ABSTRACT

HOGE, BRIANNA LEE. Characterization of the Genetic Diversity and Developmental Biology of the *Colletotrichum gloeosporioides* Species Complex in NC Apple Orchards. (Under the direction of Drs. Marc A. Cubeta and David F. Ritchie).

Glomerella leaf and fruit spot (GLFS) is an economically important disease of apple. It was first reported in North Carolina (NC) in the early 2000s, and optimal environmental conditions in subsequent years led to severe epidemics. *Colletotrichum gloeosporioides* (Cg) is the causal agent of GLFS. In recent years, several other species in the Cg species complex have been shown to infect apples, such as *C. alienum, C. fructicola,* and *C. siamense*. These pathogens cause lesions on apple leaves and fruit, leading to premature defoliation and reduced yield. Cultivars from the 'Delicious' lineage, such as 'Red Delicious' and 'Fuji' have complete or partial resistance to GLFS, while the most susceptible cultivars are from the 'Golden Delicious' lineage. This includes cvs. 'Gala', 'Golden Delicious', and 'Pink Lady', which are among the most commonly grown varieties in NC. Currently, management strategies for GLFS are limited to the use of fungicides, a few recently developed resistant cultivars, and cultural practices to remove suspected inoculum sources from orchard floors. Cultural practices consist of removal of mummified and infected fruit, pre-abscission or post-harvest application of 5% w/v urea, and burning or shredding leaf litter. The premise for the use of urea to suppress Cg is based on reduction of inoculum of the apple scab pathogen *Venturia inaequalis*. However, previous studies suggest that Cg prefers an alkaline environment that can result from treatment of leaves with urea. To implement the most effective management strategies, accurate pathogen identification and information on the response of specific pathogens to treatments is critical. The objectives of this research

were to 1) identify species in the Cg complex involved in GLFS epidemics in NC, 2) determine species occurrence on three commonly grown apple cultivars, and 3) evaluate the effect of post-harvest urea application to apple leaf litter on perithecial development of *Colletotrichum* spp.

To determine species occurrence in NC, isolates were collected from diseased leaves and fruit from a total of 15 apple orchards in 2014 and 2015. Four distinct morphotypes were identified based on assessment of morphological characteristics. Morphotype and rDNA-*ITS* sequence data were inadequate for separating species within the complex, but were used to eliminate isolates of *C. acutatum sensu lato* from this study. Multi-locus sequence analysis identified five species of *Colletotrichum gloeosporioiodes sensu lato* involved in GLFS epidemics in NC. No statistical association of multi-locus haplotype was observed with cultivar, geographic location, or year sampled.

Field and laboratory experiments were conducted to determine whether urea influences perithecial and ascospore development. The addition of urea to lima bean agar enhanced radial vegetative hyphal growth of *Colletotrichum gloeosporioides sensu lato* isolates, but had no effect on mycelial biomass at concentrations of 2.5, 5 and 10 % w/v urea compared to the non-amended control treatment. Urea amendment did not affect the number of perithecia formed, but amendment with 5 and 10 % urea delayed perithecial and ascospore maturation. In two field experiments, the same concentrations of urea were applied to diseased apple leaves provided similar results. Perithecial number was enhanced following application of 5% urea at one field location. At each location, treatment with 10% urea delayed perithecial development to the greatest extent.

Characterization of the Genetic Diversity and Developmental Biology of the *Colletotrichum gloeosporioides* Species Complex in NC Apple Orchards.

by
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APPROVED BY:

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DEDICATION

To my family, friends, and partner who never let me give up on myself and encouraged me to keep pushing forward.

BIOGRAPHY

Brianna Lee Hoge was born on March 17, 1990 in Indianapolis, Indiana. She grew up in Somerville, Tennessee, a small farm town outside of Memphis. As a student at Fayette Ware Comprehensive High School, Brianna was very active in academic and extracurricular activities, holding leadership roles in the Beta Club, National Honor Society, and Student Government; playing softball and acting as captain of the cross-country team; and participating in the school band and drama club, while maintaining valedictorian status and working as a waitress and prep cook in her family's restaurant.

In August 2008, Brianna began attending Rhodes College in Memphis, TN, studying Biology. In her freshman year, she began work with *Aspergillus nidulans* under the guidance of Dr. Terry W. Hill, and a love for all things fungal began. Her research during this period was focused on delineating the key factors involved in fungal cell wall synthesis, utilizing molecular and cellular biology techniques. She was an active member in the Hill lab for seven semesters and was fortunate enough to obtain summer funding to continue her research during the summers of 2011 and 2012.

Seeking to determine the best use of her research skills for plant protection, she worked from 2012-2014 as a laboratory technician under the direction of Dr. Jim Walgenbach for the entomology lab at NCSU's Mountain Horticultural Crops Research Extension Center in Mills River, NC. Here she reared colonies of seven insect species and tested current and experimental pesticides on insect pests of peppers, tomatoes, and stone fruit. During this time, Brianna discovered a passion for agriculture, interacting with growers, and the blending of theoretical and applied science.

In 2014, Brianna began her graduate career in the research program of Dr. Marc A. Cubeta working with the causal agents of Glomerella leaf and fruit spot, *Colletotrichum gloeosporioides* for her Master of Science degree. Her time spent in Dr. Cubeta's lab allowed her to develop as a researcher and gain more experience interacting with growers and implementing lab and field experiments. Brianna hopes to continue her career in agriculture in extension after graduation.

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I would like to thank my committee members, Drs. Marc A. Cubeta, David Ritchie, and Sara Villani for their patience, guidance, and understanding. I would like to especially thank them for pushing me to seek opportunities to speak with growers at annual meetings and in informal settings, to attend professional meetings, and to participate in educational outreach events. Because of these experiences, I have been able to further develop my ability to interact with a wide variety of audiences, from school children to established scientists, about various aspects of mycology and plant pathology. I would like to acknowledge my funding source for this work, the North Carolina Agricultural, Tobacco, and Dairy Foundation. This project would not have been possible without their support. Also, a huge thank you to all the members of the Blue Ridge Apple Growers' Association and to all the growers who interacted with me and let me into their orchards over the last three years. You have been wonderful, and I will greatly miss working with each of you.

Working with current and past members of the Cubeta lab, including Megan Miller, Marie Meckman, Elizabeth Thomas, Lindsey Becker, Maxton Collins, Ian Vander Schel, and my REU undergraduate Tara Rickman has been a delight. I can't express how thankful I am to have you all as friends and colleagues. It's been a wild ride, but working with you guys has all made it much more enjoyable.

Last, but certainly not least, I would like to thank my family, friends, and partner for supporting me along the way. Mom and Dad, thank you guys for deciding that having kids could be fun; raising me to be a strong-willed, independent woman and supporting me along the way; believing in me even when I sometimes didn't; and letting me crash whenever I

needed to travel to Asheville for sampling trips, even if that meant we barely saw each other. Cassandra, thanks for being weird with me, maintaining inside jokes that not even we understand sometimes, and reminding me to not take myself so seriously all the time. Michael Crowley, thank you for continually supporting me, for getting take out and letting me sleep through movies that I picked after a 12-14 hour day at a microscope, helping me set up field trials and being amazingly precise in following my instructions, and most importantly for loving me and our cats on our bad days. Loki, Moriarty, and I are very lucky to have you in our lives. Finally, I would like to thank the community of friends I've found here in Raleigh that have given me endless joy and kept me sane through this experience through outdoor adventures, table-top Sundays and game nights, and exploring the city, including, but not limited to: Nathan Miller, Megan Miller, Michael Cannon, Emma Wallace, Nick Noel, Cody Zane, Elise and James Calentino, and Will Lehman. Without you guys Raleigh would not have felt like home, and I would certainly have had a very different experience in graduate school.

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CHAPTER I. Literature Review

Apple Production

More than 57 M metric tons of apples (*Malus domestica* L.) are produced worldwide. China produces the most apples in the world, yielding 43 M metric tons, followed by the United States (US), yielding 4.7 M metric tons in 2016 with a farm-gate value of nearly $4 billion USD (26). In the US, at least 200 cultivars are commercially grown in 32 states. North Carolina (NC) had 2,064 hectares dedicated to apple production as of 2016 (54). NC ranks seventh in apple production (46,720 metric tons) with an estimated value of roughly $24.5 million USD per year (54). Approximately 46.6% of NC apples harvested are marked for fresh market sale totaling $17.42 M USD, while the remaining 53.4% are used for processing, valued at $7.04 M USD (54). The most commonly grown cultivars in NC are 'Gala', 'Golden Delicious', 'Granny Smith', 'Honeycrisp', 'Pink Lady', 'Red Delicious', and 'Rome' (47). With rising consumer preference for these cultivars and prevalence of monoculture in agriculture, new diseases such as Glomerella leaf and fruit spot have rapidly increased in incidence and severity worldwide (18, 20). This has led to increased costs of production, higher risk of crop loss, and a greater need for effective cultural and chemical controls.

Glomerella Leaf and Fruit Spot

Glomerella leaf spot and fruit spot (GLFS), caused by members of the *Colletotrichum gloeosporioides* species complex, is currently the most economically important and intensely managed fungal disease of apple in North Carolina. The disease was first reported in the US in a 'Golden Delicious' planting in Georgia in 1970 (52). Ascospores have been shown to serve

as the primary inoculum, with mature ascospores being forcibly ejected from mid-April through July (51, 52). Perithecia can infect apple leaves and fruit, though leaf symptoms tend to be visible before fruit symptoms, occurring after petal fall. Leaf symptoms begin as small purple-brown flecks, which rapidly expand to irregularly shaped oblong lesions with characteristic light and dark brown concentric rings in optimal environmental conditions (25 - 28°C, relative humidity above 95%, and frequent rainfall) (48). The formation of asexual fruiting structure (acervuli) and spores (conidia) on leaf lesions in apple orchards, suggest that conidia function as a source of secondary inoculum (52). Late in the growing season, chlorosis radiates from the leading edge of the lesion, eventually covering the entire leaf surface, and up to 75-100% premature abscission occurs (18, 19). Unlike *Colletotrichum acutatum*, the causal agent of bitter rot, fruit symptoms caused by *C. gloeosporioi*des are variable in phenotype. Small circular fruit lesions (1-2 mm diameter) have a gray-brown center surrounded by a black border that does not extend into the apple flesh. Expanded lesions (1.5 cm in diameter) are light to medium brown, and a brown discoloration often develops below the endocarp, though it does not extend into the flesh as deeply as lesions caused by *C. acutatum*. A red or yellow halo may or may not form around the lesion. Only minimal production of asexual fruiting structures and conidia has been reported from fruit lesions caused by Cg, unlike lesions typical of bitter rot caused by *C. acutatum* (52).

Fruit lesions reduce the wholesale marketability and value of the harvest. Fruit with severe disease symptoms may be unsalable to processing plants due to health and safety concerns. Annual losses to GLFS can lead to permanent damage that reduces tree health and yield (18, 19, 20). North Carolina farmers have reported up to 100% loss in years where optimal

environmental conditions occur (41). This can translate to upwards of hundreds of thousands of dollars in crop loss, excluding production expenses, such as irrigation, fungicide applications, and labor.

Colletotrichum gloeosporioides **Species Complex**

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. 1884 was first proposed by Panzig and Saccardo from a specimen described on citrus (*Citrus medica*) as *Vermicularia gloeosporioides* Penz. (1882). Morphological similarities to *Colletotrichum spp.* and plant host preference criteria were used as justification for changing the nomenclature (61). Over the next century, the "von Arxian" taxonomic concept utilized morphological features such as colony color, size and shape of conidia and appressoria, presence or absence of setae, presence of a sexual stage (teleomorph), and plant host species for characterization and identification (8, 30, 48, 61). Unfortunately, morphological characteristics are inadequate and variable for classification, and may be lost or altered with repeated subculturing (24, 25, 31, 61). In addition, several studies have indicated that sequence analysis of the ribosomal DNA internal transcribed spacer (rDNA-ITS) region is not adequate for differentiating species of *Colletotrichum*, which is further exacerbated by low quality, inaccurate and non-curated sequences in GenBank and other databases (7, 14, 15, 25, 39). Nine species originally designated as C. *gloeosporioides* (Cg) have been associated with the *C. acutatum*, Cg, and *C. orbiculare* species aggregates, based on DNA multi-locus sequence analysis of four loci: glutamine synthetase (GS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DNA lyase (*Apn2*), and mating (*MAT*) regions of the genome (14, 15, 24, 25, 44, 49, 55, 61, 67). *C. gloeosporioides* is now recognized as a species aggregate or species complex comprised of at

least 22 species and one subspecies, some of which can infect up to 20 species of plants (15, 61).

Population Genetic Diversity of *Colletotrichum* in Apple Orchards

Most of our knowledge about *Colletotrichum* spp. in apple orchards is based on *Colletotrichum acutatum* (Ca) Simmonds 1965. While Ca and Cg overlap in epidemiological characteristics on apple, they produce distinct diseases and differ in a few requirements for infection to occur, such as pH preference (17, 37, 38, 57). It should be noted that while this author distinguishes the fruit symptoms associated with GLFS from those associated with bitter rot, historically, the term bitter rot has been associated with fruit symptoms caused by both Ca and Cg. For the sake of continuity in discussing population diversity here, the historical approach will be maintained and expanded upon.

Recent molecular advances have led to the description of several species within the *C. acutatum* and *C. gloeosporioides* species complexes being associated with bitter rot and GLFS worldwide (1, 2, 4, 28, 39, 55). In Kentucky, a multi-locus phylogenetic analysis of approximately 500 isolates from fruit lesions indicated that the prevalent species recovered are *C. fioriniae* (Marcelino &Gouli) Pennycook 2017, *C. nymphaeae* (Pass.) Aa 1978, *C. siamense* Prihast, L. Cai & K.D. Hyde 2009, *C. fructicola*, *C. theobromicola* Delacr. 1905, *C. gloeosporioides sensu stricto*, and *C. acutatum sensu stricto* (38). *C. fioriniae* Prihast, L. Cai & K.D. Hyde 2009, *C. acutatum, and C. nymphaeae* currently belong to the *C. acutatum* species complex, while the others are designated as members of the *C. gloeosporioides* species complex (14, 39, 59, 61). A similar study of 38 isolates sampled from infected leaves and fruits in New Hampshire, New York, and New Jersey yielded one isolate designated *C. nymphaeae*,

while the remaining 37 isolates were *C. fioriniae* (59). C. *fioriniae* was the most frequently recovered species from fruit in both studies (39, 59). These studies support the results and first report of *C. fioriniae* causing post-harvest decay on cv. 'Nittany', of the 'Golden Delicious' lineage, grown in Pennsylvania (28).

Much of our knowledge about the genetic diversity of *Colletotrichum* populations in apple orchards in temperate and tropical climates comes from the southeastern US, Brazil, and Uruguay. In the southeastern US, research investigations from 1992-2006 used morphological characteristics, vegetative compatibility groups (VCGs) and molecular techniques to infer population genetic diversity, before the advent of species complexes (18, 19, 20, 49). A common experimental approach was used to differentiate isolates based on their ability to form perithecia in culture. Using this criterion, isolates that formed perithecia were tentatively identified as *Glomerella cingulata*, while those unable to form perithecia were designated as *C. gloeosporioides.* For this discussion, the historical designations of *C. gloeosporioides* and *G. cingulata* will be adopted.

Shi et al. studied the frequency of *Colletotrichum* spp. causing bitter rot of apple fruit in Arkansas, North Carolina, and Virginia utilizing conidial and cultural morphology and hyphal growth rate (49). The results suggested that Ca was the predominant species, though Cg represented 31.3% of the sample and was the predominant species isolated from diseased fruit in one North Carolina orchard in 1992 and 1993 (49). In addition, *G. cingulata* was isolated from fruit in 5 of 8 orchards sampled and was the predominant species recovered from one orchard in North Carolina in 1993. Following this study, Gonzalez and Sutton performed a similar study in two orchards in North Carolina utilizing morphology, VCG, and

molecular characterization (18, 19, 20). *G. cingulata* was the predominant species in all orchards surveyed, while Cg was less widespread and recovered from three orchards (18, 19, 20). A phylogenetic analysis of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genomic region provided support for two independent evolutionary clades: Cg/*Glomerella cingulata* and *C. acutatum* (18).

Recent studies from Brazil and Uruguay provided increased resolution of *Colletotrichum* species sampled from infected leaves and fruit with restriction fragment length polymorphism (RFLP) and multi-locus sequence analyses. Results from these studies revealed that *C. fructicola* was the predominant species associated with GLFS and bitter rot (35, 55). Isolates recovered from infected fruit and leaves in Brazilian orchards were identified as members of three distinct species complexes (*C. fructicola* (Cg complex), *C. nymphaeae* (Ca complex), and *C. karstii* Yang, Liu, Hyde & Cai 2011 (*C. boninense* complex)) (35, 55). Isolates sampled from diseased fruit and leaves from orchards in Uruguay represented genetically distinct populations of *C. fructicola* and *C. theobromicola* (Cg complex), and *C. melonis* Damm, Cannon & Crous 2012 (Ca complex) (34, 52). A correlation of species complex with geographical region, but not host tissue preference (fruit or leaf) was documented with principal component analysis (35). The authors hypothesized that these observed genetically differentiated populations may be partially explained due to differences in apple cultivars grown in each country. For example, ‘Golden Delicious’ varieties, such as ‘Gala’, represent the primary cultivars grown in Brazil, while ‘Delicious’ varieties, such as ‘Red Delicious’ are the primary cultivars grown in Uruguay (35).

Disease Management

Fungicides

Management of GLFS depends heavily on protective and systemic fungicides. Applications targeting GLFS begin at petal fall and continue every 7-14 days until harvest (58). Quinone outside inhibitors (QoIs) are currently the only single-site fungicide group that provide a commercially acceptable level of control for GLFS and are mixed with protective, multi-site fungicides during summer cover applications. The current recommended fungicides for managing GLFS are: Captan (captan), Ziram 76DF (ziram), mancozeb, Prophyt (potassium phosphite), Sovran (kresoxim-methyl), Flint 50WG (trifloxystrobin), Pristine (boscalid + pyraclostrobin), Merivon (fluxapyroxad + pyraclostrobin), Omega (fluazinam), and Aprovia (benzovindiflupyr) (58). In years when optimal environmental conditions persist utilizing the shortest spray interval is suggested for orchards with high disease pressure, though it is unlikely to completely prevent leaf and fruit symptoms.

Host Plant Resistance

Developing cultivars with resistance to *Cg* is another management strategy for the control of GLFS. Research has shown that cultivars developed from the parental lineage 'Golden Delicious', such as 'Gala' and 'Pink Lady', are highly susceptible to GLFS, while those from the 'Delicious' lineage, such as 'Fuji' and 'Red Delicious' have complete or partial resistance (35, 55). In Brazil, optimal climate conditions for GLFS epidemics and the lack of efficient fungicide regimens have yielded renewed interest in breeding hybrid cultivars for disease resistance. The Instituto Agronómico do Paraná (IAPAR) and Epagri have developed three cultivars with GLFS resistance which have fruit characteristics (coloration, acidity,

crispness, and sweetness) like cv. ‘Gala’. Rather than traditional qualitative or quantitative resistance strategies, these cultivars rely on disease escape or avoidance to reduce the impact of GLFS epidemics (6, 22, 42). IAPAR 74-Eva is a low-chill requiring variety that can be grown in areas not suitable for traditional cultivars. It is derived from a cross of ‘Gala’ and ‘Anna’, produces large yields, resembles the ‘Gala’ parent in appearance and flavor profile, and shows high levels of GLFS resistance (42). IAPAR ‘Julieta’ has similar environmental tolerances and resistance to GLFS. It is derived from a cross between ‘Mollie’s Delicious’ and ‘Anna’, has an appearance and flavor profile like ‘Anna’, ripens approximately two weeks before Eva, and is recommended for planting alongside of Eva, as it is partially self-fertile and serves as a pollinator (22). ‘Julieta’ is also moderately tolerant to powdery mildew, apple scab, and spider mites (42). Epagri’s ‘MonaLisa’ is another apple resistant to GLFS, apple scab, and spider mites. It is derived from a cross between ‘Gala’ and the non-patented ‘Malus4’, resembles ‘Gala’ in flavor and texture, has a low-chill requirement, and produces uniform red/purple fruits (6). Continued success of newly established orchards in northern Brazil will likely inform breeding programs elsewhere in the pursuit of GLFS management through host plant resistance.

Cultural Practices

Due to the limited options for managing GLFS with host plant resistance and high costs of fungicides, the deployment of cultural practices is critical. These practices include removal of mummified and infected fruit from trees and orchard floor, pre-abscission or post-harvest application of urea, and burning or shredding leaf litter (58). While general orchard sanitation is a prudent management practice, it is labor intensive and expensive. Post-harvest applications

of urea to reduce overwintering populations of *Venturia inaequalis*, have been an important component in apple scab management programs for decades (9, 20, 33, 34, 40, 43, 50, 57, 58). The success of urea in reducing primary inoculum of *V. inaequalis* suggests that applications of urea could also influence perithecial and ascospore development of *Colletotrichum* spp. populations in overwintering on apple leaves.

Principles of Urea Application for Disease Control

Applications of nitrogen (N) containing compounds, including commercial and fertilizer grade urea, have been incorporated into crop health and disease management programs for centuries (30). Recently, with pressure to shift toward more biological and "green" means of disease control, researchers have begun to focus on the mechanisms behind these methods that have the common goal of reducing loss from various plant diseases, increasing crop yield, and lowering pesticide resistance risk. Application of N rich compounds has been shown repeatedly to negatively affect the severity of fungal phytopathogens (5, 9, 10, 12, 16, 20, 21, 23, 27, 30, 33, 34, 40, 43, 50, 56, 57). At the same time, examination of the local microflora has shown a marked increase in saprobic species, with some studies reporting a 100-1000-fold increase in the microbial population (30). The following studies suggest that amendment, regardless of nitrogen source, could function in a two-fold way, by enhancing competition for space and by creating a less hospitable environment for some fungi.

Soil Nitrogen Amendment for Fertilization and Disease Control

Current knowledge of the effectiveness of amending soil with nitrogen (N) sources for the control of plant pathogens is in conflict. A meta-analysis of 57 studies tested the

nitrogen disease hypothesis, which states that plant growth at high N availability may result in increased susceptibility to pathogens (56). Their results indicated that *Solanum* spp. were the only plant species for which a reduction of disease severity was significant after N fertilization, specifically when interacting with *Verticillium* spp. In addition, while disease severity due to necrotrophic pathogens initially increased, severity due to hemibiotrophs and biotrophs increased by a significantly greater amount, particularly when phosphorus was simultaneously added (56). These results are like those found by Datnoff and colleagues, who found that N is readily utilized by plants while variable in the effect it has on pathogens, depending on rate and pathogen preference for the type of N available (12).

In other studies, urea treatment of soil has also been found to be toxic to *Phellinus noxius* (Corner) G. Cunn. 1965, some *Ganoderma* spp., and several other wood and root rotting fungal species (10). Regardless of mechanism, it has been postulated that pathogens negatively influenced by urea and high N containing organic compounds are displaced by the influx of saprobic species in the microbiome, many of which have been implicated as potential biocontrol agents (29, 55). The implication of these studies on soil-borne pathogen control is selective management and preservation of beneficial organisms. Another study, conducted by Lazarovits in 2001, utilized *Verticillium dahliae* Kelb. 1913 and *Streptomyces scabies* populations in potato production to assess the mechanism of pathogen control with addition of high-nitrogen organic matter (30). Results indicated that degradation of organic matter by microorganisms prompted the release of ammonia into the soil (30). Ammonia quickly converts to ammonium (NH_4^+), which increases soil pH, making it less hospitable toward certain microbes. Further conversion by bacteria leads to the production of nitrite

(NO_2^-) and nitrous acid (HNO_2), the latter being highly toxic to plant pathogens including *V. dahliae*, *S. scabies*, *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen 1940, and *Sclerotinia sclerotiorum* (Lib.) de Bary 1884, as well as some weed species (30). In addition, a study conducted by Veverka et al. demonstrated that volatile NH_3 released after hydrolysis of urea was found to be lethal to several soil-borne pathogens including *Phytophthora* spp., *Pythium ultimum* Trow 1901, *Thelaviopsis basicola* (Berk. et Br.) Ferraris 1912, and *Macrophomina phaseolina* (Tassi) Goid. 1947 (57).

Urea for Fertilization and Disease Control in the Phylloplane

Foliar applications of nitrogen, specifically in the form of urea, have been utilized to manage plant pathogens and fertilize tree crops. Spring shoot and leaf growth is positively correlated with the amount of N reserves for many tree fruit species, including apple, peach, and nectarine (16, 27, 46, 60). Urea can be applied in the spring in low concentrations (1-2% w/v), though mild phytotoxicity is a concern. As a result, autumn applications are favored, because a higher rate can be applied without concern for damaging the tree (13, 16, 27, 46, 60). Experiments conducted on apple, peach, and nectarine have shown that recovered N is greater when the source was applied to leaves rather than soil, and that fall foliar application can improve flowering and fruit set the following season (16, 46, 65). In addition to these growth benefits, there is some evidence that foliar application can reduce severity of ash dieback and cherry leaf spot diseases (5, 21, 23). It should be noted, that efficacy of foliar urea treatment for fertilization or disease control is dependent on tree N content prior to application. It is also recommended that growers use low-biuret urea, as biuret, found in

fertilizer grade urea, has some phytotoxic effects when combined with higher rates of urea (13, 27).

Urea and Apple Crop Protection: A Tale of two Genera

Our current understanding of the use of urea as a cultural practice for managing GLFS stems from research conducted for *Venturia inaequalis*. In both diseases, a single small lesion on an apple can yield the fruit unmarketable (9). *V. inaequalis* overwinters in apple leaf litter, forming pseudothecia and ascospores which serve as the primary source of inoculum (9, 33, 43, 50). Successful phytosanitary practices revolve around the removal, degradation, or treatment of leaf litter, primarily through the application of 5% urea and shredding, mulching, or burning (9, 33, 34, 43, 50). The application of 5% urea to trees post-harvest in combination with leaf shredding reduces leaf scab severity and incidence of fruit scab by 42-65% (33, 50, 65). Urea limits the development of *V. inaequalis* ascospores by accelerating the rate of leaf decomposition and enhancing activity of earthworms and beneficial saprobic microbes (9, 40, 43, 50). The application of urea also increases leaf pH, inhibiting pseudothecial production and ascospore maturation (33 43). Production of pseudothecia by *V. inaequalis* is reduced at pH $\leq$4 and pH $\geq$ 7.5 (43).

Similar to *V. inaequalis*, apple leaf tissue provides an overwintering location for the development of perithecia and ascospores of *Colletotrichum* spp. (13, 51, 52). Several studies have indicated that *Colletotrichum* spp. prefer alkalinized environments for growth and reproduction (3, 17, 37, 38). In a study comparing nutritional and abiotic factors affecting the growth of Cg and sexual reproduction, ammonium phosphate, urea, and casein resulted in the greatest biomass and vegetative growth (29). The results of this study also suggested that the

optimal pH for hyphal growth was 5.5-7.0, and the optimal pH for asexual spore production was 6.0 (29). In addition, nitrogen amendment and a pH ≥4.9 enhanced secretion of pectate lyase, a pathogenicity factor, with optimal production detected at pH 6.0 (17). In nature, Cg actively alkalinizes the ambient environment via ammonia secretion during colonization through the activation of glutamine synthetase, glutamate dehydrogenase, ammonia exporter, and urease genes (3, 37, 38). In a study of Cg on avocado mesocarp, a pH increase from 5.7 to 7.5 was observed which induced appressorium formation (37, 38). The demonstrated preference of Cg for a higher pH environment than *V. inaequalis* challenges the assumption that treatment of leaves infected with Cg will suppress perithecia and ascospore development in litter.

Perithecial Development

To our knowledge, no comprehensive studies have been conducted to examine the effect of urea application on perithecia and ascospore development in Cg. Much of the research into perithecial formation in *Glomerella* was conducted from the early 1900s through 1960s. In 1920, Dastur examined the sexual stage of *Gloeoesporium piperatum* (=*Colletotrichum coccodes*) on chilies and found variation in perithecial production among isolates (11). The formation of perithecia was either scattered or aggregated, with the cortex formed by large, thin-walled cells and dark, smaller, tightly packed cells comprising the medulla (11). Asci and ascospores were hyaline and varied in size (45-66 µ x 7.7-11 µ and 12-19 µ x 4.4-6.6 µ, respectively). Sterile elongated cells, rather than true paraphyses, were observed between asci, which were hypothesized to be aborted asci (11). The seminal research of Edgerton, Wheeler, and colleagues investigated the genetics, plasmogamy, and reproductive strategies of

Glomerella. Edgerton and colleagues reported in 1944 that some strains produced perithecia and viable ascospores while other strains were nearly sterile (32). These were designated as plus and minus, with the plus strain consistently producing ascospores of both types in a 1:1 ratio (32, 64). McGahen and Wheeler examined stages of perithecial development, from the development and differentiation of the initial coils to the development of the ascogenous system. Their results suggested that: 1) two genes were responsible for sexual reproduction in *Glomerella* (A and B); 2) wild isolates are self-fertile and cross-fertile; and 3) perithecial production is reduced in the presence of an unidentified exudate from conidia, though a mating of perithecial cultures and conidial cultures produced viable perithecia (36, 62, 63, 64).

Perithecial development begins with the formation of two lateral initials, which grow in parallel for a short time, until one elongates more rapidly than the other and coils around the other, forming an inner and outer coil (36). The inner coil consists of 3-5 short, thick cells, while the outer coil is comprised of many elongated, uninucleate cells. Once the inner coil is fully enclosed in the outer coil, this structure develops into a peridium, and plasmogamy occurs (36). After plasmogamy, development is rapid, with the cells of the peridium branching profusely and becoming thick and dark, and thin multinucleate cells filling the interior of the perithecium, which disintegrate as asci mature and are hypothesized to serve a nutritive function (36). The ascogonial (central) coil develops slowly, containing fewer than a dozen cells by the time the perithecium is mature in size (36). As development progresses, the ascogonial coil produces lateral branches and crozier hooks, which leads to nuclear fusion, meiotic and equational division, and the production of eight haploid nuclei in each ascus (36).

Once ascospores reach maturity, they are forcibly ejected from the neck of the perithecium and asci walls break down (36, 11).

When *G. cingulata* was first reported on apple in the US, perithecia were observed in overwintering leaves early in the summer (May), and mature, viable ascospores were observed by mid-June (52). The fungus is capable of infecting fruit, but failed to produce conidia or ascospores on fruit in the lab and field. The fungus did sporulate on leaves, suggesting that ascospores may be the primary source of inoculum for GLFS epidemics (51, 52). In a subsequent experiment in North Carolina in 1979, fruit infection was observed as early as late May (51). In addition, perithecial strains of *G. cingulata* have been shown to kill chili pepper seedlings more rapidly than conidial or sterile strains, and that these strains cause greater symptom severity on apples in field trials (11, 51). Because ascospores function as the primary source of inoculum and may potentially lead to increased disease severity, further investigation into ascocarp developmental biology within apple leaf litter treated with urea in the field is needed.

Research Objectives and Hypotheses

The overarching goals of this research project were to 1) characterize and identify species in the *Colletotrichum gloeosporioides* species complex (CgSC) involved in GLFS epidemics in North Carolina and 2) investigate the role of urea on sexual fruiting body (perithecial) development. We sampled diseased fruits and leaves from multiple apple cultivars in North Carolina in 2014 and 2015 and subjected 70 single conidium isolates to multilocus DNA sequence analysis. We tested the hypotheses that 1) multiple phylogenetically distinct species within the Cg complex will be present and 2) species

occurrence will be associated with cultivar and geographic location. To investigate the role of urea on perithecial development of Cg in apple leaves, we established a field experiment at two field locations in North Carolina to test the hypothesis that urea will delay perithecial development and ascospore maturity.

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Chapter II.

Title: Characterization of the *Colletotrichum gloeosporioides* species complex associated with Glomerella leaf spot and fruit rot of apple in North Carolina

Abstract

Glomerella leaf and fruit spot (GLFS) is one of the most important diseases of apple (*Malus domestica*). Historically, *Colletotrichum gloeosporioides* (Cg) and its teleomorph *Glomerella cingulata* have been identified as the causal agents. Since the discovery of species complexes within Colletotrichum, several other species have been attributed to GLFS. In this study, we characterized and identified species within the Cg complex sampled from apple leaves and fruits with GLFS symptoms in North Carolina orchards in 2014 and 2015. Four distinct morphotypes based on macroscopic and microscopic characteristics were identified. Morphotype and rDNA-ITS sequence data were inadequate for resolving species within the complex. Multi-locus sequence analysis identified at least five species and 21 haplotypes. No association of species with cultivar, geographic location, or year was observed through primary component analysis.

Introduction

Glomerella leaf and fruit spot (GLFS) is an economically important disease in North Carolina apple orchards. *Colletotrichum gloeosporioi*des (Penz.) Penz. & Sacc. (teleomorph = *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) has historically been identified as the causal agent of GLFS. Since the discovery of species complexes within the genus *Colletotrichum*, several other closely related species have been attributed to GLFS epidemics worldwide including *C. alienum* B. S. Weir & P. R. Johnst. 2012, *C. fructicola* Prihast., L.

Cai, & K. D. Hyde 2009, *C. nymphaeae* (Pass.) Aa 1978, *C. siamense* Prihast, L. Cai & K.D. Hyde 2009, and *C. theobromicola* Delacr. 1905 (13, 15, 18, 25, 26, 28). Multiple species occur in the same orchard and across locations, though populations can be geographically differentiated particularly when different cultivars are grown in separate locations (15, 18, 26). Management of these pathogens is heavily dependent on fungicides and there is evidence suggesting that species within the *C. gloeosporioides* complex (aggregate) respond differently to fungicides (18, 27, 28). To this end, accurate species identification is critical for the implementation of effective management practices for GLFS in areas where apple production is concentrated.

Historically, identification of *Colletotrichum* spp. has been performed utilizing colony and conidial morphology, vegetative compatibility groups (VCGs), or sequence analysis of rDNA-ITS and single copy genes (8, 9, 23, 28). Colony morphology and pathogenicity are highly variable and traits may be lost with repeated subculturing (28). In addition, previous studies have shown that rDNA-ITS sequence analysis is not sufficient for differentiating *Colletotrichum* fungi in a species complex (2, 5, 10, 11, 18). The combination of morphological classification coupled with multilocus sequence analyses is needed for accurate identification and elucidation of relationships among closely related fungal species (18, 28).

The objectives of this study were to characterize and identify species within the *Colletotrichum gloeosporioides* species complex associated with GLFS epidemics in western North Carolina (NC) apple orchards and to investigate their geographic distribution and occurrence on three commonly grown apple cultivars. We hypothesized that multiple species

within the Cg species complex will be present in NC apple orchards. In addition, we tested the hypothesis that the distribution of species found will vary among cultivar and county.

Materials and Methods

Isolate source

Diseased apple leaves and fruit demonstrating symptoms of GLFS were collected from cultivars Gala, Golden Delicious, and Pink Lady from 15 distinct conventional orchards in Henderson, Polk, Cleveland, Moore, and Wake County, NC during summer 2014 and 2015. Nine orchards were sampled per year, with some sampled both years (Table 2.1). To control for differences in orchard size and composition (small, single cultivar or large, multiple cultivar), ten trees were sampled per cultivar in each orchard. Trees were divided into quadrants, and two leaves and two fruit demonstrating GLFS symptoms were collected per quadrant for each tree. For isolation from apple fruit, diseased tissue was surface disinfested by rinsing for 20 s with 70% EtOH, blotted dry with sterilized paper towels, and 10-mm sections were excised from the leading edge of a necrotic lesion using a sterile scalpel after removal of the exocarp and plated on Lima Bean Agar (LBA). The medium was prepared by boiling 286 g of lima beans in 1L of SDW for 1.5 h and filtering through cheesecloth before adding 15 g agar and autoclaving. This medium was chosen over more commonly used media such as potato dextrose, as colony color is consistent across *Colletotrichum* spp., sporulation is enhanced, and *Colletotrichum* is readily distinguishable from other fungi frequently recovered from leaf and fruit tissue. Spore shape and size on LBA is consistent with those produced on PDA.

For leaf tissue, 5-mm sections were excised from the leading edge of lesions, rinsed with sterile distilled H_2O (SDW), soaked in 10% bleach (6.15% NaClO) for 30 s, rinsed with SDW, and placed on a sterilize paper towel to dry. Dry, disinfested sections were plated on LBA. Monoconidial isolates were obtained from the resulting colonies according to the procedure of Du et al. (7) and pure culture isolates were prepared for storage by adding three mycelial plugs from each culture to a cryovial containing 500 µL potato dextrose broth (PDB, Difco). Cultures were grown at room temperature (approximately 20°C) for 5 d before adding 500 µL 50% glycerol and storing at -80°C. Of the 150 monoconidial isolates obtained above, several were excluded from this study due to morphological and rDNA-ITS similarity to the *C. acutatum* species complex, and others were excluded due to an inability to consistently isolate and amplify DNA. The seventy remaining isolates were utilized in morphological and phylogenetic comparisons. Reference strains of six members of the *C. gloeosporioides* species complex were obtained for use as controls for all experiments from Dr. Stephen Rehner (USDA-ARS, Systematic Mycology and Microbiology Laboratory, Beltsville, MD) and the Fungal Biodiversity Institute culture collection in Utrecht, The Netherlands (Table 2.2)

Morphological characterization

Seventy isolates collected from leaves and fruit demonstrating GLFS symptoms were subjected to macroscopic and microscopic morphological examination. Morphological traits evaluated included colony color, size and shape of conidia, and perithecial production on LBA at 23°C with a 16 hr light/8 hr dark cycle (48 μ/m^2). Colony color was determined after 7 d incubation. Conidia were harvested from a 10-d-old culture of each isolate by flooding

each plate with 750 µL SDW, gently scraping the upper surface of the culture with a sterile metal spreader, and suspending conidia in 5 mL SDW. Conidia shape was determined for all isolates visually by adding 20 µL of each conidial suspension to a glass slide and observing at 40x utilizing a Nikon compound microscope. Perithecial production was noted after 16 d. A subsample of isolates (n=12) were selected to determine conidial size using a weighted sampling strategy based on the relative frequency of isolates collected in 2014 and 2015 from cv. 'Gala', 'Golden Delicious', and 'Pink Lady'. The length and width of 50 arbitrarily selected conidia for each of the twelve representative isolates were measured at 40x with an optical stage micrometer. Isolates were assigned to one of four morphotypes, and average length, width, and standard error were determined for each morphotype based on the complied isolate data. An arbitrary subset of ascospores from 10 observed perithecia per representative isolate (n=12) were plated on LBA to test viability.

DNA extraction and sequence analysis

Seventy isolates of *C. gloeosporioides* sensu lato and six reference strains (Tables 2.1 and 2.2) were grown in 100 mL PDB at 23°C with a 16 h light/8 h dark cycle (48 µ/m^2). Mycelium was harvested by filtration after 7 d incubation, frozen at -80°C, and lyophilized. Genomic DNA was extracted from lyophilized mycelium with either CTAB or an OmniPrep for Plant DNA isolation kit (GBiosciences, St. Louis, MO) following the manufacturer's protocol. DNA amplification with PCR was performed with Qiagen HotStarTaq master mix (Qiagen Inc., CA) in an Eppendorf Mastercycler thermo cycler. Amplification of ribosomal DNA internal transcribed spacer (rDNA-ITS), actin (ACT), β-tubulin (TUB2), and DNA lyase (*APN2*) genomic regions was performed with the primer pairs ITS-F (5′-

CTTGGTCATTTAGAGGAAGTAA-3′) and ITS-R (5′-TCCTCCGCTTATTGATATGC-3′) for rDNA-ITS, ACT-F (5′-ATGTGCAAGGCCGGTTTCGC-3′) and ACT-R (5′-TACGAGTCCTTCTGGCCCAT-3′) for ACT, TUB-F (5′-AACATGCGTGAGATTGTAAGT-3′) and TUB-R (5′-TAGTGACCCTTGGCCCAGTTG-3′) for TUB2, and AP-F (5′-AGAAACCCRTTYGGATACCAA-3′) and AP-R (5′-TGGGATACATCATCAAGAGGC-3′) for APN2 (25). PCR conditions for the four regions consisted of an initial heat activation step at 95°C for 15 min. The primer pair specific conditions for the four regions used in this study are as follows: for rDNA-ITS, 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min; for ACT, 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min; for TUB2, 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min; and for *APN*, a touchdown procedure was used with 40 cycles of denaturation at 94°C for 1 min, annealing at 62-50°C ramped down every 15 sec for 1 min, and extension at 72°C for 2.4 min. This was followed by a final extension at 72°C for 10 min. ExoSAP cleanup and Sanger sequencing of PCR products were conducted by the Genomic Sciences Laboratory at North Carolina State University. Forward and reverse sequences were trimmed for quality and aligned to obtain a consensus sequence for each isolate using Sequencher (Gene Codes Corp., MI). Basic Local Alignment Search Tool (BLAST) was used to examine nucleotide sequence similarity for the rDNA-ITS region to sequences deposited in GenBank. Consensus sequences for each of the four regions were aligned with MUSCLE for multiple alignment, alignments of the four regions were concatenated, and a phylogeny was inferred using the random axelerated

maximum likelihood function (RAxML) in Geneious 10.2.3 (https://www.geneious.com, Kearse et al., 2012). All loci were included in the analysis and branch support was estimated with 1000 bootstrap replications. Metadata of host cultivar was visualized on the inferred phylogenetic tree. Concatenated sequences for all isolates were mapped to multi-locus haplotypes and a primary component analysis was conducted in Mobyle SNAP workbench to determine species distribution across cultivars, geographic locations, and collection years (17).

Results

Morphological Characterization.

Four morphological types (morphotypes) were identified based on colony color and appearance, conidia shape, and production of perithecia on LBA from the 70 *Colletotrichum* isolates used in this study which were sampled from diseased apple leaves and fruit with GLFS symptoms in North Carolina (Table 2.3; Figure 2.1). From this sample, a representative subsample of 12 isolates was selected based on a weighted sampling strategy for characterization of conidial size in an attempt to further distinguish morphotypes. The four morphotypes corresponded to four reference species that infect apple: *C. asianum, C. fructicola, C. gloeosporioides,* and *C. siamense* (8, 9, 18, 19, 28).

Morphotype 1 was characterized by production of white, tufted mycelia (Figure 2.1). Orange masses of conidia were submerged in the colony. Conidia were cylindrical with rounded ends and ranged in size from 12.5-16.25 µ x 3.75-6.25 µ (Figure 2.1). Mean conidial length was 14.75± 0.73 µ and mean width was 5.0 ± 0.40 µ. No perithecia were observed

after 16 d incubation. Based on these characteristics, isolates in Morphotype 1 corresponded with *C. siamense* (19, 28).

Morphotype 2 isolates were characterized by white-to-grey non-aerial mycelia (Figure 2.1). Conidia were cylindrical with rounded ends and ranged 10-17.5 µ x 5-7.5 µ in size. Mean conidial length was 13.75± 1.21 µ and average width was 6.25± 0.32 µ. Perithecia containing asci with 8 ascospores (15-17.5 µ x 4.5-6.25 µ) were observed after 16 d incubation (Figure 2.1). From these characteristics, Morphotype 2 isolates were consistent with the description for *C. gloeosporioides* (teleomorph = *Glomerella cingulata*) (8, 9, 12, 28).

Morphotype 3 produced white tufted mycelia. Conidia were cylindrical, frequently with one rounded and one acute end, ranging 8.75-16.25 µ x 5-7.5 µ in size (Figure 2.1). Mean conidial length was 12.75± 1.33 µ and average width was 6.25± 0.56 µ. No perithecia were observed after 16 d. Isolates with these characteristics were consistent with *C. fructicola* (12, 28).

Isolates of Morphotype 4 were distinguished by white, tufted mycelia that became grey with age. Conidia were cylindrical with rounded ends, similar to Morphotype 1, ranged in size from 11.25-15µ x 6.25-7.5µ, and submerged in the mycelia (Figure 2.1). Mean conidial length was 13.5± 0.73 µ and average width was 7.25± 0.25 µ. Perithecia were observed in culture containing asci with 8 hyaline ascospores (17.5-19.5 x 4.25-5 µ). Based on these characteristics, Morphotype 4 isolates corresponded with *C. alienum* (12, 24, 28).

DNA sequence analysis.

Genomic sequences for the rDNA-ITS region of each isolate were subjected to BLAST analysis in GenBank. All 70 isolates were tentatively placed in the *C. gloeosporioides* species complex based on 95-100% sequence similarity with sequences deposited in GenBank. The multi-copy genes for the ribosomal DNA internal transcribed spacer (rDNA-ITS), actin (ACT), and β-tubulin (TUB2), and DNA lyase (*Apn2*) regions were successfully amplified from 70 isolates and six reference strains. PCR products from amplification of these regions were approximately 607, 260, 700, and 840 bp, respectively. Five species within the *C. gloeosporioides* species complex were identified from the sample. One isolate (L28) sampled from a diseased leaf of cv. 'Pink Lady' in 2014 was most closely related to *C. siamense* (100% bootstrap support). Two isolates (GA19L and GA21L) sampled from a diseased leaf of cv. 'Gala' and one isolate (GD16F) sampled from fruit of cv. 'Golden Delicious' were placed in a clade with *C. alienum* with bootstrap support ≥75%. Two leaf isolates from cv. 'Golden Delicious' were closely related to *C. gloeosporioides* (100% bootstrap support), and 12 isolates from fruit and leaves of all cultivars were assigned to a clade with *C. fructicola* based on bootstrap support ranging 75.5-100%. The remaining 52 isolates grouped in a clade with the most similarity to *C. tropicale* (97.8-100% bootstrap support). Twenty-one multi-locus haplotypes (MLH) were found in the sample, with MLH 15 and MLH 1, respectively as the first and second most frequently recovered. The remaining MLHs contained only one or two isolates each. The distribution and occurrence of Cg, *C. fructicola*, *C. alienum*, *C. siamense*, and *C. tropicale* on apple fruit and leaves in NC is not correlated with geographic location according to primary component analysis. In addition, no

significant cultivar or host tissue type preference was found among isolates examined in this study. Isolates originating from leaves and fruit of all three cultivars were sampled across each of the five identified species. In addition to host-interaction phenotypes (symptomology, data not shown), colony morphology was not useful for distinguishing species within the *C. gloeosporioides* species complex.

Discussion

A major objective of this research was to determine which species in the *C. gloeosporioides* (Cg) complex are involved in Glomerella leaf and fruit spot epidemics in North Carolina apple orchards. We hypothesized that multiple species and multilocus haplotypes of *Colletotrichum* (Cg, *C. fructicola,* and *C. alienum*) would be recovered in our sample. To test this hypothesis, we characterized the morphology of isolates and assigned them to four morphotypes based on colony morphology, conidia size and shape, and occurrence of perithecia. Substantial overlap was found among the morphotypes, and when combined with multi-locus sequence data, morphotype was not an accurate predictor of species within the Cg species complex. These results are consistent with previous findings, as colony morphology is a highly variable character within a species, and traits can be lost with subsequent culturing (10, 11, 14, 20, 28).

Prior to this study, only *C. gloeosporioides* (Cg) was described from apple leaves and fruits associated with GLFS symptoms in North Carolina, but those research findings occurred before the discovery that Cg was not a single species but rather an aggregate or complex of closely related cryptic species. Our hypothesis was based on the fact that these studies identified Cg species designated as ‘perithecial’ and ‘non-perithecial’ types. Within

the species complex there are several species that infect apple and vary in their ability to produce perithecia, including *C. fructicola and C. alienum* (28). Cg, *C. fructicola*, and *C. siamense* have also been identified as causal agents of GLFS epidemics in New Hampshire and Kentucky apple orchards (18, 28). In Brazil and Uruguay, *C. fructicola, C. asianum, C. alienum,* and *C. siamense* have been identified as the causal agents of leaf spot and fruit anthracnose of apple and mango (15, 16, 25, 26). Based on this information, we expanded our hypothesized species diversity from three to five species which cause GLFS. Based on our results, we accept this revised hypothesis.

DNA sequences analysis of the rDNA-ITS region with BLAST was used as a proxy to tentatively identify isolates as *Colletotrichum gloeosporioides sensu lato* and differentiate it from *C. acutatum,* a pathogen of apple fruit that causes bitter rot. However, the rDNA-ITS region did not provide resolution of species in the Cg complex (2, 3, 4, 5, 11, 18, 28). Therefore, we conducted experiments with three additional single copy genes, actin, β-tubulin, and DNA lyase, to infer a multilocus phylogeny. Our phylogenetic analyses provided evidence for the occurrence of five different species associated with GLFS epidemics in NC. The *C. siamense* clade was highly similar to *C. asianum,* highlighting the genetic similarity among members of the complex. The majority (n=52) of isolates were closely related to *C. tropicale* which suggests that typically endophytic *Colletotrichum* spp. may also play a role in GLFS epidemics.

Based on our sampling of diseased leaves and fruit from 15 orchards in NC, there are at least five different species in the *Colletotrichum gloeosporioides* species complex: *C. alienum, C. fructicola, C. gloeosporioides, C. siamense,* and *C. tropicale*. All species, except

C. tropicale, have been previously reported as disease causing agents on apple leaves and/or fruit (8, 9, 15, 16, 18, 26, 28). The ability of all reference cultures used in this study to produce GLFS fruit symptoms was tested through wounding inoculations on 'Gala' and 'Pink Lady'. All species, with the exception of *C. tropicale* were able to produce fruit lesions (data not shown). We did not conduct similar experiments with apple leaves.

While several species of *Colletotrichum* can survive as asymptomatic endophytes in leaves of various host species, on apple, many maintain a pathogenic lifestyle. It has been noted that endophytic fungi co-occur with pathogenic fungi and can influence the expression of host defense genes in a positive or antagonistic manner (1, 16). Additionally, this interaction among endophytes, pathogens, and host species is dependent on abiotic and biotic environmental factors (1). Depending on a host plant's physiological condition and genotype as well as environmental conditions, the interaction between endophytes and host plants can switch from mutualistic to pathogenic (6). *C. tropicale* was represented in the greatest frequency in our isolate collection from apple leaves and fruit collected in 2014 and 2015from the orchards surveyed. While C. *tropicale* has not been shown previously to infect apple leaves and fruit, it is a commonly recovered endophytic species associated with a wide variety of host species and with rotting custard apple fruit (*Annona squamosa* L. and *Annona muricata*) in Panama and other tropical regions (16, 21, 25). It has also been shown to be pathogenic on guava, mango, papaya, and pepper (13). It has been suggested that *C. tropicale* may asymptomatically infect cacao leaves and fruit, but may switch to active colonization causing ripe rot after shifts occur in the fruit during ripening (20). Our results suggest that either *C. tropicale* is co-infecting with other pathogenic *Colletotrichum* spp. or a shift in

lifestyle from asymptomatic endophyte to pathogen is occurring due to host or environmental factors.

Our results suggest that populations of Cg in NC apple orchards are more diverse than previously demonstrated (8, 9). This is likely due to variation introduced through the presence of a sexual stage in many of the isolates selected for this study (18).

Accurate pathogen identification is crucial for the development of effective strategies to manage GLFS. Past methods of identification have included vegetative compatibility and use of individual genes (ITS, GAPDH) to determine species identity of fungi sampled during GLFS epidemics, which have since been shown to be ineffective in resolving species within the complex. Future studies should investigate species distribution within individual orchards across the state and among apple growing regions in the southeast. In addition, examination of the relative pathogenicity and aggressiveness of the five identified species, and their respective response to currently recommended fungicides is warranted.

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Table 2.1. Source of isolates of *Colletotrichum gloeosporioides sensu lato* used in this study.

Isolate	Sample Date[a]	Cultivar[b]	Host Tissue	Location	Orchard	Morphotype	Species ID
L24	23/9/14	GA	Leaf	Moore	8	4	*C. fructicola*
F22	18/9/14	GD	Fruit	Henderson	2	2	*C. tropicale*
F23	18/9/14	GD	Fruit	Henderson	2	2	*C. tropicale*
F24	18/9/14	GD	Fruit	Henderson	2	2	*C. tropicale*
F26	18/9/14	GD	Fruit	Henderson	2	2	*C. tropicale*
F29	18/9/14	GD	Fruit	Henderson	2	2	*C. fructicola*
F45	9/10/14	GD	Fruit	Henderson	9	2	*C. tropicale*
L34	9/10/14	GD	Leaf	Henderson	9	2	*C. gloeosporioides*
F47	9/10/14	GD	Fruit	Henderson	9	2	*C. fructicola*
F34	9/10/14	GD	Fruit	Henderson	9	4	*C. tropicale*
L37	9/10/14	GD	Leaf	Henderson	9	2	*C. gloeosporioides*
F31	26/9/14	PL	Fruit	Henderson	2	2	*C. tropicale*
L28	9/10/14	PL	Leaf	Henderson	9	2	*C. siamense*
L31	9/10/14	PL	Leaf	Henderson	9	2	*C. tropicale*
GD2L	3/7/15	GD	Leaf	Polk	5	2	*C. tropicale*
GA3L	22/7/15	GA	Leaf	Henderson	11	4	*C. tropicale*
GA4L	22/7/15	GA	Leaf	Henderson	11	2	*C. tropicale*
GA6L	22/7/15	GA	Leaf	Henderson	11	1	*C. tropicale*
GA9L	3/7/15	GA	Leaf	Polk	12	2	*C. tropicale*
GA15L	23/7/15	GA	Leaf	Polk	12	1	*C. tropicale*
GA16L	23/7/15	GA	Leaf	Polk	12	4	*C. tropicale*
GA18L	23/7/15	GA	Leaf	Polk	12	2	*C. tropicale*
GA19L	22/7/15	GA	Leaf	Henderson	11	4	*C. alienum*
GA21L	22/7/15	GA	Leaf	Henderson	11	2	*C. alienum*
PL1L	21/7/15	PL	Leaf	Henderson	2	4	*C. fructicola*
PL2L	21/7/15	PL	Leaf	Henderson	2	2	*C. fructicola*
GD2F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
GD3F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
GD7F	23/7/15	GD	Fruit	Polk	5	4	*C. tropicale*
GD8F	23/7/15	GD	Fruit	Polk	5	4	*C. tropicale*
GD10F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
GD11F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
GD12F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
GD15F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*

Table 2.1 Continued

GD16F	23/7/15	GD	Fruit	Polk	5	2	*C. alienum*
GD17F	23/7/15	GD	Fruit	Polk	5	4	*C. tropicale*
GD18F	23/7/15	GD	Fruit	Polk	5	4	*C. tropicale*
GD20F	23/7/15	GD	Fruit	Polk	5	4	*C. tropicale*
GD21F	23/7/15	GD	Fruit	Polk	5	4	*C. fructicola*
GD22F	23/7/15	GD	Fruit	Polk	5	3	*C. tropicale*
GD24F	23/7/15	GD	Fruit	Polk	5	2	*C. fructicola*
PL2F	23/7/15	PL	Fruit	Polk	14	2	*C. fructicola*
GA1F	22/7/15	GA	Fruit	Henderson	11	4	*C. tropicale*
GA2F	23/7/15	GA	Fruit	Polk	12	2	*C. tropicale*
GA3F	23/7/15	GA	Fruit	Polk	12	4	*C. tropicale*
GA5F	22/7/15	GA	Fruit	Henderson	11	4	*C. tropicale*
GA6F	23/7/15	GA	Fruit	Polk	12	2	*C. tropicale*
GA7F	22/7/15	GA	Fruit	Henderson	11	2	*C. fructicola*
GA8F	22/7/15	GA	Fruit	Henderson	11	4	*C. tropicale*
GA9F	23/7/15	GA	Fruit	Polk	12	4	*C. fructicola*
GA10F	23/7/15	GA	Fruit	Polk	12	4	*C. tropicale*
GA11F	23/7/15	GA	Fruit	Polk	12	2	*C. tropicale*
GA14F	3/7/15	GA	Fruit	Polk	12	2	*C. fructicola*
GA16F	3/7/15	GA	Fruit	Polk	12	2	*C. tropicale*
GA17F	3/7/15	GA	Fruit	Polk	12	4	*C. tropicale*
GA18F	3/7/15	GA	Fruit	Polk	12	3	*C. tropicale*
GA25F	22/7/15	GA	Fruit	Henderson	15	3	*C. tropicale*
GA26F	22/7/15	GA	Fruit	Henderson	15	3	*C. tropicale*
GA28F	22/7/15	GA	Fruit	Henderson	15	2	*C. tropicale*
GA29F	22/7/15	GA	Fruit	Henderson	15	3	*C. tropicale*
GA30F	22/7/15	GA	Fruit	Henderson	15	3	*C. tropicale*
GA31F	22/7/15	GA	Fruit	Henderson	15	1	*C. fructicola*
GA32F	22/7/15	GA	Fruit	Henderson	15	3	*C. tropicale*
GA34F	21/7/15	GA	Fruit	Henderson	2	1	*C. tropicale*
GA35F	21/7/15	GA	Fruit	Henderson	2	3	*C. tropicale*
GA36F	21/7/15	GA	Fruit	Henderson	2	3	*C. tropicale*
GD25F	22/7/15	GD	Fruit	Henderson	10	1	*C. tropicale*
GD26F	30/7/15	GD	Fruit	Henderson	7	3	*C. tropicale*
GD27F	23/7/15	GD	Fruit	Henderson	7	3	*C. tropicale*
PL4F	21/7/15	PL	Fruit	Henderson	2	3	*C. tropicale*

[a] Collection date is formatted as day/month/year

[b] Cultivar designation: Gala (GA), Golden Delicious (GD), and Pink Lady (PL).

Table 2.2. Species, isolate designation, and source of reference strains used in this study.

Reference Species	Isolate Designation	Source
C. alienum	CBS112991	CBS-KNAWa, Netherlands
C. asianum	CBS130418	CBS-KNAW, Netherlands
C. fructicola	SAR10-38	Stephen Rehner, USDA-ARS, Maryland
C. gloeosporioides	SAR10-72	Stephen Rehner, USDA-ARS, Maryland
C. siamense	CBS130417	CBS-KNAW, Netherlands
C. tropicale	SAR10-213	Stephen Rehner, USDA-ARS, Maryland

[a] CBS-KNAW is now known as the Fungal Biodiversity Institute culture collection in Utrecht, The Netherlands

Table 2.3. Description of morphotype for 70 isolates based on colony color and appearance, conidia shape and size, and presence or absence of perithecia grown on lima bean agar for 7-14 d at 23 C.

Morphotype	Colony	Conidial Shape	Perithecia	Species
Morphotype 1	White, dense, aerial mycelium; no pigment change with age	Cylindrical with rounded ends	-	*C. siamense*
Morphotype 2	White, non-aerial mycelium, becoming grey with age	Cylindrical with rounded ends	+	*C. gloeosporioides*
Morphotype 3	White, thin, aerial mycelium; no pigment change with age	Cylindrical with one rounded end one acute end	-	*C. fructicola*
Morphotype 4	White dense, aerial mycelium, becoming grey with age	Cylindrical with rounded ends	+	*C. alienum*

Table 2.4. Representative subset of *Colletotrichum* spp. isolates from symptomatic fruit and leaves in North Carolina.

Isolate	Year of Isolation	Cultivar	Host Tissue	County	Orchard	Morphotype	Species ID
F22	2014	Golden Delicious	Fruit	Henderson	2	2	*C. tropicale*
GA15L	2015	Gala	Leaf	Polk	12	1	*C. tropicale*
GA18L	2015	Gala	Leaf	Polk	12	2	*C. tropicale*
GA5F	2015	Gala	Fruit	Henderson	11	4	*C tropicale*
GA9L	2015	Gala	Leaf	Polk	12	2	*C. tropicale*
GD10F	2015	Golden Delicious	Fruit	Polk	5	2	*C. tropicale*
GD18F	2015	Golden Delicious	Fruit	Polk	5	4	*C. tropicale*
L24	2014	Gala	Leaf	Moore	8	4	*C. fructicola*
L28	2014	Pink Lady	Leaf	Henderson	9	2	*C. siamense*
L34	2014	Golden Delicious	Leaf	Henderson	9	2	*C. gloeosporioides*
PL1L	2015	Pink Lady	Leaf	Henderson	2	4	*C. fructicola*
PL4F	2015	Pink Lady	Fruit	Henderson	2	3	*C. tropicale*

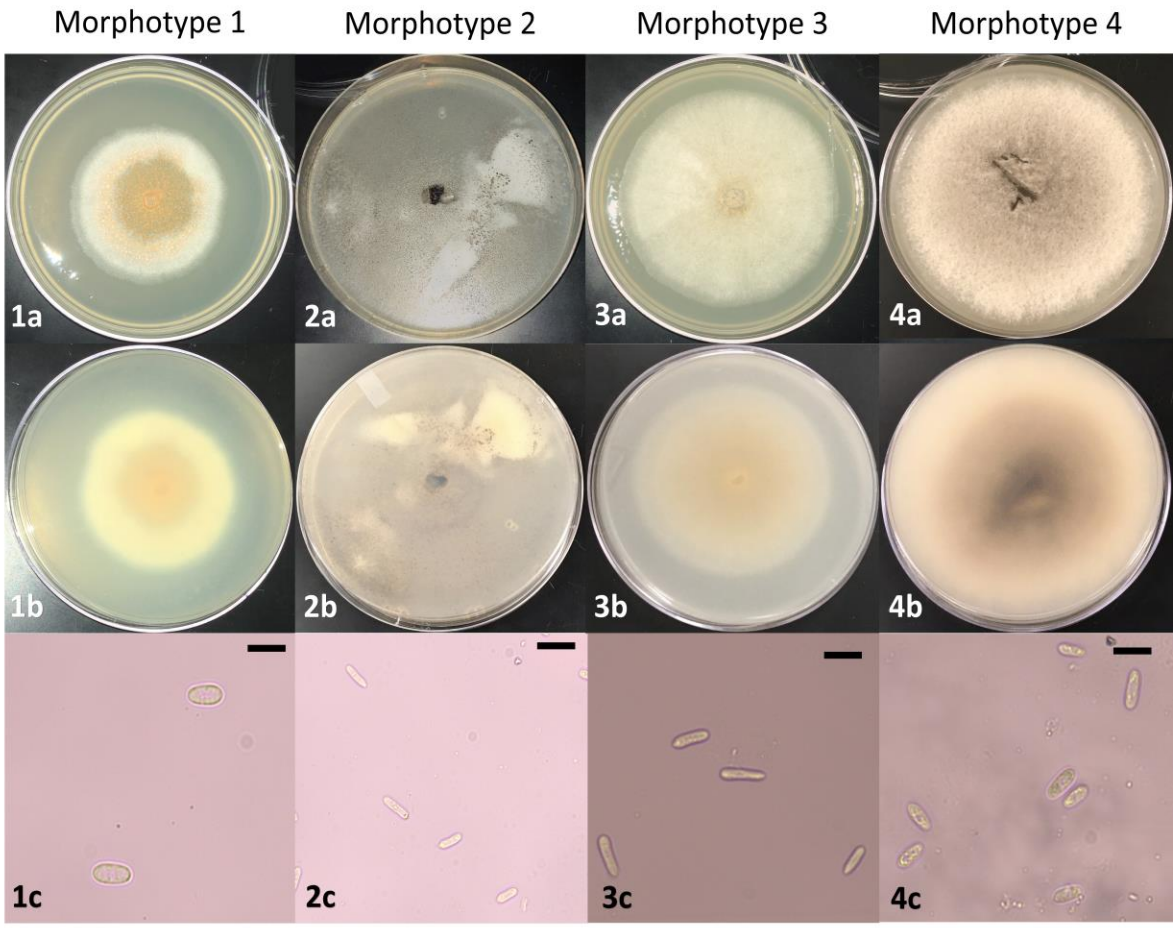

Figure 2.1. Morphotypes of isolates of *Colletotrichum gloeosporioides sensu lato* isolated from apple fruit and leaves demonstrating symptoms of GLFS in North Carolina. Characterization was based on colony color and appearance, conidia shape, and formation of perithecia. Colony color of Morphotype 1 upper and lower surface, respectively, and conidial shape (1a-c); Morphotype 2 (2a-c); Morphotype 3 (3 a-c); and Morphotype 4 (4 a-c). Colonies were observed after 7 d, conidia after 10 d, and perithecia after 16 d incubation on lima bean agar at 23 C. Black bars= 15 µm.

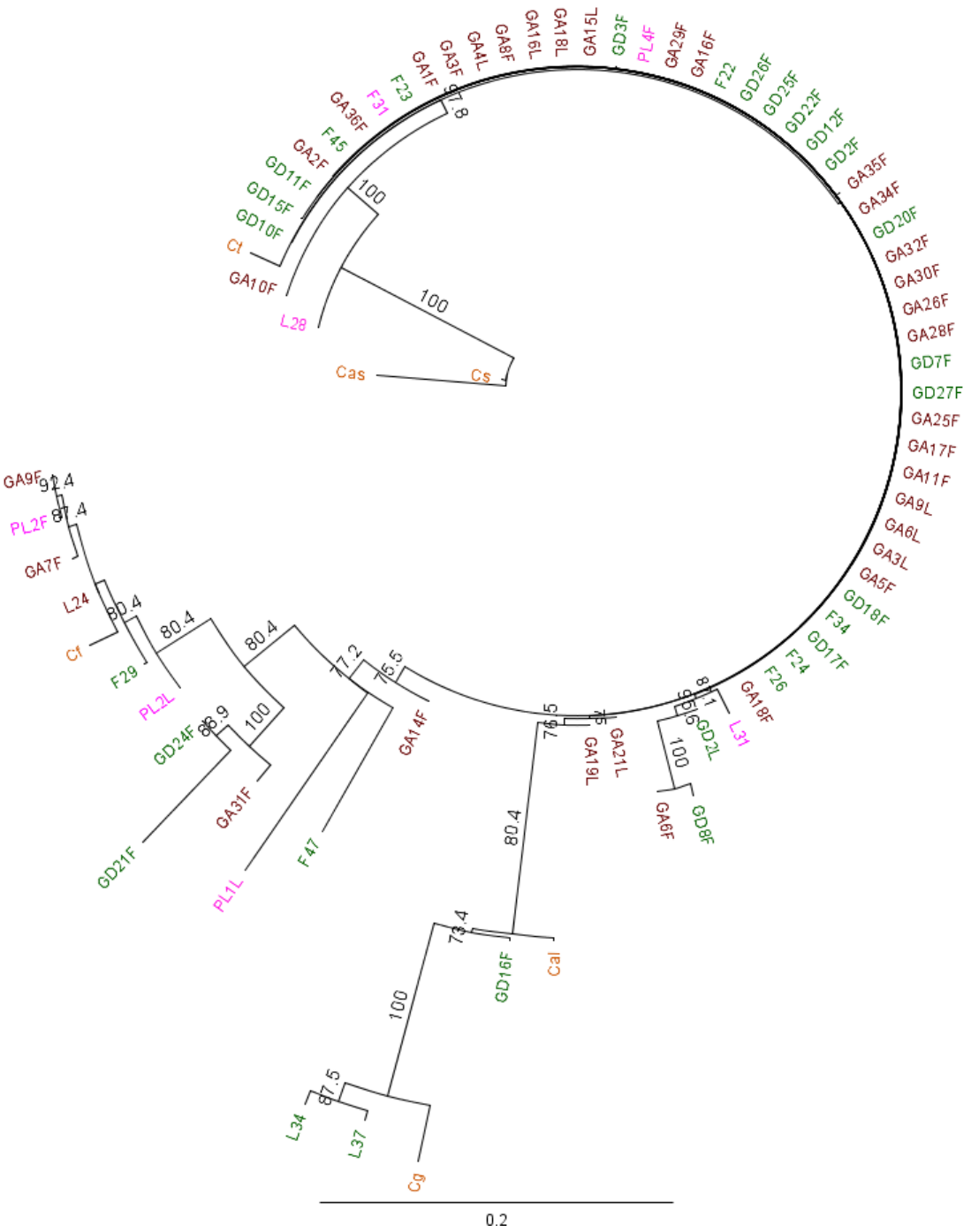

Figure 2.2. Unrooted phylogenetic tree based on four genomic regions (rDNA-ITS, ACT, TUB2, and APN2). The tree was inferred using the random axelerated maximum likelihood function (RAxML) in Geneious v. 10.2.3 with 1000 bootstrap replications. Numbers at each branch indicate percentage of branch support values based on maximum likelihood. Color of isolate name is indicative of cultivar or origin with red= 'Gala', green= 'Golden Delicious', pink= 'Pink Lady', and black= reference strain.

Chapter III.

Urea increases mycelial growth and reduces perithecia development of *Colletotrichum gloeosporioides*

Abstract

Fungi in the *Colletotrichum gloeosporioides* species complex cause Glomerella Leaf and Fruit Spot (GLFS). A current cultural practice for managing GLFS in North Carolina (NC) involves the application of 5% urea to leaf litter after harvest, but little is known about the effect(s) of urea on fungal growth and development in the context of disease suppression. In this study, we investigated the effect of three concentrations of urea (2.5, 5, and 10% w/v) on mycelial growth and perithecial development in laboratory experiments with lima bean agar (LBA) and field experiments with diseased apple leaves, respectively. Amendment of LBA at all concentrations of urea increased radial mycelial growth compared to the non-amended control, but had no effect on mycelial biomass or number of perithecia produced. Addition of 5 or 10% urea to LBA delayed perithecial and ascospore development. In field experiments conducted at two locations in NC, application of the above concentrations of urea to apple leaves with disease symptoms had no effect on the number of perithecia formed on leaves at the first field location, but 5% significantly increased the number produced at the second. At each location, treatment of apple leaves with 10% urea delayed perithecial maturation to the greatest extent at both locations.

Introduction

Glomerella leaf and fruit spot (GLFS) is an economically important disease of apples in the southeast US. It was first reported in Georgia in 1970 on cultivar ‘Golden Delicious’

(20). Under optimal conditions, with extended periods of warm, humid weather, GLFS can cause 75-100% defoliation, weakening trees and reducing yield (6). Severe epidemics of GLFS occurred in eastern Tennessee and North Carolina (NC) in 1998 and 2001, respectively, resulting in renewed efforts to elucidate pathogen ecology and population diversity in the context of disease management (5, 6, 7). Historically, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph =*Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) has been identified as the causal agent of GLFS, but after the identification of species complexes by Weir and colleagues, several other species in the Cg complex have been found to infect apple. Management of these pathogens is heavily dependent on fungicides, but there are few fungicide classes that are efficacious in controlling the disease (14, 21, 22).

Due to the limited options for managing GLFS, deployment of cultural practices is critical. These include removal of mummified and infected fruit from trees and the orchard floor, pre-abscission or post-harvest application of 5% with urea (w/v), and burning or shredding leaf litter. The premise for the use of urea to suppress Cg is based on research conducted on the apple scab pathogen *Venturia inaequalis* (5, 10, 17, 18). These studies demonstrated that ascocarp (pseudothecia) and ascospore development were reduced by application of 5% urea to apple leaves at 10% defoliation. The mechanisms for suppression of apple scab involved accelerated degradation of leaf litter and enhanced activity of earthworms and beneficial saprobic microorganisms. In addition, application of urea increased leaf pH beyond the range in which *V. inaequalis* produces pseudothecia (16). However, several studies suggest that species of *Colletotrichum* prefer alkalinized

environments for infection (1, 3, 12, 13). Secretion of pectin lyase, a virulence factor of *Colletotrichum* spp., has been shown to increase with the addition of nitrogen and a pH ≥ 4.9 (3). Additionally, ammonium phosphate, urea, and casein have been shown to enhance vegetative growth and biomass of Cg *in vitro* (8). To our knowledge, the hypothesis that 5% urea will reduce perithecial and ascospore development of *Colletotrichum gloeosporioides sensu lato* has not been investigated.

The objective of our research was to determine the effect of post-harvest urea application to apple leaf litter on the vegetative mycelial growth and perithecial production of *Colletotrichum* spp. We tested the hypothesis that addition of three rates of urea will increase radial vegetative growth and biomass of Cg. Our second hypothesis was that treatment with urea will decrease the number of perithecia formed on nutrient medium in the laboratory and on diseased apple leaves in the field. Our final hypothesis was that urea treatment will delay perithecia and ascospore maturation. Within these hypotheses, our objectives were to determine the effect of the concentration of urea that is currently used in NC orchards (5% w/v) compared to a water control treatment and to compare the effect of half and double concentration to 5 % w/v.

Materials and Methods

Isolate source and storage

Diseased apple leaves and fruit with symptoms of GLFS were collected from cultivars Gala, Golden Delicious and Pink Lady from 15 distinct orchards (9 total per year) in Henderson County, NC during summer 2014 and 2015. For isolation from apple fruit, diseased tissue was surface disinfested by rinsing for 20 s with 70% EtOH and blotted dry with a sterilize paper

towel. Ten-millimeter sections were excised from the leading edge of a necrotic lesion with a sterile scalpel after removal of the exocarp and plated on Lima Bean Agar (LBA). The medium was prepared by boiling 286 g of lima beans in 1L of SDW for 1.5 h and filtering through cheesecloth before adding 15 g agar and autoclaving. For leaf tissue, 5-mm sections were excised from the leading edge of lesions, rinsed with sterile distilled H_2O (SDW), soaked in 10% bleach (6.15% NaClO) for 30 s, rinsed with SDW, and placed on a sterile paper towel to dry. Disinfested sections were plated on LBA. Monoconidial isolates were obtained from the resulting colonies according to the procedure of Du et al. (4) and pure culture isolates were prepared for storage by adding three mycelial plugs from each culture to a cryovial containing 500 µL potato dextrose broth (PDB, Difco). Cultures were grown at room temperature (approximately 20°C) for 5 d before adding 500 µL 50% glycerol and storing at -80 C.

Relative mycelial growth, conidia, and perithecia production on urea medium

Twelve monoconidial isolates of Cg *sensu lato* were selected on the basis of perithecial production and host-interaction phenotype (leaf or fruit source; apple cultivar source) (Table 3.1). Isolates recovered from storage at -80 C were grown on LBA in a 9-cm diameter plastic petri plate in a 24°C incubator (Perceval Scientific) with a 12 h light/dark cycle (48 µE m^{-2} s^{-1}). For each isolate, a 5-mm diameter plug taken from the advancing edge of the colony was placed in the center of LBA amended with 5% w/v urea or SDW (2016) and 2.5, 5, or 10% w/v urea or SDW (2017) (CAS #57-13-6, Sigma -Aldrich, MO). Addition of 10% urea raised the pH of the medium to 6.15, so all other concentrations and the control were adjusted to match with 10M NaOH. This adjustment was made to ensure that the effect of varying urea concentration would not be masked by the effect of pH. There were three

technical replicate plates for each isolate x treatment combination and plates were arranged in a completely randomized design in a 24°C incubator. Colony diameter was measured every 24 h for 14 d until a maximum possible colony diameter of 83 mm (petri dish diameter) was obtained for all treatments. After attaining a maximum colony diameter, the number of perithecia on each replicate plate was counted and developmental stage of 10 arbitrarily selected perithecia per isolate-treatment combination replicate was assessed every 3 d for 27 d after maximum plate diameter was obtained (15 days post inoculation (dpi) -42 dpi). The experiment was conducted twice. Perithecial development was rated using a modification of a 1-8 scale developed from a combination of the scale used by Sutton and colleagues for *Venturia inaequalis* and the seminal research on perithecial development and plasmogamy in *Glomerella* by McGahan and Wheeler (10, 11, 18). Stages 1-4 were associated with exterior perithecium development where Stage 1 presented as coiling of melanized hyphae; Stage 2, coiled melanized hyphae but lacking a defined perithecium flask shape; Stage 3, exterior of perithecium thickening and ostiole forming; and Stage 4, exterior perithecial development complete with a thick (1.25-2µ) exterior and ostiole (Figure 3.1). Stages 5-8 consisted of the internal perithecium structural developmental characteristics: Stage 5, paraphyses observed; Stage 6, paraphyses and asci initials present; Stage 7, ascospore initials observed within developing asci; and Stage 8, mature ascospores observed (Figure 3.1). The experiment was conducted twice, except that 2.5 and 10% urea concentrations were included in the second experiment.

In a separate experiment, fungal biomass for each of the 12 Cg isolates was assessed by adding a 5-mm diameter mycelial plug of the fungus into 100 ml of lima bean broth (LB)

amended with 0, 2.5, 5, or 10 % urea (pH adjusted to 6.15 as above). LB was made following the same protocol as above, without the addition of agar. For each isolate x treatment combination, three technical replications were incubated at 24°C (100 rpm) in a Series 25 shaker (New Brunswick Scientific) in a completely randomized design. After 12 d of incubation, mycelium was harvested by vacuum filtration on pre-weight Whatman #1 filter paper, dried at 40°C in a Thelco lab oven (Precision Scientific), and weighed to determine biomass. The experiment was conducted twice.

Perithecia production on urea treated apple leaves.

Apple leaves (cv. 'Gala') of similar physiological age and GLFS leaf symptom severity (50-70%) were removed from trees at 20% defoliation on 21 October 2016 at the Mountain Horticultural Crops Research Station (MHCRS) in Henderson County, NC. No perithecia were present on leaves at the beginning of the experiment. Leaf discs (2-cm diameter) were punched from leaves, sprayed until runoff with water (control) or 2.5, 5, or 10% urea w/v (CAS #57-13-6, Sigma -Aldrich, MO), and 10 arbitrarily selected discs were placed in 10.16 x 7.62 cm sheer organza mesh bags (Amazon). Bags were stored at room temperature (approximately 20°C) before being placed on the soil surface in a field plot established on 12 Nov 2016 at MHCRS and on 19 Nov 2016 at Lake Wheeler Road Research Station (LWRS) in Raleigh. There were six and four replicates of each treatment at MHCRS and LWRS, respectively, arranged in a split plot design. Bags were collected on 3 Dec 2016, 24 Dec 2016, 14 Jan 2017, 4 Feb 2017, 25 Feb 2017, 18 Mar 2017, 8 Apr 2017, 29 Apr 2017, 20 May 2017, and 10 Jun 2017 at MHCRS and 10 Dec 2016, 31 Dec 2016, 21 Jan 2017, 11 Feb 2017, 4 Mar 2017, 25 Mar 2017, 15 Apr 2017, 6 May 2017, 27 May 2017, and 17 Jun

2017 at LWRS. Environmental data for daily average air and soil temperature, daily relative humidity, and daily total precipitation was collected for each location over the course of the study and averaged across each three week interval. Leaf discs were assessed for the total number of perithecia formed per replicate. Fifty perithecia were arbitrarily selected from each treatment replication and assessed for perithecial developmental stage using the rating scale described above.

Data analysis

In vitro and field data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC). For all experiments, an analysis of variance (ANOVA) was conducted to determine treatment effects over the course of the experiments, and means were compared using the Tukey multiple comparison test. Field data were further analyzed for each location at each date for collection dates 7-10 (8 Apr-10 Jun (MHCRS) and 15 Apr-17 Jun (LWRS)) to determine treatment effects during the hypothesized infection window. Mean developmental stage and the number of perithecia rated at stage 7 were further investigated for the final four collection dates for each location (MHCRS- 8 Apr, 29 Apr, 20 May, 10 Jun; LWRS- 15 Apr, 6 May, 27 May, 17 Jun) to better understand the effect of a fall application of urea on perithecial count and maturation during and after bloom (typically mid-April in NC). A square root transformation was conducted on the count response variable to reduce skewness in the model. Dunnett's multiple means comparison test was conducted at each date to compare each rate of urea to the control.

Results

In vitro mycelial growth rate and biomass experiments

Results from the ANOVA with radial mycelial growth as the dependent response variable indicated a significant increase in mycelial growth ($P \leq 0.0001$) for isolates grown on LBA amended with 2.5, 5, and 10% urea compared to the non-amended control. Radial mycelial growth was measured every 24 h for 14 d. Enhanced growth was observed as early as 1 dpi. Isolates on control and 2.5% urea plates averaged 10 mm; on 5%, 20 mm; and on 10%, 30 mm. This trend continued until isolates reached 60 mm, at which point no significant difference was observed among the treatments (Figure 3.2). All treatments (2.5, 5, and 10% w/v) were significantly different from the control ($P \leq 0.05$), but were not significantly different from each other over the course of the experiment ($P \geq 0.05$). For mycelial biomass, no significant ($P \leq 0.05$) treatment or treatment x isolate interaction effects were found.

In vitro perithecial count and development

Treatment with urea was found to have no significant effect on the number of perithecia formed in culture ($P = 0.1103$) compared to the non-amended control. Perithecial development was rated every three days from 14-42 dpi. Variation was observed among isolates in their response to urea treatments over the course of time. In order to simplify the model, the final three rating dates (36, 39, and 42 dpi) were analyzed. These dates were selected because mature perithecia were not observed before this time. At 36 dpi, amendment of LBA plates with all three rates of urea significantly reduced perithecial development ($P = 0.001$) compared to the non-amended control. Dunnett's multiple comparison test of the

square root transformation of the number of mature perithecia (Stage 8) observed in each treatment revealed that treatment with 5 and 10% urea significantly delayed perithecial maturation, compared to control and 2.5% urea treatment (Figure 3.3). At 39 dpi, comparison of the number of mature perithecia revealed no significant difference compared to the control treatment, but Dunnett's test of the mean developmental stage suggested that treatment with 10% urea was significantly different from the control ($P \leq 0.05$). At 42 dpi, mature perithecia were observed in all treatment x isolate combinations. No significant differences among treatments were found through analysis of the number of mature perithecia or mean developmental stage (Figure 3.3).

Perithecial development on apple leaves in the field.

At MHCRS, treatment with urea did not influence the number of perithecia formed, but increased the number of perithecia formed at LWRS. Based on Dunnett's multiple comparison test, treatment of apple leaves with 5% urea significantly increased the number of perithecia formed (Figure 3.4). Because mature perithecia were not recovered in the field study, stage 7 was utilized in analyses as the most advanced stage of development, as opposed to immature perithecia in stages 1-6.

Over the course of this study at both locations, urea treatment varied in its effect on perithecial maturation (Figure 3.5). To better assess treatment effects during and after bloom, typically beginning early to mid-April in NC, we further examined development data from collection dates 7-10 for each location. During these dates, leaves are fully expanded and fruit begins to develop, providing an abundance of potential infection courts. At MHCRS, on 8 and 29 Apr, treatment with urea significantly reduced perithecial development ($P \leq 0.0001$).

Dunnett's multiple comparison test of the square root transformation of the number of perithecia at Stage 7 revealed that a fall application of 10% urea significantly ($P \leq 0.0004$) delayed perithecial maturation (Figure 3.6). Treatment with 10% urea significantly delayed perithecial development, while 2.5% enhanced perithecial development by 20 May (Figure 3.6).

At LWRS, a fall application of urea to apple leaves significantly reduced perithecial development among samples rated on 15 Apr ($P \leq 0.0051$). Treatment with 10% urea significantly delayed perithecial maturation compared to the control, according to Dunnett's multiple comparison test of the square root transformation of the number of perithecia at Stage 7 (Figure 3.7). No significant difference was found among treatments through analysis of the number of perithecia at Stage 7 or the average developmental stage of samples rated on 6 May (Figure 3.7). Treatment with 2.5% urea significantly increased perithecial development among samples rated on 27 May (Figure 3.7).

Discussion

In this study, we found that urea influences the growth and development of perithecia of *Colletotrichum* spp. responsible for Glomerella leaf and fruit spot epidemics in North Carolina apple orchards. *In vitro* experiments performed in 2016 and 2017 to test the effect of various concentrations of urea on vegetative growth and biomass indicated that treatment with urea enhanced radial mycelial growth, but had no effect on mycelial biomass. These results are consistent with previous findings, suggesting that urea is readily utilized by *C. gloeosporioides* (Cg) as a source of nitrogen and carbon (3). In the same study, the optimal range of pH for hyphal growth was 5.5-7.0 and the optimal for asexual spore production was

6.0 (3). In this experiment, the addition of 10% urea to LBA increased pH to 6.15, well within the optimal range for Cg growth. All other treatments were adjusted to this level to control for the effect of pH, and treatment effects were still observed. This suggests that nitrogen levels, rather than pH increase alone, may potentially enhance vegetative mycelial growth of *Colletotrichum* spp.

To test the hypothesis that treatment with urea will reduce the number of perithecia formed by *Colletotrichum* spp., *in vitro* and field experiments were performed in 2016 and 2017. In laboratory experiments, amendment of LBA with 2.5, 5, or 10% urea exhibited no effect on the number of perithecia formed. In field trials, the effect of urea treatment on the number of perithecia observed varied by location. At location 1, MHCRS, no significant difference was found among the treatments over the course of the experiment. However, at LWRS, treatment with 5% urea (current field rate) significantly increased the number of perithecia observed over the course of the experiment compared to the control treatment. The results at LRWS are not consistent with results from a similar study of *V. inaequalis* where application of 5% urea to apple leaves reduced the number of pseudothecia and ascospores formed (5, 10, 17, 18).

The hypothesis that treatment with urea will delay perithecial maturation was also tested in laboratory and field experiments in 2016 and 2017. *In vitro* analysis indicated that urea significantly delayed perithecial maturation of 12 isolates of Cg *sensu lato* selected to represent species recovered from NC orchards. At 36 and 39 dpi, when mature perithecia were first observed, LBA amended with either 5 or 10% urea reduced perithecial development, although no significant difference was found among treatments at the final

observation date of 42 d. Mature ascospores sampled from all treatments were capable of germinating on non-amended LBA plates (data not shown).

Treatment with urea delayed and enhanced perithecial maturation in the field experiments at various time points throughout the experiment. At MHCRS, by 8 Apr, ascospore initials were observed. Apple bloom generally occurs in mid-April in North Carolina (NC), and the dates selected for further analysis in this study were selected based on this timeframe. On 8 April and 29 Apr, treatment with urea significantly delayed ascospore development. Treatment with 10% urea significantly delayed perithecial maturation, but 2.5 and 5% urea were not different from the control. By 20 May, when disease symptoms were first observed on leaves in western NC orchards, including the experimental field site, treatment with 10% urea significantly delayed maturation, while 2.5% urea increased perithecial development. By 10 Jun, no differences were observed among treatments assessed in this experiment. These results indicate that fall application of urea may not be as effective as presumed at ameliorating GLFS severity.

At LWRS, on 15 Apr, results identical to those found at MHCRS. Urea applied to diseased apple leaves at 10% was the most effective treatment in for delaying perithecial development, as treatment with 2.5 and 5% were not significantly different from the control treatment. After this sampling date, no treatments significantly delayed perithecial maturation, but on 27 May, 2.5% urea increased perithecial development. More variation in perithecial development was observed at this location, though treatment with urea significantly reduced perithecial development over the course of the study. This may be due to differences in environmental factors between sites (Figure 3.8). During this experiment,

differences were observed at various collection dates in air temperature, relative humidity, and total precipitation between sites. Soil temperature was not found to differ throughout the experiment. Previous research indicates that temperature and relative humidity impacts Cg appressoria formation and growth *in vitro*, with high RH and 12-32°C (19, 22). In addition, previous field experiments indicate that the greatest number of Cg ascospores are trapped during periods of high relative humidity or immediately after rain (19).

Fall application of all three rates of urea delayed perithecial production compared to the control group, but it did not halt development or reduce the viability of ascospores. This could be explained by the natural process by which urea breaks down, particularly when in contact with soil. Urea has been found to control various fungal phytopathogens, including *V. inaequalis* in a two-fold manner: by enhancing the rate of leaf litter degradation and by enhancing the activity of saprobic microbes and earthworms (2, 15, 16, 18). As organic matter breaks down through the activity of saprobic microbes, ammonia is released and quickly converted to ammonium, which increases pH. Further conversion by bacteria leads to the production of nitrite (NO_2^-) and nitrous acid (HNO_2), which is highly toxic to several fungal species (9). Eventually, much of the nitrogen is leached into the soil and carbon is released in the form of CO_2. Our results suggest that the level of ammonia or other toxic forms of nitrogen generated through the application of urea to leaf litter is not substantial enough or remain in the environment long enough to inhibit Cg development.

Our results support previous findings that the introduction of a readily available source of nitrogen and the enhanced pH of the ambient environment caused by this provide an ideal environment for infection and growth *in vitro* and in host tissues by *Colletotrichum*

spp. (1, 12, 13). It is unclear from this study why the same external chemical stimulant delays the process of sexual reproduction. The variation in response of perithecial development to treatment with urea over the course of the experiment could be due to the fact that there are likely several species within the Cg species complex which are active in GLFS epidemics in NC. In a separate experiment, we identified five species from infected leaves and fruit in NC orchards: *C. alienum, C. fructicola,* Cg, *C. siamense,* and *C. tropicale.* Previous research has shown that closely related *Colletotrichum* spp. vary in their response to chemical-based control measures (fungicides), and therefore we develop our hypotheses that response to urea will also vary based on these observations (14, 22). Perithecia were not observed in culture until after a maximum colony diameter was obtained (data not shown), and no significant difference was observed among treatments at the final collection date at either site in our field study. Future research should examine which pathways are activated during growth and reproduction phases in the presence of urea and determine the effect of timing on urea application post-harvest. Studying the timing of nitrogen breakdown in this pathosystem could better inform this application timing. The effect of a second urea application in the spring before greentip and combined effect of urea treatment and leaf shredding on the abundance of ascospores in future growing seasons would also provide beneficial information for controlling GLFS.

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Table 3.1: Isolates selected for use in *in vitro* experiments on the effect of urea on mycelial growth and reproduction of members of the Cg species complex. Date format is day/moth/year. Cultivar is as follows: Gala=GA, Golden Delicious=GD, and Pink Lady=PL.

Isolate	Collection Date	Cultivar	Host Tissue	County	Orchard	Morphotype	Species ID
F41	9/10/14	PL	Fruit	Henderson	9	2	
L24	9/10/14	GD	Leaf	Henderson	8	4	*C. fructicola*
GD2L	3/7/15	GD	Leaf	Polk	5	2	*C. tropicale*
GD12F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
L31	9/10/14	PL	Leaf	Henderson	9	2	*C. tropicale*
F43	9/10/14	PL	Fruit	Henderson	9	2	
GD10F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
L30	3/7/15	GA	Leaf	Polk	11	2	
F36	9/10/14	PL	Fruit	Henderson	9	2	
L35	9/10/14	GD	Leaf	Henderson	9	2	
GD2F	23/7/15	GD	Fruit	Polk	5	2	*C. tropicale*
L33	3/7/15	GA	Leaf	Henderson	9	2	

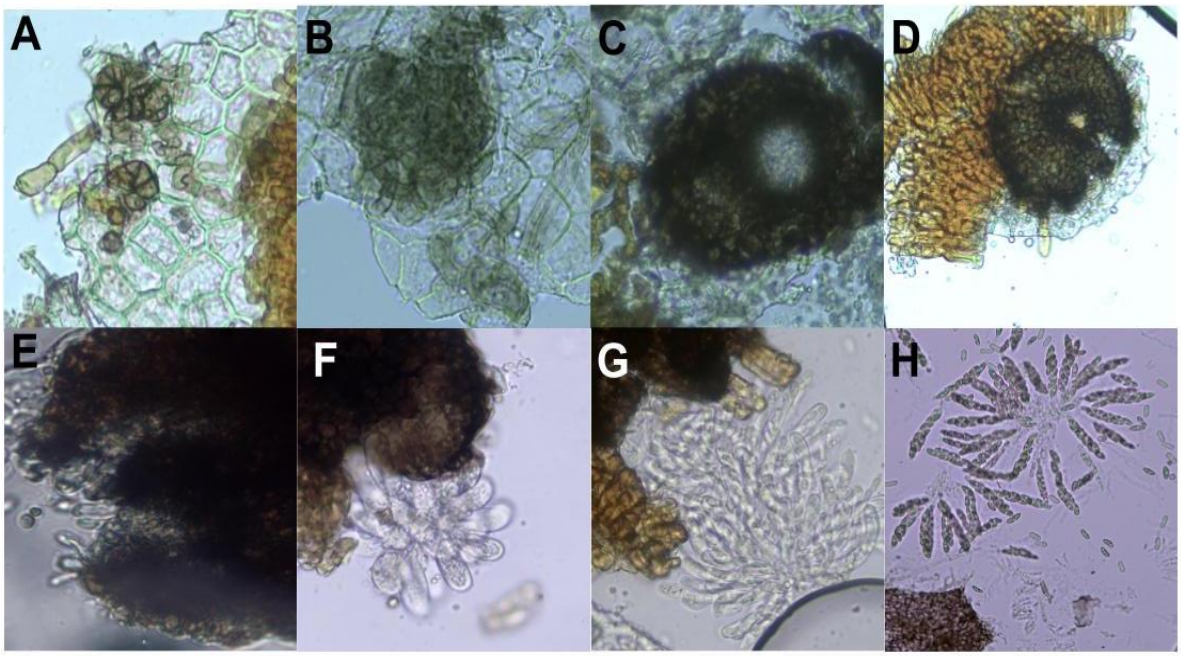

Figure 3.1: Rating scale for perithecial development used in this study. **A)** Stage 1, melanized hyphae beginning to coil, **B)** Stage 2, coiled, melanized hyphae lacking the defined flask shape of a perithecium, **C)** Stage 3, perithecium exterior is thick and an ostiole forming, **D)** Stage 4, perithecial exterior and ostiole are formed, **E)** Stage 5, paraphyses visible, **F)** Stage 6, paraphyses and asci initials present, **G)** Stage 7, ascospore initials present within asci, and **H)** Stage 8, mature ascospores present.

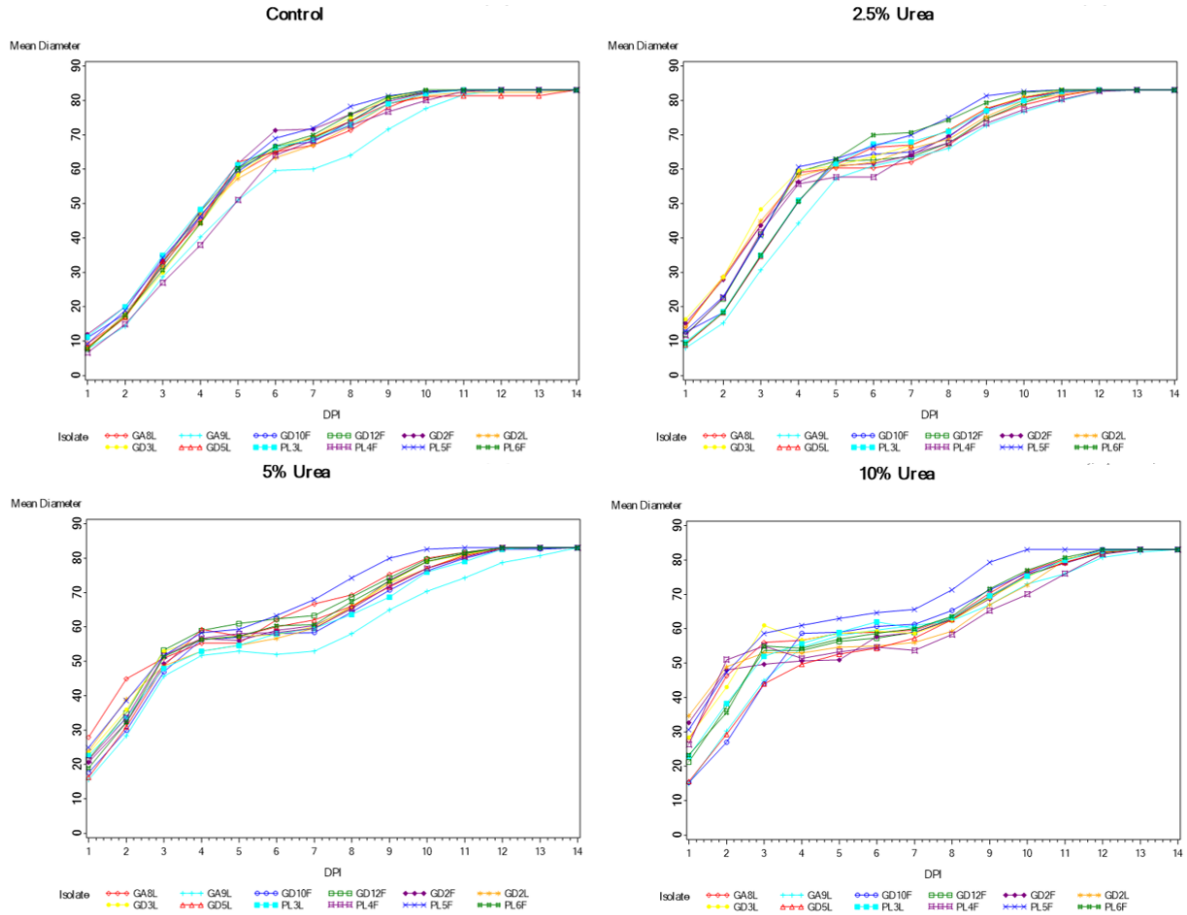

Figure 3.2: Mean mycelial growth (colony diameter) of 12 representative isolates of *Colletotrichum gloeosporioides sensu lato* on lima bean agar amended with 2.5, 5 or 10% urea (w/v) and non-amended control.

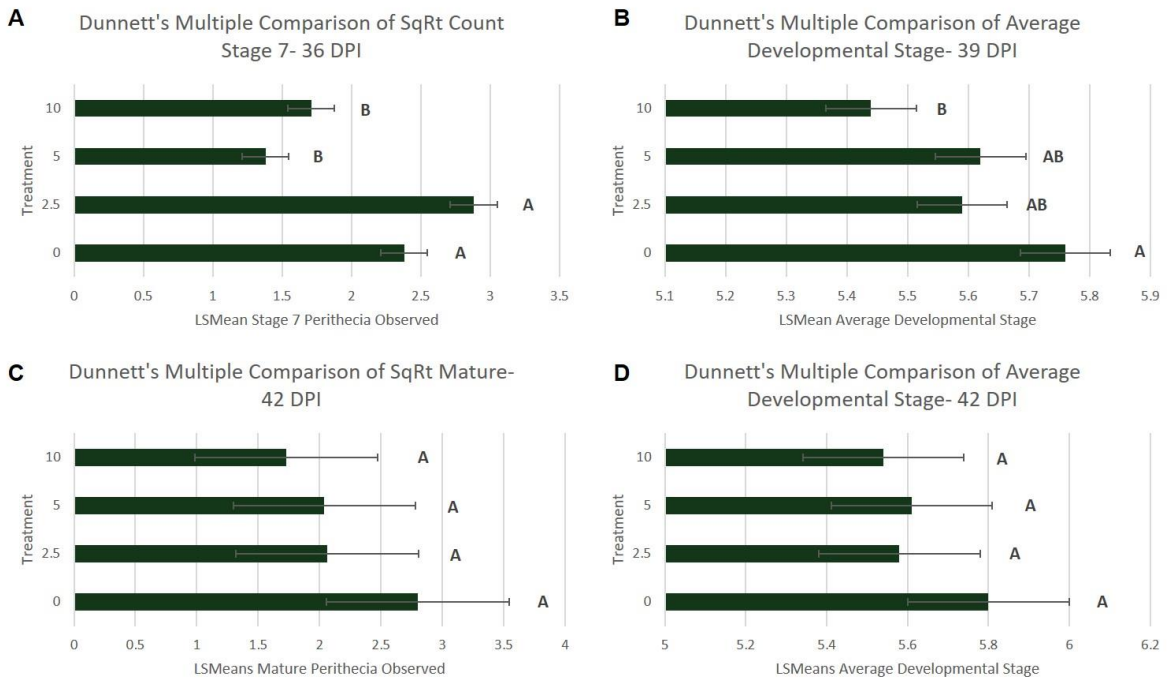

Figure 3.3. Analysis of the effect of urea on perithecial development *in vitro*. Treatments are 0, 2.5, 5, and 10% urea and a non-amended urea control. **A)** Multiple comparison of the square root transformation of the number of mature perithecia (Stage 8) at 36 dpi **B)** Multiple comparison of the mean developmental stage 39 dpi **C)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia at 42 dpi **D)** Multiple comparison of the mean developmental stage 42 dpi.

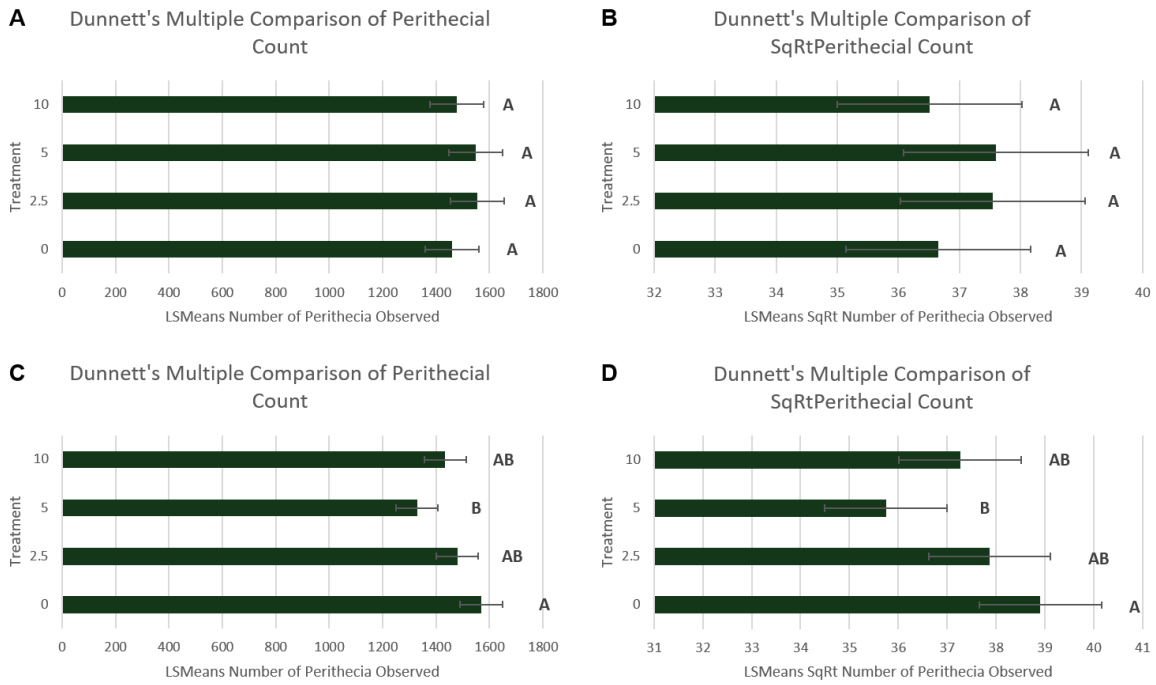

Figure 3.4: Analysis of the effect of urea on the number of perithecia formed *in vivo.* **A-B:** Multiple comparison of the number of perithecia formed and the square root transformation of that data at MHCRS. **C-D:** Multiple comparison of the number of perithecia formed and the square root transformation of that data at LWRS.

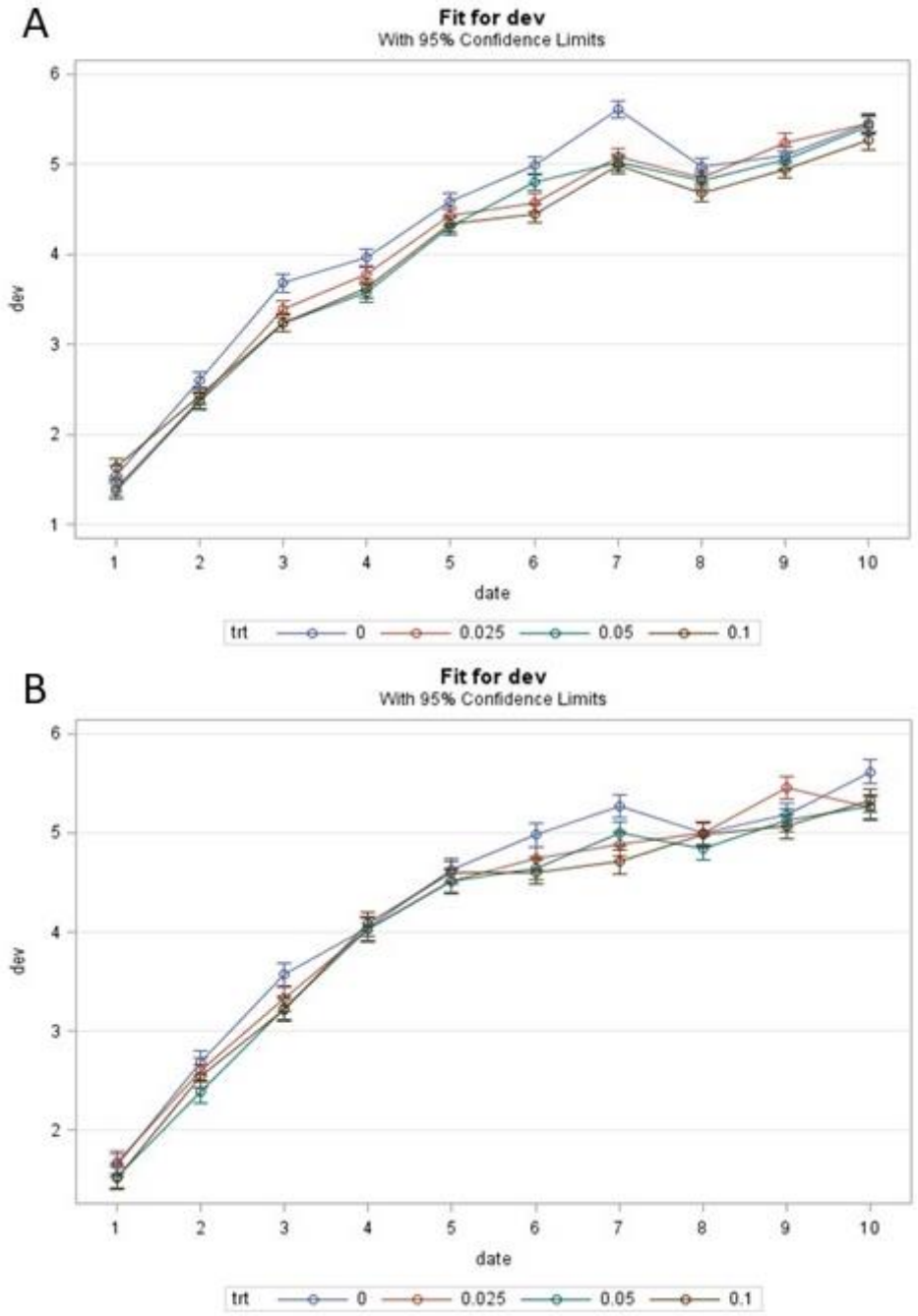

Figure 3.5: Perithecial development over time. Treatments have been converted from % urea to decimal format. **A)** Mean perithecial developmental stages (dev) observed at MHCRS from 3 Dec through 1 July. **B)** Mean perithecial developmental stages observed at LWRS from 10 Dec through 8 July.

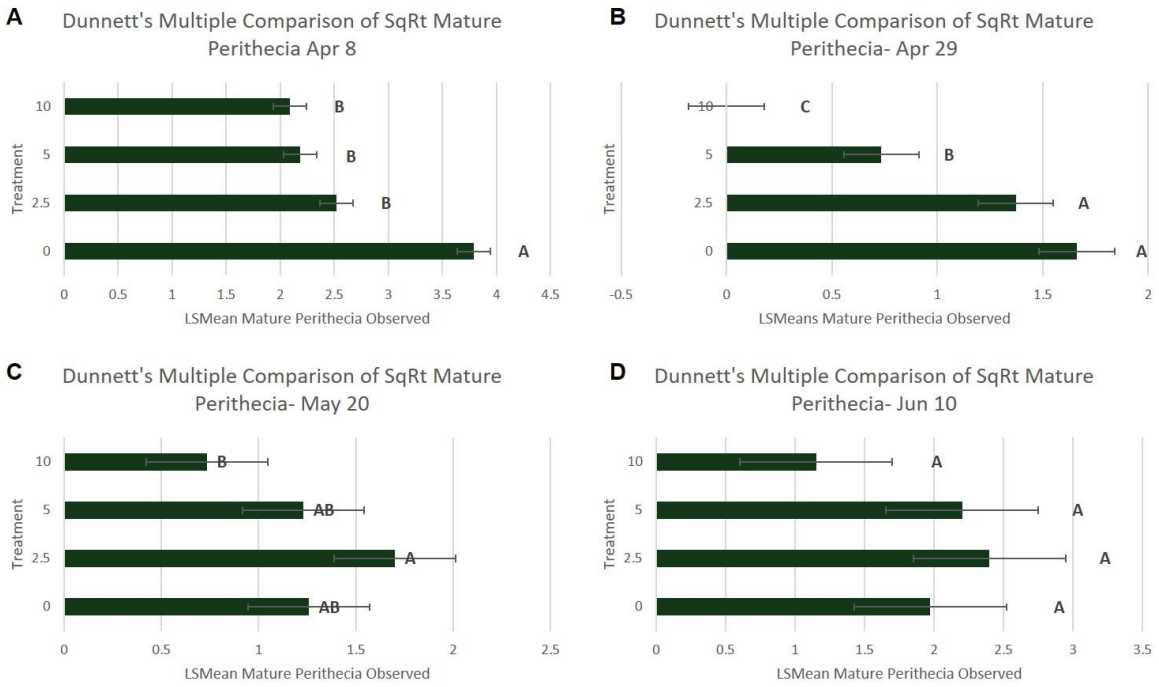

Figure 3.6: Analysis of the effect of urea on perithecial development *in vivo* at MHCRS. Treatments are 0, 2.5, 5, and 10% urea. **A)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 8 April **B)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 29 April **C)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 20 May **D)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 10 Jun.

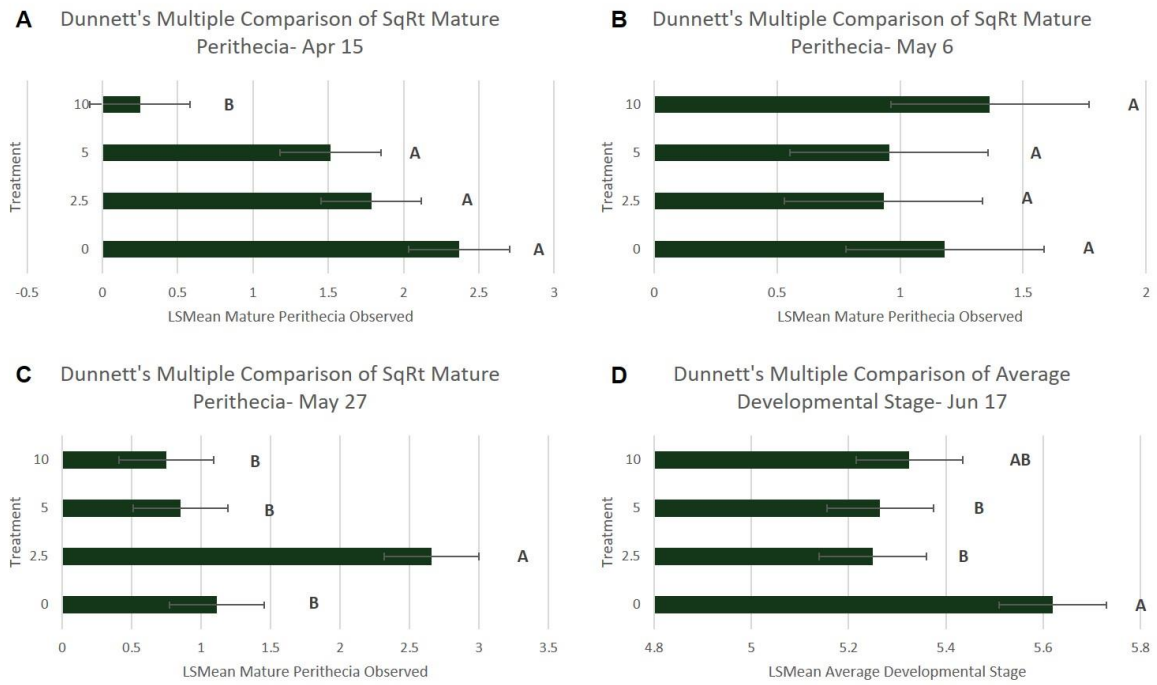

Figure 3.7: Analysis of the effect of urea on perithecial development *in vitro* at LWRS. Treatments are 0, 2.5, 5, and 10% urea. **A)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 15 April **B)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 6 May **C)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 27 May **D)** Multiple comparison of the square root transformation of the number of mature perithecia to immature perithecia on 17 June.

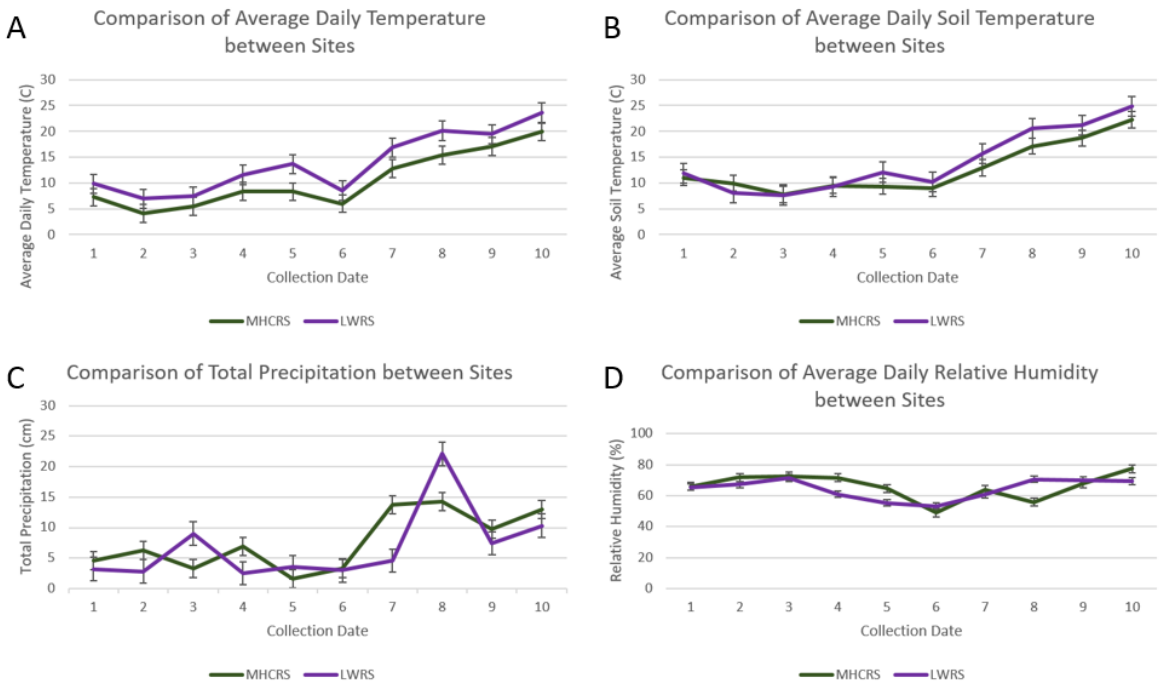

Figure 3.8: Comparison of environmental factors between experimental sites (MHCRS and LWRS). **A)** Mean daily air temperature (C), **B)** Mean daily soil temperature (C), **C)** Total precipitation (cm), **D)** Mean daily percent relative humidity. Collection dates were 1) 3 Dec 2016, 2) 24 Dec 2016, 3) 14 Jan 2017, 4) 4 Feb 2017, 5) 25 Feb 2017, 6) 18 Mar 2017, 7) 8 Apr 2017, 8) 29 Apr 2017, 9) 20 May 2017, and 10) 10 Jun 2017 at MHCRS and 1) 10 Dec 2016, 2) 31 Dec 2016, 3) 21 Jan 2017, 4) 11 Feb 2017, 5) 4 Mar 2017, 6) 25 Mar 2017, 7) 15 Apr 2017, 8) 6 May 2017, 9) 27 May 2017, and 10) 17 Jun 2017 at LWRS