58.1	ab
Milestone	56.7	bcde	ns	60.8	cg	51.5	ad
Bradley	59.9	bcde	ns	64.6	cf	53.5	ad
Redwing	60.7	bcde	**	71.8	bd	48.6	bd
Livingston	62.7	abcd	ns	61.7	cg	64.7	a
Candy	63.8	abce	ns	73.3	bc	55.9	ac
Stanley	64.7	abcde	**	84.7	ab	43.3	bd
Prince	65.7	ab	ns	73.7	bc	56.7	ab
Highlander	76.1	a	**	94.9	a	47.3	bd

[z]Mean percentage of infected plants per cultivar.

[y]For each cultivar, asterisks indicate that final incidence differed significantly on the cultivar x year interaction alpha= 0.05.

[x]Means within a column followed by the same letter are not significantly different (LSD).

Leaf and neck anthracnose severity progression over time (represented by the mean SAUDPC values) was significantly different among cultivars (*P*<0.001), and a significant interaction among cultivars and years was observed (*P*=0.02). However, the number of onion plants per plot was a significant covariate with slopes different from zero (P=0.0089) but with similar slopes among cultivars (*P*=0.1262). After the covariate was included in the model, the interaction between cultivar and year changed (*P*=0.057). Only two cultivars,

Hendrix, and Marco, had differences in SAUDPC values between years with higher SAUDPC values in 2012 compared with 2011 (Table 10).

In both years, 'Highlander' and 'Candy' consistently had the highest mean SAUDPC values when compared with the other 14 cultivars (Table 10). The values for these two cultivars were significantly different from each other in 2012, with 'Highlander' having the highest SAUDPC value; while in 2011 their mean SAUDPC values were similar (Table 10).

Table 10. Mean area under the disease progress curve (SAUDPC) values in onion cultivars inoculated with *Colletotrichum coccodes* during 2011 and 2012.

	Mean SAUDPC[z]						
Cultivar	Combined years		Differences by year[y]	2011 Mean		2012 Mean	
Hendrix	51.5	f[x]	**	41.2	a	61.9	ab
Talon	52.9	ef	ns	57.3	abc	48.4	a
Polo	55.1	ef	ns	58.4	abc	51.9	a
Redwing	56.5	ef	ns	61.7	bc	51.2	a
Vespucci	57.4	ef	ns	51.6	abc	63.2	ab
Hamlet	57.6	ef	ns	66.3	bc	48.9	a
Infinity	58.1	ef	ns	56.5	ab	59.7	ab
Livingston	59.6	ef	ns	62	bc	57.1	ab
Pulsar	61.9	ef	ns	61.6	bc	62.2	ab
Prince	62.3	ef	ns	64.5	bc	60.1	ab
Stanley	64.1	def	ns	71.5	bc	56.8	ab
Milestone	64.2	de	ns	65.6	bc	62.9	ab
Bradley	79.1	dc	ns	80.7	c	77.6	bc
Marco	79.8	c	**	65	bc	94.6	c
Candy	102.3	b	ns	105.7	d	98.8	c
Highlander	125.0	a	ns	123.5	d	126.6	d

[z]Mean SAUDPC calculated using whole plot severity rating assessed 12, 21, 27, 35, 42, 49 and 56 DAI in 2011 and 16, 35, 42, 47 and 56 DAI in 2012.

[y]For each cultivar, asterisks indicate that final incidence and mean AUDPC differed significantly on the cultivar- year interaction alpha= 0.01

[x]Means within a column followed by the same letter are not significantly different (LSD).

After plating, *C. coccodes* colonies developed from foliar lesions five days after isolations were conducted, and microsclerotia were observed 14 days later. Among cultivars, onion roots did not bear microsclerotia at collection time. After incubating onion root isolations for two weeks, microsclerotia were observed on cultivars Bradley (15% of the roots), Marco (15%), Livingston (10%), Stanley (10%), Vespucci (10%), Hendrix (5%), Infinity (5%) and Prince (5%) in 2011. In 2012 microsclerotia were observed on the roots of cultivars Candy (10%), Polo (10%), Prince (5%), Livingston (5%) and Stanley (5%). Six cultivars (Talon, Redwing, Pulsar, Milestone, Highlander and Hamlet) showed no evidence of root infection.

Leaf and neck anthracnose symptoms were first observed 14 and 16 DAI for the 2011 and 2012 fungicide trials, respectively, and disease incidence increased over time. Mean IAUDPC values were significantly different among fungicide treatments ($P<0.0001$) between years ($P=0.002$) and with a significant year and fungicide treatment interaction ($P=0.0099$). Plants treated with propiconazole, pyrimethanil or fluopyram + pyrimethanil had higher IAUDPC values in 2011 compared to 2012 (Table 11). In contrast, pyraclostrobin was the only treatment that had significantly higher IAUDPC value in 2012 compared with 2011 (Table 11).

According to IAUDPC data from 2011, pyraclostrobin was similar to chlorothalonil and azoxystrobin. However, azoxystrobin and chlorothalonil treatments were significantly different from each other. Pyrimethanil, difenoconazole, and mancozeb were similar to chlorothalonil, but also similar to the untreated inoculated control, fluopyram + pyrimethanil, and propiconazole (Table 11).

In 2012, an untreated non-inoculated control and two additional fungicide treatments, fluxapyroxad + pyraclostrobin and boscalid (Table 8), were included in the experiment. Unexpectedly, the untreated non-inoculated plots showed symptoms of the disease 16 DAI at the same time symptoms were observed on untreated inoculated and other treatments. IAUDPC values for plots treated with fluxapyroxad + pyraclostrobin, azoxystrobin, pyraclostrobin, mancozeb, chlorothalonil, and difenoconazole were significantly lower than the untreated inoculated control (Table 11). IAUDPC values were similar for azoxystrobin and pyraclostrobin, and were significantly lower than all of the other fungicide treatments tested, except for chlorothalonil (Table 11).

Table 11. Effect of fungicide treatment on onion leaf and neck anthracnose on disease incidence progression in time (IAUDPC).

	IAUDPC[z]				
Treatment	2011		2012		Year
Untreated non-inoculated	-	-	459.1	ac	-
Untreated inoculated	1196.0	a	576.9	a	ns
Chlorothalonil	416.1	bc	391.8	bc	ns
Pyraclostrobin	281.9	cd	361.4	bc	**
Boscalid	-	-	571.8	a	-
Difenoconazole	641.4	ab	400.9	bc	ns
Fluopyram + Pyrimethanil	995.3	a	449.9	ac	**
Mancozeb	567.8	ab	380.3	bc	ns
Fluxapyroxad + Pyraclostrobin	-	-	300.9	c	-
Azoxystrobin	281.0	d	303.0	bc	ns
Pyrimethanil	878.0	ab	464.5	ab	**
Propiconazole	891.1	a	419.0	ac	**

[z]IAUDPC was calculated using number of infected plants assessed 12, 21, 27, 35, 42 and 49 DAI in 2011 and 16, 35, 42 and 47 DAI in 2012.
Within columns, values with a common letter do not differ significantly (LSD at alpha= 0.05, except for the 2012 final incidence alpha= 0.1)

The mean SAUDPC values differed between years (P=0.005) and therefore the data were analyzed as separate data sets. In 2011 fungicide treatments had significantly different

SAUDPC values (*P*=0.0012). Azoxystrobin and pyraclostrobin had mean SAUDPC values that were significantly lower than the untreated control (Table 12). Chlorothalonil, difenoconazole, and mancozeb had SAUDPC values similar to azoxystrobin and pyraclostrobin, but were not significantly different from the untreated inoculated control (Table 12). No significant differences were detected in 2012 in SAUDPC values (*P*=0.16) among treatments (Table 12).

Table 12. Effect of fungicide treatment on onion leaf and neck anthracnose severity area under the disease progress curve (SAUDPC).

	SAUDPC[z]			
Treatment	2011		2012	
Untreated non-inoculated	-	-	38.8	ns[y]
Untreated inoculated	65.1	a	47.3	
Chlorothalonil	39.1	ad	35.9	
Pyraclostrobin	29.4	dc	26.6	
Boscalid	-	-	45.8	
Difenoconazole	37.4	ad	31.9	
Fluopyram + Pyrimethanil	51.9	ab	36.0	
Mancozeb	45.6	ad	31.8	
Fluxapyroxad + Pyraclostrobin	-	-	26.6	
Azoxystrobin	26.9	d	33.6	
Pyrimethanil	56.3	ab	44.8	
Propiconazole	49.8	ac	35.3	

[z]SAUDPC was calculated using disease severity rating of inner 4 m plot assessed 12, 21, 27, 35, 42 and 49 DAI in 2011 and 16, 35, 42 and 47 DAI in 2012. Within columns, values with a common letter do not differ significantly (LSD at alpha= 0.05).
[y]ns, not significant differences at alpha= 0.05.

The effect of *C. coccodes* on total yield was significantly different among fungicide treatments in 2011 (*P*<0.001). Azoxystrobin had the highest total and marketable yield and was significantly different than the untreated inoculated control (Table 13). The total and marketable yield of the remaining fungicide treatments did not differ from azoxystrobin or the untreated inoculated control (Table 13). In 2012, significant differences were not

observed among treatments. Plots treated with fluopyram + pyrimethanil had the lowest total yield while treatments of pyrimethanil, boscalid, chlorothalonil and the untreated non-inoculated control had the highest (Table 14).

DISCUSSION

Onion cultivars suitable for Michigan were screened under field conditions and all were susceptible to *C. coccodes*. Disease symptoms were less severe on 'Hendrix' and more severe on 'Highlander' and 'Candy' in both years. Both 'Candy' and 'Highlander' require a relatively short period to maturity (Table 7) and are ready for harvest 2 to 3 weeks earlier than other cultivars. These early maturing cultivars can be planted later in the spring when field operation have been delayed by poor environmental condition such excessive rain. 'Highlander' lodges early and has superior neck closure when compare with other cultivars (194), but has been rated as an onion with thin skin that cracks easily, produce an uneven shaped bulb with an overall poor appearance (194).

The uneven plant stand across cultivars was a significant covariate that was included in the statistical analysis (ANCOVA) and the slopes per cultivar in ANCOVA were not significantly different with a positive slope. This indicates the higher the plant density the higher the disease severity. The number of onions per area and the spatial arrangement of the plants in the plot (clumped plants vs gaps) have an effect on the microclimate of the plots (temperatures, moisture distribution, leaf wetness duration) and can impact the inoculum dispersal in space (36,47,205). The effect of planting density on the severity of onion leaf and neck anthracnose could be further investigated, as it has the potential to be used as a cultural disease management tool.

Table 13. Effect of fungicide treatment on onion yield of 'Infinity' onions when plants were inoculated with *Colletotrichum coccodes* in the field.

	2011 Yield (Kg/4.6 m^2)						2012 Yield (Kg/4.3 $x10^{-4}$ ha)					
Treatment	Non marketable[z]		Marketable[y]		Total[x]		Non marketable		Marketable		Total	
Untreated non-inoculated	-		-		-		21.1	ns	5.4	ns	26.4	ns
Untreated inoculated	12.3	ab	5.3	b	17.6	b	22.8		3.5		26.3	
Chlorothalonil	13.0	ab	6.7	ab	19.7	ab	22.0		5.0		27.1	
Pyraclostrobin	12.3	ab	8.8	ab	21.1	ab	21.2		4.6		25.8	
Boscalid	-	-	-	-	-	-	20.9		5.7		26.6	
Difenoconazole	10.7	b	6.2	ab	16.9	b	20.3		3.8		24.1	
Fluopyram and Pyrimethanil	11.3	ab	9.1	ab	20.4	ab	20.0		2.6		22.6	
Mancozeb	14.8	a	4.8	b	19.6	ab	19.9		4.8		24.6	
Fluxapyroxad and Pyraclostrobin	-	-	-	-	-	-	20.5		5.3		25.9	
Azoxystrobin	13.7	ab	10.8	a	24.5	a	20.7		3.6		24.3	
Pyrimethanil	11.7	ab	4.8	b	16.5	b	19.9		5.9		25.8	
Propiconazole	13.5	ab	8.1	ab	21.6	ab	20.6		2.4		23.0	

[z]Onion bulbs less than 5 cm in diameter, [y]Onion bulbs greater than 5 cm in diameter. Within columns, values with a common letter do not differ significantly (LSD at alpha = 0.05). [x]Total bulbs weight (marketable and non marketable). [w]ns, no significant difference at alpha = 0.05.

Final onion leaf and neck anthracnose incidence varied between years for cultivars Pulsar, Hamlet, Redwing, Stanley and Highlander, where incidence was significantly higher in 2011 compared with 2012 (Table 9). Two factors could explain those differences: the uneven plant stand and differences in weather conditions. Cultivars Pulsar and Stanley had significant differences in the plant stand between years, but cultivars Redwing and Highlander had homogeneous densities.

For 14 out of the 16 cultivars, the onion seed used for 2011 originated from growers and may have been from the previous year's seed lot. Onion seed is known to be short lived (201), and old seed (stored for 1 - 2 seasons or more) could have resulted in deceased germination and an unequal plant stand.

Disease incidence observed on 'Hamlet', 'Redwing' and 'Highlander' differed between years and could be the result of lower air temperatures in August 2012 compared to the same month in 2011 (Figure 8). *Colletotrichum* spp. optimal mycelial growth occurs from 18 to 30°C (80,272,273,285). Lesion formation and disease severity occurs in a similar temperature range but varies among *Colletotrichum* spp. (159,172,205,273). *Colletotrichum orbiculare* optimal infection in watermelon occurs from 21 to 24°C (204), while lesion formation in cucumber foliage occurs from 20 to 28°C. (273). *Colletotrichum truncatum* optimal anthracnose development in lentil occurs at temperatures between 20 to 24°C (59). In onion, *C. coccodes* infected and caused lesions from 15 to 30°C, but disease severity was greater at 25°C (Rodriguez-S and Hausbeck, unpublished data).

The degree of susceptibility observed among onion cultivars and the differences in severity for some cultivars between years, suggests the environment has an effect on their response to *C. coccodes.* Horizontal resistance is controlled by multiple genes, and is known to be influenced by environmental conditions (6). Similarly, horizontal resistance mechanisms had been postulated for the *Allium* spp. and *C. gloeosporioides* pathosystem (113). Screening commercial onion cultivars for their susceptibility to *C. coccodes* is a first step to identify any existing cultivars with resistance to onion leaf and neck anthracnose. Additional sources of resistance could be available in wild relatives of *Allium cepa*. For instance, *A. cepa* relatives *A. fistulosum, A. galanthum* and *A. roylei* showed partial levels of resistance to *C. gloeosporioides* (113). Further research could characterize the response of *A. cepa* wild relatives to *C. coccodes.* Sources of resistance within *A. cepa* could expedite development of new cultivars; sexual barriers exist between *A. cepa* and wild *Allium* spp. that make breeding challenging (8,156,161). In addition, cultivars resistant to *C. coccodes* need the desired market quality characteristics suitable for Michigan.

Colletotrichum coccodes was recovered from roots of several onion cultivars even though microsclerotia were not observed at collection time. Instead microsclerotia were observed 14 days after plating the roots, with *C. coccodes* recovery percentage ranging from 5 to 15%. Although onion is a low organic residue crop, *C. coccodes* could overwinter in onion roots left behind after harvest. Microsclerotia in roots will contribute to soilborne inoculum, as it does in tomatoes and potatoes (82,175). Survival and persistence of this inoculum can be influenced by residue location in the soil profile and soil management after onion harvest, as described in tomatoes (82). Number of *C. coccodes* infected root

segments decreased with foliar fungicide applications (83) and therefore foliar fungicide applications could reduce *C. coccodes* soilborne inoculum.

SAUDPC values for the fungicide treatments were lower in 2012 compared to 2011 (Table 12). A similar effect was observed for IAUDPC; fluopyram + pyrimethanil, propiconazole, and pyrimethanil had significantly lower IAUDPC values in 2012 than in 2011 (Table 11). Temperatures in July 2011 and 2012 (Figure 8) were conducive to *C. coccodes* infection and symptom development (Rodriguez, L. M, unpublished data) but temperatures in August 2012 were cooler (Figure 8) and resulted in decreased leaf and neck anthracnose severity and incidence.

Complete control of onion leaf and neck anthracnose was not achieved with the fungicides included in this study. Azoxystrobin, and pyraclostrobin limited incidence in both years. Fluoxapyroxad + pyraclostrobin had a similar effect comparable to other QoI fungicides azoxystrobin and pyraclostrobin. Therefore, the QoI active ingredient in fluoxapyroxad + pyraclostrobin was likely responsible for the efficacy of this product. QoI fungicides have a translaminar movement in the plant tissues (154,288,293). Protectant, residual fungicides such as chlorothalonil and mancozeb are broad spectrum fungicides with a multisite mode of action (126). The efficacy of protectant fungicides is influenced by their lack of mobility on plant tissues, weathering, and need for frequent reapplication (46,100,126). In other studies, repeated applications of chlorothalonil alone has shown to have a negative effect on onion yield, an effect that has not been observed with repeated applications of mancozeb (198,264).

Mixture or alternation of QoI fungicides, DMI (difenoconazole) and protectants, should be included in fungicide programs to manage onion leaf and neck anthracnose. This strategy

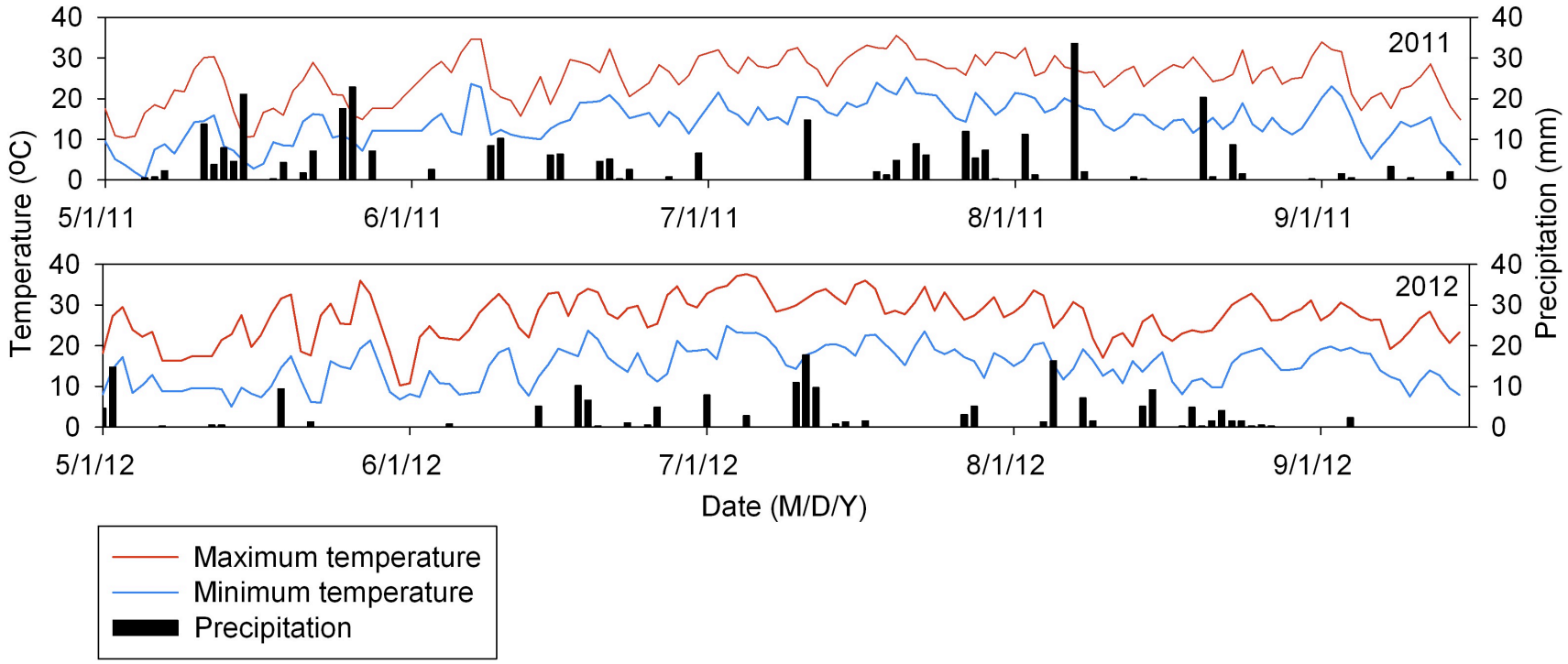

Figure 8. Air temperature and precipitation at the Southwest Michigan research and extension center, Benton Harbor, MI during 2011 and 2012 summer season. Blue series represent minimum air temperature; red series represent maximum air temperature, solid bar represent precipitation events. Solid circles denote fungicide application, open squares mark planting date and dash circle denote inoculation date.

can help delay resistance build up to the strobilurin chemistry as resistance has been reported in other pathosystems (20,23,117). In Ohio, *C. coccodes* and other *Colletotrichum* spp. isolated from tomato fruit were sensitive to axozytrobin, mancozeb and chlorothalonil (128). However, resistance is a growing concern as shifts in fungicide sensitivity on *C. coccodes* isolated from potato has been observed (18).QoI fungicides are currently labeled and recommended in Michigan to control key onion foliar blights (32) and are also effective in controlling leaf and neck anthracnose. Difenoconazole was similar to the protectant fungicides in efficacy against *C. coccodes*, while propiconazole performed similar to the non-efficacious fungicides in the FRAC groups 7 (carboximides) and 9 (AP). The results of this study are in contrast with studies in chili pepper anthracnose incited by *C. capsici*, where both propiconazole and difenoconazole reduced severity of this disease; the propiconazole treatment resulted in lower disease incidence than difenoconazole (119). Treatments of pyrimethanil, fluopyram + pyrimethanil, propiconazole and boscalid did not limit *C. coccodes* in onions, and were similar to the untreated inoculated control.

In onion, the orientation and waxy cuticle of the leaves require a fine droplet size that can be achieved with the proper spray equipment calibration, volume and pressure (261). Larger spray volumes are desirable as coverage increases by using a higher volume of the carrier (120). For fungicide application and inoculation a flat fan nozzle was used to take advantage of its spray pattern and minimize spray drift (120). Lack of efficacy is attributed mainly to the active ingredient, but formulation, deposition, actual coverage and retention in foliage could play a role. Evidence supports that adjuvants increase azoxystrobin absorption in onion (115). The use of a fungicide mixed with a spreader sticker adjuvant resulted in a significant reduction of *Botrytis* spp. and *Alternaria porri*

incidence (163) while some adjuvants cause injury on onion foliage (115). Testing mixtures of fungicides with and without adjuvants against *C. coccodes* may provide additional options to limit leaf and neck anthracnose.

Despite the differences among fungicides on total yield in 2011, the effect of *C. coccodes* on yield needs to be further investigated. The untreated non-inoculated was included in 2012 as an attempt to observe differences in relative yield caused by *C. coccodes*. However, leaf and neck anthracnose was observed on the untreated non-inoculated onion plots shortly after inoculated plots showed disease symptoms. Therefore, the untreated non-inoculated plots were cross infected due to i) inoculum drift at inoculation time or ii) potential soil bone inoculum from the 2011 trials. Splash dispersal of inoculum may initiate disease epidemics in *Colletotrichum* spp. (186,219,224) when precipitation events or irrigation move inocula within a field. The small droplet size used to inoculate the plots could have drifted to nearby plots. The 2012 trials were planted in the same location as the *C. coccodes* inoculated trials of 2011. Therefore soilborne inoculum could have also contributed to the untreated non-inoculated plots developing onion leaf and neck anthracnose symptoms.

As new fungicides are developed and registered and other onion cultivars become available, additional testing could be helpful. Combining cultivar resistance with effective fungicides would provide the most effective management strategies for onion growers. In conclusion, QoI fungicides limited onion leaf and neck anthracnose compared with the other FRAC groups included in these studies. Protectant multisite fungicides and one of the DMI fungicides tested (difenoconazole) provided some control but not to the level of the QoI fungicides. However, multiple active ingredients should be included in a fungicide rotation

program to manage onion leaf and neck anthracnose in the field. The combination of host resistance, efficacious fungicides sprays and cultural practices (pathogen free seed, crop rotation, weed control, plant spacing, timed and limited irrigation) are key for the management of onion leaf and neck anthracnose. Michigan growers should avoid planting cultivars such 'Highlander' and 'Candy', apply fungicides, and rotate onions with crops that are not hosts of *C. coccodes*.

CHAPTER IV

CHARACTERIZATION, VIRULENCE, EPIDEMIOLOGY, AND MANAGEMENT OF *COLLETOTRICHUM ACUTATUM* SENSU LATO CAUSING ANTHRACNOSE IN CELERY

ABSTRACT

Colletotrichum acutatum sensu lato caused leaf curling and petiole twisting in several commercial fields in five Michigan counties in 2010 through 2012. Based on conidial morphology and species-specific primers, the isolates belong to the *C. acutatum* species complex with the exception of one isolate (C57-8) that was designated as *Colletotrichum* sp.. Inter Simple Sequence Repeat ISSR banding patterns were similar between isolates from celery (n=51) and blueberry (n=1), but different from the negative controls (*C. dematium* and *C. gloeosporioides*). Four ISSR primers resulted in 4% polymorphism when tested on isolates from celery. *C. acutatum* transcriptome derived Simple Sequence Repeat (SSR) marker from Expressed Sequence Tags (ESTs) were generated, with SSR markers showing 12% polymorphism. Pathogenicity of *C. acutatum* s.l. isolated from celery (n=81), tomato (n=2) and blueberry (n=1) were evaluated in greenhouse experiments. Differences in virulence were found among isolates, but no significant differences specific to collection year, county, or field were observed. Studies were conducted in dew and growth chambers to determine the effect of temperature (15, 20, 25, 30°C) and wetness duration (0, 12, 24, 48, 72 and 96 h) on disease (lesions and leaf/petiole distortion). High temperatures (>25°C) or long wetness duration (>24h) resulted in increased disease incidence. An interaction between temperature and wetness duration was not found. Twelve fungicides were tested in field studies in two consecutive years to determine their efficacy against celery anthracnose. The fungicides azoxystrobin, pyraclostrobin, mancozeb, and chlorothalonil significantly reduced disease compared to the untreated control. Rotation of a strobilurin fungicide (azoxystrobin or pyraclostrobin) with

a protectant fungicide (chlorothalonil) is recommended to limit celery anthracnose in the field.

INTRODUCTION

In the U.S., 12,000 hectares of celery (*Apium graveolens* L. var. *dulce* (Mill.) Pers.) were planted in 2012. A total of 57,406 tons of celery were harvested in Michigan during the same year, accounting for approximately $22 million (15). Celery is grown in organic soils in the west side of Michigan's lower peninsula (274), and is cultivated for both the fresh and processing markets.

Early blight (*Cercospora apii*), late blight (*Septoria apiicola*), and bacterial blight (*Pseudomonas syringae* pv. *apii*) commonly occur in celery. Management includes using pathogen-free, treated seed, and fungicide sprays (39,74,164). In 2010, inward curling of the leaves and inconspicuous elliptical sunken lesions along the petioles were observed and determined to be caused by *Colletotrichum acutatum* sensu lato and the disease is named celery leaf curl and petiole anthracnose (238).

Colletotrichum acutatum s.l. has a broad host range (97,224) that includes strawberry, citrus, apple, peach, blueberry, cranberry, olive, avocado, pine , and pepper (97,177,224). Common symptoms incited by this pathogen include necroses and lesions in various tissue types either pre- and post-harvest (224,285). *Colletotrichum acutatum* inoculum can be present in the soil, plant debris, weeds (89,104), and this pathogen has been reported to be seed-borne (235). This pathogen is splash dispersed (187,292) and known to establish biotrophic, hemibiotrophic and necrotrophic host infections (224).

Colletotrichum orbiculare (97,255,282) and *C. acutatum* (290) were reported as pathogens of celery in Australia. Recently, *C. acutatum* has been reported as a pathogen of

celery in the U. S. with reports from Michigan (238) and Pennsylvania (227). *Colletotrichum simmondsii* was reported causing celery stunt anthracnose in Japan (110). Other *Colletotrichum* species are reported to infect members of the carrot and celery family (Apiaceae) but are considered minor pathogens. In carrot, *C. acutatum* was reported in Poland (97,207) and *C. gloeosporioides* in Brazil (97,195). *Colletotrichum truncatum* was reported in India and *C. gloeosporioides* in Brazil (97) as a pathogens of cilantro.

Multiple studies have characterized *C. acutatum* s.l. isolates based on conidial and appresorial morphology and various molecular markers (107,153,171,260). Species-specific diagnostic markers have been widely used to differentiate among *C. acutatum*, *C. gloeosporioides* and other *Colletotrichum* spp. (3,95,101,254). Pathogen population and genetic diversity studies have combined species-specific primers with sequencing of various genes to infer phylogenetic relationships (85,123,183). Based on the sequence of six different genes, the *C. acutatum* s.l. or complex is considered a monophyletic unit (54). The complex comprises closely related species that group on five phylogenetic clades and 29 subclades/species (71). Damm et al. confirmed the *C. lupini* (210), *C. simmondsii* and *C. fioriniae* (252) species designations and described 25 additional species not previously recognized within the *C. acutatum* clade (71).

Multiple studies have explored techniques that use arbitrary random molecular markers such Inter Simple Sequence Repeat (ISSR (200,233)) and Randomly Amplified Polymorphic DNA (RAPD (286)) to study genotypic diversity in pathogen populations. ISSR and RAPD banding patterns reproducibility issues can be addressed using Simple Sequence Repeat (SSR) markers, which are more reliable markers, highly abundant, polymorphic and codominant yielding with specific and reproducible bands. SSR markers

identification has evolved from preparation of enriched libraries (60) to identification of SSR from Expressed Sequence Tags (ESTs) in plants (88,284) and plant pathogens (58,84,114). Due to the lack of *C. acutatum* genome wide sequences, SSR markers based on transcriptome sequences were generated in this study to investigate genic variability of *C. acutatum* s.l. isolated from celery.

The epidemiology of *C. acutatum* has been extensively studied in strawberry (159,172,187,188,218,287), and most recently in almonds (78) and olives (205). In general, disease incidence increased as wetness duration increased in strawberry fruit (287) and almond blossoms and leaves (78). Optimal infection occurred at 25 to 30°C in strawberry (287), 15 to 20°C in almonds (78), and 17 to 24°C in olives (205). Infection models and disease thresholds have incorporated environmental conditions to forecast strawberry fruit infection and time fungicide applications (184,287). Environmental conditions conducive to celery anthracnose symptoms and disease progression are not known but are needed to develop effective management strategies.

In 2011 and 2012, fungicides including copper, thiram, mancozeb, and chlorothalonil, limited celery leaf curl under glasshouse and field conditions in Australia (135). New fungicides are now available to limit diseases caused by *Colletotrichum* spp. on various crops including the QoI inhibitors or strobilurins, (178,278), demethylation Inhibitors-DMI (108) and multi site protectant fungicides such as chlorothalonil, and mancozeb (2,102,108,128,148,185).

The objectives of this study were i) to investigate pathogenic and genetic variability within the *C. acutatum* s.l. isolated from celery, ii) to determine the effects of temperature and leaf wetness duration on *C. acutatum* s.l. infection and disease progress on celery

petioles and leaves, and iii) to evaluate different fungicides active ingredients to limit celery leaf curl and petiole anthracnose.

MATERIALS AND METHODS

Isolate collection and maintenance

In 2010, 2011 and 2012, 58 fields distributed in five counties in Michigan were scouted for leaf curling and lesion on the petioles (Appendix B, Table B1). In each field, symptomatic celery petioles were collected and transported to the lab where the pathogen was isolated onto 25% strength potato dextrose agar (PDA). A *C. acutatum* isolate from blueberry, *C. dematium* and *C. gloeosporioides* (used as negative controls) all courtesy of A. Schilder; and two *Colletotrichum* spp. isolated from tomato were also included in the culture collection.

Monosporic cultures of all isolates were maintained in long-term storage as mycelial plugs in 700 µl of 30% glycerol at -20°C, and as 200 µl of conidial suspension mixed with 1000 µl of conservation media (20% glycerol, 0.04% yeast extract, 0.1% malt extract, 0.04% glucose, 0.02% K2HPO4) at -80°C on 2.0ml cryogenic vial (Sigma-aldrich St. Louis, MO); both techniques modified from Dhingra et al. (77).

Morphological characterization

Microscopic characteristics were examined for 62 *Colletotrichum* spp. isolated from celery in Michigan. Monoconidial isolates were grown in 25% strength PDA and incubated at 21 ± 2°C under fluorescent light for seven days. Conidial masses were then scraped from the colonies with a sterile toothpick and mounted onto a glass slide with 15 µl of lactophenol solution (100ml lactophenol and 10ml glacial acetic acid) and sealed with

Cytoseal 60 (Thermo Scientific; Waltham, MA). Permanent slides were then inspected under a light microscope (Leica Microsystems, Wetzlar, Germany) and photographs were taken with a Leica Digital camera DC 300 (Leica Microsystems, Wetzlar, Germany). For each isolate, conidial length and width of 10 randomly chosen conidia were measured. The experiment was conducted twice.

DNA extraction

Colletotrichum spp. isolates were transferred into 35ml of 50% strength potato dextrose broth and incubated on a rotary shaker (MaxQ™ 4000 Thermo Scientific; Waltham, MA) for 7 days at 23 ± 2°C. Mycelial masses were then vacuum-filtered and lyophilized overnight on a FreeZone 1 Liter Benchtop Freeze Dry System (Labconco, Kansas city, MO). Dry mycelium was then macerated and 500 µl of extraction buffer (AutoGen, Inc; Holliston, MA) was added. The samples were submitted to the MSU Research Technology Support Facility (East Lansing) for robotically assisted DNA extraction using AutoGen 740 (AutoGen Inc; Holliston, MA). DNA concentration was quantified using NanoDrop ND 1000 spectrophotometer (Thermo scientific; Wilmington, DE) and NanoDrop 2.4.7c software (Thermo scientific; Wilmington, DE). DNA quality and integrity was analyzed by electrophoresis in 1% (wt/vol) agarose gel in 0.5X Tris-borate-EDTA buffer (52), stained with ethidium bromide (5 µg/ml) for visualization (190). DNA was diluted to 10ng/µl for PCR amplification.

Species-specific primers and ISSR markers

DNA from 113 *C. acutatum* s.l. isolates was used as a template to amplify the *C. acutatum* specific primers CaInt2 and ITS4 as previously described (199), except that the annealing temperature used was 60ºC. The 490 bp PCR fragment was visualized in a 2%

agarose gel, stained with ethidium bromide as described in the previous section and compared with a 100 bp DNA ladder (New England biolabs Inc; Ipswich, MA). A survey of isolates including an isolate from celery in 2010, celery 2011, tomato, a positive (*C. acutatum* from blueberry) and negative controls (*C. dematium* and *C. gloeosporioides*) were used to evaluate five ISSR motifs (GTC5, CAG5, GACAC3, GACA4 and TCC5). Four ISSR were amplified on a bigger panel of 56 isolates including *C. acutatum* s.l. isolated from celery (n=51), tomato (n=2) and isolate controls (n=3). PCR reactions were carried out on a 20 µl final volume containing 50 ng of genomic DNA, 1 mM of primer, 1 x PCR buffer (Promega Corp; Madison, WI), 250 mM of total dNTP and 1.25 unit of Go Taq polymerase (Promega Corp; Madison, WI). Markers were amplified on a Eppendorf mastercycler ep systems thermal cycler (Eppendorf, Westbury, NY) using the following program: hot start of 95°C for 1 min; then 30 cycles of 95°C denaturing for 30 seconds; 48°C (GACAC3, GACA4 and TCC5) or 60°C (CGA5 and GTG5) annealing for 30 sec and 72°C extension for 1.5 min. ISSR PCR fragments were separated on 2% agarose gels stained with ethidium bromide and a 100 bp or a 1 Kb DNA ladder (New England biolabs Inc; Ipswich, MA).

RNA extraction, purification and sequencing

Five, seven and fourteen-day-old cultures of *C. acutatum* s.l. isolate C26-1-2 were used for RNA extraction. Mycelium was scraped from the surface of the cultures growing on PDA using RNase free water and sterile tongue depressors. Mycelia were transferred into 1.7 ml microcentifuge tubes. Tubes were kept on dry ice while retrieving scrapings, and stored at -80°C until lyophilized. Mycelia were lyophilized as described for DNA

extraction above. Total RNA was extracted using Triazol (Invitrogen, Life Technologies; Grand Island, NY) following the procedure described by Hallen et al. (131). Total RNA was DNase treated using the turbo DNA-free Kit (Ambion- Applied biosystems, Carlsbad CA). RNA concentration and quality was determined using the Bioanalyzer 2100 (Agilent Technologies, San Diego CA). A mixture of RNA obtained from the different growth stages was mixed in a 1:1:1 proportion and submitted to MSU Research Technology Support Facility (East Lansing MI) where RNA-Sequencing (RNA-Seq) libraries were constructed and sequenced using Illumina HiSeq platform (Illumina, San Diego, CA).

Transcriptome data analysis, SSR finding

RNA-Seq reads were analyzed using FastQC (Babrahan Bioinfomatics, Cambridge, UK) and FastXTool kit (Cold Spring Harbor Laboraroty, Cold Spring Harbor NY) for base call quality, trimming of barcodes and primers. Cleaned reads were used to construct a transcriptome assembly and search for longer isoforms were performed using Oases (246). The assembled *C. acutatum* transcriptome was compared with the annotated *C. graminicola* genome (223) and the uniref database (269) using BlastX (10). SSR motifs were identified and primers that flank the SSRs were designed as described in Hamilton et al (132).

SSR marker selection and amplification

Only SSR loci with perfect repeat units of 2-6 nucleotides (SSR type) were selected. A set of 28 SSR markers (Appendix B, Table B4) was tested for amplification in *C. acutatum* s.l. isolated from celery, tomato and blueberry. The 28 primers were selected as follows: trinucleotides (10) hexanucleotides (8), pentanucleotides (6), dinucleotides (2) and tetranucleotides (2). PCR conditions were used throughout the initial (6 isolates) and pilot surveys (96 isolates). The PCR reactions were carried out on a 25 µl final volume

containing 30 ng of genomic DNA, 0.1 mM of each of the forward and reverse primers, 1 x PCR buffer (Promega Corp; Madison, WI), 250 mM of total dNTP and 0.5 unit of Go Taq polymerase (Promega Corp; Madison, WI). Markers were amplified on a Eppendorf mastercycler ep systems thermal cycler (Eppendorf, Westbury, NY) using the following program: hot start of 93°C for 4 min; then 30 cycles of 93°C denaturing for 1 min; 55°C annealing for 1 min and 72°C extension for 2 min; and final extension at 72°C for 5 min. PCR fragments were visualized in 4% agarose and stained with ethidium bromide as previously described for ISSR markers.

Plant material used in greenhouse, and growth chamber experiments

Celery seedlings 'Green Bay' were grown in research greenhouses located on the campus of MSU with a 15 h light/ 9 h dark photoperiod and temperatures of 23 ± 6°C. Celery seedlings were started from seeds planted in 288 in cell flats (12 cm^3) containing Suremix perlite media (Michigan grower products, Inc., MI). Seedlings were grown in flats until they developed 2 to 3 petioles and then they were transplanted into 176 cm^3, seedlings were watered as needed and fertilized biweekly with 20-20-20.

Pathogenicity experiments

A total of eighty-five *C. acutatum* s.l. isolates were included in the pathogenicity experiments. Humid chambers (Length 178 cm x Width 100 cm, x Height 63 cm) consisting of a balsa wood frame wrapped in clear plastic bags were placed on a greenhouse bench and used for these experiments. The inside of the tents were sprayed with distilled water; damp paper towel was placed on the border and across the center of the tents to ensure high (95 ± 5 %) relative humidity (RH). A Watchdog (Spectrum technologies Inc;

Plainfield, IL) was placed inside each humid chamber to measure temperature and RH. Five-week old celery seedlings were placed in the humid chambers overnight prior to inoculation.

Temperature and RH experiments

The effect of temperature and wetness duration was tested on six-week-old celery seedlings. Celery seedlings were moved from the greenhouse and placed in a dew chamber (Model I35-DL, Percival Scientific Inc, Boone, IA) at 96 ± 3% RH and 25°C ± 2(day)/ 20°C ± 2 (night) overnight for 19 hours. Celery plants were retrieved from the dew chamber, and leaves and petioles spray-inoculated with a *C. acutatum* conidial suspension. Plants were returned to the dew chamber immediately after inoculation. Plants randomly assigned to 0 hours of wetness were placed in the growth cambers (Model CMP3244, Conviron, Pembina, ND) set at 15, 20, 25 or 30°C. After each wetness period (12, 24, 48,72, 96 h) was completed in the dew chamber, two plants were moved to the growth chambers at four different temperatures.

Fungicide experiments

Field plots were located at the MSU Plant Pathology Farm, East Lansing, MI, in a field of Houghton muck. Blocks 3 and 4 were pure Houghton muck while blocks 1 and 2 contained 13% sandy loam (14). The field was previously planted to tomato (2010) and pepper (2011). On 3 June 2011 and 23 May 2012, five-week old 'Green Bay' celery seedlings were transplanted in the field 16 cm apart in rows that were 0.9 m apart. The treatment plots were 6.1 m long with a buffer row in between fungicide treatment plots and 0.7 m between blocks. Ten (2011) and twelve (2012) fungicide treatments, untreated

Table 14. Fungicides and rates evaluated for managing celery leaf curl and petiole anthracnose on 'Green Bay' celery inoculated with *Colletotrichum acutatum* s.l. in field trials conducted in 2011 and 2012.

	Treatments[z]	Active ingredient (a.i.)[y]	Formulation	Rate (kg a.i./ha)	FRAC code	Labeled in Celery	Company[x]
1	Untreated inoculated						
2	Untreated non inoculated						
3	Quadris	azoxystrobin	SC	0.22	11	Y	Syngenta
4	Endura	boscalid	WG	0.36	7	Y	BASF
5	Bravo Weather Stik	chlorothalonil	SC	1.68	M5	Y	Syngenta
6	Kocide	copper hydroxide	DF	0.86	M1	Y	Dupont
7	Nordox +	cuprous Oxide +	WG	1.68	M1	Y	Nordox As
	Bravo Weather Stik	chlorothalonil	SC	1.05	/M5		Syngenta
8	Inspire	difenoconazole	SC	0.13	3	N	Syngenta
9	Scala	pyrimethanil	WG	0.79	9	N	Bayer
10	Luna Tranquility	fluopyram/ pyrimethanil	SC	0.58	7/9	N	Bayer
11	Priaxor	fluxapyroxad/ pyraclostrobin	SC	0.22	7/11	N	BASF
12	Manzate Pro Stik	mancozeb	DF	2.52	M3	N	Dupont
13	Tilt	propiconazole	EC	0.23	3	Y	Syngenta
14	Cabrio	pyraclostrobin	EC	0.17	11	Y	BASF

[z]Fungicide treatments were applied on a 7 ± 2 days spray interval

[y](+)= treatments were tank mixed prior to application; (/)=premix product

[x]BASF = BASF Corp., Research Triangle Park, NC; Bayer = Bayer CropScience LP, Research Triangle Park, NC; DuPont = E. I. du Pont de Nemours and Co., Wilmington, DE; Syngenta = Syngenta Crop Protection, Inc., Greensboro, NC; Nordox As= Nordox As Oslo, Norway.

control (2011 and 2012) and untreated non-inoculated (2012) treatments (Table 14) were arranged in a randomized complete block design (RCBD) with four blocks. In the field, a mist irrigation system was used to maintain water requirements of the crop and promote *C. acutatum* infection, dispersal, and disease development.

The designated fungicide treatments were applied once or twice to the plots prior to inoculation with *C. acutatum.* Fungicides were applied on a 7- to 10-day interval, depending on the weather (wind speed). Prior to inoculation, the sprinkler irrigation system was operated for 2 hours to provide, leaf wetness, and high RH conducive to infection in the field.

Inoculum production and inoculation

Colletotrichum acutatum s.l. isolates C12-1-3, C26-1-2, C27-1-3, C40-1-10 and C42-1-1 isolated from celery in Michigan in 2010 were used for growth chamber and field experiments. For the pathogenicity experiment, 85 *C. acutatum* s.l. isolates were tested (Appendix B, Table B1.). Isolates were transferred onto 50% strength potato dextrose agar (PDA) from long-term storage. After incubating for seven days under fluorescent light at 22 ± 2°C, *C. acutatum* s.l. cultures were used to prepare a conidial suspension. For the pathogenicity and temperature/RH experiments, cultures were flooded with 3 ml of double distilled water, and conidia were dislodged with a sterile glass cell spreader. The suspension was collected from the plate and added to 17 ml of 0.001% Tween double distilled water. For the field experiments, 7 day-old sporulating colonies of C. *acutatum* s.l. isolates C12-1-3, C26-1-2, C27-1-3, C40-1-10 and C42-1-1 (12 plates per isolate) were placed in a blender with 300 ml of 0.001% Tween double distilled water and blended to dislodge conidia. The resulting mixture of isolates was strained through four layers of sterile cheesecloth. For all

experiments, a hematocytometer was used to determine conidial concentration; the conidial suspension was adjusted to 1.0 x 10^6 conidia/ml using sterile double distilled water with 0.001% Tween.

Disease evaluation

For the pathogenicity and growth chamber experiments celery plants were rated 5, 7, 14 and 21 days after inoculation (DAI). Number of curled leaves and lesions on the petioles were used to calculate the area under the disease progress curve (AUDPC). In the field experiments, incidence and severity of celery leaf curl and petiole anthracnose was evaluated weekly from 5 to 53 DAI on plants in the inner 5.5 m of each treatment plot. Severity was visually assessed using a zero to five scale as follows; 0: no symptoms, 1: >1 - 10%, 2: >10-25%, 3: >25- 50%, 4: >50-75% and 5: >75- 100% plant area showing leaf curl and petiole anthracnose symptoms. At the end of the field experiment, plots were hand harvested. Healthy plants were trimmed following fresh market specifications (39), weighed for the marketable yield. The diseased plants and diseased tissue were considered trimmings or non-marketable weight.

Pathogen confirmation was conducted by isolating the pathogen from 40 and 20% of the celery inoculated in the pathogenicity and temperature/RH experiments, respectively, and for the field trial by re-isolating from four plants in all plots per block.

Experimental design and statistical analysis

Three complete replications were conducted for all experiments. The greenhouse experiments were conducted using a randomized complete block design (RCBD) and one celery seedling per isolate was inoculated per humid chamber and three humid chambers were used per replication.

A split- plot design with two fixed factors was used for the growth chamber experiments. Replication was treated as a blocking factor, temperature as the whole-plot factor within each block (replication), and wetness duration as the sub-plot factor within each temperature. The field trial replications were conducted twice in 2011 and once in 2012, all using a RCBD with four blocks. Incidence, severity and yield (field trial only) were analyzed using the PROC MIXED and PROC GLIMMIX procedure of the SAS statistical analysis software (SAS Institute Inc., Cary NC). Data were tested for equal variances using Levene's test. Residual normal distribution was tested using box plot, normality plots, q-q plots and the Shapiro Wilk test. ANOVA was evaluated using type 3 effects output, significant differences ($P<0.05$) were subject to grouping by Least Square Difference (LSD).

RESULTS

Celery anthracnose was widespread across Michigan celery producing counties (Allegan, Barry, Kent, Ottawa, and Van Buren). Only eight fields appeared to be free of celery anthracnose out of the 58 fields scouted. A total of 87, 100 and 40% of the fields scouted were positive for celery anthracnose in 2010, 2011 and 2012 respectively. A culture collection of 549 *Colletrotrichum* spp. isolates was established. A total of 180, 325 and 44 isolates were collected in 2010, 2011 and 2012 respectively.

A subset of 62 *Colletrotrichum* spp. isolates had elliptical conidia, with 1 (or both) acute ends (data not shown) and conidial sizes ranging from 7.1 to 12.1 x 2.3 to 5.1 μm and average L/W ratio 3.1 (Table 15, Appendix B and Table B2). From the 113 *Colletotrichum* spp. isolates included in the diagnostic marker testing, 112 *Colletotrichum* spp., amplified the 490 bp fragment expected for the CaInt2 / ITS2 primer combination. This included 109

Colletotrichum spp. isolated from celery, and *C. acutatum* from blueberry and tomato (positive controls). The species-specific marker did not amplify in isolate C57-8 from celery, the negative controls (*C. dematium* and *C. gloeosporioides*) or the blank reaction. Based on morphological characteristics and species-specific primers, 109 *Colletotrichum* spp. isolated from celery were identified as *C. acutatum* s.l.

Table 15. Conidial size of *Colletotrichum* spp. isolated from celery with celery leaf and neck anthracnose symptoms, data grouped by county of collection.

	No.	Length (µm)		Width (µm)		L/W Ratio	
County[z]	Isolates	Mean	SE[y]	Mean	SE	Mean	SE
A	5	9.5	0.3	3.1	0.1	3.1	0.1
B	4	9.2	0.4	4.2	0.4	3.3	0.1
K	21	9.9	0.1	3.2	0.0	3.1	0.0
NA	6	8.7	0.2	3.0	0.1	2.9	0.1
O	5	9.1	0.3	3.1	0.1	3.0	0.1
VB	21	9.6	0.1	3.1	0.0	3.1	0.0

[z]Michigan county; A= Allegan, B= Barry, K=Kent, N.A.= no information available, O=Ottawa, VB=Van Buren

[y]SE= Standard error

From the initial ISSR survey, four primers yielded banding patterns. Only the TCC5 primer did not amplify. An average of four loci were amplified, with a maximum of seven loci for CAG5 primer. When primers GTC5, CAG5, GACAC3 and GACA4 were tested on additional isolates, band sizes and presence were consistent to those observed on the initial survey. Isolate C57-8 showed differential patterns or did not amplify; providing additional evidence to designate this isolate as *Colletotrichum* sp. Polymorphism was low (4%), with two isolates (C53B-2 and C24B1-1-3) showing differential banding patterns on two ISSR markers. The majority (96%) of isolates recovered from celery showed identical banding size and distribution patterns to the *C. acutatum* isolated from blueberry and tomato.

Banding patterns were different from the negative control included in the experiments (*C. dematium* and *C. gloeosporioides*).

For the transcriptome analysis and identification of SSR markers, a total of 13,213 transcripts were assembled, of which, 13,188 encoded a predicted protein. A total of 2,619 transcripts contained microsatellites sequences and suitable primers were designed for 2,423 transcripts. Trinucleotide SSRs were the most abundant motif (996 loci) in the *C. acutatum* transcriptome with 996 loci, followed by tetranucleotides with 463 loci (Appendix B, Table B3).

The rate of successful amplification was 89%, 25 primers out of the 28 primers pairs amplified the expected product size. Polymorphism was observed with 12% of the markers tested on the *C. acutatum* s.l. isolates. Polymorphic primers were CA5807, CA5317 and CA2513 with 3, 3 and 2 different alleles, respectively. The expected allele was present on 82, 89 and 80% of the isolates while the different alleles were present at frequencies of 18, 11 and 20% respectively for CA5807, CA5317 and CA2513.

Pathogenicity test showed *Colletotrichum acutatum* s.l. isolated from celery (n=81), blueberry (n=1) and tomato (n=2) were pathogenic to 'Green Bay" celery seedlings. Isolate C57-8 failed to cause symptoms on celery seedlings for the duration of the experiment. Leaf curling and lesion in petioles were the prevalent symptoms observed. Petiole twisting, adventitious root formation on lesions, or petiole galls were observed 14 to 21 DAI but were not consistently observed for the same isolate or at the same rating date across trials (data not shown).

Lesion formation in petioles was observed across rating dates (7, 21 and 28 DAI). Occasionally, lesions on petioles coalesced and caused the petiole to break. Replications

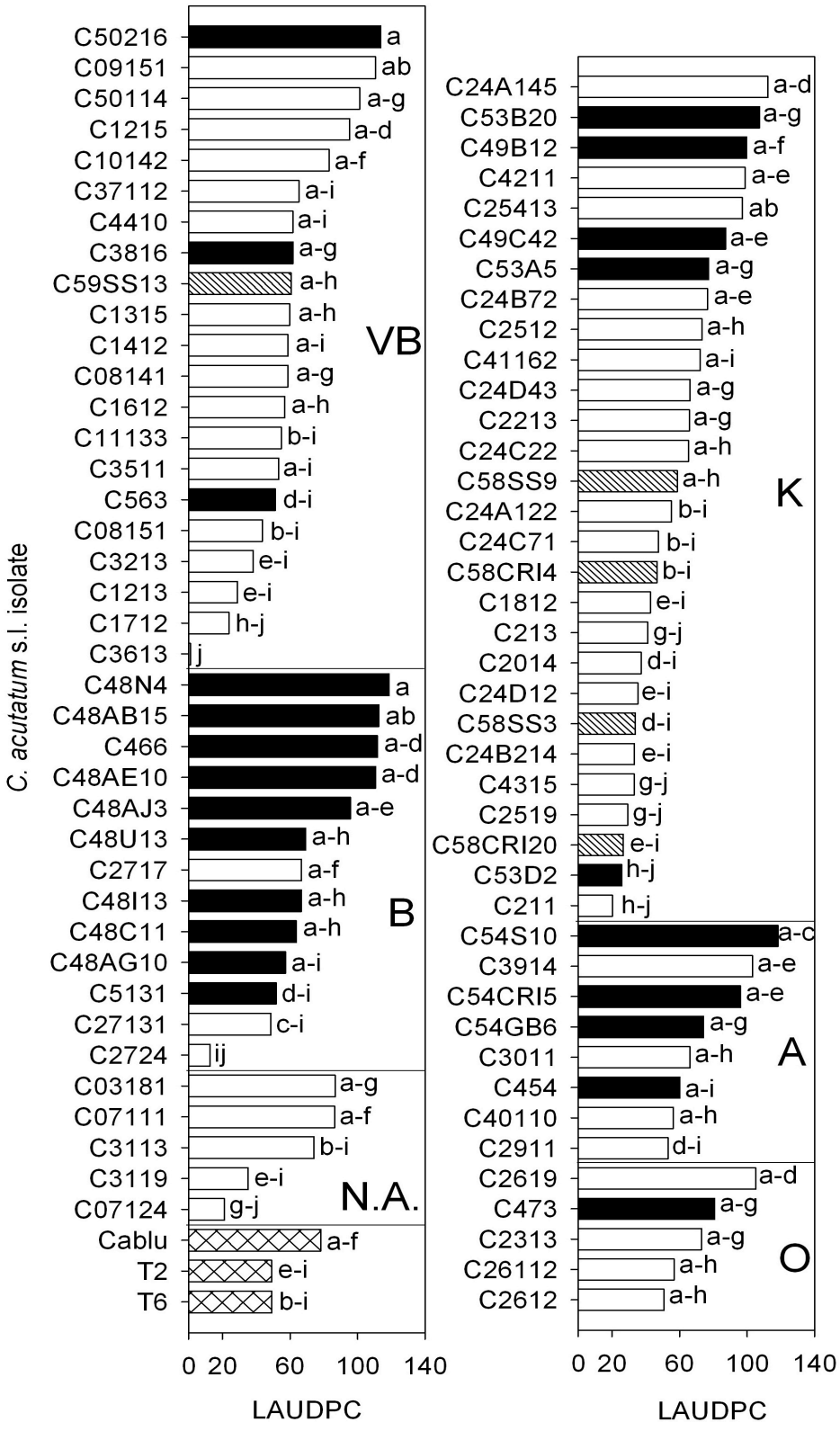

Figure 9. Celery leaf curl and petiole anthracnose lesion area under the disease progress curve (LAUDPC) on 'Green Bay' celery seedlings inoculated with one of the 84

Figure 9. (cont'd) *Colletotrichum acutatum* s.l. isolates obtained from celery, tomato (T2 and T6) and blueberry (Cablu). Graphs group celery isolates by collection county (A= Allegan, B= Barry, K= Kent, O= Ottawa, VB= Van Buren, N.A.= no information available), collection year (open bars=2010, solid bars 2011 and hatched bars 2012), and isolates from other hosts (crosshatched bars). Bars with common letters are not significantly different, LSD *P*=0.05.

were analyzed together as no significant differences were found among replications. Differences in virulence among isolates were observed for disease incidence (Figure 9). Isolates C50-2-16 and C48N4 had the highest number of lesions over time-LAUDPC (Figure 9). In contrast, isolate C36-1-3 had the smallest LAUDPC value (Figure 9). No trends in virulence were observed by year of collection or origin of collection (i.e. county or field). The *C. acutatum* s.l. isolates were successfully reisolated from the lesion on the petioles and discoloration on the margins of curled leaves (90 to 84% respectively). Only 5 to 15% of the isolations from adventitious roots, galls, or twisted petioles without lesions yielded *C. acutatum* s.l.

Celery seedlings inoculated with *C. acutatum* s. l. mixture of isolates showed leaf curling and anthracnose petiole lesions 5 DAI on the temperature and leaf wetness duration experiments. *Colletotrichum acutatum* s.l .was able to infect petioles and cause symptoms in all combinations of wetness duration and temperature tested. Non-inoculated controls remained disease free. Replications in time were analyzed as pooled data since no significant differences were found among whole experiment replications. A significant interaction of temperature and wetness duration periods was not found for lesion count (*P*=0.467) or for the number of curled leaves (*P*=0.284). However, temperature or wetness duration had a significant effect on lesion numbers and number of curled leaves (Table 16).

The highest temperature (30°C) or greatest duration of leaf wetness (96h) resulted in increased lesion AUDPC values (LAUDPC), which were significantly higher than any other temperature or leaf wetness duration tested (Table 16). LAUDPC values at 25°C were the second largest (Table 16), and significantly different from the smallest values observed at 15 and 20°C. LAUDPC values of celery plants incubated at 48h of wetness were intermediate and similar to 72 and 24h (Table 16). LAUDPC values were significantly lowest at 0 or 12 h of wetness.

Table 16. Effect of temperature and wetness duration on celery anthracnose incidence (mean AUDPC) on 'Green Bay' celery seedlings inoculated with *Colletotrichum acutatum* s. l.

Factor	Mean AUDPC (*P* value)	
Temperature (°C)	Lesion LAUDPC (*P*=0.023)	Leaf curl CAUDPC (*P*=0.0129)
15	36.4 c	27.8 c
20	71.3 c	55.0 bc
25	100.0 b	96.2 ab
30	197.5 a	120.5 a
Wetness duration (h)	LAUDPC (*P*<0.0001)	CAUDPC (*P*<0.0001)
0	20.0 d	41.5 d
12	12.5 d	30.3 cd
24	58.1 c	62.6 cb
48	112.8 bc	78.0 b
72	139.3 b	94.2 b
96	265.2 a	142.7 a

A similar trend was observed in leaf curl AUDPC (CAUDPC) values for the hours of wetness duration. At the highest level of temperature or wetness duration CAUDPC values were higher (Table 16). CAUDPC values at 25 and 30°C were similar; while values at 20°C were not significantly different from CAUDPC values at 25 or 15°C (Table 16).

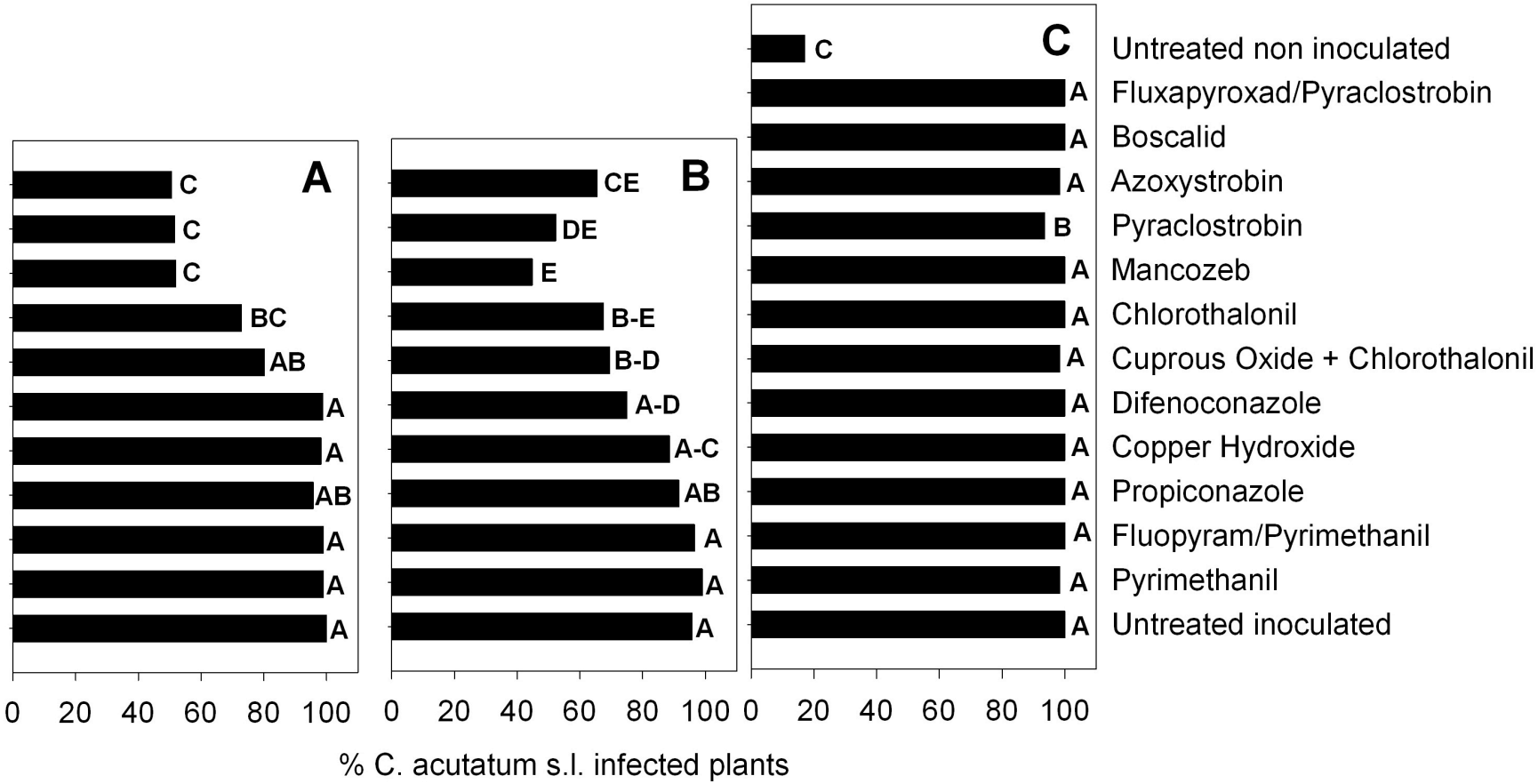

Figure 10. Effect of fungicide treatments on final incidence of celery anthracnose in 'Green Bay' celery inoculated with *Colletotrichum acutatum* s.l. mixture of isolates the field. Field trials year identification: A, 2011 trial A; B, 2011 trial B; and C, 2012 trial C. Bars with common letters are not significantly different (LSD *P*=0.05) within year. Fungicide treatments were applied on a 7 ± 2 days spray interval. (+)= Treatments were tank mixed prior to application; (/)= indicates premix product.

The first celery anthracnose symptoms were observed in the untreated inoculated field plots 4 to 6 DAI. The initial symptoms included discoloration of theyoungest tissue in the heart of the celery plants, followed by leaf cupping that was observed 7 to 14 DAI. The untreated *C. acutatum* inoculated plots had between 96 to 100% infected plants at the end of the 2011 trials (Figure 10). In 2012, the incidence peaked to 100% at 5 DAI (data not shown).

Significant differences among treatments were found for anthracnose severity under the disease progress curve (SAUDPC) and final percentage of infected plants (both $P<0.0001$). Trials were analyzed separately as significant differences were identified among trials. In the field trial 2011-A, the plants (%) infected in the plots sprayed with mancozeb, pyraclostrobin, or azoxystrobin were significantly smaller compared to the untreated inoculated control, fluopyram/pyrimethanil, pyrimethanil alone, difenoconazole, or copper hydroxide (Figure 10A). The SAUDPC values for plots sprayed with pyraclostrobin, azoxystrobin, and mancozeb were significantly lower compared with the untreated inoculated control (Figure 11A). Plots treated with fluopyram/pyrimethanil, copper hydroxide, propiconazole and pyrimethanil were not significantly different from the untreated control SAUDPC value (Figure 11A).

In the field trial 2011-B, mancozeb had the lowest celery anthracnose incidence followed by pyraclostrobin, azoxystrobin and chlorothalonil (Figure 10B). Plots treated with azoxystrobin, chlorothalonil, cuprous oxide + chlorothalonil, mancozeb and pyraclostrobin resulted in significantly smaller SAUDPC values compared with the untreated inoculated control (Figure 11B). The SAUDPC values for the plots treated with

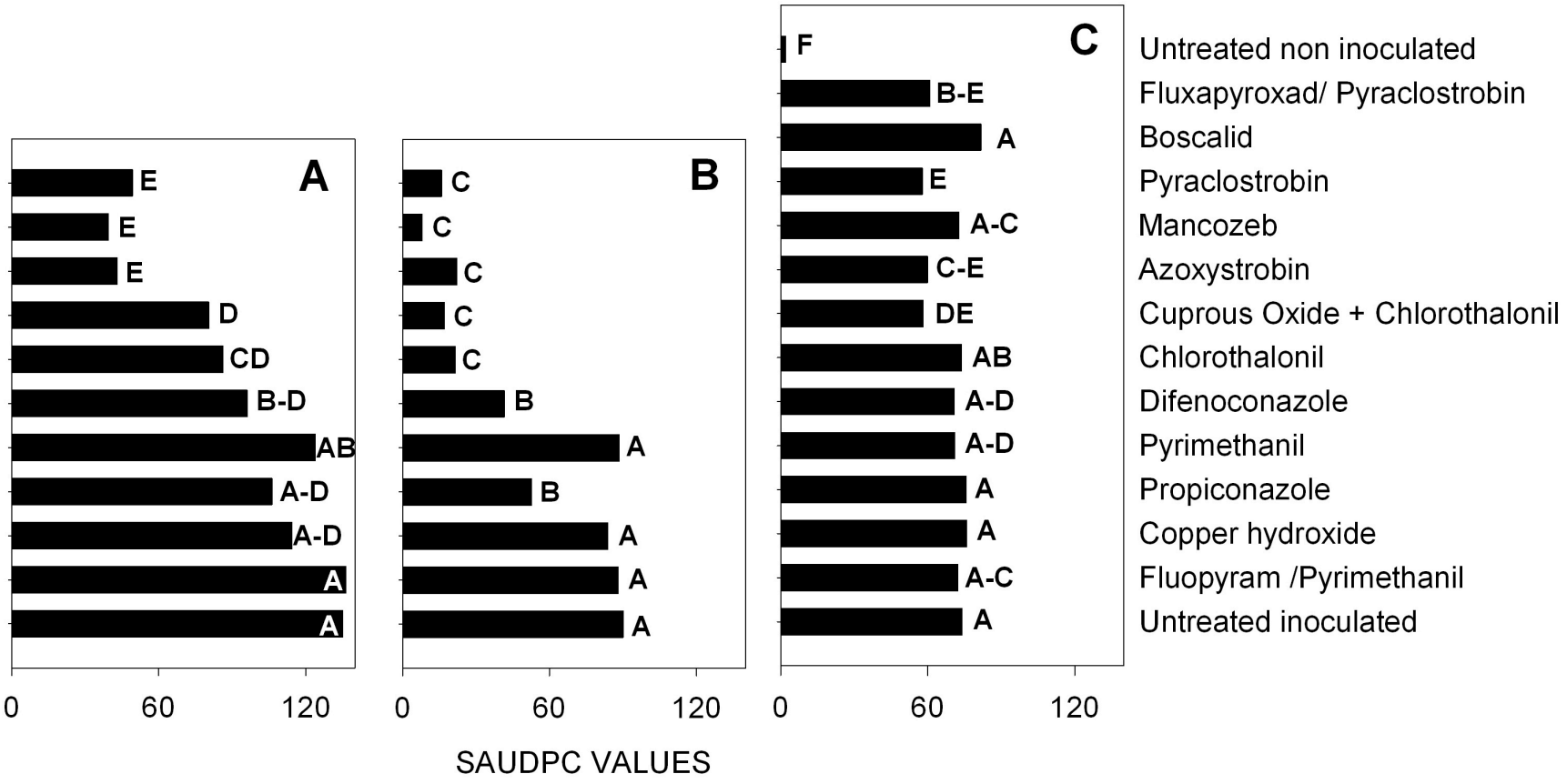

Figure 11. Effect of fungicide treatments on celery anthracnose severity progression under the disease curve (SAUDPC) in 'Green Bay' celery inoculated with *Colletotrichum acutatum* s.l. mixture of isolates. Field trials year identification: A, 2011 trial A; B, 2011 trial B; and C, 2012 trial C. Bars with common letters are not significantly different (LSD *P*=0.05) within year. Fungicide treatments were applied on a 7 ± 2 days spray interval. (+)= Treatments were tank mixed prior to application; (/)= premix product.

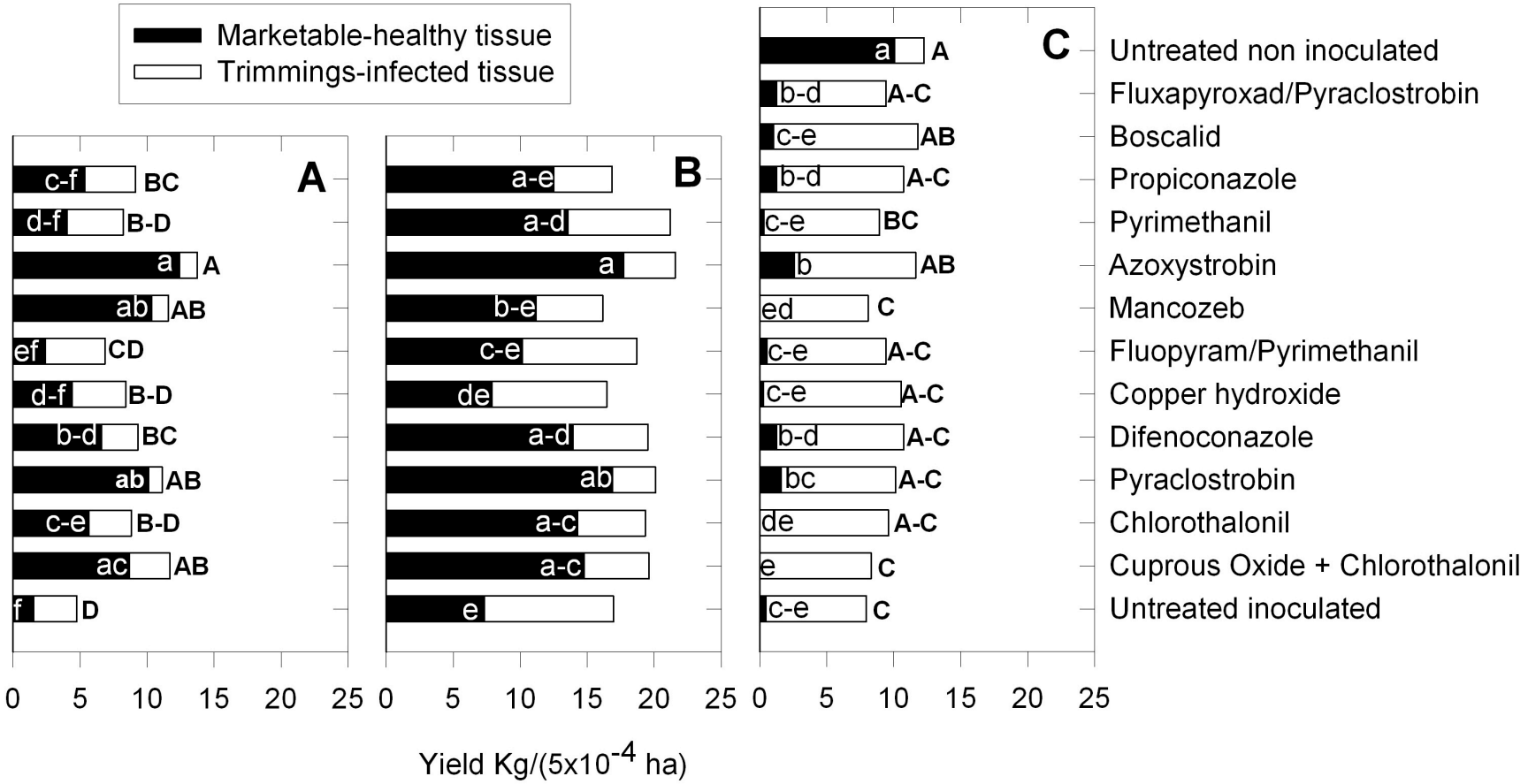

Figure 12. Yield differences among fungicides treatments in 'Green Bay" celery inoculated with *Colletotrichum acutatum* s.l. mixture of isolates. Field trial year identification: A, 2011 trial A; B, 2011 trial B; and C, 2012 trial C. White bars are infected tissue trimmed from the plant at time of harvest. Black bars represent the marketable yield. Capital letters illustrate significant differences among total yield (P=0.05), lower case letters represent significant differences among marketable yield (P=0.05). (+)= treatments were tank mixed prior to application; (/)= premix product.

copper hydroxide, fluopyram/pyrimethanil and pyrimethanil were not significantly different from the untreated control (Figure 11B).

In the 2012 field trial, pyraclostrobin was the only treatment with anthracnose incidence significantly different from the untreated inoculated control (Figure 10C). The SAUDPC values of plots sprayed with azoxystrobin, pyraclostrobin, fluxapyroxad/pyraclostrobin and the cuprous oxide + chlorothalonil were significantly lower than the untreated inoculated (Figure 11C).

The SAUDPC values for the plots sprayed with the remaining eight treatments (boscalid, copper hydroxide, propiconazole, chlorothalonil, mancozeb, fluopyram/pyrimethanil, difenoconazole and pyrimethanil) were not significantly different from the untreated inoculated control (Figure 11C).

Significant differences in fungicide treatments total yield were found for experiments 2011-A and 2012 ($P<0.0001$) but not for the experiment 2011-B. ($P=0.604$). In the 2012 trial, the majority of the total yield reported was non marketable (trimmings, Figure 12C). Azoxytrobin and pyraclostrobin had the greatest marketable yield across years, chlorothalonil and difenoconazole had intermediate marketable yield compared with the strobilurin treatments. Non-efficacious fungicide products such as copper hydroxide and fluopyram/pyrimethanil had similar marketable yields to the untreated inoculated control (Figure 12).

DISCUSSION

Celery anthracnose was present in all of the main celery production counties in Michigan, not only in 2010 when the disease was first reported in the U.S., but also in 2011

and 2012. In contrast, leaf curl in celery was sporadic in Australia but when outbreaks occurred they resulted in 25 to 50% yield losses (135). When sampling for celery anthracnose, symptoms could be clearly distinguished from other celery diseases (early, late, and bacterial blights). Lesions on the petioles were parallel along the petioles and light brown to brown in coloration. Some lesions resembled cracks in the petioles with brown edges; occasionally adventitious roots were observed in association with the lesions. Leaf curling and petiole twisting were observed on seedlings and older celery plants. The celery heart (younger tissue in the center of the plant) was discolored in affected plants and was often observed in plants with curled leaves and discolored margins.

Conidial size of *C. acutatum* s.l. isolated from celery were within the values reported in *C. acutatum* complex (71). Moreover, the *C. acutatum* species-specific marker identified 109 isolates from celery in this complex. Isolate C57-8 was the only isolate that did not amplify the species-specific marker and therefore its taxonomic designation remains as *Colletotrichum* sp. A low polymorphism rate was observed for the genome wide ISSR markers, where most of the celery isolates had banding patterns with identical fragment size and band presence. Additional ISSR makers could provide further insight on the genetic diversity of the *C. acutatum* s.l. population recovered from symptomatic celery. However, the advantage of using SSRs markers over ISSRs is that SSR amplify reproducible fragments with less PCR reagents.

The majority of *C. acutatum* s.l. isolates shared the same PCR fragment sizes using transcriptome-derived SSRs markers. Based on the primers evaluated, the low polymorphism detected could indicate that a clonal population has been favored, consistent with *C. acutatum* s.l. introduction via infected seed. In contrast, PCR fragments were

visualized on a system with relatively limited fragment size difference detection and therefore some potential alleles with differences of a single SSR unit (3, 4, 5 or 6 nucleotide) or Single Nucleotide Polymorphisms (SNPs). Finer resolution of PCR product fragment size and the presence of SNPs can be studied using fluorescently labeled primers, and PCR fragments analyzed through sequencing (48,49). Additional marker testing is needed to draw more detailed conclusions on the genetic structure of the *C. acutatum* s.l. population recovered from celery.

Differences in virulence among *C. acutatum* s.l. isolated from celery were observed, but high variability among isolate virulence resulted in an overlapping LSD grouping. The variability observed in LAUDPC could be explained by i) the specific host pathogen interaction, ii) the species composition within the culture collection, and iii) evolutionary forces driving genotype and phenotype population diversity.

C*olletotrichum* spp. are know to be epiphytes and both nonpathogenic and pathogenic (104,224). When pathogenic, this genus of fungus can establish biotrophic infections that can result in periods of latent or quiescent infections (159,224). Asymptomatic seedlings were observed on the first and second rating dates that showed symptoms later in the experiment. Additionally, symptomatic seedlings can have inconspicuous lesions on the petioles but little to no leaf curling. It appeared that celery transplants could remain asymptomatic due to latent infection periods, or symptomatic but undetected during celery transplant production. Asymptomatic and undetected symptomatic transplants can become a source of inoculum for celery fields.

Isolates from tomato and blueberry were pathogenic to celery seedlings. *Colletotrichum acutatum s.l.* is known to infect multiple hosts (71,123,260,286), but a host

can be infected by multiple *Colletotrichum* spp. (106,258). *Colletotrichum simmondssi* has been reported as a pathogen of celery (110), and could be present in the collection of isolates. The newly described species within the *C. acutatum* complex (71) may be capable of infecting celery and could be present in the collection of isolates, contributing to the virulence variability observed.

Introduction of *C. acutatum* s.l isolates to celery greenhouse and fields could occur via infected seed or transplants as a founder effect. Different isolates of *C. acutatum* could be associated with specific celery variety seeds, transplants, or greenhouses facilities. New *C. acutatum* s.l. genotypes could be introduced every year into greenhouses and fields, but a bottle neck effect is expected at harvest time, were a drastic pathogen population size reduction is expected. The hypothesized influx of *C. acutatum* isolates into the field in combination with the genotypes that may survive the winter months could result in the phenotypic variability observed.

The reaction of celery cultivars to different *C. acutatum* s.l. isolates remains to be studied in detailed, as regional cultivar specialization has been reported in other *Colletotrichum* spp. (22,189). Several studies have characterized *Colletotrichum* spp. isolate virulence using a series of cultivars or differentials with diverse genetic backgrounds (22,75,95,250), a logical next step investigating virulence of *C. acutatum* s.l. isolated from celery.

Lastly, the variability observed could be explained by sexual and non-sexual variability mechanisms. *Colletotrichum acutatum* sexual reproduction has been observed *in vitro* but not in nature (122). Mechanisms such as heterokaryosis and vegetative compatibility groups-VCG (69), conidial anastomosis tubes (CAT) formation (237) and

parasexuality (242,259), provide with somatic variability in *Colletotrichum* spp. (69,145,226,259) and other fungal plant pathogens population (217,245,263).

Celery anthracnose was influenced by temperature and leaf wetness duration similar to other *Colletotrichum* spp., in various hosts (78,205,287). Symptoms were especially severe at warmer temperatures (>25°C) or at longer wetness duration periods (>48 h). However, *C. acutatum* s.l. infected celery and caused disease at all temperatures and wetness durations tested. Infection may occur early in the celery production cycle. Celery production in Michigan, and in most U. S. celery growing regions, starts with transplants in the greenhouse at temperatures ranging between 15 and 25°C (night/day temperatures). In addition, celery seedlings in the greenhouses are watered daily, which creates periods of leaf wetness and RH conducive to infection and symptom development. Celery seedlings could become infected in the greenhouse from inoculum originating from contaminated reused plug trays, asymptomatic weeds (104,224) and infected seed (235) as for other *Colletotrichum* spp. The data indicate that the progression of symptoms would be slow as long as temperatures ranged between 15 and 20°C. Symptom development can peak in late spring in the greenhouses, or in the summer months in the field when temperatures become warmer (≥25-30°C).

Periods of leaf wetness are created in the field by rainfall or by the overhead irrigation most growers in Michigan implement. Irrigation is required for this shallow root, cool season biennial. Limiting irrigation duration to minimize periods of leaf wetness and *C. acutatum* s.l. inoculum splash, could be helpful if the water requirement of the crop is met. Drip irrigation in California celery fields has increased since the late 1990s (41,162).

Drip irrigation can meet celery water requirements (41) while minimizing *C. acutatum* s.l inoculum splashing events.

In field studies under artificially inoculated conditions, *C. acutatum* s.l. caused 100% incidence on the untreated inoculated treatment. Strobilurins (azoxystrobin, pyraclostrobin) and mancozeb reduced celery anthracnose incidence by 48 to 51% in the 2011 trials (A and B respectively). Chlorothalonil alone or tank mixed with copper reduced incidence by 20 to 30% in 2011 trials. In 2012, incidence across treatments was >80% by the second rating date and at the final rating date only pyraclostrobin was significantly different from the other fungicide treatments. Trials 2011A and 2012 were inoculated when plants were smaller (5 weeks after transplanting, Figure 13) while in trial 2011B plants were inoculated 8 weeks after transplanting (Figure 13). Therefore, the earlier the infection occurs in the field the larger the impact celery anthracnose can have on incidence, severity and yield. In trial 2011A, heavy rainfall events occurred between spray application 4 and 5 (Figure 13) and anthracnose incidence peaked rapidly as inoculum present in the plots was likely splash dispersed and there were extended periods of leaf wetness. Efficacious fungicides need to be applied at the correct time to protect new and healthy celery tissue prior to forecasted rain events that promote *C. acutatum* conidial splashing. The later inoculation of trial 2011B missed the late July precipitation (Figure 13), but also these larger celery plants received the same amount of inoculum that smaller plants received in trials 2011A and 2012.

The overhead irrigation of the field trials effectively spread the inoculum, provided periods of leaf wetness conducive to infection and disease development, and increased the incidence level in all trials. Incidence at 9 and 10 DAI ranged from 20-50% and 10 to 60% *C. acutatum*infected plants for the 2011A and 2011B trials respectively. In contrast, greater

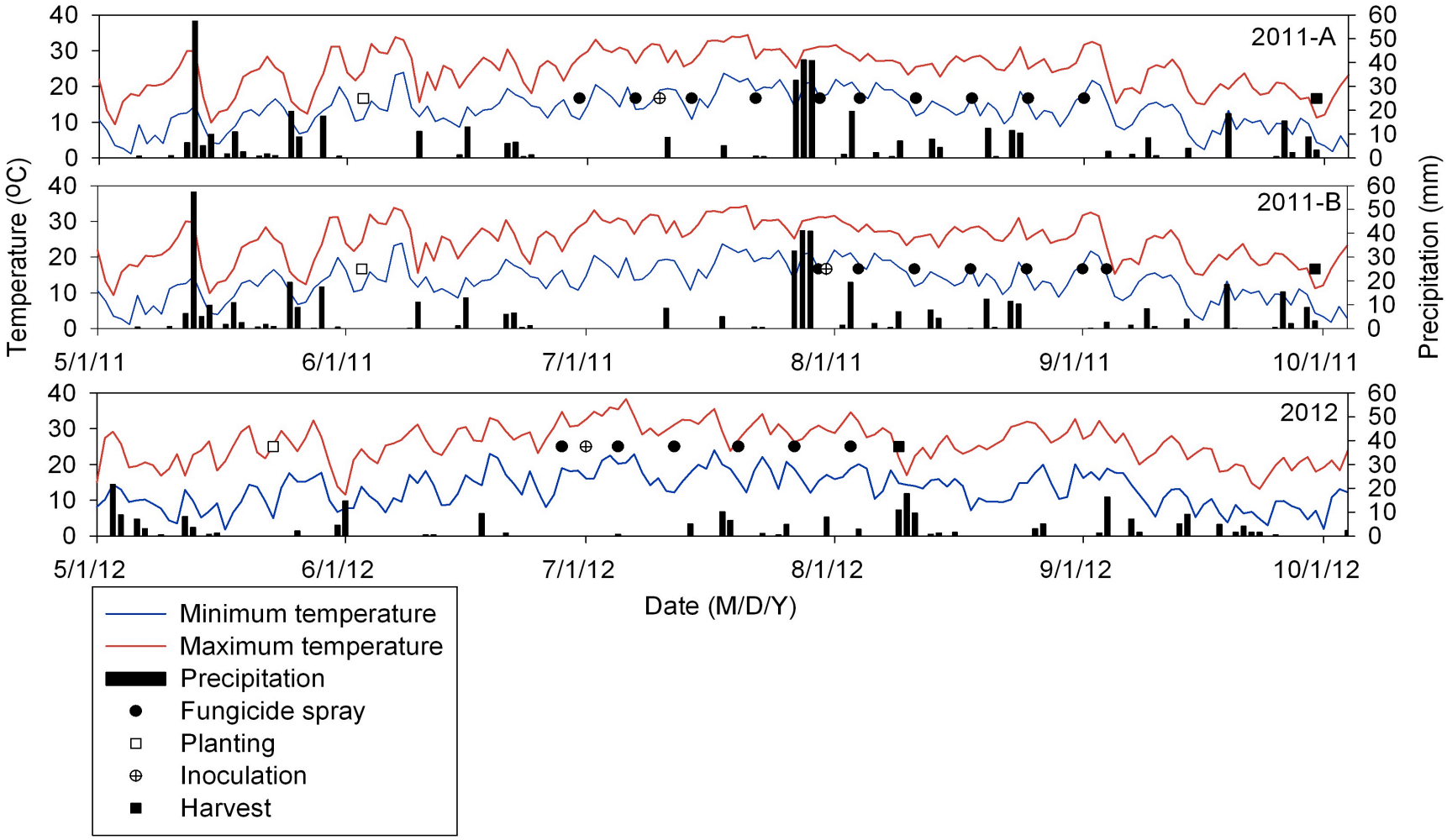

Figure 13. Air temperature and precipitation at the MSU Plant Pathology Farm, East Lansing, MI during the 2011 and 2012 summers. Blue series represent minimum air temperature; red series represent maximum air temperature, solid bars represent precipitation events. Solid circles denote fungicide applications, open squares mark planting dates; dash circles denote inoculation date and solid squares marks harvest dates.

than 80% incidence was observed 5 DAI in the 2012 trial. Compared to 2011, 2012 was a warmer and drier year and additional irrigation was needed (from twice a week schedule in 2011 to 3 to 4 times a week in 2012). The 2012 trial was terminated shortly after the untreated non-inoculated plots became infected with *C. acutatum*s.l. by splashing from nearby inoculated plots. Under the severe celery anthracnose pressure in trial 2011A (Figure 11) fungicides azoxystrobin, pyraclotrobin and mancozeb limited anthracnose severity progression, by 3, 2.7 and 3.5 fold respectively, compared with the untreated control. Under moderate celery anthracnose severity (Trial 2011B, Figure 11), chlorothalonil alone or tank mixed with copper, and DMI fungicides (difenoconazole and propiconazole) were second to strobilurins and mancozeb, in limiting celery anthracnose severity.

Celery is grown for their edible petioles, and the presence of blemishes, bruises or disease lesions will render the petioles unmarketable (38,39). In field experiments, untreated inoculated plots resulted in 68, 57 and 94% symptomatic tissue at harvest time. Even though severe celery anthracnose impacted total and marketable yields, efficacious fungicides that limited disease severity and incidence, such as strobilurins, resulted in higher total and marketable yields compared with the untreated control. Chlorothalonil, fluopyram/pyrimethanil and pyrimethanil resulted in similar total yield to the untreated inoculated. However, chlorothalonil decreased celery anthracnose incidence and severity protecting marketable yield, which was significantly different from the untreated inoculated in the 2011A trial.

In agreement to Heaton and Dullahide's findings in the 1990s (135), EBCD (mancozeb) outperformed chlorothalonil suppressing celery anthracnose in field

experiments. Mancozeb suppressed anthracnose incidence and severity second to the strobilurins, nonetheless mancozeb did not prevent yield loss as the strobilurins did and is not currently registered for use on celery. Protectant fungicides play a key role in avoiding fungicide resistance to site-specific fungicides reported in several pathosystems (20,117,144,158). Likewise, celery growers should tank mix strobilurins with protectant fungicides to prevent *C. acutatum* s.l. resistance build up to strobilurins and minimize yield losses.

The celery variety 'Green Bay' was used across experiments in this study, but at least five different celery cultivars are grown in Michigan ('Sabrosa', 'Sweet sensation', 'CR1', 'Green Bay', and 'Dutchess') and different cultivars are grown in California, Florida and other minor celery producing states in the U.S. No significant differences in the anthracnose severity was found when comparing four week old 'Sabrosa', 'Green Bay', and 'Dutchess' seedlings inoculated with a mixture of *C. acutatum* s.l. isolates (240). However, Wright and Heaton (1991) evaluated five celery cultivars commonly grown in Australia and found differences among leaf curl severity (290). Host resistance as a management tool needs to be further studied to identify tolerant or resistant cultivars that can be included in the breeding of new cultivars. Identifying celery cultivars resistant to anthracnose or with a limited number of lesions in the petioles will be crucial to minimize diseased tissue trimmings, the additional labor involved in trimming symptomatic petioles, reduce fungicide applications and the cost associated with both of these.

To conclude, celery anthracnose is widespread in the celery growing areas in Michigan. Based on morphology and molecular markers, the isolates recovered from celery are members of the *C. acutatum* s.l. complex. A set of transcriptome derived Simple

Sequence Repeat (SSR) marker specific to the *C. acutatum* s.l complex is available and can be used to further genotype and investigate species composition and population diversity. All *Colletotrichum* spp. isolates but one were pathogenic to celery, and differences in virulence among isolates were found. Different levels of isolate aggressiveness can impact epidemic development and have implications on management strategies. Higher temperatures and longer periods of wetness are conducive to leaf curling and lesion development on celery petioles. Therefore growers should minimize wetness duration in the greenhouse and the field and protect the crop from celery anthracnose with effective fungicide application. Strobilurins tank mixed with protectant fungicides, alternated with DMI under moderate disease severity in the field can limit celery anthracnose incidence, severity and yield losses.

CHAPTER V
CONCLUSION

CONCLUSION

The *Colletotrichum* spp. isolated from onion had cylindrical conidia with tapered ends consistent with the *C. coccodes* type description. This is the first report of *C. coccodes* as a pathogen of onions globally. One common genotype was observed among the 110 isolates DNA used to amplify four ISSR and 22 SSR markers, and therefore the population is clonal. Differences in virulence were found among fields were the isolates were collected, indicating a local environmental effect. No differences in virulence were identified among isolate grouped by their hosts. However, molecular marker loci differences were observed between isolates by host of origin (onion, potato or tomato). Additional studies that include *C. coccodes* from different hosts and geographical location can elucidate the relationship of the population isolated from onion in Michigan and populations in tomato and potato across the U.S.

Conidial morphology of *Colletotrichum* spp. isolated from celery were consistent to *C. acutatum* s.l. type descriptions. On four ISSR markers and 25 SSR markers 4% and 12% polymorphic loci were found respectively, indicating the *C. acutatum* isolates recovered from symptomatic celery plants is a genetically heterogeneous population with some degree of clonality. All isolates were pathogenic to celery, including *C. acutatum* s.l. from other hosts (tomato and blueberry). Differences in virulence were identified among isolates, indicating phenotypic diversity in the population recovered from celery in Michigan. A diverse population can impact the effectiveness of management approaches of celery leaf and neck anthracnose. Monitoring pathogen population changes can help adjust management practices accordingly.

Onion leaf and neck anthracnose symptoms were more severe when inoculated plants were subjected to a combination of ≥20°C and ≥24h of high relative humidity. Foliar and neck lesion development was minimal when plants were subjected to combinations of <20°C and <24h of RH duration. This indicates onion leaf and neck anthracnose can be especially severe in the warmer months of the Michigan growing season (July and August).

Celery leaf and neck anthracnose symptom development occurred in the entire range of temperatures tested (15 to 30°C) with more severe symptoms observed at ≥25°C. Similarly, symptom development occurred at low leaf wetness duration (≤12h), but symptoms were more severe when inoculated plants were incubated for more that 12 hours of leaf wetness. Therefore, *C. acutatum* infection and symptoms development can occur at the different stages of the celery growing season in Michigan, in the greenhouse on seedlings trays and in the field during the three staggered crops from May to September.

Integrated management of onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose should include pathogen avoidance and eradication. Pathogen avoidance starts with pathogen free seed to minimize introduction of inoculum into onion fields and the greenhouse for celery. *Colletrotrichum* spp. are known to be seed borne pathogens; investigation of the importance and contribution of seed borne and soilborne inoculum for onion leaf and neck anthracnose and celery anthracnose can help develop additional management strategies. Furthermore, testing techniques to detect *C. coccodes* in onion seed and *C. acutatum* in celery seed should be investigated as part of the seed certification procedure.

Reduction of the pathogen population could be accomplished by site selection and crop rotations that avoid susceptible hosts. Sanitation in the greenhouse and the field focusing on weed management and residue in the field can reduce primary inoculum. Michigan onion and celery growers should manage irrigation timing and duration to avoid extended periods of moisture conducive to disease development. This is especially important for celery growers in the greenhouse; creating unfavorable conditions for the pathogen could minimize infection in seedlings that will be transplanted in the field.

Inoculum reduction and symptoms development limitation in the field can be accomplished with preventive efficacious fungicide sprays. Strobilurin fungicides were efficacious limiting both onion leaf and neck anthracnose and celery leaf curl and petiole anthracnose and were capable of protecting yield quantity and quality. Protectants and DMI fungicides provide some control while helping decrease the risk of the pathogen population developing resistance to strobilurins. As new fungicide chemistries are developed, additional testing should be conducted.

Additional investigation of the susceptibility of onion and celery cultivars could help identify cultivars with the ability to produce satisfactory yields under severe disease conditions (tolerance) and cultivars with complete resistance. Choosing less susceptible onion and celery cultivars combined with efficacious fungicides may limit crop losses for Michigan growers.

APPENDICES

APPENDIX A

FIELD SAMPLING, MORPHOLOGICAL CHARACTERIZATION AND MOLECULAR MARKER GENERATION FOR *COLLETOTRICHUM COCCODES.*

Table A1. *Colletotrichum coccodes* isolated from onions, tomato and potato. Other *Colletotrichum* spp. were included as negative controls. Isolates collected in 2010, 2011 and 2012, number of isolates included in morphology, molecular markers and virulence experiments.

				No. Isolates			
Year	Field No	County	Host	Collected	Morphology	Markers	Virulence
2010	31	Calhoun	Onion	7	0	4	7
2011	46	Calhoun	Onion	7	0	4	6
2011	47	Calhoun	Onion	174	0	16	147
2011	60	Calhoun	Onion	1	0	1	0
2010	38	Eaton	Onion	1	0	1	1
2011	45	Eaton	Onion	5	0	3	4
2010	24	Ionia	Onion	5	3	4	5
2011	62	Ionia	Onion	19	0	4	18
2012	68	Ionia	Onion	14	0	4	20
2010	14	Kent	Onion	8	6	4	8
2010	15	Kent	Onion	2	1	2	2
2010	32	Kent	Onion	5	1	3	5
2010	34	Kent	Onion	44	8	31	40
2011	63	Kent	Onion	6	0	4	5
2012	66	Kent	Onion	19	0	4	18
2011	58	Montcalm	Onion	6	0	4	6
2010	3	Newaygo	Onion	11	8	4	11
2010	4	Newaygo	Onion	8	7	4	8
2010	5	Newaygo	Onion	2	1	2	2
2010	6	Newaygo	Onion	4	2	4	4
2010	7	Newaygo	Onion	15	11	4	15
2010	23	Newaygo	Onion	2	0	1	2
2010	26	Newaygo	Onion	1	1	1	1
2010	27	Newaygo	Onion	1	1	1	1
2010	36	Newaygo	Onion	4	1	4	4
2011	40	Newaygo	Onion	107	0	4	91
2011	41	Newaygo	Onion	43	0	5	37
2011	51	Newaygo	Onion	25	0	5	23
2011	52	Newaygo	Onion	32	0	5	28
2011	53	Newaygo	Onion	10	0	4	10
2011	54	Newaygo	Onion	93	0	5	82
2011	55	Newaygo	Onion	95	0	4	92
2011	56	Newaygo	Onion	59	0	4	59
2012	64	Newaygo	Onion	20	0	4	19

Table A1. (cont'd)

				No. Isolates			
Year	Field No	County	Host	Collected	Morphology	Markers	Virulence
2012	65	Newaygo	Onion	20	0	4	20
2012	67	Newaygo	Onion	25	0	4	20
2010	8	Ottawa	Onion	2	0	2	2
Controls	Cco	Michigan	Potato[z]	1	0	1	1
Controls	14f	Idaho	Potato[y]	4	0	4	0
Controls	Cd123	Michigan	N.a	1	0	1	0
Controls	Cg104	Michigan	N.a	1	0	1	0
Controls	TI	Michigan	Tomato	1	0	1	1
Controls	TR	Michigan	Tomato	2	0	2	2

[z]Courtesy of W. Kirk, MSU

[y]Courtesy of P. Wharton University of Idaho

Table A2. Conidial size characteristics of 51 *Colletotrichum coccodes* isolates from onion in 2010.

		Isolate	Length (µm)		Width (µm)		L/W Ratio	
County	Field	Identifier	Mean	SE[y]	Mean	SE	Mean	SE
Ionia	24	24-1-2-1	9.14	0.85	2.44	0.22	3.77	0.16
Ionia	24	24-1-4-1	11.50	0.48	3.26	0.09	3.55	0.14
Ionia	24	24-1-3-4	13.02	0.47	3.57	0.09	3.67	0.14
Kent	14	14-2-1-1	10.45	1.43	2.71	0.37	3.86	0.21
Kent	14	14-2-4-2	13.31	0.66	3.34	0.10	4.05	0.24
Kent	14	14-2-8-1	12.19	1.00	3.51	0.24	3.47	0.14
Kent	14	14-1-6-1	9.77	0.96	3.08	0.58	3.76	0.22
Kent	14	14-2-6-1	12.65	1.75	2.94	0.33	4.04	0.20
Kent	14	14-2-7-1	8.87	0.90	2.67	0.26	3.37	0.20
Kent	15	15-1-4-1	12.35	0.55	3.84	0.17	3.31	0.19
Kent	32	32-1-2-2	8.62	0.92	2.67	0.31	3.37	0.14
Kent	34	34-1-5-1	8.74	0.90	2.41	0.27	3.75	0.23
Kent	34	34-B5-2	13.39	0.62	3.18	0.09	4.22	0.17
Kent	34	34-C3-2	13.52	0.87	3.59	0.14	3.79	0.23
Kent	34	34-D2-1-2	12.82	0.67	3.46	0.13	3.75	0.18
Kent	34	34-E2-2	16.78	0.53	3.21	0.07	5.27	0.19
Kent	34	34-F1-2	11.47	0.59	3.48	0.13	3.35	0.20
Kent	34	34-G5-1	12.20	0.52	3.74	0.11	3.30	0.16
Kent	34	34-A3-2	15.09	0.79	3.61	0.16	4.20	0.19
Newaygo	3	3-3-2-1	18.33	0.73	3.84	0.13	4.98	0.35
Newaygo	3	3-1-1-2	13.25	0.82	3.51	0.12	3.80	0.23
Newaygo	3	3-1-2-2	10.22	0.66	2.60	0.28	4.46	0.31
Newaygo	3	3-3-1-1	9.25	0.94	2.66	0.31	3.67	0.15
Newaygo	3	3-4-1-2	10.25	1.01	2.54	0.26	4.26	0.31
Newaygo	3	3-4-4-2	17.85	0.72	4.02	0.12	4.60	0.30
Newaygo	3	3-4-5-1	10.66	0.90	2.48	0.26	4.67	0.35
Newaygo	3	3-4-2-1	13.33	0.82	3.58	0.24	3.82	0.21
Newaygo	4	4-1-3-3	12.27	0.46	3.08	0.11	4.12	0.24
Newaygo	4	4-1-1-1	12.23	0.50	3.45	0.14	3.71	0.28
Newaygo	4	4-1-7-2	10.57	0.72	3.02	0.31	3.91	0.26
Newaygo	4	4-2-3-2	10.99	1.33	2.78	0.30	3.91	0.21
Newaygo	4	4-2-4-2	13.08	0.54	3.72	0.10	3.59	0.18
Newaygo	4	4-2-5-0	16.14	1.17	3.86	0.18	4.32	0.35

[y]SE= Standard error.

Table A2. (cont'd).

County	Field	Isolate Identifier	Length (µm) Mean	Length (µm) SE[y]	Width (µm) Mean	Width (µm) SE	L/W Ratio Mean	L/W Ratio SE
Newaygo	4	4-2-7-1	13.69	0.62	3.59	0.17	4.05	0.32
Newaygo	5	5-2-3-2	14.01	0.79	3.83	0.21	3.82	0.29
Newaygo	6	6B1-1-1	13.88	0.72	4.34	0.21	3.33	0.23
Newaygo	6	6B-2-7	15.29	1.00	3.38	0.13	4.74	0.42
Newaygo	7	7-2-2-1	9.23	0.80	2.51	0.27	3.99	0.27
Newaygo	7	7-3-9-2	12.01	1.46	2.97	0.34	4.00	0.15
Newaygo	7	7-4-2-1	13.19	0.45	4.48	0.16	3.00	0.13
Newaygo	7	7-4-3-1	8.64	1.28	2.44	0.32	3.47	0.13
Newaygo	7	7-5-4-2	8.89	0.99	2.53	0.32	3.70	0.20
Newaygo	7	7-2-5-2	17.86	0.69	3.56	0.14	5.14	0.25
Newaygo	7	7-3-4-1	13.35	0.87	3.36	0.13	4.04	0.27
Newaygo	7	7-3-6-2	20.04	0.55	3.40	0.05	5.93	0.20
Newaygo	7	7-4-1-1	16.22	0.96	3.28	0.05	4.97	0.30
Newaygo	7	7-4-7-1	10.97	1.28	2.70	0.30	4.23	0.26
Newaygo	7	7-5-6-1	9.64	1.07	2.83	0.33	3.53	0.14
Newaygo	26	26-1-2-1	9.24	1.09	2.20	0.25	4.24	0.19
Newaygo	27	27-1-3-1	8.12	0.82	10.85	8.59	3.33	0.26

[y]SE= Standard error.

Table A3. Abundance of SSR types, size, primer design suitability, and total number loci on *Colletotrichum coccodes* isolate 38-1-3-1 transcriptome.

SSR type	SSR size	Primers		No of Loci
		Designed	None	
Mononucleotide	20-32	12	18	30
Dinucleotides	10	225	14	239
	12	62	1	63
	16	16	3	19
	18	16	1	17
	20	9	2	11
	22	4	0	4
	24	3	0	3
	26	1	1	2
	32	1	0	1
	Total	337	22	359
Trinucleotides	12	642	17	659
	15	161	4	165
	18	57	2	59
	21	37	2	39
	24	17	1	18
	27	3	0	3
	30	3	1	4
	Total	1257	49	1306
Tetranucleotide	12	314	28	342
	16	27	3	30
	20	6	1	7
	24	3	0	3
	28	1	0	1
	Total	351	32	383
Pentanucleotide	15	117	7	124
	20	18	1	19
	25	9	0	9
	30	0	2	2
	35	1	0	1
	Total	145	10	155
Hexanucleotide	18	123	35	158
	24	16	6	22
	30	4	3	7
	36	2	1	3
	0	0	0	0
	Total	145	45	190

Table A3. (cont'd).

SSR type	SSR size	Primers Designed	None	No of Loci
Complex	>24-363	198	24	222
Grand total		2300	155	2455

Table A4. Characteristics of 34 microsatellite (SSR) markers designed from *Colletotrichum coccodes* transcriptome sequences tested on 110 onion *C. coccodes* isolates DNA samples pooled by collection field (pilot), and 8 *C. coccodes* from other hosts.

SSR name	Motif	Repetition	Forward primer	Reverse primer	Expected product size (bp)
CO2530	GAGGC	5	CCAATGACATGGCTGTTGAC	TGCAGTGTCCACGTCTCTTC	324
COS196	AGATA	3	AACAATTGCACAGGTGACCA	TGAAGCCGGAGCACTTACTT	331
COS260	CAGGAA	5	GTCCGAACCATCCTTTTTGA	ACAGGTGTTTGGGCAAGTTC	355
COS191	GAGGA	4	TGCATGAACCACACCAAAGT	GCACACCTGACCTACCCACT	356
COS210	GAA	4	AGGGTTTGGGCCTCTTACAT	TTCTCCATCTCGTCCAATCC	386
CO4383	TTTCG	7	TAAGGCTGAGCCGACAAAGT	GCTCTTTGCCAAGCAACTTC	421
COS143	TCG	7	CGCCCGTATCAGTCGTTATT	TGAGGCACCTACATTGACCA	421
CO4187	GACGCC	6	TGCACGACAAGAGGAGTTTG	TCTTCCTTGACAGCCTCGTT	436
COS82	CCGAAT	4	CGGACGAAGACCGTGTTATT	ATATGATGTGTGGCGGGATT	467
COS222	AAG	4	CGGCTCCTACCACTTTGTGT	CCTGCGGGTGTAACGTAGAT	514
CO752	CAT	9	CCGCTTTCTCTTGCCTACAC	TGTGGCGGTCTTGATATTGA	525
COS240	TCG	4	GACATATCGCAGGGTCTCGT	CTTGGCGACGTGTACAGCTA	549
CO1262	GCG	4	GGTCTCGAGCAGATTGGAAG	AACTGGGTTGCTTTGGACAC	549
COS132	TCG	4	TCGGCCGTATCAAATTTCTC	CGACAAGCGACGAGTATGAA	566
COS202	AAAG	7	GACTCAGTCATCCCCCTTGA	TATTCGCAGCTGGTGAGAGA	582
COS91	CTT	4	AAAAGGTTGCACCACCAAAG	ACCAGGATTAATCGCAGCA	584
COS309	CGC	4	CAACAACTCGCGTGACAACT	CGACACCGCATAAACTCCTT	591
COS134	GCC	4	ATGCACAGTTCGAGCCTCTT	TGTCCCATATCCCGAATGAT	600
CO1681	TTGCTC	5	CCTTCGCCCCTACTACCCTA	GTTGGAGGCAAGAGTGAAGC	621
CO1634	TGGTG	5	TGTGGTGGAGATTGGACAGA	GTCTTCTTCGGCTCGTGTTC	640
CO2983	CAG	8	TTCCTCTTCGACGCTCGTAT	GCCGAACTCTTGTGAAAAGC	647
CO2096	CCAGC	4	TAAGGTCGGCTCTTGCTGTT	TGGGATCATATGGCATTTTG	652
COS300	CGCAGC	3	GGGAAGAGGAAAGGGAGATG	TAACTTTCGTCGCTGACGTG	683

Table A4. (cont'd).

SSR name	Motif	Repetition	Forward primer	Reverse primers	Expected product size (bp)
CO2290	CAC	9	TGGCCGACGTTACTCTACCT	GTCGTTGAACAAGGGTTCGT	684
CO3737	AAGA	5	CGTTGGCAGGAAGGTACAAT	CGATGACTCTACCCGACGAT	700
CO2043	ACC	10	TTGAGCGTATCGTCGAACTG	AGTCCATCATGCCCAGAAAG	723
CO3076	CGCCCA	6	TGGGACTGTCTCTGCCTCTT	ATGGGACGAATTGGTGTGTT	748
CO4230	TGC	7	CATAGGCGTTACCCAGAGGA	ACCATCGTCACAAGACACCA	824
CO2447	CCG	7	CCTCGAGGGTTCCTTCTACC	CCAGTTCTTGCCTCTGAAGG	835
CO4342	CACTCT	3	CCGTGTCTCTCCCACCATAC	GGTTACGCCAGATGTCGTTT	837
CO584	GGGGCT	3	CGCCATTCAACAACCCTACT	GACGTCTTCCCTGATGGTGT	848
COS307	CGC	4	GAGCTTTCTACCCCCGATTC	GCCGCAGATCTTACAGAAGG	860
CO1570	AGA	8	ATCAAGGCCACACGGACTAC	CCGAGTAGCATGTTCCCATT	889
CO3111	GGC	6	TCTCCTCCACCAAGACTGCT	TGGAGTGTTCCCGATTCTTC	892

APPENDIX B

FIELD SAMPLING, MORPHOLOGICAL CHARACTERIZATION, MOLECULAR MARKER GENERATION FOR *COLLETOTRICHUM ACUTATUM,* AND FUNGICIDE EFFICACY IN THE GREENHOUSE.

Table B1. Survey of *Colletotrichum* spp. in Michigan celery production areas. Isolates included in virulence and molecular marker testing.

Field No	Collection Year	County[z]	No. Isolates Collected	Morphology	Genotyping	Pathogenicity
C29	2010	A	9	1	1	1
C30	2010	A	2	1	1	1
C39	2010	A	4	1	2	1
C40	2010	A	4	1	1	1
C27	2010	B	17	3	3	3
C28	2010	B	0	0	0	0
C02	2010	K	4	2	2	2
C18	2010	K	4	1	1	1
C19	2010	K	0	0	0	0
C20	2010	K	6	2	2	1
C21	2010	K	1	0	1	0
C22	2010	K	3	1	1	1
C24	2010	K	45	7	10	8
C41	2010	K	6	1	1	1
C42	2010	K	4	1	1	1
C43	2010	K	2	1	1	1
C25	2010	K	11	1	4	3
C03	2010	N.A	1	1	1	1
C07	2010	N.A	4	2	2	2
C31	2010	N.A	5	3	2	2
C23	2010	O	3	1	1	1
C26	2010	O	10	2	3	3
C08	2010	VB	2	2	2	2
C09	2010	VB	1	1	1	1
C10	2010	VB	2	1	2	1
C11	2010	VB	3	1	2	1
C12	2010	VB	3	2	2	2
C13	2010	VB	2	1	2	1
C14	2010	VB	1	1	1	1
C15	2010	VB	0	0	0	0
C16	2010	VB	2	1	2	1
C17	2010	VB	3	1	2	1
C32	2010	VB	4	1	2	1

[z]N.A.= no information available, A= Allegan, B= Barry, K=Kent, O=Ottawa, VB=Van Buren and BE=Berrien County.

Table B1. (cont'd).

Field No	Collection Year	County[z]	No. Isolates			
			Collected	Morphology	Genotyping	Pathogenicity
C33	2010	VB	0	0	0	0
C34	2010	VB	0	0	0	0
C35	2010	VB	2	1	1	1
C36	2010	VB	4	2	1	1
C37	2010	VB	1	1	1	1
C38	2010	VB	5	2	2	1
C45	2011	A	2	1	2	1
C54	2011	A	35	0	6	3
C46	2011	B	4	1	2	1
C48	2011	B	50	0	8	8
C51	2011	B	17	0	2	1
C49	2011	K	75	2	5	2
C53	2011	K	56	2	5	3
C55	2011	N	31	0	1	1
C47	2011	O	6	2	2	1
C57	2011	O	1	0	1	1
C44	2011	VB	8	1	2	1
C50	2011	VB	34	2	3	2
C52	2011	VB	2	0	0	0
C56	2011	VB	4	0	2	1
C58	2012	K	36	0	4	4
C62	2012	K	0	0	0	0
C60	2012	N	0	0	0	0
C61	2012	O	0	0	0	0
C59	2012	VB	8	0	1	1
Controls						
Tomato	2011	BE	2	0	2	2
Blueberry	N.A.	N.A	1	0	1	1
		Total	549	62	113	85

[z]N.A.= no information available, A= Allegan, B= Barry, K=Kent, O=Ottawa, VB=Van Buren and BE=Berrien County.

Table B2. Conidial size for isolates of *Colletotrichum* spp. isolated from celery in 2010 and 2011.

			No.	Length (μm)		Width (μm)		Ratio	
Year	County[z]	Field	Isolates	Mean	SE[y]	Mean	SE	Mean	SE
2010	N.A.	3	1	11.7	0.5	4.2	0.1	2.8	0.1
2010	N.A	7	2	7.9	0.3	2.8	0.1	2.9	0.1
2010	N.A	31	3	8.4	0.3	2.9	0.1	3.0	0.1
2010	A	29	1	8.5	0.5	2.9	0.1	3.0	0.2
2010	A	30	1	10.1	0.3	3.2	0.2	3.3	0.1
2010	A	39	1	8.3	0.5	2.7	0.1	3.1	0.1
2010	A	40	1	8.9	0.8	3.3	0.2	2.7	0.2
2011	A	45	1	11.8	0.3	3.8	0.1	3.2	0.1
2010	B	27	3	8.4	0.4	4.6	0.5	3.2	0.1
2011	B	46	1	11.2	0.4	3.3	0.1	3.5	0.1
2010	K	2	2	9.3	0.3	3.2	0.1	2.9	0.1
2010	K	18	1	9.2	0.4	3.3	0.1	2.8	0.1
2010	K	20	2	9.3	0.4	3.0	0.1	3.1	0.1
2010	K	22	1	9.1	0.4	2.9	0.1	3.2	0.2
2010	K	24	7	9.9	0.2	3.2	0.0	3.1	0.1
2010	K	25	1	9.5	0.5	3.2	0.1	3.0	0.1
2010	K	41	1	8.9	0.3	3.1	0.1	3.0	0.1
2010	K	42	1	9.8	0.5	3.5	0.1	2.8	0.1
2010	K	43	1	9.1	0.3	2.8	0.1	3.3	0.2
2011	K	49	2	11.2	0.3	3.5	0.1	3.2	0.1
2011	K	53	2	11.5	0.3	3.8	0.1	3.1	0.1
2010	O	23	1	9.8	0.6	3.4	0.1	2.9	0.1
2010	O	26	2	8.7	0.5	3.0	0.1	3.0	0.1
2011	O	47	2	9.1	0.3	3.1	0.1	3.0	0.1
2010	VB	8	2	10.2	0.4	3.2	0.2	3.4	0.2
2010	VB	9	1	10.7	0.3	3.2	0.1	3.3	0.1
2010	VB	10	1	8.5	0.4	2.5	0.1	3.4	0.1
2010	VB	11	1	9.0	0.7	3.0	0.2	2.9	0.1
2010	VB	12	2	7.9	0.2	3.1	0.1	2.6	0.1
2010	VB	13	1	9.7	0.5	3.1	0.1	3.2	0.2
2010	VB	14	1	10.7	0.5	3.4	0.2	3.2	0.1
2010	VB	16	1	9.6	0.4	3.0	0.1	3.2	0.1
2010	VB	17	1	10.0	0.3	3.0	0.1	3.3	0.1
2010	VB	32	1	9.7	0.7	3.0	0.2	3.2	0.1

[z]N.A.= no information available, A= Allegan, B= Barry, K=Kent, O=Ottawa, VB=Van Buren

[y]SE= Standard error

Table B2. (cont'd).

Year	County[z]	Field	No.	Length (μm)		Width (μm)		Ratio	
			Isolates	Mean	SE[y]	Mean	SE	Mean	SE
2010	VB	35	1	7.4	0.3	2.4	0.1	3.1	0.1
2010	VB	36	2	9.5	0.3	3.1	0.1	3.1	0.1
2010	VB	37	1	7.5	0.3	2.6	0.1	2.9	0.1
2010	VB	38	2	9.4	0.3	3.4	0.1	2.8	0.1
2011	VB	44	1	11.7	0.4	3.7	0.1	3.1	0.1
2011	VB	50	2	11.4	0.3	3.6	0.1	3.2	0.1

[z]N.A.= no information available, A= Allegan, B= Barry, K=Kent, O=Ottawa, VB=Van Buren

[y]SE= Standard error

Table B3. Abundance of SSR types, size, primer design suitability, and total number loci on *Colletotrichum acutatum* s.l. isolate C26-1-2 transcriptome.

		Primers		
SSR type	SSR size	Designed	None[z]	No. of Loci
Mononucleotide	20-82	22	27	49
Dinucleotides	24	2	2	4
	22	7	1	8
	20	13	1	14
	18	21	6	27
	16	19	4	23
	14	34	1	35
	12	76	7	83
	10	279	13	292
	Total	451	35	486
Trinucleotides	27	4	0	4
	24	25	5	30
	21	34	3	37
	18	70	3	73
	15	209	9	218
	12	654	24	678
	Total	996	44	1040
Tetranucleotide	32	1	0	1
	28	1	2	3
	24	5	0	5
	20	10	3	13
	16	50	2	52
	12	396	29	425
	Total	463	36	499
Pentanucleotide	30	1	0	1
	25	6	0	6
	20	31	3	34
	15	135	22	157
	Total	173	25	198
Hexanucleotide	42	1	0	1
	36	1	0	1
	30	4	0	4
	24	28	1	29
	18	141	6	147
	Total	175	7	182
Complex	>82	318	29	347
	Grand total	2423	196	2619

[z]Primer design script did not find suitable primer sequence flanking SSR motif.

Table B4. Characteristics of 28 microsatellite (SSR) markers designed from transcriptome sequences of *Colletotrichum acutatum* s.l tested on 113 *C. acutatum* s.l. isolates.

SSR ID	Motif	Rep[z]	Forward	Reverse	Product size (bp)	Loci[y]
CA2593	CTACA	4	GGTACATCCTTGGGTCATCG	CTTGGAATTGTCGTCGACCT	391	M
CA4548	TGG	7	CGAAGGCGAGCAAATAAGAC	TTCCTCCTCTTCCTCCTCCT	291	M
CA4846	TGCTGG	3	CGTAGCAGACGAGAGCTGTG	CCAGGGAAACTGACGAAAAA	356	M
CA5317	CTT	4	TTCCATCTCACCTTCGAACC	CCGTGGACGGAGTATGAAGT	366	P
CA5807	TTTC	8	GGGCTGGAGATTCAGGACTT	TGGGACGGAAACTGTCTACC	379	P
CAS23	GTC	4	ACCCAATGTTTGCTTTCCAG	GTCAAGCGACTGCATCAAAA	397	M
CAS231	GCAAG	5	CCAGCTAGGTCTTGCTTTGG	CTGCTAGAGAATGCGGTTCC	530	M
CAS301	GT	7	GCCTGGACTTCAGAGTGAGG	GTGACCTTGCTCGTGTCTCA	683	M
CAS346	GGC	9	ATTGGGAGAGGTTGTTCACG	CTTCCTTCACTCCCCTTTCC	460	M
CAS519	CGTCT	3	GAGTAGCCTCGGGGAATAGG	CACTGCCCAAGTGGAAGATT	348	M
CA2719	TGAACC	3	GGCATTTGCTTTTTCTCTGC	TACATGTTGGTGCCATTGCT	886	M
CA2987	CGCAT	5	TTTTCTCCCCTCTCCCATCT	TCCTCTTTGTGGGGTACTGG	535	M
CA4685	ACGC	7	GTCCTCCACTGCATCATCCT	TGCAAATCTTTGGGGAAGAC	485	M
CA5665	GC	5	CAAAGCCCCCTTTTTATCGT	GGCAGCTTTTACCTGCAAAG	479	M
CA5788	GCGGCA	4	CGTCACTTCCGAGAGACTCC	CCTCAGATCGCTGGATGTTT	701	NA
CA913	ACCGC	6	GCAGACTGGAGGAAAACAGC	GTGTAGCGCTTTGGAGGAAG	691	M
CA1251	TAAGG	5	ATGAACGTAGGTGGCCTGAC	TCCACGTCAATTCATCCTGA	702	M
CA2896	AGA	9	CCGAGCAAGAAACAGAGGAC	TGCGGAGGACTTCGTAGACT	887	M
CAS471	GAT	6	TGGCAAGGAAGGAGAAGAGA	TGACCAGACGTCCATCATGT	507	M
CA1786	GATCGT	3	GTGGTTGAGGTCTGGCAGTT	GTCCAACGTGCCTTCGTAAT	878	NA
CA6614	GGAGCC	7	CTCCCTCAAGGACGAGTCTG	AACTGACGAACGGGAACATC	441	M

[z]Rep= number of SSR motif repetitions on *C. acutatum* s.l. isolate C26-1-2.

[y]M=monomorphic, P=Polymorphic, NA= did not amplify.

Table B4. (cont'd).

SSR ID	Motif	Rep[z]	Forward	Reverse	Product size (bp)	Loci[y]
CAS532	GCAGAC	6	TCCAATCCGAGATCCTCAAC	TTACGGTGCACCACAATGAT	831	M
CA2513	GGTGCT	5	ACAACAACACCAGCAAACCA	TCCCTACCACCAGCACTACC	318	P
CA5464	GCA	8	TCCATTCTGTTCTGCTGTCG	TTGTGCGTTGAGATGAGAGG	863	M
CA2046	TGC	7	CGTCTTCGTCAACTTCGTCA	GCCTTACATCAAGCCAGAGC	593	M
CA3496	AGA	9	CGATTATGTGGCAGTGGTTG	GACGAATACTAGCGCCTTCG	879	M
CAS136	TACAAC	3	TGACAGAACGAGCAAAATGC	AACAAGAGCCGAGTCTCGAA	314	NA
CAS546	CTT	6	CGGATCGGTAGATGGTGAGT	GCAAGCTGGAGGATCTCAAC	514	M

[z]Rep= number of SSR motif repetitions on *C. acutatum* s.l. isolate C26-1-2.

[y]M=monomorphic, P=Polymorphic, NA= did not amplify.

Celery (*Apium graveolens)*
Celery Anthracnose;
Colletotrichum acutatum s.l.

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Evaluation of fungicides to control celery anthracnose in the greenhouse

This study was conducted in the greenhouses using 5 weeks old 'Green Bay' celery seedlings. Treatments were replicated six times in randomized complete design. Fungicides were applied as foliar sprays. Treatments were applied 28 Mar, and plants inoculated on Mar 29 with *C. acutatum* conidial suspension. Disease severity was evaluated (0 to 5; 0= no disease, 1=1 to 12% symptomatic tissue, 2= 13- 24%, 3= 25- 50%, 4= 50- 74%, 5= 75 - 100%) on April, 4, 11 and 18.

Disease severity mid point of the scale was used as final severity. The untreated inoculated control had 4 symptomatic plants and 2 scapes. When taking into account only symptomatic plants, the mean final severity of the untreated inoculated treatment was 41 ± 20.3%. The mean of all 6 replicates of the untreated inoculated was 27.73 ± 15.5%, and 0 for the untreated inoculated. Based on the ANOVA, treatments had a significant effect on final severity. In ascending order, Quadris + Bravo, and Omega had the lower final severity values. Fungicides Fontelis, Medallion Catamaran and Rovral were not significantly different with the untreated control.

Treatment, formulation and rate/100gal	Apr 18 Severity (%)	Std error	LSD grouping[1]
Untreated uninoculated	0.0	0.0	c
Untreated inoculated	27.3	15.5[2]	a
Quadris SC 15.5 fl oz + Bravo Weather Stik SC 3 pt	1.2	1.2	bc
Fontelis SC 24 fl oz	19.0	9.1	a
Rovral L 2 pt	12.7	10.0	abc
Medallion WP 7 oz	17.8	9.5	a
Omega 500F 1 pt	1.2	1.2	bc
Catamaran 5 pt	4.7	1.5	ab

[1]Letter grouping based on log transformed data. Data was transformed to fulfill normal distribution of residuals and variances homogeneity. Columns with common letters are not significantly different, LSD *P*=0.05

[2] Two celery seedlings out of the six were infection scapes.

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