

ABSTRACT

JACOBS, RAYMOND LEO, III. Inheritance of Rate-Limiting Foliar Resistance to Anthracnose Crown Rot and Fruit Rot in Cultivated Strawberry. (Under the direction of Dr. Jeremy A. Pattison and Dr. G. Craig Yencho.)

Anthracnose crown rot and fruit rot caused by *Colletotrichum gloeosporioides* and *C. acutatum* are two of the most prominent and destructive diseases of cultivated strawberry (*Fragaria x ananassa* Duchesne). Both species are capable of establishing hemibiotrophic infections (HBI) in leaf tissue, encouraging undetected dissemination of diseased nursery plants to fruit production fields and inciting subsequent crown- and fruit-rotting epidemics. The purpose of this research was to explore a novel mechanism in strawberry foliage, which may confer rate-limiting resistance to multiple *Colletotrichum* species in that tissue. Germplasm from the NC State strawberry breeding program were screened for resistance to *C. gloeosporioides* HBI. Preliminary variation in resistance and significant differences among genotypes were observed. Methods were developed to increase the accuracy and precision of percent sporulating leaf area (PSLA) measurements, including the use of imaging software for quantification of PSLA. Direct visual estimates were strongly correlated ($r=0.91$) to image-based quantification of PSLA, but the accuracy of visual estimation varied with different patterns of sporulation and tended to underestimate higher infection severities. A larger panel of 18 cultivars and NC State selections were screened for resistance to hemibiotrophic foliar infections of both *C. gloeosporioides* and *C. acutatum*. Genotype PSLA means were significantly different and ranged from 8.5% to 26.5%, though means separation was poor for moderate genotypes. These 18 genotypes were also evaluated for resistance to anthracnose crown rot (ACR) by applying *C. gloeosporioides* inoculum directly to the crown and observing wilt symptoms over ten weeks. ACR means separated

distinctly over a wide range of resistance phenotypes. Findings of moderate correlation of resistance to *C. gloeosporioides* and *C. acutatum* HBI in leaf tissue suggested resistance to these *Colletotrichum* species may be shared in this common tissue type. However, weak correlation of resistance to *C. gloeosporioides* HBI and ACR in leaf and crown tissue, respectively, suggested that resistance to a single *Colletotrichum* species may operate independently between tissue types. A population was constructed to examine the inheritance of rate-limiting resistance to *C. gloeosporioides* and *C. acutatum* HBI in strawberry leaf tissue as well as the inheritance of resistance to ACR in crown tissue. Low dominance to additive variance ratios for *C. acutatum* HBI and *C. gloeosporioides* ACR indicate strong additive genetic control of resistance to these traits. *C. gloeosporioides* HBI had a much higher dominance to additive variance ratio which suggested this trait was under nearly equal dominance and additive genetic control. Heritability estimates were low for *C. acutatum* HBI (0.25) and *C. gloeosporioides* HBI (0.16) but were moderate for *C. gloeosporioides* ACR (0.61). Resistance gains from selection were predicted to be high for ACR, moderate for *C. acutatum* HBI, and low for *C. gloeosporioides* HBI. A strong genetic correlation ($r_A = 0.98$) between resistance to *C. acutatum* HBI and *C. gloeosporioides* HBI suggested that resistance to these two *Colletotrichum* species was controlled by the expression of common genes in strawberry leaf tissue. Selecting for increased resistance to HBI for one *Colletotrichum* species should produce gains in resistance to the other. Negative genetic correlations between ACR and both HBI traits ($r_A = -0.85$ and -0.61) suggested that resistance in crown tissue is inherited independently of resistance in leaf tissue in the examined population. These results recommend independent evaluation and advancement of resistance to HBI and ACR within breeding programs.

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Inheritance of Rate-Limiting Foliar Resistance to Anthracnose Crown Rot and Fruit Rot in
Cultivated Strawberry

by
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DEDICATION

To my closest friend, who braved all of the emotions that graduate school brings. You stood by my side every step of the way and for that I will always be grateful.

To my sister, for her unconditional love and support. You never failed to keep my outlook positive and spirits high.

To my grandparents, for always letting me know how proud they were of me.

Finally, to my parents, who taught me to be humble and kind. You urged me to seize every opportunity, encouraged excellence in my work, and provided the freedom to forge my own path in the world.

BIOGRAPHY

Raymond Leo Jacobs III was born in Green Pond, South Carolina on December 24, 1986 to Ray and Paula Jacobs. Just 13 months later his sister and lifelong friend Rebecca Jacobs was born. He spent his childhood roaming the expansive forests and wetlands of White Hall Plantation where his family lived and his father served as property manager. Raymond was curious, mischievous, and had a talent for getting into trouble.

During his childhood, Raymond's favorite time of year was spring. His morning ritual included an inspection of his parents' garden to see what progress had been made from the day before. He learned the magic of pollination at an early age and could be seen pulling cotton swabs out of his pockets on spring and summer mornings, competing with the bees to set a good crop.

Raymond's first foray into the world of plant breeding occurred in his mother's daylily garden where there were an amazing diversity of daylily shapes, sizes, and colorations. He discovered that he could make controlled crosses among these plants, collect the seed, and within a year could grow the plants to flowering size and view his new creations. Many beautiful and hideous daylilies resulted from these crosses. Regardless, he was fascinated and a career was born. His mother still grows the daylilies from these first crosses – even the ugly ones.

Raymond whizzed through Colleton County High School where he was honored to graduate valedictorian of his class and to accept an offer to study at Cornell University. Going from Zone 8b in South Carolina to 5b in upstate New York wasn't the only shock of heading to college at Cornell. Raymond (now Ray) was met and welcomed by a diversity of cultures and ideas completely new to him. Ray majored in Plant Biology with a focus in

Plant Breeding and Genetics and minored in Business. He taught himself how to study at a college level and excelled at his coursework. Some of Ray's most influential experiences, however, were outside of the classroom. He was an active member and president of Hortus Forum, Cornell's undergraduate horticulture club. The club grew houseplants on campus that were sold to students and faculty at weekly plant sales to fund educational trips. During his four years at Cornell Ray traveled with the club to six countries to explore agricultural operations around the globe. He also spent a semester abroad studying at the University of Melbourne in Victoria, Australia – a place he fell in love with and hopes to return to someday. Ray graduated with a B.S. under the guidance of Dr. Mark Sorrells in May 2009.

Ray then ventured west to Woodland, CA to spend a year working at Seminis Vegetable Seeds and gaining experience in private industry. He picked up many practical skills during his time there and thoroughly enjoyed the experience. He knew he wanted to pursue a career in plant breeding but would first need a graduate-level education to master the subject. Ray enrolled in the Department of Horticultural Science at North Carolina State University in August 2010.

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I would like to thank a number of people for helping me during my time in graduate school: Dr. Jeremy Pattison for taking me on as a student, working closely with me to develop this project, teaching me to keep an open mind, and showing me how to “see the trees through the forest”; Elizabeth Clevinger for all of the time and hard work she contributed to seeing this project a success; Megan Bame for her dedicated care of my plants and for going beyond the call of duty to help me when I needed it most; Dr. Craig Yencho for his excellent breeding advice and willingness to step into the role of committee chair; Dr. Frank Louws and Dr. Tika Adhikari for their help in developing this project and understanding the complex nature of these pathogens; Dr. Dennis Werner for showing me how much fun teaching can be as well as his encouragement and willingness to join my committee; Dr. Jason Osborne for his continued statistical advice during this project; the crew at the Piedmont Research Station in Salisbury, NC for their hard work and dedication to the strawberry breeding program; and finally to all of the faculty and staff at the Plants for Human Health Institute at the NC Research Campus in Kannapolis, NC for being a pleasure to work with and always coming to my aid. I fully appreciate the work that each and every one of you have provided over the years and would not have pulled this off without you.

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Chapter One:

GENERAL INTRODUCTION

Strawberry

Taxonomy and Origin

Strawberries are members of the family Rosaceae, subfamily Rosoideae, and genus *Fragaria*. In addition to strawberries, subfamily Rosoideae includes familiar species such as roses, the caneberries blackberry and raspberry, and many ornamental species, making it one of the most economically important groups of horticultural crops (Hummer and Janick, 2009).

Fragaria species display an impressive range of natural ploidies, from diploid to decaploid and many in between. Polyploidy in *Fragaria* likely arose through the unification of unreduced $2n$ gametes, a relatively common occurrence observed in *Fragaria* species (Hancock, 1999; Janick and Moore, 1996). The pattern and distribution of ploidy levels within continents reflects the history and evolution of endemic *Fragaria* species. Analyses of such patterns have been some of the strongest tools for differentiation of species within the genus. Günter Staudt (1999) utilized the evolutionary fingerprint of polyploidization to suggest East Asia as a center of origin for diploid and tetraploid species. The evolution of *Fragaria* species has been debated for many decades but current consensus of strawberry

phylogeny remains largely based on these systematic studies of Günter Staudt. The development and use of molecular tools has greatly aided in the resolution of strawberry phylogeny but has not yet clarified all uncertainties. Rousseau-Gueutin *et al.* (2008) used *GBSSI-2* and *DHAR* nuclear gene sequences to investigate the phylogenetic origins of polyploid *Fragaria* species. They found evidence of multiple polyploidization events within *Fragaria* as well as evidence suggesting that hexaploid and octoploid species are of allopolyploid origin. Evidence of the origin of tetraploid species is less conclusive but several reports point to morphological similarities of diploid species and their geographic proximity in suggesting an autopolyploid origin (Hummer and Hancock, 2009).

Phylogenetic analyses of nuclear genes (Rousseau-Gueutin *et al.*, 2008) and chloroplast genomes (Njuguna *et al.*, 2013) consistently resolve two clades within *Fragaria*. The “China clade” is comprised of nine diploid and tetraploid species distributed across China, Nepal, and Japan. The “vesca clade” is comprised of eleven species ranging from diploid to decaploid which are distributed from northern Eurasia to Hawaii and North and South America. Attempts to resolve the phylogenetic positions of three diploid species, Eurasian *F. viridis*, southeast Asian *F. nilgerrensis*, and Japanese *F. iinumae*, have so far been unsuccessful (Liston *et al.*, 2014).

Based on the most recent summary of strawberry evolution and speciation by Liston *et al.* (2014) there are currently understood to be 1 cultivated (*F. x ananassa*) and 24 wild species in the genus *Fragaria*, including 12 diploids, 5 tetraploids, 1 hexaploid, 4 octoploids, and 2 decaploids. Other naturally occurring ploidies have been observed but are infertile (Hummer and Hancock, 2009). Diploids ($2n=2x=14$) include *F. bucharica* Losinsk., *F. chinensis* Losinsk., *F. daltoniana*, J. Gay, *F. hayatai* Makino, *F. iinumae* Makino, *F.*

mandshurica Staudt, *F. nilgerrensis* Schltdl. Ex J. Gay, *F. nipponica* Makino, *F. nubicola* Lindl., *F. pentaphylla* Losinsk., *F. vesca* Duchesne, and *F. viridis* Duchesne. *F. hayatai*, a species native to Taiwan, was recently recognized as distinct from *F. nilgerrensis* based on microscopic observations of pollen morphology (Liston et al., 2014). *F. vesca*, also known as the woodland strawberry, is the most widespread of all native strawberry species and is found throughout the Northern Hemisphere. The small stature, short generation time, ease of propagation, and small genome size (~240 Mb) of *F. vesca* made it an ideal candidate for genome sequencing. An international consortium of seventy-five researchers from thirty-eight institutions completed the project in 2010 (Liston et al., 2014). Tetraploids ($2n=4x=28$) include *F. corymbosa* Losinsk., *F. orientalis* Losinsk., *F. gracilis* Losinsk., *F. moupinensis* (Franch.) Cardot, and *F. tibetica* Staudt & Dickore. The single hexaploid ($2n=6x=42$) species, *F. moschata* Duchesne, is endemic to western Eurasia where it was domesticated in the early 1600's. Octoploid ($2n=8x=56$) species include *F. chiloensis* (L.) Duchesne, *F. iturupensis* Staudt, *F. virginiana* Duchesne, and *F. x ananassa* Duchesne. *F. x ananassa* is the commercially grown dessert strawberry but this hybrid also occurs naturally where the native range of its parents *F. chiloensis* and *F. virginiana* overlap. Two identified decaploid ($2n=10x=70$) species are *F. cascadenis* Hummer and *F. iturupensis*, mentioned previously. *F. cascadenis* was recently described from populations in the Cascade Range of western Oregon where it was previously confused with the morphologically similar *F. virginiana* (Hummer et al., 2009). *F. iturupensis* occurs as both an octoploid and decaploid on Iturup, one of the Kurile Islands northeast of Japan. The decaploid form is limited to just a few colonies growing on the rocky outcroppings of the eastern flank of Volcano Atsunupuri on the island of Iturup. It is thought that the geography of Iturup may have sheltered these

species and provided refuge from glaciation during the most recent glacial maximum (Hummer et al., 2009; Hummer and Hancock, 2009).

The origin of the North American octoploid strawberries is obscured by the fact that diploid *F. vesca* and decaploid *F. cascadiensis* are the only other *Fragaria* species found in North America (Hancock et al., 2004). The vast majority of diploids are distributed throughout Eurasia and the only other octoploid and decaploid species are strictly confined to the Iturup Islands northeast of Japan (Hancock et al., 2004; Hummer et al., 2009). Njuguna *et al.* (2013) estimate the origin of the octoploid clade to be between 0.370 and 2.050 million years ago. This timing is consistent with the only fossil evidence of *Fragaria*, a single achene collected on Prince Patrick Island, Canada dating to 2.9 ± 0.4 million years ago (Matthews et al., 2003; Topel et al., 2012). This island was once part of Beringia, a region surrounding the Bering Strait which included the Bering land bridge during periods of glacial maxima. This fossil evidence and the current distribution of known ancestors suggests the octoploid clade may have originated in Beringia during the eastward spread of *Fragaria* species from Eurasia to North America.

After its arrival in the American Northwest, the octoploid strawberry likely evolved differential adaptations and diverged into the two ecologically distinct octoploid species found in America today (Hancock et al., 2004; Staudt, 1999). *Fragaria chiloensis*, also known as the beach strawberry, is divided into four subspecies which adapted to the Pacific coastal climates of western American shores from Alaska south to California (subsp. *pacifica* and subsp. *lucida*), coastal Chile inland to the Andes Mountains (subsp. *chiloensis*), and the mountains of Hawaii (subsp. *sandwicensis*) (Hummer et al., 2011). *F. chiloensis* is typified by thick, leathery leaves that are glossy and dark green in color. Vigorous, low-spreading

plants produce large flowers that yield large fruit, dull red in appearance with firm white flesh and pungent aroma. Plants of *F. chiloensis* are generally evergreen and dioecious (Hancock, 1999; Liston et al., 2014). *Fragaria virginiana*, also known as the “scarlet” strawberry, is divided into four subspecies which adapted to the mountainous continental climates of eastern North America. *F. virginiana* subspecies can be found throughout eastern North America spreading northwest to British Columbia (subsp. *virginiana* and subsp. *glauca*), northwestern Texas to Nebraska, Iowa, and Illinois (subsp. *grayana*), and British Columbia south to northern California (subsp. *platypetala*) (Hummer et al., 2011). *F. virginiana* is typified by thin leaves that are dull bluish to light green in color. Tall, slender plants produce small flowers that yield small fruit, scarlet to crimson in color with soft red flesh. Plants of *F. virginiana* are generally deciduous and subdioecious (males, females, and hermaphrodites) (Hancock, 1999; Liston et al., 2014).

History of Domestication

The cultivated strawberry, *Fragaria x ananassa*, is one of the most recently domesticated plants. The first reference to strawberry cultivation in Europe appears in French literature from the 1300s and describes the planting of strawberries in the royal gardens of the Louvre as well as in gardens of the Dukes of Burgundy. These plants likely originated from wild strawberry populations that were transplanted into these and other gardens (Darrow, 1966; Hummer et al., 2011). Strawberries grew in popularity through the Middle Ages in spite of the abbess St. Hildegard von Bingen who claimed strawberries brought mucus to those who eat it, denouncing strawberries as dangerous and unfit for consumption as its fruit were borne near the ground in putrid air (Bühler, 1960). By the

1500s, *F. vesca* was being grown throughout Europe and strawberry consumers were becoming more discerning. By the mid-1500s, distinctions were being made between wild and garden strawberries while botanists were identifying albino and everbearing forms of *F. vesca* and collecting them for cultivation (Hancock, 1999). The musk strawberry, *F. moschata* was domesticated during the 1600s and grown alongside *F. vesca* for its distinct flavor. By the mid-1600s, production of native European strawberries had reached their peak and many of the agronomic practices used in strawberry production today were being developed and refined. Europeans had become expert strawberry growers and the stage was set for the arrival of novel species from the New World (Hancock, 1999).

No domestication efforts are known to have been made by the native people of North America. They enjoyed the fruit both fresh and made into bread (Sauer, 1993; Wilhelm and Sagen, 1974), but the natural abundance of native strawberries provided no incentives for its cultivation (Hancock, 1999). Based on the timing of his voyage and entries from his diary it is believed that Jacques Cartier, who discovered the St. Lawrence River in 1523, was most likely the first to bring *F. virginiana* to the Old World (Hancock, 1999; Wilhelm and Sagen, 1974). *F. virginiana* was likely introduced to Europe multiple times during the late 1500s and 1600s, a period of rapid exploration in the New World. However, very little cultivation of *F. virginiana* occurred in Europe until the late 1700s (Hancock, 1999).

Unlike their neighbors to the north, strawberries were highly utilized by the indigenous people of South America for well over 1,000 years. The Mapuches and Picunches tribes of central Chile used strawberry fruits fresh, dried, as fermented juice, or as medicinal infusions. Strawberry cultivation was mostly limited to garden plots, but the Mapuches also planted small plots of strawberries in open spaces of the forest and used them

as traps during the Spanish conquest. The Mapuches waited for Spanish soldiers to lay down their arms to pick the fruit before attacking and killing them (de Nájera, 1866). A clone of *F. chiloensis* found its way to Europe via the return voyage of a French spy, Captain Amédée Frézier (Darrow, 1966; Wilhelm and Sagen, 1974), who was amazed by the size of the fruit he observed near Concepción, Chile during his inspections of Spanish fortifications in the area. He collected some of the largest fruiting types and returned to Marseilles in 1716. Five of these plants survived the voyage and were propagated for distribution to European growers. Unfortunately, early plantings of *F. chiloensis* in European gardens were barren and unproductive, generating highly negative criticism of the new introduction (Hancock, 1999). Several years passed before a young French botanist by the name of Antoine Nicholas Duchesne determined that Captain Frézier had collected only female plants of the dioecious species and discovered that *F. chiloensis* produced large, beautiful strawberries when pollinated by *F. moschata* or *F. virginiana* (Darrow, 1966; Hummer et al., 2011).

Now interplanted with other species, *F. chiloensis* grew in popularity across Europe, reaching its highest acclaim in Brittany, France in the mid-1800s. *F. chiloensis* thrived in the cool maritime climate of Brittany and at this time it is estimated that cultivation in France exceeded that of its native Chile, with over 2,500 acres in production in Brittany alone (Hummer et al., 2011; Wilhelm and Sagen, 1974). During the mid-1700s unusual seedlings with new combinations of fruit and morphological characteristics began to appear in the gardens of Brittany. Duchesne (1766) correctly determined these seedlings to be hybrids of *F. chiloensis* x *F. virginiana*. He named them *Fragaria* x *ananassa*, attributing the perfume of the fruit to the scent of pineapple (*Ananas*). The first selections of *F. x ananassa* were likely made in the commercial fields of Brittany and in botanical gardens across Europe. The

modern strawberry *Fragaria x ananassa* was born (Hummer et al., 2011).

Anatomy

The strawberry is a herbaceous perennial plant with a modified central stem known as a crown from which leaves, roots, stolons (runners), and inflorescences emerge (Hancock, 1999). The crown is composed primarily of pith, surrounded by a thin cambial layer and a vascular ring. Roots emerge from the base of the crown where it meets the soil. Leaves, arranged in a spiral around the crown, are generally pinnate and trifoliate. Leaves are joined to the crown by petioles and above each of these junctions on the crown is an axillary bud. Axillary buds can remain dormant or produce runners or branch crowns (Hancock, 1999). A terminal meristem located at the top of the crown can develop leaves or become an inflorescence. If an inflorescence is produced, continuing vegetative growth will displace the original terminal axis to one side (Galletta et al., 1990). The strawberry inflorescence is a modified stem and typically has one primary, two secondary, four tertiary, and eight quaternary flowers. Flowers have ten sepals, five petals, 20-30 stamens, and 60-600 pistils (Hancock, 1999). Strawberries produce an 'aggregate' fruit composed of numerous ovaries, each with a single ovule. The seed produced by each are called 'achenes' and are the true fruit of the strawberry (Hancock, 1999).

Production

The dessert strawberry, *F. x ananassa*, now dominates strawberry production in nearly all arable regions of the world. Strawberries are a regular part of the diets of millions of people and are appreciated for their delicate flavor, distinctive aroma, and rich nutritional

value (Hancock, 1999). Consumer demand for strawberries in the United States climbs every year and is influenced by Americans' access to fresh strawberries year-round. Annual consumption has experienced dramatic increases, from 1.6 pounds per capita in 1970 to 7.9 pounds in 2013 (ERS/USDA, 2014b). Increased consumption in recent years has been fueled by increased supply, lower retail prices, and the increasingly health-conscious consumer (ERS/USDA, 2014a; Pinto et al., 2010).

The strawberry industry as a whole is composed of two industries that work in tandem: the nursery industry that produces clonal plant propagules (bare root or plug plants) and the fruiting industry that produces harvestable fruit (Rahman et al., 2013). The nursery industry occupies only a small percentage of total strawberry acreage but plays a critical role in the success and profitability of the strawberry industry. The focus of the nursery industry is to provide true-to-type and pathogen-free clonal plant material to strawberry fruit producers and other nursery systems. Nursery operations are often located in cooler climates and at higher elevations than the fruiting industry, largely due to decreased disease pressure in these areas appealing to nurseries' critical need to maintain clean plant material. Strawberry nurseries generally operate on a matted row production system. Matted row production is an old method of growing strawberries and was once the preferred method of the fruiting industry. This system utilizes wide, flat beds where mother plants are generously spaced and allowed to produce runners (Hancock, 1999). Runners root into the open spaces of the row and establish daughter plants that will produce fruit and runners of their own. Flowers and fruit are generally stripped from the plant in the nursery industry to encourage runner production. Strawberry nurseries annually plant source stocks (mother plants) in fumigated beds, root the runners that form, and harvest these runner plants for shipment to

strawberry growers. The fruiting industry is made up of strawberry growers who occupy the vast majority of strawberry acreage. The focus of this industry is a familiar one – to grow plant material received from the nursery industry to a mature size capable of substantial strawberry production and to harvest this fruit for sale. The fruiting industry in California and the southeastern United States operates on an annual hill production system with raised beds, plastic (polyethylene) mulch, drip irrigation, and overhead irrigation for frost protection where necessary (Hancock, 1999).

Annual strawberry production has increased steadily during most of the 20th century with a slight increase in the rate of growth over the past few decades. The last 20 years have seen production more than double from 3.5 million tons in 1993 to over 8.5 million tons in 2013 (FAOSTAT, 2014). The vast majority of strawberry production (96.4%) occurs in the northern hemisphere, but production is beginning to expand into the southern hemisphere and lower latitudes. There are no barriers preventing this expansion from continuing (Hancock, 1999). Expanding production in these regions contribute to strawberry's increasingly year-round availability in many parts of the world.

The United States is the world's largest producer of strawberries, accounting for approximately 29% of global production in 2013, followed by Mexico (8.0%), Turkey (7.8%), Spain (6.6%), and Egypt (5.4%) (FAOSTAT, 2014). Planted acreage has increased steadily in recent decades, with 61,310 acres planted in the United States in 2014 (USDA-NASS, 2015a). Strawberry production in the United States totaled 1.5 million tons in 2014 carrying a farm-gate value of nearly \$2.9 billion USD (USDA-NASS, 2015a). California is the prevailing leader of strawberry production in the United States where plants thrive under moderate climates with warm days, cool nights, and low humidity. In 2014 California

strawberry growers planted 41,500 acres or nearly 68% of the national total. California planted acreage is followed by Florida with 11,000 acres (18%), Oregon with 2,000 acres (3%), Washington with 1,300 acres (2%), and North Carolina with 1,200 acres (2%) (USDA-NASS, 2015b). California's 12-month growing season contributes to higher strawberry yields per acre than any other region averaging 66,500 pounds per acre. California is followed by Florida at 19,000 pounds per acre, North Carolina at 14,000 pounds per acre, and Oregon at 9,100 pounds per acre (USDA-NASS, 2015b). Likewise, California leads total strawberry production with 1.4 million tons or approximately 91% of national production in 2014. Florida ranked a distant second in total production with 102,558 tons (7%) and is followed by Oregon with 7,750 tons (0.5%), and North Carolina with 7,700 tons (0.5%) (USDA-NASS, 2015b).

The strawberry industry in the southeastern U.S. has seen great growth over the last decade. This is especially true in North Carolina as tobacco quotas have been cut and farms traditionally dedicated to tobacco have chosen to diversify their crops, often including strawberry in their selections. With the exception of larger industry production in Florida, the southeastern fruiting industry is largely represented by family farms that utilize alternative marketing channels such as you-pick operations, roadside stands, and farmers markets to bring consumers to their product (Boriss et al., 2014). North Carolina ranked 4th nationally in 2014 for strawberry production, harvesting 7,700 tons worth approximately \$23.4 million USD (USDA-NASS, 2015b), making strawberries the second most valuable fruit crop in the state (NCDA&CS, 2015). The majority of North Carolina's strawberry acreage is planted in the annual hill or plasticulture production system. In this system freshly dug or plug plants are planted in the early fall in double rows on raised, fumigated beds

covered in black plastic mulch.

Plasticulture became feasible in North Carolina largely due to the release of two June-bearing strawberry varieties developed by the University of California: ‘Chandler’ and ‘Camarosa’. These varieties are widely adapted throughout North Carolina and produce excellent yield and fruit quality for six weeks or more in plasticulture production systems (Poling, 1993). However, many people have questioned the wisdom of North Carolina’s dependency on cultivars developed in a dry Mediterranean climate that is very different from the humid subtropical climate of the southeastern United States. In fact, the southeast has the ideal climate for proliferation of fungal diseases and crops grown in this region are under varieties and intensities of biotic stress rarely seen in strawberry-growing regions of California. As such, ‘Chandler’ and ‘Camarosa’ are highly susceptible to anthracnose crown rot (ACR) and anthracnose fruit rot (AFR), incited by *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*, respectively, and commonly suffer losses to these fungal pathogens (Rahman et al., 2013).

Colletotrichum

Anthracnose of Strawberry

Anthracnose, from the Greek roots “anthrak-“ (coal) and “-nosos” (disease), is defined as a disease that appears as black, sunken lesions on leaves, stems, or fruit and is caused by fungi that produce asexual spores in acervuli (Agrios, 1988). Members of the genus *Colletotrichum* occur as endophytes, epiphytes, saprobes, and even human pathogens

(Hyde et al., 2009a). *Colletotrichum* is one of the most economically important genera of fungi, causing anthracnose symptoms on a wide variety of plants, especially tropical and subtropical crops and fruit trees (Hyde et al., 2009b; Jeger and Bailey, 1992). *Colletotrichum* species are broad-range pathogens: a single host species may be infected by many *Colletotrichum* species and a single *Colletotrichum* species is capable of infecting numerous hosts (Freeman et al., 1998).

Anthracnose crown rot (ACR) and anthracnose fruit rot (AFR) of strawberry can be incited by any of three *Colletotrichum* species, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (telomorph: *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk), *C. fragariae* A. N. Brooks, and *C. acutatum* J. H. Simmons (Freeman and Katan, 1997; Howard et al., 1992; Smith, 2008). While all species are capable of producing similar symptoms on nearly every strawberry tissue, each species preferentially targets certain tissues for necrotrophic infection and is generally only of economic concern as it relates to that tissue (Smith, 2008). *C. acutatum* is primarily considered a fruit-rotting pathogen, causing dark sunken lesions on developing fruit that render them unmarketable. *C. fragariae* and *C. gloeosporioides* are two closely-related species that produce a reddish-brown (often marbled or banded) necrosis of crown tissue leading to the eventual wilt and collapse of infected plants (MacKenzie et al., 2006; Smith, 2008). *C. gloeosporioides* has emerged as the primary causal agent of ACR in the southeast (Howard et al., 1992; Lewers et al., 2007; Urena-Padilla et al., 2002) and many investigators now question the classification of *C. fragariae* as a distinct species due its close genetic relatedness to many isolates of *C. gloeosporioides* (Mills et al., 1992; Sreenivasaprasad et al., 1992). Many if not all isolates once believed to be *C. fragariae* have recently been reclassified as *C. gloeosporioides* in the updated species definition of Gunnell

and Gubler (1992).

Technically, the symptoms of ACR (wilt and plant collapse) are not true anthracnose symptoms (restricted dark lesions). The name ‘anthracnose crown rot’ was proposed in the 1980s by Barbara Smith (1987) to distinguish the symptoms produced by *C. fragariae* in strawberry crowns from AFR symptoms appearing on fruit. The name has become widely used to describe all *Colletotrichum* diseases affecting crown tissue, even when not produced by *C. fragariae* or describing true anthracnose symptoms (Urena-Padilla, 2001). In the interest of simplified discussion, this dissertation will continue using the name ‘anthracnose crown rot’ or ‘ACR’ to describe the symptoms of crown infection by *C. gloeosporioides*, but recognizes its misrepresentation of true anthracnose symptoms.

Species Identification

Classically, identification of *Colletotrichum* species has been based largely on host specificity and cultural characteristics as well as morphology of conidia, appressoria, and setae (Leandro, 2002; Smith, 2013). However, morphological traits are highly variable among isolates and often quite ambiguous. Generally, *C. gloeosporioides* isolates develop beige to olive to dark gray colonies and produce conidia which are rounded on both ends. *C. gloeosporioides* produces dark black setae in acervuli in culture and on lesion surfaces (Peres et al., 2005; Smith, 2013). *C. acutatum* isolates develop initially into white colonies which later become covered in pink to orange conidial masses. Conidia of *C. acutatum* are fusiform with one or both ends tapered and setae are sparse if produced at all (Peres et al., 2005; Smith, 2013). Other characters, such as colony growth rate on artificial media and sensitivity to the fungicide benomyl have been useful in differentiating *C. gloeosporioides* and *C.*

acutatum (Peres et al., 2005). The growth rate of *C. acutatum* on artificial media is much slower than *C. gloeosporioides*, especially at temperatures at or above 25°C (Smith, 2013). *C. acutatum* is tolerant of media with moderate levels (0.1 to 1.0 µL/mL) of benomyl which are completely inhibitory to other species of *Colletotrichum*, including *C. gloeosporioides* (Peres et al., 2005; Smith, 2013). More recently a number of molecular methods have been developed to aid in identification of *Colletotrichum* species. These methods include the use of arbitrary primed polymerase chain reaction, restriction fragment length polymorphisms (RFLPs) of PCR-amplified ribosomal DNA (rDNA), nucleotide sequences of the internal transcribed spacer (ITS) regions of rDNA, and more informative sections of chromosomal DNA (Freeman et al., 2000; Smith, 2008; Smith, 2013).

Anthracnose Symptoms

Anthracnose crown rot (ACR) caused by *C. gloeosporioides* is most prevalent in the southeastern United States where warm subtropical conditions and abundant rainfall favor the pathogen's rapid growth and dissemination (Gupton and Smith, 1991; MacKenzie et al., 2006; Rahman et al., 2015). ACR tends to be most severe in nurseries where close spacing of mother and daughter plants promotes pathogen dispersal and in production fields planted with infected nursery stock. Often the first visible symptom of ACR is the wilting of the youngest leaves during the warmest part of the day (Smith et al., 1998). The severity of wilting increases each day as infection spreads through crown tissue and disrupts water transport to aboveground tissues. Wilted leaves may recover slightly as turgor pressure is regained overnight, but most leaves wilt beyond recovery and collapse within days of initial wilt symptoms (Smith, 2013). Lengthwise cross-sections through the crown of wilted plants

reveals a characteristic firm rot with reddish-brown marbled or banded discoloration in contrast to the uniform ivory background color of healthy crown tissue. It is not uncommon for *C. gloeosporioides* to produce symptoms on other strawberry tissues such as leaves and runners. Splash-dispersed conidia of *C. gloeosporioides* can cause leaf spotting in extremely warm and humid conditions. Anthracnose leaf spot due to *C. gloeosporioides* is characterized by small (< 5 mm), round, gray or light black non-necrotic spots, which often resemble 'ink spots' splattered across the leaf surface. Observations of anthracnose leaf spot by Rahman *et al.* (2015) suggest leaf spot symptoms are an indication of an aggressive fungal strain and conducive environmental conditions but are not predictive of the amount of foliar infection in a field. *C. gloeosporioides* is also capable of causing stolon lesions which may girdle stolons, killing developing runners and unrooted daughter plants (Freeman, 2008). Runner tips and daughter plants are easily infected by diseased mother plants and can result in heavy losses in nursery production settings if large areas must be destroyed due to widespread infection (Rahman et al., 2015).

C. acutatum is occasionally isolated from infected strawberry crown or root tissue (Rahman et al., 2015; Smith, 2013), but such infections by *C. acutatum* usually result in stunted plants rather than total collapse and death (Smith, 2013). *C. acutatum* is the primary pathogen responsible for anthracnose fruit rot (AFR) of strawberry and causes the most economic damage to ripe and unripe fruit tissues (Peres et al., 2005). Initial symptoms will differ depending on whether infection occurs during flowering, fruit development, or ripening. Flower blight can appear if infection occurs during flowering and will result in the desiccation of buds, pedicels, peduncles, and flowers. Infection occurring shortly after pollination will result in small, hard, or misshapen fruit. Infection occurring during fruit

ripening develop into circular or elliptic sunken lesions, appearing brown to black in color and sometimes covered in orange-colored spore masses in humid conditions. Conidia from sporulating fruit lesions are easily splash-dispersed by rain or overhead irrigation and readily establish secondary infections in nearby fruit. AFR tends to be most severe in plasticulture production systems due to greater splash-dispersal of inoculum between ripening fruit on the surface of plastic mulch (Rahman et al., 2013; Yang et al., 1990).

Origin and Impact

ACR and AFR first affected the Florida and Louisiana strawberry industries long before appearing in North Carolina. ACR caused by *C. fragariae* or potentially misclassified *C. gloeosporioides* was first reported in Florida nurseries in 1931 (Brooks, 1931). Since the 1930s, *C. fragariae* and/or *C. gloeosporioides* spread throughout the southeastern United States inciting ACR in nurseries and fruit production fields as it traveled (Smith, 2008). *C. gloeosporioides* impacted the North Carolina strawberry industry for the first time in 1975 when it reached epidemic proportions and caused severe plant loss due to ACR (Delp and Milholland, 1980). The fungicide benomyl was used intensively by strawberry growers for years to suppress disease but benomyl-resistant strains of *Colletotrichum* were soon identified and the fungicide lost its effectiveness (Delp and Milholland, 1980). *C. acutatum*, the causal agent of AFR, was first reported on strawberry in the United States in Mississippi in 1983 (Smith and Black, 1986). North Carolina's strawberry industry was nearly ruined by this pathogen in the spring of 1990 as fruiting fields became overwhelmed with AFR. No fungicides were available to suppress the spread of disease on highly susceptible cultivars as the registration for the only effective fungicide expired years earlier (Poling, 1993). For

many years successful production from anthracnose-susceptible cultivars depended largely on the health of nursery plants as well as sanitation and manipulation of the plant environment. However, weather and disease pressure are not easily controlled and epidemics cannot be excluded by cultural methods alone. Epidemics struck North Carolina again in the fall of 2003 as Canadian planting stock infected with *C. acutatum* collapsed from AFR after planting (Schwegel et al., 2004) and forced growers to destroy an estimated eight million plug plants (Poling, 2008). In 2007 and 2008 North Carolina's strawberry nurseries were scrutinized after distributing nursery stock infected with *C. gloeosporioides* to growers throughout the southeast, leading to a widespread ACR epidemics in the region. Serious losses were incurred from these epidemics and plant mortality in some fields approached 100% (MacKenzie et al., 2009). North Carolina's field-based, certified nursery plant production program was terminated in response to this event and many of the certified strawberry nurseries who were just beginning to develop viable businesses were unable to recover (Poling, 2008). Anxious growers began purchasing from nurseries in the northern United States, Canada, or high elevation nurseries believed to lie outside of the range of *C. gloeosporioides* and *C. acutatum*.

North Carolina has not been the only state affected by ACR and AFR in recent years. It is estimated that the Florida strawberry industry incurs losses due to anthracnose averaging \$3 million USD per year, climbing to \$10 to \$15 million USD during severe outbreaks (Poling, 2008). Although anthracnose diseases of strawberry tend to be more virulent in warm climates where damage can be catastrophic, it frequently has its origins in temperate climates where nursery material is grown (Jeger and Bailey, 1992; Wilson et al., 1990). Significant mortality of plants from these areas is rare but losses still occur (MacKenzie et

al., 2009). *C. acutatum* is able to proliferate in cooler conditions than *C. gloeosporioides* and has now been reported in nearly every strawberry-growing region of the world (Peres et al., 2005; Smith, 2008), including the moderate Mediterranean climate of coastal California's strawberry industry. California has experienced several epidemics of AFR within the last decade which have cost the nursery and fruiting industries millions of dollars (Poling, 2008; Sjulín, 2008).

Losses due to ACR and AFR are not typically experienced annually at the same location. Rather, the location and timing of epidemics depend on the health and distribution of nursery stock and the presence of environmental conditions conducive to disease development. In epidemic years it is estimated that *C. gloeosporioides* has been responsible for 50-80% of plant loss in nurseries and 40-50% of yield loss in fruiting fields (Howard et al., 1992; Xie et al., 2010). Similarly, 50% fruit loss due to *C. acutatum* is not uncommon (Howard et al., 1992) and is the greatest source of economic loss due to anthracnose on strawberry (Peres et al., 2005; Smith, 2008). In extreme cases of optimal disease pressure and conducive environmental conditions there have been reports of entire fields lost to either ACR or AFR (MacKenzie et al., 2009; Poling, 2008). Around the country and world, anthracnose diseases of strawberry have a history of pernicious, destructive, and unpredictable behavior and are an increasing threat to strawberry nurserymen and growers.

Environmental Requirements for Infection

Epidemics of ACR and AFR are difficult to predict and control due to the polycyclic nature of anthracnose pathogens as well as their tendency to persist asymptotically for extended periods of time. Environmental variables important to anthracnose disease

progression include temperature, wetness duration, and relative humidity.

Temperature has been shown to have a significant effect on the success of tissue colonization and duration of quiescence. Several studies have been conducted to determine the optimum temperatures for conidia germination and secondary sporulation. These studies generally agree that the optimum temperatures for germination of *C. acutatum* conidia are between 23 and 27°C with most studies pinpointing 25°C as the optimum temperature (King et al., 1997; Wilson et al., 1990). A study by King *et al.* (1997) compared conidia production of *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* at different temperatures and found conidia production was greatest at 25°C for all three species. Quiescent periods were shortest (2 to 3 days) and similar across these three species at 25°C. King *et al.* (1997) also confirmed the ability of *C. acutatum* to proliferate at lower temperatures than *C. fragariae* and *C. gloeosporioides*. At 5 to 10°C, *C. acutatum* isolates produced 40 and 400 times more conidia than isolates of *C. fragariae* and *C. gloeosporioides*, respectively. Furthermore, at 5°C the quiescent period of *C. acutatum* lasted 6 to 11 days, much shorter than the 16 to 17 days required of *C. fragariae* and *C. gloeosporioides*.

Conidia of *C. gloeosporioides* and *C. acutatum* require free moisture or high relative humidity for successful germination. Therefore, wetness duration has been shown to directly affect the success of strawberry tissue colonization. Wilson *et al.* (1990) found that *C. acutatum* disease incidence increased with increased wetness durations (up to 48 hours) except at extreme temperatures ($\leq 10^{\circ}\text{C}$ and $\geq 30^{\circ}\text{C}$). At 25°C disease incidence was negligible below 6 hours wetness duration, increasing greatly to a maximum of nearly 100% disease incidence at 13 hours wetness duration. This finding was supported by Leandro *et al.* (2003b) who also found that conidial germination was greater at increasing wetness durations

and very low at wetness durations of 4 hours or less. The relationship between relative humidity and disease development of *C. gloeosporioides* has been investigated in mango (Dodd et al., 1991). This study determined that conidia were able to germinate and form appressoria at relative humidity values of 95% to 100%, even though free water was only visible at 100% relative humidity. Thus, nights when relative humidity can climb near 100% may play an important role in the germination of *Colletotrichum* conidia in the absence of free water.

Disease Cycle on Strawberry

The disease cycles of *C. gloeosporioides* and *C. acutatum* are complex and in many host-pathogen systems are still poorly understood. These pathogens are able to reproduce on many different tissue types across numerous species and can exist as necrotrophs, biotrophs, hemibiotrophs, endophytes, or epiphytes depending on the particular species and tissue inhabited (Peres et al., 2005). *C. gloeosporioides* and *C. acutatum* are believed to be broad-range pathogens, but have developed incredibly intimate and unique relationships with individual host species. Within each host species *C. gloeosporioides* and *C. acutatum* have evolved tissue-specific infection strategies, highly specialized relationships at the cellular level which are reflected in their disease cycles (Peres et al., 2005). *C. gloeosporioides* and *C. acutatum* have very similar disease cycles on strawberry due to the many similarities of their tissue-specific infection strategies.

The management and cultivation of strawberry as an annual affects the disease cycles of *C. gloeosporioides* and *C. acutatum*. These pathogens do not persist in soil between seasons (Peres et al., 2005; Urena-Padilla et al., 2002) but are known to survive on non-

cultivated (alternate) host species (Freeman et al., 1998; Hyde et al., 2009b; Smith, 2013). In addition to inoculating transplant nurseries, alternate hosts near production fields bridge the off-season gap and provide a source of inoculum the following season (Freeman, 2008; Hyde et al., 2009b; Peres et al., 2005). In North Carolina, ACR can be linked to local plant sources (Rahman et al. 2015) but this is rare for problems associated with AFR (F.J. Louws, personal observations).

Nursery operations are conducive to anthracnose persistence due to overlapping production cycles and the vegetatively-propagated nature of strawberry production. During summers in strawberry nurseries, conidia are produced in acervuli on nearby alternate hosts or previously infected nursery stock. Conidia are splash-dispersed by rain or overhead irrigation to daughter plants and uninfected tissue. The close spacing of established nursery plantings is ideal for the plant-to-plant spread of inoculum and contributes to the tendency of nurseries to experience widespread infections. Germinating conidia may cause ACR in daughter plants within the nursery but are arguably more destructive as quiescent hemibiotrophic infections, permitting the widespread dissemination of infected nursery material to production fields. Although inoculum sources for plants in fruiting fields can be diverse, asymptomatic planting stock carrying quiescent *Colletotrichum* infections are often the most important source of inoculum in production fields (Leandro et al., 2003b; Smith, 2008).

Infected nursery material can remain asymptomatic for extended periods, often until planting in production fields and in many cases until fruit ripening the following spring (Rahman et al., 2013). Nursery transplants infected with *C. gloeosporioides* commonly fail to establish after transplanting due to conducive disease conditions of transplant

establishment (Rahman et al., 2015). Prolonged wetness from overhead irrigation and warm temperatures in the weeks following transplanting trigger rapid production on leaves and promote its dissemination to new tissues. Conidia splashed into the crown invade this tissue and cause necrotrophic crown rot followed by wilt and plant collapse.

Once inoculum is introduced to a production field it can continue to proliferate through secondary conidiation to initiate new hemibiotrophic infections or can survive epiphytically on leaf tissue (Leandro et al., 2001; Legard, 2000). As winter brings cold weather and dormancy to strawberries, targeted host tissues become less susceptible to necrotrophic infection. In response, hemibiotrophic infections of *C. gloeosporioides* and *C. acutatum* arrest their growth in synchrony with the physiological state of their host and enter a quiescent state to await more favorable disease conditions (Latunde-Dada, 2001).

Termination of the quiescent phase and a switch to necrotrophic behavior is triggered by physiological and biochemical changes that occur in the host during growth, flowering, and fruit ripening in spring. Rapid inoculum proliferation on strawberry leaf tissue causes lesion formation on fruit, petioles, and stolons and the production of even greater quantities of conidia (Latunde-Dada, 2001; Leandro et al., 2003a). Secondary infections occur through direct contact with necrotrophic lesions or by rain splash-dispersal of conidia from sporulating lesions to adjacent tissues. Fruit and crown tissue are readily infected during this period, either by inoculum from leaf tissue or subsequent secondary infections. Both *C. gloeosporioides* and *C. acutatum* are polycyclic pathogens and if left unchecked will continuously infect new tissue for the duration of the season.

The Infection Process

C. gloeosporioides and *C. acutatum* exhibit an intracellular hemibiotrophic infection strategy shared by a group of agronomically significant fungal pathogens (Gan et al., 2013; Muench et al., 2008). Hemibiotrophic fungal pathogens employ a two-phase infection process: a preliminary symptomless or biotrophic phase where fungi obtain nutrients from living host cells, followed by a necrotrophic phase where nutrients are obtained from host cells that have been killed by the fungus (Arroyo et al., 2005; Gan et al., 2013; Muench et al., 2008; Perfect et al., 1999).

It is thought that some *Colletotrichum* species evolved a hemibiotrophic infection strategy as a means of avoiding host detection until conditions are conducive for necrotrophic infection of target tissues (Latunde-Dada, 2001). A nice example of the advantage of hemibiotrophy is provided by Latunde-Dada (2001) in their discussion of fruit-infecting pathogens such as *C. acutatum*. Unripe fruit contain extremely high concentrations of pre-formed antimicrobial compounds, a passive defense mechanism which is toxic to most fungi and inhibit successful infection. The concentration of these compounds decreases dramatically as fruit ripen. Hemibiotrophy provides *C. acutatum* with a solution to bypass strawberry's active (inducible) as well as passive (pre-formed) defenses to successfully infect fruit to cause AFR. The fungus is able to gain a foothold on host leaf tissue as hemibiotrophic infections to avoid triggering an active defense response and remains in this quiescent state until ripening begins and passive chemical defenses have dissipated. Thus, hemibiotrophy is a clever strategy used by these fungi to bide time, undetected but in close proximity to target tissues, and increases the chance of launching a successful necrotrophic

infection when conditions are optimal.

C. gloeosporioides and *C. acutatum* share this hemibiotrophic lifestyle and are capable of producing asymptomatic, biotrophic infections in leaf tissue and subsequent necrotrophic infections in other parts of the strawberry plant. The fate of germinating conidia and the infection strategy taken depend upon the tissue on which they land and its physiological state (Peres et al., 2005). Conidia germinating on leaves and petioles initiate the first stage of a hemibiotrophic infection strategy by establishing biotrophic infections in these tissues. Germinating conidia form appressoria that become melanized with pores through which an infection peg penetrates the cuticle and cell wall of epidermal cells. Once a cell has been penetrated, specialized infection vesicles form and develop biotrophic intracellular hyphae that invaginate the plasma membrane to obtain nutrients from the living cell (Gan et al., 2013; Muench et al., 2008). Initiating infections with a biotrophic phase avoids activation of defense responses and ensures successful establishment of the fungus in host tissue. The biotrophic phase is typically brief (2 to 3 days) and under ideal conditions will transition immediately to necrotrophy. However, hemibiotrophic pathogens such as *C. gloeosporioides* and *C. acutatum* possess the ability to pause between biotrophic and necrotrophic phases in the absence of preferred tissue or ideal conditions (Prusky et al., 2013). This ability is perfectly adapted to the mechanics of the strawberry industry and is realized in two physiologically distinct ways: quiescent infection and secondary conidiation.

Quiescence contributes to the overall success of a hemibiotrophic infection strategy and is defined as the period from host infection to the activation of fungal development and symptom expression (Prusky et al., 2013). Quiescent infections are host- and tissue-specific and these factors determine if and how quiescence will be induced (Prusky et al., 2013).

Quiescent periods are associated with many stages of infection and may be initiated during appressoria or penetration peg formation or after biotrophic establishment within host cells (Prusky et al., 1991). Studies exploring the induction of quiescence have been inconclusive, but three hypotheses have been proposed: i) deficiency of host nutritional resources necessary for pathogen development; ii) presence of pre-formed or inducible antifungal compounds; and iii) unfavorable environment for activation of fungal pathogenicity factors (Prusky, 1996). Quiescent periods permit an extension of the biotrophic phase of hemibiotrophic infections to remain undetected until target tissues are susceptible to necrotrophic infection. Termination of the quiescent phase is signaled by physiological changes in the host such as ripening, senescence, or flowering (Latunde-Dada, 2001; MacKenzie et al., 2010; Mertely and Legard, 2000; Prusky et al., 2013).

Secondary conidia play a major role in *Colletotrichum*'s hemibiotrophic avoidance of host defenses by entirely sidestepping the need to penetrate host tissue. Secondary conidiation, also known as microcyclic or precocious conidiation, is defined as conidiation that occurs directly after conidial germination with little or no mycelial growth (Leandro et al., 2001). This phenomenon has been reported in *C. acutatum* and *C. gloeosporioides*, as well as other *Colletotrichum* species (Leandro et al., 2001; Lingappa and Lingappa, 1969; Slade et al., 1987). Leandro *et al.* (2001) observed that secondary conidiation on inoculated leaves and glass cover slips increased the total number of conidia on both surfaces. These results indicate that host penetration and the presence of nutrients are not required for secondary conidiation to occur. In reference to this result, Stanley Freeman (2008) questions our knowledge of the host range and specificity of *Colletotrichum* species and suggests that this epiphytic strategy may extend the host range of anthracnose pathogens well beyond

known susceptible hosts. Leandro *et al.* (2003a) also investigated the role of strawberry flower extracts in triggering the production of secondary conidia. They found that flower extracts resulted in as much as 10- and 16-fold increases in conidia production over water on leaves and coverslips, respectively (Leandro *et al.*, 2003a). These studies suggest that secondary conidiation is a strategy employed by *Colletotrichum* species to increase inoculum when susceptible host tissue is unavailable. Additionally, these results indicate that flowering may trigger rapid inoculum production on asymptomatic leaves and this could serve as primary inoculum for anthracnose infections (Leandro *et al.*, 2003a; Leandro *et al.*, 2001; Pantidou and Schroeder, 1955; Timmer and Brown, 2000).

The necrotrophic phase is characterized by the production of infection hyphae that spread rapidly throughout the tissue, killing cells as they advance by secreting depolymerases to degrade cell walls (Muench *et al.*, 2008; Prusky and Lichter, 2007). Degradation of the cell walls liberates a variety of sugars that become available to invading fungi and fuel their growth. The primary biotrophic strategy of avoiding host defenses is replaced with a necrotrophic strategy of active and rapid degradation of host cells. *Colletotrichum* species secrete toxins to disrupt membrane integrity and generate reactive oxygen species to induce programmed cell death (Muench *et al.*, 2008).

Necrotrophy is the final phase of hemibiotrophic infections and is the period during which economic losses due to ACR and AFR are incurred. The evolution of a hemibiotrophic infection strategy by *Colletotrichum* pathogens of strawberry makes them well-equipped to evade many of strawberry's natural defenses. Unfortunately, this strategy also limits detection by humans and facilitates the distribution of infected nursery material. The complex hemibiotrophic lifestyle of *C. gloeosporioides* and *C. acutatum* is the primary

reason ACR and AFR are two of the most important diseases impacting strawberry.

Cultural Control Measures

Many anthracnose epidemics experienced in fruit production fields in the southeastern United States can be traced back to asymptomatic nursery material (Delp and Milholland, 1980; MacKenzie et al., 2009; Poling, 2008; Smith, 2008). Thus, industry-wide control of anthracnose begins in the nursery and continues in fruit production fields (Rahman et al., 2015; Urena-Padilla, 2001).

The movement of many strawberry nursery operations from the southeast to northern latitudes or high elevation has dramatically decreased the number of quiescently infected transplants distributed to production fields, but disease incidence is unpredictable and losses still occur (MacKenzie, 2005; Osorio et al., 2014). Total elimination of *C. gloeosporioides* and *C. acutatum* from nursery production cycles is unlikely due to the susceptibility of current cultivars, clonally-propagated nature of the crop, difficulty in detecting quiescent infections, and presence of alternate hosts. However, certain management practices can be utilized to minimize anthracnose infections and avoid widespread dispersal of infected nursery material. Removal of weeds in and around nursery fields reduces the risk that alternate hosts may spread inoculum to strawberries. The use of agronomic systems that reduce free moisture and limit the splash-dispersal of conidia will restrict the spread of anthracnose. Drip irrigation is an efficient method to deliver water and fertilizer directly to plant root zones and reduces free water on foliage compared to overhead irrigation. Various agricultural technologies, such as low and high tunnels add another layer of control and protect from rain events and subsequent splash-dispersal of inoculum (Freeman, 2008).

Nursery material should be scouted regularly to identify visible symptoms of anthracnose infection. Additionally, leaf samples should be periodically assayed using the paraquat method described by Cerkauskas *et al.* (1982) to detect asymptomatic quiescent infections. Herbicides should not be used to destroy weeds or infected strawberry plants as some herbicides are known to induce sporulation of latent infections (Cerkauskas and Sinclair, 1982). Instead, infected plants should be physically removed from the field and burned or buried. Rahman *et al.* (2015) found that removing plants in a four-meter radius from diseased material most successfully reduced the risk of distributing infected transplants from nursery plantings.

The use of disease-free transplants is the primary and most effective method to control ACR and AFR in fruiting fields (Rahman *et al.*, 2015). However, distribution of infected nursery material is not uncommon and inoculum is also spread from many alternate host species adjacent to fruiting fields throughout the growing season (MacKenzie *et al.*, 2007; Osorio *et al.*, 2014; Xiao *et al.*, 2004). Management practices similar to those recommended for nurseries should be adopted by fruiting operations to reduce infection risks.

On low-growing crops such as strawberry, *Colletotrichum* conidia have a relatively steep dispersal gradient (Madden and Boudreau, 1997) and sharply decline beyond one meter from inoculum origin (Rahman, 2015). Plastic mulches are known to increase dispersal distance and result in higher anthracnose incidence over textured organic mulches such as wheat straw and pine mulch (Coelho *et al.*, 2008; Madden *et al.*, 1993; Madden and Boudreau, 1997). The use of living mulches (grasses) in row middles is also effective at reducing the splash-dispersal and spread of *Colletotrichum* (Coelho *et al.*, 2008). In addition

to short-distance dispersal by splashing water, *Colletotrichum* conidia are disseminated over longer distances by the movement of workers, equipment and animals (Norman and Strandberg, 1997). Transfer of conidia between infected and healthy plants and fruit increases during harvest or plant maintenance activities due to the movement of workers through the field (Legard, 2000). Such work should be avoided when plants are wet as free moisture promotes the transfer and germination of conidia. Strawberries grown in soils with high nitrogen levels, especially the ammonium form, are more susceptible to anthracnose than those grown under lower nitrogen fertility (Smith, 2008; Smith, 2009). Nitrogen levels should be monitored and kept at a minimum required level to reduce risk in problematic fields.

Chemical Control Measures

The use of fungicides to treat ACR and AFR has a history of overuse by growers leading to fungicide resistance development in *Colletotrichum* populations (Smith, 2013). New fungicide labels address the issue of pathogen resistance development by restricting the number of total and consecutive applications that can be made per season (Rahman et al., 2013; Smith et al., 2013; Smith, 2013).

Current recommended fungicides are protectant or systemic in nature and are most effective at limiting the buildup of inoculum in the field before symptoms are present. Protectant fungicides do not inhibit the development of symptoms in already-infected tissue (Peres et al., 2005), making anthracnose difficult to control after symptoms appear (Louws and Rahman, 2012). Ongoing epidemics of AFR may respond to fungicide applications within 10 to 14 days, but there are currently no protocols available to effectively treat

ongoing crown infections (Louws et al., 2014).

Anthracnose management in central Florida is based on weekly variable-rate applications of Captan to maintain continuous coverage of this broad-spectrum protectant fungicide throughout the season. The season begins with applications at the lowest label rates and this rate is increased throughout the season in accordance with weather and disease pressure. Additional fungicides with curative activity (Quinone outside Inhibitors, or QoI) may be needed for increased control during periods favoring disease development (MacKenzie et al., 2009). An integrated pest management (IPM) program was developed for anthracnose at NC State University that aims to balance satisfactory control of anthracnose with minimized risk of selecting for fungicide resistance in pathogen populations (Louws and Rahman, 2012). This IPM program recommends early season fungicide applications only when problems are identified. The program also notes that QoIs are the most effective fungicides for controlling anthracnose but are prone to resistance development in the pathogen and should be reserved for high disease pressure situations only.

A major challenge in the control of anthracnose is knowing if and when precautionary management strategies need to be taken. In an effort to cut down on unnecessary fungicide applications, Pavan *et al.* (2011) developed the Strawberry Advisory System to advise growers in making fungicide application decisions. The system uses an algorithm to interpret leaf wetness and temperature data in central Florida to predict disease outbreaks and can send text or email alerts to users when certain thresholds are crossed. The predictive nature of the advisory system allows growers to make management decisions in advance of increasing disease pressure for better disease control. Field-based tests of the system showed the number of fungicide applications could be reduced by 50% without significant loss of yield

or quality (Pavan et al., 2011).

The development of IPM programs and best management practices have substantially improved the control of ACR and AFR in both nursery and fruit production settings. Reductions in anthracnose epidemics have been due to strategies such as the production and use of disease-free planting stock, early scouting for visible symptoms, roguing of diseased plants, altered irrigation practices, and carefully-timed fungicide applications (Poling, 2008; Rahman et al., 2013). However, these management strategies are labor- and chemical-intensive and at times can be largely ineffective. These strategies not only increase production costs, but reliance on frequent fungicide applications also carries environmental hazards, risks development of fungicide resistance in these and other pathogens, and is not a viable option to organic strawberry growers.

When highly susceptible cultivars are grown in disease-prone regions, rigorous management strategies and regular fungicide applications are necessary to suppress disease development. The successful deployment of genetic resistance is expected to be one of the most durable, effective, and sustainable means of controlling ACR and AFR in the future.

Resistance

Sources of Resistance

One portion of a complete anthracnose IPM program that has never been successfully deployed is the development of host resistance. Very little information on anthracnose resistance in *Fragaria* germplasm was available before the 1980s when anthracnose became

a major disease of strawberries in the southeastern United States (Smith, 2013). It was during this period that strawberry breeding programs at NC State, the University of Florida, and the United States Department of Agriculture–Agricultural Research Service in Poplarville, MS, began developing resistant cultivars adapted to this region (Ballington et al., 2002; Galletta et al., 1993; Howard et al., 1992; Smith and Spiers, 1982; Smith and Black, 1990). Germplasm developed by these programs vary in resistance to ACR and AFR (Denoyes-Rothan et al., 1999; MacKenzie et al., 2006; Rahman et al., 2013) but generally fail to combine resistance to either disease with desirable fruit quality and agronomic traits (Rahman et al., 2013).

Previous studies have evaluated breeding lines and commercial cultivars for resistance to anthracnose and report a general trend of susceptibility, especially to ACR (Ballington et al., 2002; Chandler et al., 1997; Denoyes-Rothan et al., 1999; Galletta et al., 1995; Giménez and Ballington, 2002; Gupton and Smith, 1991; MacKenzie et al., 2006; Osorio et al., 2014; Rahman et al., 2013; Smith and Spiers, 1982; Smith et al., 1998). Only a few genotypes are known to possess resistance to both ACR and AFR (Hancock et al., 2008). Conclusions drawn by these studies are often conflicting and are dependent upon complex experimental parameters such as environmental conditions, plant tissue evaluated, inoculation method, *Colletotrichum* species/race/isolate, method of resistance evaluation, and strawberry genotype (Shuman, 2001). Furthermore, strong genotype x environmental interactions affect the expression of resistance to these pathogens, leading Smith and Black (1987) to suggest that apparent anthracnose “field resistance” may not be representative of true genetic resistance.

Resistant cultivars are an excellent means of controlling diseases, however the highly

susceptible cultivars ‘Chandler’ and ‘Camarosa’ with their desirable fruit quality traits remain the popular choice of growers and regularly suffer losses due to anthracnose (Poling, 2008; Smith, 2008). Cultivars resistant to ACR and AFR will require competitive fruit quality and agronomic traits to be successful in the southeast.

Inheritance of Resistance

Resistance phenotypes (and disease symptoms) are the end result of complex interactions between host, pathogen, and the environment over time. Apart from physical barriers, plants primarily have two types of defense against pathogens governed by two kinds of genes: minor gene resistance and major gene resistance. Race-non-specific, or minor gene resistance is effective against all isolates of a pathogen, whereas race-specific or major gene resistance is only effective against a subset of isolates (Van der Plank, 1968). In pathosystems where race-specific resistance occurs, the interaction between the host and pathogen is determined by the presence or absence of major host resistance genes and pathogen avirulence genes as described in the gene-for-gene hypothesis (Gabriel, 1999) and tends to be simply inherited (Van der Plank, 1968). Race-non-specific resistance is governed by many minor host genes that contribute incrementally to overall host resistance and is usually inherited quantitatively (Parlevliet and Zadoks, 1977). In statistical analyses, absence of a cultivar by isolate interaction is evidence of race-non-specific resistance while a significant cultivar by isolate interaction would suggest race-specific resistance mechanisms are active (Van der Plank, 1968).

Several studies on the inheritance of resistance to anthracnose diseases have found a broad range of resistance among strawberry cultivars and pathogenicity among

Colletotrichum isolates (Delp and Milholland, 1981; Denoyes-Rothan et al., 2005; Horn et al., 1972; Lewers et al., 2007; MacKenzie et al., 2006; Osorio et al., 2014; Smith and Black, 1990). However, there is a lack of agreement in their reports of race-specificity and mode of gene action. Some studies have reported race-specific resistance or major gene action (Delp and Milholland, 1981; Horn et al., 1972; Lewers et al., 2007; Smith and Black, 1990) while others have reported race-non-specific resistance or minor gene action (Ballington et al., 2002; MacKenzie et al., 2006; Osorio et al., 2014). The contrasting results of these studies may be due to differences in *Colletotrichum* species or isolate, strawberry germplasm, or differences in the statistical interpretation of results. Analysis of variance (ANOVA) is often used to determine whether race-specific resistance is present based on the statistical significance of cultivar x isolate interactions. However, conclusions drawn in this way should be made cautiously as they are sensitive to deviations from additivity. Such deviations can be caused by the scale used to measure disease severity and can result in falsely reported significant interactions (Parlevliet, 1976).

Additionally, a few studies note aspects of both major and minor gene inheritance (Denoyes-Rothan et al., 2005; Giménez and Ballington, 2002; Gupton and Smith, 1991). This is not surprising as both types of inheritance, major gene and minor gene resistance, often occur together (Parlevliet, 1995). For example, Denoyes-Rothan *et al.* (2005) discovered that a single dominant gene (*Rca2*) controlled high-level resistance to *C. acutatum*, but that the resistance of intermediate genotypes was controlled by minor genes. Studies such as these with both major and minor gene inheritance often recommend a two-stage breeding approach of progeny testing followed by individual selection within progenies (Giménez and Ballington, 2002; Gupton and Smith, 1991).

Many of the dissonant conclusions mentioned above are based on contrasting estimates of additive and dominance variance. Previous studies of inheritance of anthracnose resistance have been conducted with both large and small populations inoculated with one or more *Colletotrichum* species. In a large population of 40 parents and 87 full-sib families, Gupton *et al.* (1991) estimated dominance variance to be 6 to 10 times higher than additive variance with moderate estimates of narrow-sense heritability. Gupton *et al.* (1991) also examined a smaller population of six parents and nine full-sib families, finding additive variance to be four times larger than dominance variance and very high estimates of narrow-sense heritability (0.89). The Gupton *et al.* (1991) studies demonstrate the potential range of conclusions drawn populations of different size and composition.

Gimenez et al (2002) constructed a small population of six parents and 14 full-sib families to study the inheritance of resistance to *C. acutatum* on strawberry runners. This study found both additive and dominance variance to be important. Larger estimates of specific combining ability (SCA) than general combining ability (GCA) suggest that major gene action is of greater importance in this experiment. A recent study by Osorio *et al.* (2014) was the first to estimate the genetic parameters governing inheritance of resistance to *C. gloeosporioides* in strawberry. This study developed a population of 11 parents and 42 full-sib families that were screened for resistance to ACR. Estimates of additive variance were approximately three times larger than dominance variance, indicating a greater importance of minor gene action in this experiment. The variables determining race-specificity and mode of inheritance are unique to every set of experimental conditions and ultimately depend on the collection of host genotypes and pathogen isolates under examination (Van der Plank, 1968).

Resistance in Differential Host Tissues

Some of the discrepancies in the previously-mentioned inheritance studies may be explained by spatial-temporal considerations in pathosystems similar to the strawberry-*Colletotrichum* pathosystem. Several previous studies have examined the differential expression of host resistance to late blight (*Phytophthora infestans*) in potato (*Solanum tuberosum*) foliage and tubers, but this relationship is not clear and is often contradicting. Platt and Tai (1998) and Stewart *et al.* (1994) reported that foliar and tuber resistance were correlated, but the association found by Stewart *et al.* (1994) could not be confirmed in later studies (Douches *et al.*, 2002; Kirk *et al.*, 2001). On the other hand, several studies report genetic evidence of little or no correlation between tuber and foliar resistance to late blight (Kirk *et al.*, 2001; Liu and Halterman, 2009; Simko *et al.*, 2006). Park *et al.* (2005) examined the inheritance of tuber blight resistance in four mapping populations and compared these data to foliar blight resistance. This study found expression of resistance was strongly correlated in some populations but not in others, indicating that any correlation between foliar and tuber resistance is dependent on the *R* gene(s) involved. It is likely that some regions of the potato genome are involved in both foliar and tuber resistance, while others are specifically associated with one tissue or the other (Park *et al.*, 2005; Simko *et al.*, 2006). Studies on the differential expression of late blight resistance in potato recommend separate evaluations of foliar and tuber resistance and that breeding programs should include multiple accessions to incorporate resistance from both tissue types (Kirk *et al.*, 2001; Liu and Halterman, 2009).

Published reports on resistance to *C. gloeosporioides* and *C. acutatum* have

demonstrated usable genetic variation in strawberry, however very few studies have compared resistance across different tissue types. Casado-Diaz (2006) investigated gene expression responses to strawberry fruit and crown tissues inoculated with *C. acutatum*. This study found differential gene expression across tissues in response to infection and suggests that strawberry tissues vary in resistance to pathogen infection. Denoyes-Rothan *et al.* (2005) identified a single dominant gene (*Rca2*) responsible for whole-plant resistance of seedlings to *C. acutatum*. When compared to anthracnose fruit rot resistance from an earlier study (Denoyes-Rothan *et al.*, 1999), fruit resistance appeared to be controlled partially but not entirely by the presence of *Rca2* (Denoyes-Rothan *et al.*, 2005). This result is very similar to genetic control of late blight in the potato population RH94-076 described by Park *et al.* (2005) in which tuber and foliar resistance to late blight was under the dual genetic control of an *R* gene (*R1*) and minor quantitative trait loci (QTL).

Resistance in Strawberry Foliage

Limited work has been done to determine the mechanisms and heritability of resistance to anthracnose in strawberry foliage, a common and critical host tissue in the disease cycles of *C. gloeosporioides* and *C. acutatum* (Gan *et al.*, 2013; Rahman *et al.*, 2013). A recent study by Rahman *et al.* (2013) evaluated the resistance of 14 strawberry genotypes to hemibiotrophic foliar infections and AFR resistance caused by *C. acutatum*, finding that resistance to hemibiotrophic foliar infections was not well correlated to resistance to AFR. Similar to studies on the differential expression of resistance to late blight in potato, these results support a model where distinct genetic mechanisms control resistance to *C. acutatum* in foliage and fruit (Kirk *et al.*, 2001; Liu and Halterman, 2009; Park *et al.*, 2005; Simko *et*

al., 2006).

Foliar resistance such as the resistance to hemibiotrophic *Colletotrichum* infection described by Rahman *et al.* (2013) is often characterized as rate-limiting (or rate-reducing) resistance. Rate-limiting resistance is a form of partial resistance wherein a host is susceptible to a degree but possesses the capacity to resist or limit the infection, colonization, or subsequent reproduction of a pathogen (Parlevliet, 1979). Rate-limiting resistance is generally a durable and quantitatively inherited form of resistance that impedes epidemics through reduced pathogen fecundity (Ballington *et al.*, 2002; Ram, 2014; Van der Plank, 1968).

While the previous study by Rahman *et al.* (2013) suggests distinct foliar and fruit resistance mechanisms to *C. acutatum*, there have been no similar studies published on resistance to hemibiotrophic foliar infections of *C. gloeosporioides* or its correlation to resistance in crown tissue. Additionally, there are no known publications on the comparative resistance to hemibiotrophic foliar infections across multiple *Colletotrichum* species, despite indications that resistance mechanisms could be shared against multiple *Colletotrichum* species in common tissue types (Gupton and Smith, 1991).

Total ACR and AFR incidence are likely impacted by several resistance mechanisms including direct resistance of the crown and fruit tissue as well as mechanisms that limit any component of the pathogen life cycle such as colonization of foliar tissue followed by proliferation through secondary conidiation. A shared mechanism conferring rate-limiting resistance to anthracnose-causing *Colletotrichum* species would effectively limit a critical phase in the disease cycles of *C. gloeosporioides* and *C. acutatum* and provide a component of total resistance to ACR and AFR. This may be accomplished through a rate-limiting

resistance mechanism that, once incorporated into strawberry germplasm, would limit inoculum buildup in nurseries during transplant production and in fruit production fields (Ciancio and Mukerji, 2007). Genotypes with rate-reducing resistance would increase the effectiveness of disease management practices such as sanitation and strengthen previously-established anthracnose IPM programs. Additionally, a shared rate-limiting resistance mechanism in strawberry foliage could be combined with crown rot and fruit rot resistance traits to achieve optimum field-level anthracnose resistance, further reducing the risk of epidemics.

This dissertation was designed to address some of the fundamental questions that remain regarding inheritance of resistance to hemibiotrophic foliar infections of *C. gloeosporioides* and *C. acutatum* in strawberry. Original research is presented in the following four chapters. Chapter 2, the first chapter following this introduction, evaluated a panel of NCSU strawberry germplasm for resistance to hemibiotrophic foliar infections of *C. gloeosporioides*. Variability in resistance to *C. gloeosporioides* hemibiotrophic infections would complement similar observations of *C. acutatum* made by Rahman *et al.* (2013) and support further investigation of a shared foliar resistance mechanism. Chapter 3 aims to refine methods for successful establishment and quantification of hemibiotrophic *Colletotrichum* infections in order to achieve greater means separation. Methods are identified to induce high levels of infection in the susceptible cultivar ‘Chandler’ in order to differentiate resistant genotypes. In Chapter 4, a diverse panel of 7 commercial cultivars and 11 NCSU selections were screened for hemibiotrophic foliar and crown resistance to *C. gloeosporioides* and *C. acutatum*. This experiment provided data on resistances within common and divergent tissues and guided the selection of parents for studying the

inheritance of resistance in crown and leaf tissue. In Chapter 5, a population was developed to investigate the inheritance of resistance to hemibiotrophic infections of *C. gloeosporioides* and *C. acutatum* in strawberry leaf tissue and was screened for resistance to ACR caused by *C. gloeosporioides* to permit comparisons across tissue types. Data from these studies will further our understanding of the inheritance of resistance to *Colletotrichum* species within and across strawberry tissue types and will aid in defining breeding strategies for these traits.

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Chapter Two:

SPECIES IDENTIFICATION OF *COLLETOTRICHUM* ISOLATES USING REAL-TIME PCR AND EVALUATION OF STRAWBERRY GENOTYPES FOR VARIATION IN HEMIBIOTROPHIC INFECTION SEVERITY

Abstract

Resistance of strawberry genotypes to hemibiotrophic foliar infection of *Colletotrichum gloeosporioides* was characterized in a proof-of-concept study following evidence of variation in resistance to *C. acutatum* in a prior study. Ten *Colletotrichum* isolates were selected for use in this study based on known pathogenicity in strawberry. Six *Colletotrichum* isolates were obtained from the F.J. Louws plant pathology lab at NC State and four isolates were collected from infected strawberry tissue showing typical anthracnose symptoms. Genomic DNA was extracted from each isolate and run using species-specific primers for real-time PCR detection of *C. acutatum* and *C. gloeosporioides*. Results from real-time PCR confirmed putative classification of isolates based on colony and conidia morphology, identifying six isolates as *C. gloeosporioides* and four as *C. acutatum*. Eight selections from the NC State strawberry breeding program were inoculated with a 1.0×10^6 conidia·mL⁻¹ suspension applied to leaf surfaces. Leaf samples were collected every three days for eight sample dates and the severity of hemibiotrophic *Colletotrichum* infections were evaluated using a paraquat protocol to induce sporulation of asymptomatic fungal

colonists. Percent sporulating leaf area was visually estimated and used to calculate area under the disease progress curve (AUDPC) scores for comparison of genotypes. The main effect of genotype was significant and genotype AUDPC means ranged from 8.48 to 23.15. These data provided preliminary indication of variation in resistance to hemibiotrophic foliar infections of *C. gloeosporioides* and confirmed that methods previously used to evaluate *C. acutatum* foliar infections could be applied to *C. gloeosporioides*. However, high variation within these data suggested that the development of refined methods for evaluating resistance to these pathogens in strawberry germplasm would be beneficial.

Introduction

The cultivated strawberry (*Fragaria x ananassa* Duchesne) is a major fruit crop in North Carolina and the southeastern United States. North Carolina ranked 4th nationally in 2014 for strawberry production, harvesting 7,700 tons worth approximately \$23.4 million USD (USDA-NASS, 2015), making strawberries the second most valuable fruit crop in the state (NCDA&CS, 2015). The majority of North Carolina's strawberry acreage is planted in the annual hill or plasticulture production system. This system takes advantage of raised, fumigated, double beds, drip irrigation, and black plastic mulch covering the beds. Plasticulture became feasible in North Carolina largely due to the release of two June-bearing strawberry cultivars, 'Chandler' and 'Camarosa', developed by the University of California. These cultivars are widely adapted throughout North Carolina and produce excellent yield and fruit quality for six weeks or more in plasticulture production systems (Poling, 1993). However, the humid subtropical climate of North Carolina and the southeast feature varieties

and intensities of biotic stress that are rarely experienced in the Mediterranean climate where these cultivars were developed. As such, ‘Chandler’ and ‘Camarosa’ are highly susceptible to anthracnose crown rot (ACR) and anthracnose fruit rot (AFR) incited by any of three *Colletotrichum* species and commonly suffer losses to these fungal pathogens (Rahman et al., 2013).

ACR is predominantly caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and to a lesser extent by *C. fragariae* A.N. Brooks (Freeman and Katan, 1997; Rahman et al., 2015). These fungi kill plants by rapidly invading and producing a reddish-brown marbled necrosis of crown tissue, resulting in wilt and eventual collapse of the plant (Rahman, 2015). *C. gloeosporioides* has emerged as the primary causal agent of ACR in North Carolina and much of the southeast (Howard et al., 1992; Lewers et al., 2007; Urena-Padilla et al., 2002) and many investigators now question the classification of *C. fragariae* as a distinct species due to its close genetic relatedness to *C. gloeosporioides* (Mills et al., 1992; Sreenivasaprasad et al., 1992). AFR is caused by *C. acutatum* J.H. Simmons and produces dark sunken lesions on developing fruit that render them unmarketable (Freeman and Katan, 1997; Rahman et al., 2013).

ACR and AFR are most prevalent in the southeastern United States where warm subtropical conditions and abundant rainfall favor the pathogens’ rapid growth and dissemination (Gupton and Smith, 1991; MacKenzie et al., 2006; Rahman et al., 2015). Losses due to ACR and AFR are not typically experienced in the same location annually. Rather, the location and timing of epidemics depend on the health and distribution of nursery stock and the presence of environmental conditions conducive to disease development. In epidemic years it is estimated that *C. gloeosporioides* has been responsible for 50-80% of

plant loss in nurseries and 40-50% of yield loss in fruit production fields (Howard et al., 1992; Xie et al., 2010). Similarly, reports of 50% fruit loss due to *C. acutatum* are not uncommon (Howard et al., 1992) and are the greatest source of economic loss due to anthracnose on strawberry (Peres et al., 2005; Smith, 2008).

The management and cultivation of strawberry (a perennial plant) affects the disease cycles of *C. gloeosporioides* and *C. acutatum*. These pathogens do not typically persist in soil between experiments (Freeman et al., 2002; Peres et al., 2005) but re-introduction of inoculum to nursery or fruiting fields is accomplished through other channels. In the southeast, non-cultivated hosts and indigenous sources of *C. gloeosporioides* inoculum may be present and contribute to infestations in nurseries and fruiting fields (MacKenzie et al., 2007; Xiao et al., 2004). In North Carolina and Florida, *C. gloeosporioides* has commonly been isolated from wild grape (*Vitis* spp.), Virginia creeper (*Parthenocissus quinuefolia*), and oak species (*Quercus* spp.) (MacKenzie et al., 2007; Rahman et al., 2015). *C. acutatum* infestations have been associated with indigenous sources and non-cultivated hosts in other parts of the world (Freeman et al., 2001; Parikka and Lemmetty, 2011), but there is little if any evidence of this occurring in the southeastern United States. Rather, *C. acutatum* infestations in this region are typically attributed to infected transplants from strawberry nurseries (Legard, 2000; Peres et al., 2005). Nursery operations are highly conducive to anthracnose persistence due to overlapping production cycles or the re-introduction of inoculum on new plants and the vegetative nature of strawberry propagation. During summers in strawberry nurseries, conidia are produced in acervuli on nearby non-cultivated hosts or asymptomatic nursery stock and are splash-dispersed by rain or overhead irrigation to daughter plants. The close spacing of established nursery plantings is ideal for the plant-

to-plant spread of inoculum and contributes to the tendency of nurseries to experience widespread infections. Germinating conidia may produce disease symptoms immediately but are arguably more destructive when symptom appearance is delayed by the establishment of quiescent hemibiotrophic infections, permitting the widespread dissemination of asymptomatic transplants to fruiting fields (Leandro et al., 2003; Smith, 2008).

Hemibiotrophic pathogens employ a two-phase infection strategy: a preliminary asymptomatic biotrophic phase where fungi obtain nutrients from living host cells, followed by a necrotrophic phase where nutrients are obtained from host cells that have been killed by the fungus (Arroyo et al., 2005; Gan et al., 2013; Muench et al., 2008; Perfect et al., 1999). Quiescence contributes to the overall success of a hemibiotrophic infection strategy and is defined as an asymptomatic period between initial host infection and the activation of necrotrophic behavior and symptom expression (Prusky et al., 2013). Infected nursery plants can remain asymptomatic for extended periods, often until planting in production fields and in many cases until fruit ripening the following spring (Rahman et al., 2013). Once inoculum is introduced to a production field, it can continue to proliferate through secondary conidiation to initiate new hemibiotrophic infections or can survive epiphytically on leaf tissue (Leandro et al., 2001; Legard, 2000). Asymptomatic planting stock carrying quiescent *Colletotrichum* infections are often the most important source of inoculum in fruit production fields and have been implicated in many anthracnose epidemics in the southeastern United States (Delp and Milholland, 1980; Leandro et al., 2003; MacKenzie et al., 2009; Poling, 2008; Smith, 2008). Thus, industry-wide control of anthracnose begins in the nursery and continues in fruit production fields (Rahman et al., 2015; Urena-Padilla, 2001).

Very limited work has been conducted to investigate resistance to hemibiotrophic

Colletotrichum infections in strawberry foliage, a common and critical host tissue in the disease cycles of both *C. gloeosporioides* and *C. acutatum* (Gan et al., 2013; Rahman et al., 2013). A recent study by Rahman *et al.* (2013) evaluated 14 strawberry genotypes for resistance to hemibiotrophic foliar infections and AFR caused by *C. acutatum*, finding that resistance to hemibiotrophic foliar infections was not well correlated to AFR resistance. Similar to studies on the differential expression of resistance to late blight in potato foliage and tubers, these results support a model where distinct genetic mechanisms control resistance in strawberry foliage and fruit (Kirk et al., 2001; Liu and Halterman, 2009; Park et al., 2005; Simko et al., 2006).

Foliar resistance such as the resistance described by Rahman *et al.* (2013) is often characterized as rate-limiting resistance, a form of partial resistance wherein a host is susceptible to a degree but possesses the capacity to resist or limit the infection, colonization, or subsequent reproduction of a pathogen (Parlevliet, 1979). Rate-limiting resistance effectively slows the progress of *Colletotrichum* epidemics through reduced pathogen fecundity, suppressing inoculum production during a critical phase in the disease cycle (Ballington et al., 2002; Ram, 2014; Van der Plank, 1968).

While the recent study by Rahman *et al.* (2013) suggests distinct foliar and fruit resistance mechanisms to *C. acutatum*, there have been no similar studies published on resistance to hemibiotrophic foliar infections of *C. gloeosporioides* despite indications that resistance could be shared against multiple *Colletotrichum* species in some strawberry tissues (Gupton and Smith, 1991). We hypothesize that a shared rate-limiting mechanism conferring resistance to *C. gloeosporioides* and *C. acutatum* would reduce overall ACR and AFR incidence by restricting the spread and buildup of inoculum in nurseries and fruiting fields.

The objectives of this study were to 1) identify the species level of a subsample of our current collection of *Colletotrichum* isolates, 2) pilot the methods of Rahman *et al.* (2013) using *C. gloeosporioides* and 3) conduct an initial assessment of genetic variation in resistance to *C. gloeosporioides* hemibiotrophic foliar infections in a sample of strawberry genotypes from the NC State breeding population.

Materials and Methods

Species identification. Ten *Colletotrichum* isolates with known strawberry pathogenicity were acquired from different sources within North Carolina. Several isolates were provided by the F.J. Louws plant pathology lab at NC State, while others were personally collected from infected strawberry tissues in Salisbury and Kannapolis, NC. Some isolates were obtained from long-term storage on dried filter paper and revived by transferring a small (10 mm²) piece of filter paper to new plates of half strength potato dextrose agar (PDA). Isolates maintained as living cultures were simply transferred to new plates of half strength PDA. Cultures were grown in an incubator at 25°C for approximately seven days under 12 hour fluorescent lighting to encourage new mycelial growth and sporulation.

The species level of each *Colletotrichum* isolate was confirmed by morphological attributes and real-time PCR. Morphological observations were made of colony color, growth rate on PDA at 25°C, and microscopic observations of conidia morphology. Morphological observations were compared to previous reports of *Colletotrichum* species pathogenic to strawberry (Gunnell and Gubler, 1992; Smith and Black, 1990).

Approximately 200 mg of mycelium were collected by scraping the surface of each plated culture with a scalpel. Mycelia were placed into 2.0 mL microcentrifuge tubes, freeze-dried, and ground to a fine powder using a sterile glass rod. A modified cetyltrimethylammonium bromide (CTAB) DNA extraction protocol was used to extract genomic DNA from each fungal isolate. Approximately 50 mg of powdered mycelium was transferred to a sterile 1.5 mL microcentrifuge tube and rehydrated with 750 μ L CTAB extraction buffer [50 mM Tris (pH 8.0), 700 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 1% (w/v) CTAB, and 1% (w/v) β -mercaptoethanol] for cell lysis. The CTAB mixture was incubated in a water bath at 65°C for 60 min with periodic shaking. Following cell lysis, tubes were centrifuged at 10,000 g for 5 minutes to pelletize cellular debris and the supernatant removed to a clean 1.5 mL tube. The supernatant was extracted once with chloroform:isoamyl alcohol (24:1). Genomic DNA (gDNA) was precipitated by adding $\frac{1}{2}$ volume of 7.5M sodium acetate followed by 2 volumes of 100% isopropanol. gDNA was pelleted by centrifugation at 10,000 g for 10 minutes. The pellet was washed once with 80% ethanol, dried, and suspended in 100 μ L 1 x TE buffer. gDNA yield and quality were checked with a NanoDrop™ 2000 spectrophotometer. All gDNA samples were stored at -20°C until required for real-time PCR.

Species-specific primers designed by Garrido *et al.* (2009) based on highly divergent internal transcribed spacer (ITS) regions of the *Colletotrichum* genome were used for detection of *Colletotrichum* species. Species-specific primers and probes were designed for TaqMan® real-time PCR assays for differentiation of *C. gloeosporioides* and *C. acutatum* (Table 2.1). All TaqMan® assays were set up using BIOLINE® reagents. Reaction conditions consisted of 2.5 μ L 10x NH₄ reaction buffer, 1 μ L 50 mM MgCl₂ solution, 0.25

μL 100 mM dNTPs, 0.375 μL BIOLASE™ DNA Polymerase, and 8.875 μL double-distilled water (ddH₂O). Primers and probes (10 μL) were added to give a final concentration of 300 nM and 100 nM, respectively. DNA (2 μL) was added to give a final volume of 25 μL per reaction.

Real-time PCR reactions were set up in triplicate in 96-well reaction plates. Negative controls containing nuclease-free water instead of DNA were included. Reactions were amplified using an ABI 7500 Fast Real-Time PCR System. Cycling parameters consisted of 40 cycles of [denaturing: 95°C for 15 sec, annealing: 51°C for 30 sec, and extension: 72°C for 30 sec].

Inoculum preparation. Two weeks prior to inoculation a mixed isolate inoculum was prepared to represent the diverse strains found in strawberry plantings. Isolates #28, #58, and #84 were selected for use in this study based on confirmation of *C. gloeosporioides* species identity. Isolates #28 and #58 were originally collected from infected strawberry crowns near Sanford, NC. Isolate #84 was collected in Franklin, NC from an infected crown of cultivar ‘Treasure’. Fresh cultures were made from the original plate of each isolate by transferring mycelia with a sterile probe to new plates of half strength PDA. Ten plates of each isolate were made and cultures were incubated at 25°C for 10 – 14 days under 12 hour fluorescent lighting. Conidia were collected from mature cultures of *C. gloeosporioides* isolates by flooding the plate with distilled water containing 5 drops of Tween 20 per liter of water and disturbing the mycelium with a glass rod to suspend conidia. Conidial suspensions were filtered through a doubled layer of cheesecloth to remove cellular debris. Mixed isolate inoculum was prepared by adjusting the conidial concentration of each isolate to 1.0×10^6 conida·mL⁻¹ using a hemocytometer and compound microscope, then combining equal

volumes of the three isolates. Combining 500 mL of each isolate yielded a total volume of 1.5 liters of inoculum. Concentration of the mixed isolate suspension was checked by hemocytometer to confirm a final concentration of 1.0×10^6 conidia·mL⁻¹.

Inoculation. Fungal suspensions were applied via handheld sprayer (Solo® model 419) as a foliar mist on Aug. 28, 2012. Inoculum was applied just until runoff was achieved to ensure even inoculum density across all leaf surfaces. The inoculation was conducted in the early evening to take advantage of high overnight humidity in the greenhouse to encourage conidia germination. Plants were left undisturbed for three days to allow time for conidia germination and infection to occur. The greenhouse environment was maintained at $25 \pm 5^\circ\text{C}$ for the duration of the experiment.

Disease assessment. Although plant material used in this study appeared clean, it was sourced from a field where *C. gloeosporioides* does occur and quiescent infections may have been present. Therefore, ‘time zero’ samples were collected just prior to inoculation to give a baseline level of quiescent colonization of the leaf tissue. Samples consisted of four individual leaflets that were arbitrarily collected from each four-plant plot. Only inoculated leaflets were collected and new or emerging leaves were not sampled. Leaflets from each plot were placed into coin envelopes and temporarily held on ice until they were stored in a 4°C refrigerator. Samples were collected on a total of eight sample dates. In addition to time zero, samples were also collected at 3, 6, 9, 12, 15, 18, and 21 days after inoculation (DAI). Once leaflets were collected across all dates, samples were transported to the F.J. Louws plant pathology lab at NC State for further analysis. Samples were stored in a refrigerated room at 4°C until assayed.

Leaflets were evaluated for severity of fungal colonization by following a paraquat

protocol successfully used by Rahman *et al.* (2013) to visualize hemibiotrophic infections of *C. acutatum* in strawberry leaf tissue. The herbicide paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride) has been used to quantify the presence of *Colletotrichum* species on symptomless plant tissue since the 1980s (Cerkauskas and Sinclair, 1982). This chemical rapidly induces senescence of leaf tissue and triggers sporulation of quiescent fungal colonists that are not otherwise visible. Leaflets were surface sterilized by immersing in 70% ethanol for 15 seconds, 10% bleach for 60 seconds, and rinsed twice in ddH₂O. Leaflets were then treated with paraquat by immersing them for one minute in a solution of 20 mL Gramoxone® (30.1% paraquat dichloride) / 1 liter ddH₂O and rinsing a final time in ddH₂O. Excess moisture was allowed to drip off of leaflets before incubation. Leaflets were placed adaxial side up on wire screens in clear plastic incubator boxes lined with moist paper towels. Incubator boxes were stored at 25°C under fluorescent room lighting for five days to allow time for senescence to occur and acervuli to develop on their adaxial surface. After five days the samples were evaluated for presence of orange-colored acervuli. Percent sporulating leaf area (PSLA) was visually estimated as the percentage of total leaf area covered by acervuli.

Colletotrichum species identity was checked for low-level sporulation observed on time zero samples of each genotype. Four sporulating leaflets from the time zero sample of each genotype were selected and conidia from visible acervuli were transferred from each leaflet to new plates of PDA. The resulting 32 plates were incubated at 25°C for approximately seven days under 12 hour fluorescent lighting to encourage new mycelial growth. Mycelium was collected from the four plates of each genotype and bulked into a single 2.0 mL microcentrifuge tube. The previously mentioned protocols for DNA extraction and real-time PCR analysis were followed to detect species level of these quiescent

Colletotrichum infections.

Plant production. Eight advanced selections from the NC State strawberry breeding program were selected for use in this study based on field observations of ACR severity in 2011 and 2012 and represent a range of resistance and susceptibility to ACR (R. Jacobs and J. Pattison, unpublished). NC State selections chosen for this study include: NCS 10-019, NCS 10-028, NCS 10-037, NCS 10-080, NCS 10-086, NCS 10-092, NCS 10-142, and NCST 10-032. Positive (susceptible) controls were not included based on field observations of susceptibility of some included genotypes and the presence of susceptible cultivars ('Chandler' and 'Camarosa') among their parents. Apparent disease-free runner tips of each genotype were collected from a field planting at the Piedmont Research Station in Salisbury, NC on May 22, 2012 and transported to a greenhouse in Kannapolis, NC for propagation. Tips were planted on May 24, 2012 in 50-cell plug trays containing Fafard® 3B mix (Sun Gro Horticulture, Agawam, MA) and rooted under periodic mist (30 second duration, 10 minute interval) in a $21 \pm 5^{\circ}\text{C}$ greenhouse for one week until roots developed. After two additional weeks, all plants were disease-free (symptomless) and rooted plug plants were transplanted into 10 cm pots containing Fafard® 3B potting mix and grown to maturity from June to August in a $25 \pm 5^{\circ}\text{C}$ greenhouse. The plants were hand watered daily and fertilized with a solution of Jack's Professional® LX Ca-Mg 15-5-15 fertilizer (JR Peters Inc., Allentown, PA) via a Dosatron® injector system calibrated to dispense 100 ppm N. Prior to inoculation plants were arranged in a randomized complete block design (RCBD) with eight blocks and eight genotypes randomized to four-plant plots within each block. A total of 32 clonal plants of each genotype were included in the experiment.

Analysis. The cycle threshold (C_T) value of each real-time PCR reaction was

assessed using the 7500 Software v. 3.2 (Life Technologies™). Mean Ct values and standard deviations were calculated and are presented in Table 2.2.

Disease progress curves (Figure 2.1) were constructed by plotting genotype average PSLA scores over the eight sample dates. Area under the disease progress curve (AUDPC) scores were calculated and provided a summary statistic for comparing disease intensity over time across genotypes (Campbell and Madden, 1990). AUDPC is represented by the following equation of Shaner and Finney: $AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$, where “t” is time in days between each sample date, “y” is the percentage of sporulating leaf area at each sample date, and “n” is the total number of sample dates. AUDPC was used to approximate the area beneath the disease progress curve of each four-plant plot and was calculated using the midpoint rule of Campbell and Madden (1990). AUDPC scores are expressed as percentage-days, or in terms of this experiment as the daily accumulation of PSLA. Higher AUDPC scores were produced by susceptible genotypes that exhibited greater symptom intensity or displayed symptoms in early sample dates. Lower AUDPC scores were produced by genotypes that exhibited few to no symptoms or became symptomatic only in late sample dates.

A square root transformation was applied to non-normal AUDPC data and mean transformed values were used for analysis. Data were analyzed using the generalized linear mixed model procedure (PROC GLIMMIX) in SAS v. 9.4 (SAS Institute, Cary, NC). Block was considered a random effect and genotype was considered a fixed effect. Means separation of significant main effects were generated using the DIFF LINES option to perform pairwise t-tests of least-square means ($\alpha = 0.05$). Summary statistics were used to

construct box plots of genotype distributions (Figure 2.2). Least-square means were back-transformed for comparison of genotype means with t-test letter groupings (Table 2.4).

Results

Species identification. Positive species identification was indicated by rapid signal amplification (low C_T mean values) and consistent response among technical replicates (low C_T standard deviation). Non-matching primer sets and isolate gDNA were not amplified and produced no detectable signal, appearing as dashed lines in Table 2.2. The species-specific primers (Table 2.1) were successfully able to differentiate the 10 isolates examined. Six isolates were identified as *C. gloeosporioides* (#28, #46, #58, #84, #91, #92) and four as *C. acutatum* (#34, #40, #89, #90). Real-time PCR species level identification was supported by observations of colony and conidia morphology.

The morphology of *C. gloeosporioides* and *C. acutatum* isolates identified above were identical to those reported previously for each species (Gunnell and Gubler, 1992; Smith and Black, 1990). *C. gloeosporioides* colonies were grey to olive in color with areas of visible orange-colored acervuli. Colonies expanded rapidly on PDA at 25°C and reached the edge of plates in less than seven days. Conidia were cylindrical with both ends rounded. *C. acutatum* colonies were beige to pink in color, turning darker shades of pink to olive over time. *C. acutatum* colonies expanded at a fraction of the rate of *C. gloeosporioides* on PDA at 25°C, requiring approximately 12 to 14 days to reach the edge of plates. Conidia were fusiform in shape and smaller than those of *C. gloeosporioides*, but were produced more abundantly.

Disease assessment. Strawberry plants showed no anthracnose disease symptoms during propagation or maturation in the greenhouse. However, the paraquat assay indicated that low-level quiescent infections were present in strawberry leaf tissue collected prior to inoculation (0 DAI). Quiescent infections were not detected in every sample, but were detected in one or more samples from each genotype. Genotype average PSLA scores at 0 DAI ranged from 1.3% (NCS 10-037) to 6.3% (NCS 10-019). Real-time PCR analysis of isolates from sporulation on paraquat-treated leaflets detected *C. gloeosporioides* in all genotypes but did not detect *C. acutatum* (Table 2.3).

Inoculated plants showed no symptoms after inoculation or for the duration of the experiment. The paraquat protocol allowed visual estimation of PSLA. Disease progress curves were constructed from PSLA scores to chart average disease severity of each genotype across all sample dates (Figure 2.1). PSLA scores of some genotypes rapidly increased after 3 DAI while other genotypes showed relatively flat curves with little increase in disease severity over time.

Analysis of transformed AUDPC means with PROC GLIMMIX indicated a highly significant ($P < 0.0001$) main effect of genotype. Separation of genotype least-squares means by t-test letter grouping ($\alpha = 0.05$) indicated significant differences between genotypes with higher mean AUDPC scores, but genotypes with lower AUDPC means were not significantly different (Table 2.4). Mean AUDPC scores ranged from 8.48 for NCS 10-080 to 23.15 for NCS 10-086. Summary statistics of AUDPC data were used to construct box plots of the distribution of AUDPC mean scores for all eight genotypes (Figure 2.2). The coefficient of variation of the AUDPC data was high at 79.4%.

Discussion

Species identification. Real-time PCR identified the species of most isolates as expected. Isolates from crown tissue and many of the isolates from quiescent leaf infections were identified as *C. gloeosporioides*. Isolates from leaf, petiole, and fruit tissue were identified as *C. acutatum*. Morphological observations of isolates supported the putative results of real-time PCR, matching established characteristics of each species' colony and conidia morphology.

After species confirmation, isolates #28, #58, and #84 were selected for use in this experiment. Their selection was based on observations of elevated pathogenicity in strawberry plantings (M. E. Carnes, personal communication). Isolates #28 and #58 were both isolated from crowns infected during severe ACR epidemics in eastern North Carolina. Isolate #58 in particular is known to be highly pathogenic and has been successfully used in previous inoculation studies (M. E. Carnes, personal communication). Isolate #84 has been previously used in ACR resistance studies and was originally isolated from an infected crown of the cultivar 'Treasure' which is moderately resistant to ACR (Osorio et al., 2014). These highly pathogenic isolates were selected to apply sufficient disease pressure for differentiation of anthracnose resistance phenotypes in this and future studies.

Disease assessment. The paraquat protocol permitted estimates of sporulating leaf area to be made on a panel of eight strawberry genotypes. Although symptoms were not observed in the plant material used in this work, PSLA data suggests the presence of quiescent hemibiotrophic *Colletotrichum* infections at 0 DAI (Fig. 2-1). Average PSLA prior to inoculation varied with genotype but fell between 1.25% and 6.26%. Some samples had

no visible sporulation, but others were observed with PSLA as high as 20%. Real-time PCR analysis of conidia from sporulating paraquat samples from 0 DAI identified the infecting species as *C. gloeosporioides* in all genotypes. While *C. gloeosporioides* sporulation was not present in every sample from 0 DAI, these real-time PCR and paraquat results indicate that *C. gloeosporioides* was present at low levels in plant material of all genotypes prior to inoculation. It is unclear whether prior infection occurred in the field where runners were collected or in the greenhouse during propagation and plant maturation. Observation of these infections in asymptomatic plant material is indicative of the stealthy nature of hemibiotrophic *Colletotrichum* infections and emphasizes the importance of starting with clean plant material in future studies.

Artificial inoculation of plant material was followed by a rapid increase in PSLA of many genotypes between 0 and 6 DAI (Figure 2.1). The genotype most rapidly colonized by *C. gloeosporioides* was NCS 10-086, increasing from an average PSLA of 1.9% at 0 DAI to 31.9% at 6 DAI. Not surprisingly, NCS 10-086 also had the highest mean AUDPC score of 23.15 and was significantly different than all other genotypes produced by the t-test LSMEANS separation in PROC GLIMMIX (Table 2.4). NCS 10-086, NCST 10-032, and NCS 10-080 had wide distributions, which were reflected in the high coefficient of variation (79.4%) of these data (Figure 2.2). A high coefficient of variation signifies low precision of measurement, which could be due to inconsistent inoculation of tissue, non-uniform disease pressure, or errors in visual PSLA estimation.

The four genotypes with lowest AUDPC means were not significantly different from one another which may be due to insufficient disease pressure for means separation of less-susceptible genotypes. NCS 10-080 had the lowest leaf colonization of the eight genotypes

screened with a mean AUDPC of only 8.48. PSLA increased over time in all other genotypes, but no increase was detected in NCS 10-080 during the 21-day screen. In fact, leaf colonization of NCS 10-080 decreased slightly over the course of the experiment from 5.0% prior to inoculation to 3.75% on day 21. A link to NCS 10-080 in the previous study by Rahman *et al.* (2013) may offer a genetic explanation in support of its performance in this study. NCS 10-080 is the open-pollinated progeny of NCC 02-63, the most resistant of 14 genotypes evaluated for hemibiotrophic foliar infection of *C. acutatum* by Rahman *et al.* (2013). The relatedness of NCS 10-080 and NCC 02-63 and their performance in respective screens is noteworthy as they were screened against two different *Colletotrichum* species. While not conclusive evidence of genetic control of foliar resistance, this genetic relatedness supports our finding of low AUDPC scores for NCS 10-080 in the current study and warrants further investigations of resistance to *Colletotrichum* species within strawberry foliage.

Results of this study provide proof-of-concept that the methods used by Rahman *et al.* (2013) to evaluate resistance to hemibiotrophic foliar infections of *C. acutatum* can be applied to evaluate resistance to *C. gloeosporioides*. These results also provide preliminary evidence of variation in resistance to hemibiotrophic foliar infection of *C. gloeosporioides* in the NC State germplasm, but low precision of estimates from the current study suggests improvements in experimental methods are necessary prior to further research.

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Table 2.1. Primer and probe sequences used to identify *Colletotrichum* species level.

Target	Primer/Probe	Orientation	Sequence 5' - 3'	Target region (nucleotides)
<i>Colletotrichum acutatum</i> ^a	ACUT-F1	Forward	CGG AGG AAA CCA AAC TCT ATT TAC A	122-146
	ACUT-R1	Reverse	CCA GAA CCA AGA GAT CCG TTG	192-212
	ACUT-PB	Probe	CGT CTC TTC TGA GTG GCA CAA GCA AAT AAT TAA A	150-183
<i>Colletotrichum gloeosporioides</i> ^a	GLOE-F1	Forward	GGC GGG TAG GGT CYC CG ^b	52-68
	GLOE-R2	Reverse	ACT CAG AAG AAA CGT CGT TAA ATC AG	128-153
	GLOE-PB	Probe	CTC CCG GCC TCC CGC CYC ^b	75-91

^aGarrido *et al.* (2009)

^bY = (C + T)

Table 2.2. Species identification of North Carolina-collected *Colletotrichum* isolates using real-time PCR.

Sample ID	Location isolated	Tissue of origin	Putative ID ^a	<i>C. acutatum</i>		<i>C. gloeosporioides</i>		Real-time PCR outcome
				C _T mean ^b	C _T SD ^c	C _T mean	C _T SD	
NTC	N/A	N/A	N/A	--- ^d	---	---	---	N/A
28	Sanford, NC	crown	<i>C.g.</i> ^e	---	---	23.12	0.30	<i>C.g.</i>
34	Bunn, NC	leaf – quiescent	<i>C.a.</i> ^f	20.53	0.22	---	---	<i>C.a.</i>
40	unknown	clinic sample	<i>C.a.</i>	23.93	0.28	---	---	<i>C.a.</i>
46	Wake Co., NC	leaf – quiescent	<i>C.g.</i>	---	---	23.16	0.06	<i>C.g.</i>
58	Sanford, NC	crown	<i>C.g.</i>	---	---	23.10	0.29	<i>C.g.</i>
84	Franklin, NC	crown	<i>C.g.</i>	---	---	24.36	0.09	<i>C.g.</i>
89	Salisbury, NC	fruit	<i>C.a.</i>	21.14	0.31	---	---	<i>C.a.</i>
90	Kannapolis, NC	petiole	<i>C.a.</i>	20.33	0.31	---	---	<i>C.a.</i>
91	Kannapolis, NC	leaf – quiescent	<i>C.g.</i>	---	---	25.40	0.24	<i>C.g.</i>
92	Salisbury, NC	leaf – quiescent	<i>C.g.</i>	---	---	24.01	0.26	<i>C.g.</i>

^aPutative identification based on host symptomology and isolate morphology.

^bC_T mean = mean C_T score of three technical replicates.

^cC_T SD = standard deviation of three technical replicates.

^dDashed lines indicate target was not detected or did not exceed threshold.

^e*C.g.* = *Colletotrichum gloeosporioides*

^f*C.a.* = *Colletotrichum acutatum*

Table 2.3. Real-time PCR species identification of sporulating leaflets sampled prior to inoculation.

Sample ID	Collected from genotype	Number of bulked plates ^a	<i>C. acutatum</i>		<i>C. gloeosporioides</i>		<i>Colletotrichum</i> species ID
			C _T mean ^b	C _T SD ^c	C _T mean	C _T SD	
NTC	N/A	N/A	--- ^d	---	---	---	not detected
01	NCS 10-019	4	---	---	20.15	0.13	<i>C. gloeosporioides</i>
02	NCS 10-028	4	---	---	22.24	0.34	<i>C. gloeosporioides</i>
03	NCS 10-037	3	---	---	26.18	0.11	<i>C. gloeosporioides</i>
04	NCS 10-080	4	---	---	24.16	0.07	<i>C. gloeosporioides</i>
05	NCS 10-086	4	---	---	28.17	0.09	<i>C. gloeosporioides</i>
06	NCS 10-092	2	---	---	24.78	0.39	<i>C. gloeosporioides</i>
07	NCS 10-142	4	---	---	21.55	0.20	<i>C. gloeosporioides</i>
08	NCST 10-032	3	---	---	22.21	0.06	<i>C. gloeosporioides</i>

^aNumber of bulked plates = number of biological replicates bulked for DNA extraction and real-time PCR analysis. Only non-contaminated plates were bulked.

^bC_T mean = average C_T score of three technical replicates.

^cC_T SD = standard deviation from the mean of three technical replicates.

^dDashed lines indicate target was undetected or did not exceed threshold.

Table 2.4. t-test letter grouping of genotype least-squares means for AUDPC scores. AUDPC scores estimate disease severity over time and are expressed in percentage-days (i.e., the daily accumulation of percent sporulating leaf area). Least-squares means with the same letter are not significantly different ($\alpha = 0.05$).

Genotype	N ^a	Mean AUDPC ^b score	t-test letter groups	
NCS 10-086	8	23.15	A	
NCST 10-032	8	18.65	B	
NCS 10-028	8	15.73	B	C
NCS 10-019	8	14.67	D	C
NCS 10-037	8	11.76	D	E
NCS 10-092	8	11.60	D	E
NCS 10-142	8	11.19	D	E
NCS 10-080	8	8.48	E	

^aN = number of four-plant plots evaluated per genotype on each sample date.

^bAUDPC = Area Under the Disease Progress Curve.

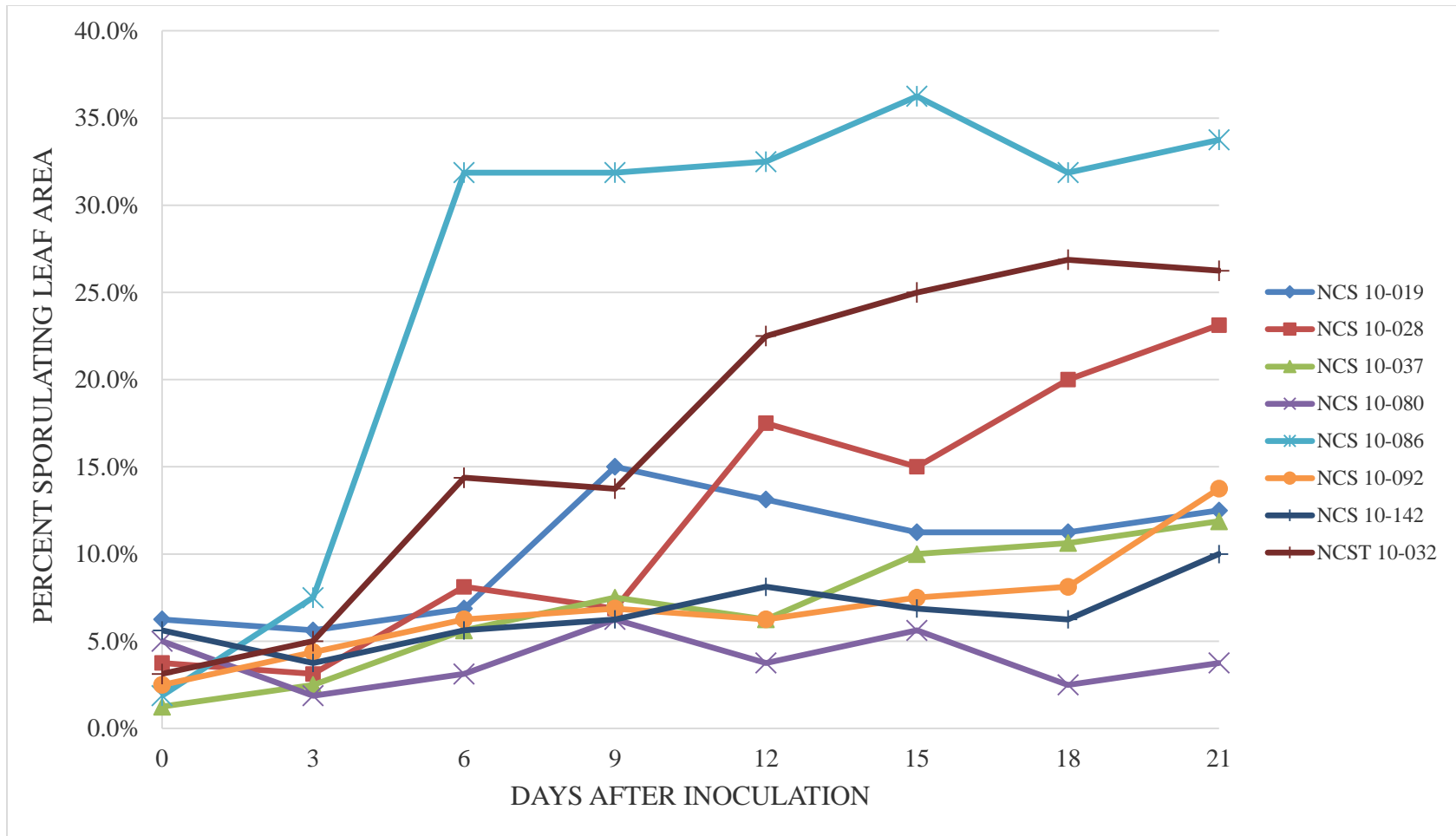


Figure 2.1. Disease progress curves of eight strawberry genotypes inoculated with *C. gloeosporioides*.

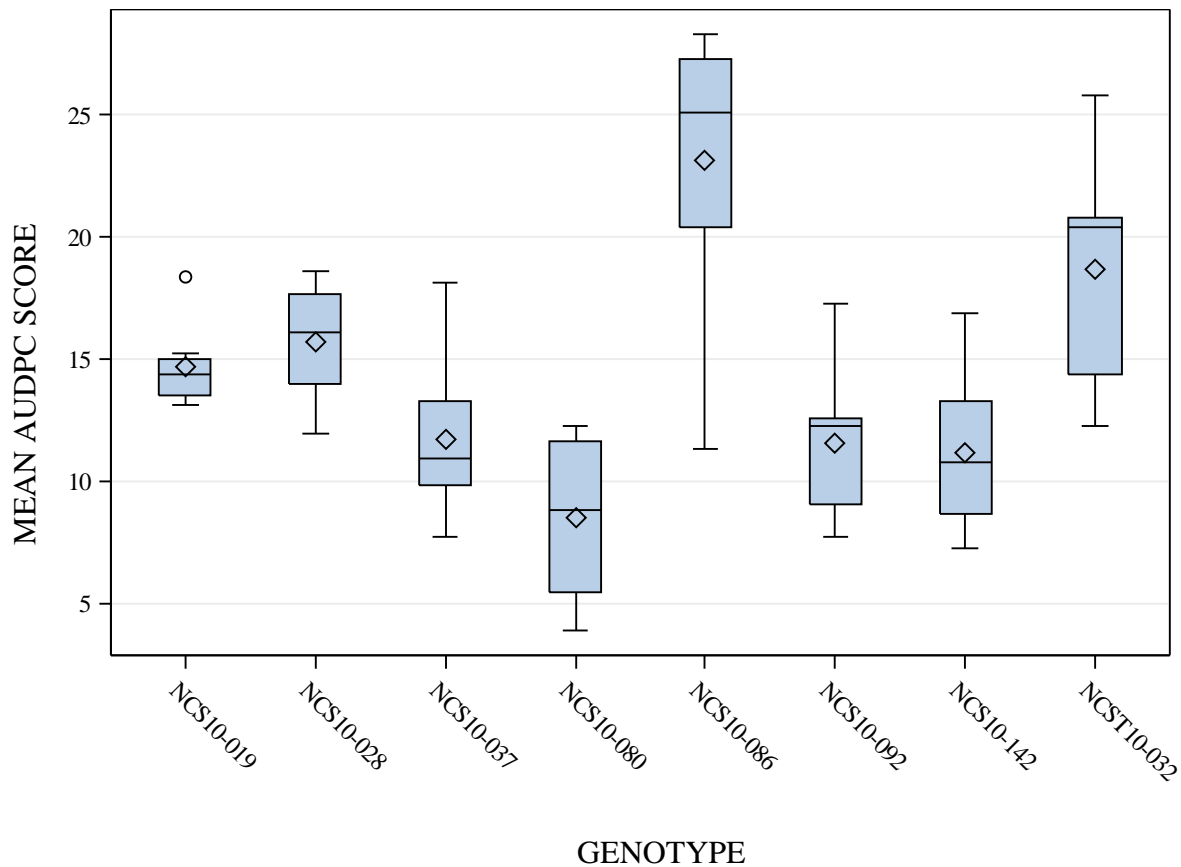


Figure 2.2. Box plot of mean area under the disease progress curve (AUDPC) of eight genotypes evaluated for foliar colonization by *C. gloeosporioides*. For each genotype, the box represents the interquartile range (the range from 25% to 75% of scores), diamonds represents the mean, terminal horizontal lines represent the minimum and maximum values, and circles represent outliers. AUDPC scores are an estimate of disease severity over time and are expressed in percentage-days (i.e., the daily accumulation of percent sporulating leaf area). Higher AUDPC scores represent greater susceptibility to infection. Lower AUDPC scores represent greater resistance to infection.

Chapter Three:

IMPROVED ESTIMATES OF HEMIBIOTROPHIC *COLLETOTRICHUM* INFECTIONS IN STRAWBERRY FOLIAGE THROUGH IMAGE-BASED ANALYSIS OF PERCENT SPORULATING LEAF AREA

Abstract

Clonal plants of the cultivar ‘Chandler’ were inoculated with two *Colletotrichum* species (*C. acutatum* and *C. gloeosporioides*) at four inoculum concentrations (UTC, 1×10^4 , 1×10^5 , and 1×10^6 conidia·mL⁻¹) to identify experimental parameters to uniformly induce high-level infection of both *Colletotrichum* species. An incubation period was implemented with 48-h of increased temperature and humidity to encourage germination of conidia and establishment of hemibiotrophic foliar infections. Leaf samples were collected every three days for 10 sample dates. The experiment was conducted twice. Imaging technology was utilized to improve precision and accuracy of percent sporulating leaf area (PSLA) measurements. Images were captured and digitally enhanced to increase contrast between *Colletotrichum* acervuli and the background leaf color of paraquat-treated leaves. The macro ‘Phenotype Quant’ developed for the image-processing software ‘ImageJ’ was used to analyze and measure diseased and total leaf surface area in each image. PSLA was calculated from the ratio of disease to total leaf surface area. Image-based measurements were strongly correlated ($r=0.91$) to visual estimates. However, the accuracy of visual

estimation varied with different patterns of sporulation and tended to underestimate higher infection severities. Analysis of image-based measurements found significant differences between experiments, which were likely due to differences in environmental conditions and resulting effects on plant physiology. Inoculation during the winter (Experiment 1) produced only a fraction of the disease severity compared to inoculation during the spring (Experiment 2). Better separation of means due to the effect of inoculum concentration in Experiment 2 suggest that infections are more uniformly established and differences in severity more easily distinguished in leaf tissue from actively-growing plants. The 1.0×10^6 conidia·mL⁻¹ level of inoculum concentration produced high levels of disease severity for *C. gloeosporioides* (55.80%) and *C. acutatum* (56.66%) in the cultivar ‘Chandler’. This suggests that inoculations at this level will best evaluate the severity of hemibiotrophic infections across both *Colletotrichum* species and should provide adequate inoculum density to evaluate a range of resistance phenotypes in future studies.

Introduction

Anthracnose crown rot (ACR) and anthracnose fruit rot (AFR) are two of the most challenging diseases of strawberry production around the world and are a limiting factor to strawberry production in North Carolina (Poling, 2008). Symptoms of ACR and AFR can be incited by any of three *Colletotrichum* species, though two of these are predominantly responsible for anthracnose incidence in North Carolina. ACR is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and this pathogen kills plants by rapidly invading and producing a reddish-brown marbled necrosis of crown tissue, resulting in wilt and eventual

collapse of the plant (Rahman et al., 2015). AFR is caused by *C. acutatum* J.H. Simmons and produces dark sunken lesions on developing fruit that render them unmarketable (Freeman and Katan, 1997; Rahman et al., 2013).

ACR and AFR are most prevalent in the southeastern United States where warm subtropical conditions and abundant rainfall favor the pathogen's rapid growth and dissemination (Gupton and Smith, 1991; MacKenzie et al., 2006; Rahman et al., 2015). Losses due to ACR and AFR are not typically experienced in the same location annually. Rather, the location and timing of epidemics depend on the health and distribution of nursery stock and the presence of environmental conditions conducive to disease development. In epidemic years it is estimated that *C. gloeosporioides* has been responsible for 50-80% of plant loss in nurseries and 40-50% of yield loss in fruit production fields (Howard et al., 1992; Xie et al., 2010). Similarly, reports of 50% fruit loss due to *C. acutatum* are not uncommon (Howard et al., 1992) and are the greatest source of economic loss due to anthracnose on strawberry (Peres et al., 2005; Smith, 2008).

C. gloeosporioides and *C. acutatum* are hemibiotrophic pathogens that employ a two-phase infection strategy: a preliminary asymptomatic biotrophic phase followed by a necrotrophic phase in which tissue damage and economic impacts are incurred (Arroyo et al., 2005; Gan et al., 2013; Muench et al., 2008; Perfect et al., 1999). Quiescence contributes to the overall success of a hemibiotrophic infection strategy and is defined as an asymptomatic period between initial host infection and the activation of necrotrophic behavior (Prusky et al., 2013). Germinating conidia may produce disease symptoms immediately but are arguably more destructive when symptom appearance is delayed by the establishment of quiescent hemibiotrophic infections, permitting the widespread dissemination of

asymptomatic transplants to fruiting fields (Leandro et al., 2003; Smith, 2008).

Asymptomatic nursery stock carrying quiescent *Colletotrichum* infections are often the most important source of inoculum in fruiting fields and have been implicated in many anthracnose epidemics in the southeastern United States (Delp and Milholland, 1980; Leandro et al., 2003; MacKenzie et al., 2009; Poling, 2008; Smith, 2008). Once inoculum is introduced to a production field, it can continue to proliferate through secondary conidiation to initiate new hemibiotrophic infections or can survive epiphytically on leaf tissue (Leandro et al., 2001; Legard, 2000).

The majority of North Carolina's strawberry acreage is planted in the annual hill or plasticulture production system that became feasible in North Carolina largely due to the release of two June-bearing strawberry cultivars, 'Chandler' and 'Camarosa', developed by the University of California. These cultivars are widely adapted throughout North Carolina and produce excellent yield and fruit quality for six weeks or more in plasticulture production systems (Poling, 1993). However, 'Chandler' and 'Camarosa' are highly susceptible to ACR and AFR and commonly suffer losses to these fungal pathogens (Rahman et al., 2013).

Overall incidence and severity of ACR and AFR are impacted by several resistance mechanisms including direct resistance of the crown and fruit tissue as well as rate-limiting mechanisms that inhibit proliferative components of the disease cycle such as colonization of foliar tissue or proliferation through secondary conidiation. Previous studies have identified usable genetic variation in resistance to ACR and AFR based on crown and fruit tissues phenotypes (Ballington et al., 2002; Gupton and Smith, 1991; MacKenzie et al., 2006; Osorio et al., 2014; Smith and Spiers, 1982). However, few studies have characterized resistance to *Colletotrichum* colonization in strawberry leaf tissue. Rate-limiting resistance has been

described in leaf tissue of other crop species and has been deployed in resistant germplasm to inhibit the spread of inoculum to economically valuable tissues to reduce the risk of disease epidemics (Kirk et al., 2001; Liu and Halterman, 2009; Reddy and Singh, 1993; Simko et al., 2006; Tooley and Grau, 1984).

Very limited work has been conducted to investigate resistance to hemibiotrophic *Colletotrichum* infections in strawberry foliage, a common and critical host tissue in the disease cycles of both *C. gloeosporioides* and *C. acutatum* (Gan et al., 2013; Rahman et al., 2013). A recent study by Rahman *et al.* (2013) evaluated 14 strawberry genotypes for resistance to AFR and hemibiotrophic foliar infections caused by *C. acutatum*. This study found that severity of hemibiotrophic foliar infections was not well correlated with severity of AFR and suggested that resistance to *C. acutatum* may be controlled independently in fruit and leaf tissue (Rahman et al., 2013).

In a proof-of-concept study conducted by Jacobs *et al.* (unpublished), we determined that the methods described by Rahman *et al.* (2013) could be used to evaluate hemibiotrophic infections of *C. gloeosporioides* in strawberry leaf tissue. A paraquat protocol was used to estimate percent sporulating leaf area (PSLA) of assayed asymptomatic leaf tissue. Area under the disease progress curve (AUDPC) scores were calculated from PSLA values and were used to compare *C. gloeosporioides* intensity over time for a small panel of NC State germplasm. Genotypes differed significantly for AUDPC mean scores and preliminary estimates of variation in resistance were observed within the genotypes tested. Significant separation of high AUDPC means versus poor separation of low AUDPC means may indicate that genotypes with low AUDPC scores exhibit similar (resistant) responses to infection. However, this result could also be produced by insufficient disease pressure to

challenge more resistant genotypes and encourage separation of low-end AUDPC genotype means.

The *Colletotrichum*-strawberry pathosystem is inherently variable and is predisposed to non-uniform expression of disease symptoms. Smith and Black (1987) suggested that apparent field-level resistance may not be representative of true genetic resistance due to high genotype x environment interactions, though previous resistance studies have been successfully carried out in both field and greenhouse settings. Greenhouses offer many advantages to disease resistance studies when there is a critical need to minimize genotype x environment effects or to meet certain parameters for disease establishment. Previous studies of resistance to *Colletotrichum* species have utilized greenhouses or other controlled environments to increase disease pressure and reduce environmental variability which can be problematic in field studies (Giménez and Ballington, 2002; Lerceteanu-Köhler et al., 2005; Lewers et al., 2007; Rahman et al., 2013; Smith and Black, 1987). Inoculum concentrations used in these studies have ranged from 5.0×10^4 to 6.0×10^6 conidia·mL⁻¹, but most use concentrations close to 1.0×10^6 conidia·mL⁻¹. An incubation period is generally used after inoculation to increase disease pressure and encourage penetration of host tissues. Most incubation periods last for 48 hours during which temperature is held between 25 and 32°C and humidity is increased to maintain leaf wetness.

The study by Rahman *et al.* (2013) established a preliminary protocol for scoring resistance to *Colletotrichum* foliar infections, but specific parameters required to reliably induce and evaluate this phenotype remain undetermined. A high coefficient of variation in the study by Jacobs *et al.* (unpublished) indicated low precision of PSLA estimates in that work. Precision may be increased through replication, refined inoculation protocols, and

standardized environmental parameters. Additional consideration should be given to improve upon the accuracy of direct visual estimates of PSLA that were used by Rahman *et al.* (2013) and Jacobs *et al.* (unpublished). The observed phenotype, PSLA, occurs on a continuum and is more accurately assessed using a quantitative continuous scale (Lynch, 1998; Parlevliet, 1979). In an effort to improve the accuracy and precision of estimates of PSLA, the objectives of this study were to 1) identify experimental parameters to uniformly induce high-level hemibiotrophic infections of *C. gloeosporioides* and *C. acutatum* in leaves of cultivar ‘Chandler’ and 2) develop methods to evaluate PSLA on a quantitative continuous scale.

Materials and Methods

Plant production. Runner tips were collected in July, 2012 from apparent disease-free mother plants of the cultivar ‘Chandler’ in a field planting at the Piedmont Research Station in Salisbury, NC. Tips were transported to a greenhouse in Kannapolis, NC and all but the youngest expanded leaf were removed to minimize importing inoculum from the field. Cleaned tips were planted in 50-cell plug trays containing Fafard® 3B mix (Sun Gro Horticulture, Agawam, MA) and rooted under periodic mist (30 second duration, 10 minute interval) in a $21 \pm 5^{\circ}\text{C}$ greenhouse for one week until roots developed. After two additional weeks, disease-free (symptomless) rooted plug plants were transplanted into 10 cm pots containing Fafard® 3B potting mix, grown to maturity during the fall, and overwintered in a $10 \pm 5^{\circ}\text{C}$ greenhouse. The plants were hand watered 3 to 4 times per week and fertilized with a solution of Jack’s Professional® LX Ca-Mg 15-5-15 fertilizer (JR Peters Inc.,

Allentown, PA) via a Dosatron® injector system calibrated to dispense 50 ppm N. Plants were cleaned a second time one month prior to inoculation by removing the oldest leaves, further reducing the risk of quiescent infections in starting material. At this time plants were moved to a $25 \pm 5^\circ\text{C}$ greenhouse to encourage vegetative growth.

Experiment design. The experiment was designed as a randomized complete block split-split plot design with 320 clonal plants of ‘Chandler’ divided equally between 4 blocks. Each block was divided into 2 main plots to which *Colletotrichum* species (*C. gloeosporioides* and *C. acutatum*) were randomly assigned. Main plots were each divided into 4 split-plots to which inoculum concentrations (UTC, 1×10^4 , 1×10^5 , and 1×10^6 conidia·mL⁻¹) were randomly assigned. Sample dates were treated as a split-split plot factor in this experiment and were randomly assigned to individual plants. The experiment was run twice, from March 22, 2013 to April 22, 2013 (Experiment 1) and again from May 17, 2013 to June 13, 2013 (Experiment 2). Treatments were randomly assigned to main plots, split-plots, and split-split plots separately within and between experiments.

Inoculum preparation. Two weeks prior to inoculation a mixed isolate inoculum of *C. gloeosporioides* and *C. acutatum* was prepared to represent diverse strains found in strawberry plantings. Isolates #28, #58, and #84 were selected for *C. gloeosporioides* and isolates #34, #40, and #89 were selected for *C. acutatum* based on confirmation of *Colletotrichum* species level identity in previous work (Jacobs et al., unpublished). The selected isolates were originally collected from infected strawberry tissue in multiple locations across North Carolina and are known to be highly pathogenic on strawberry (M.E. Carnes, personal communication). Four of the six selected isolates were provided by the F.J. Louws plant pathology lab at NC State where they were kept in long-term storage to

maintain pathogenicity. Stored isolates were revived by transferring a small (10 mm²) section of infested filter paper to new plates of half strength PDA and incubating these at 25°C for four days under 12-hour fluorescent lighting. New cultures were made from the original plate of each isolate by transferring mycelia with a sterile probe to new plates of half strength PDA. Eight plates of each isolate were made and cultures were incubated at 25°C for 10 to 14 days under 12 hour fluorescent lighting. Conidia were collected from mature cultures of all isolates by flooding the plate with distilled water containing 5 drops of Tween 20 per liter of water and disturbing the mycelium with a glass rod to suspend conidia. Conidial suspensions were filtered through a doubled layer of cheesecloth to remove cellular debris. Serial dilutions of each isolate were prepared using a hemocytometer to achieve 1×10^6 , 1×10^5 , and 1×10^4 conida·mL⁻¹. Mixed isolate inoculum was made of each *Colletotrichum* species by combining equal volumes of the three isolates at each dilution level. This produced 750 mL of inoculum at 1×10^6 , 1×10^5 , and 1×10^4 conida·mL⁻¹ for each *Colletotrichum* species. The mixed isolate suspensions were checked by hemocytometer to confirm final inoculum concentrations.

Inoculation. Two disease chambers with PVC frames and vinyl plastic sides were constructed prior to inoculation to create a high-humidity microenvironment around plants during incubation. These chambers were sized to fit on greenhouse benches and had adjustable sides to regulate airflow. Chambers were mounted with fog nozzles (Netafim™ USA, Fresno, CA) connected to a greenhouse irrigation controller. These nozzles emitted a fine (65 micron) fog, allowing humidity to be increased and leaf wetness maintained without splashing or washing conidia from leaves.

Experiment 1 plants were inoculated on March 22, 2013 and Experiment 2 on May

17, 2013. To prevent cross-contamination from inoculum drift, plants assigned to each treatment combination were placed on separate benches and greenhouse fans were turned off during inoculation. Inoculations were performed by misting the foliage of each treatment combination with inoculum of the appropriate species and concentration. Inoculum was applied via handheld sprayer (Solo® model 419) just until runoff to ensure even disease pressure across all leaf surfaces. Untreated controls (UTC) were the first treatment applied and were inoculated with ddH₂O. *C. gloeosporioides* was applied next, starting with lowest concentration and ending with the highest. *C. acutatum* was applied last, starting with the lowest concentration and ending with the highest. The sprayer was completely purged of inoculum between applications of the same species and was washed with 70% ethanol and dried between applications of different species. Greenhouse fans were turned on once inoculations were complete and leaves were allowed to dry for 20 minutes to prevent cross-contamination during placement in disease chambers according to the experiment design. Plants were placed in order of decreasing inoculum concentration, ending with the placement of UTCs to avoid residual inoculum dripping onto them. Space was left between treatment combinations to avoid leaves touching and cross-contamination.

Disease chamber sides were closed and plants were incubated for 48 hours at 28 ± 2°C with periodic fog (3 sec duration, 5 min interval) to maintain humidity near 100%. Disease chamber conditions were monitored using two digital household temperature and humidity gauges. Following incubation, fogging was discontinued and disease chamber sides were raised to increase air-flow. Relative humidity was not controlled post-incubation. Day/night temperatures were set to 28/20°C for the remainder of the experiment to keep environmental conditions within the range of the North Carolina piedmont during May and

June when ACR and AFR are problematic. Temperature and humidity data were collected via a Vantage Pro2™ weather station module (Davis Instruments Corp., Hayward, CA) located in the greenhouse. Plants received natural daylight and photoperiod during the course of the experiment.

Disease assessment. Starting plant material was subsampled and screened for pre-existing quiescent infections one week prior to inoculation. Samples were collected on 10 sample dates during Experiment 1 (3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 DAI) and eight sample dates during Experiment 2 (3, 6, 9, 15, 18, 24, and 27 DAI). The 4th and 7th sample dates were eliminated from Experiment 2 due to insufficient plant material. A single plant was collected from split-split plots assigned to each sample date. All inoculated leaves were removed from sampled plants, placed in labeled plastic bags, and temporarily held in a 4°C refrigerator prior to analysis.

Leaves were evaluated for severity of fungal colonization using a modification of the paraquat protocol successfully used by Rahman *et al.* (2013) and Jacobs *et al.* (unpublished) to detect hemibiotrophic infections of *C. acutatum* and *C. gloeosporioides* in strawberry leaf tissue. In this protocol the herbicide paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride) was used to rapidly induce senescence of leaf tissue and trigger the production of orange-colored acervuli from quiescent and otherwise asymptomatic fungal colonists (Cerkauskas and Sinclair, 1982). Leaves were surface sterilized by immersing in 70% ethanol for 15 seconds, 10% bleach for 60 seconds, and rinsed twice in ddH₂O. Leaflets were then treated with paraquat by immersing them for one minute in a solution of 20 mL Gramoxone® (30.1% paraquat dichloride) / 1 liter ddH₂O and rinsing a final time in ddH₂O. To increase throughput, 26 x 30 cm plastic zipper bags were used in place of incubator

(crisper) boxes. Bags were labeled and lined with a doubled layer of 25 x 25 cm paper towel. Paraquat-treated leaves were placed adaxial side up in a single layer inside prepared bags and bags were left unsealed to allow gas exchange (Figure 3.8A). Bags were incubated in a growth room (Harris Environmental Systems Inc., Andover, MA) set to maintain 25°C and 40% relative humidity. Twenty-four hours of strong supplemental lighting ($175 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was provided to encourage rapid and uniform senescence of leaf tissue (personal observation) followed by 72 hours of ambient room lighting for acervular development (Figure 3.8B). Samples were removed from the growth room after 96 hours for visual and image-based evaluation of PSLA.

Image analysis. Imaging software was tested in addition to direct visual estimation to determine if it could be used to increase the accuracy and precision of PSLA measurements. A macro plugin called “Phenotype Quant” was developed by Ahmed Abd-El-Haliem (2012) as an unbiased and semi-automated method to quantify visible disease symptoms using the imaging software “ImageJ”. Phenotype Quant was coded to quantify infection severity of transgenic GUS-stained *Cladosporium fulvum* in cleared (destained) tomato leaves, but it can be used to quantify most diseases that produce visible symptoms in contrast to healthy tissue (Abd-El-Haliem, personal communication). Phenotype Quant uses filters to quantify the area (in pixels) of diseased leaf tissue and total leaf tissue, distinguishing each by user-defined colors. These two values can be used to calculate the diseased percentage of total leaf area. Though leaf and disease colors are easily modified to accommodate other tissues and symptoms, the program is unable to differentiate symptoms that are close to the background leaf color.

In preliminary tests of *Colletotrichum* infections in strawberry leaf tissue, the

Phenotype Quant macro was able to quantify PSLA in some samples images but not consistently across a set of sample images. The colors of *C. gloeosporioides* and *C. acutatum* acervuli (orange to brown) were too close in color to paraquat-treated leaves (copper to brown). Thresholds could be adjusted to accurately estimate PSLA of a single image, but sample-to-sample variation in background leaf color did not accommodate quantification of a set of images. A workaround was implemented in which sporulating lesions were digitally highlighted prior to ImageJ analysis, allowing accurate quantification of PSLA in sample images (Figure 3.9).

Samples were placed on a blue background with plot number and sample date visible. PSLA was visually estimated using a linear scale (0-100%) with equal 5% intervals. Each sample was photographed using a Panasonic Lumix DMC-ZS10 point-and-shoot camera (Panasonic Corporation, Osaka, Japan) under standardized lighting. Images were transferred to a tablet (Samsung, Seoul, South Korea) and sporulating lesions highlighted using a stylus. Processed images were analyzed using the Phenotype Quant macro in ImageJ to compute the diseased and total leaf areas. The program analyzed each image in succession and output a list of area measurements.

Analysis. PSLA was calculated from ImageJ data in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA) as follows: $PSLA = \left(\frac{\text{Diseased Leaf Area}}{\text{Total Leaf Area}} \right) \times 100$. The relationship between actual disease severity (PSLA) and visual estimates was analyzed using the correlate procedure (PROC CORR) in SAS v. 9.4 (SAS Institute, Cary, NC). A square root transformation was applied to non-normal ImageJ data and transformed PSLA values were used for analysis. Data were analyzed using the mixed model procedure (PROC

MIXED) in SAS. Block was considered a random effect, while experiment, *Colletotrichum* species, inoculum concentration, and DAI were considered fixed effects. Significant interactions were further analyzed using the SLICE option. Significant effects were compared using least squares means, which were back-transformed for comparison.

Results

Experiment 1 disease chambers maintained $25 \pm 2^\circ\text{C}$ and 100% relative humidity during the incubation period. The post-incubation environment for Experiment 1 averaged $25 \pm 2^\circ\text{C}$ with 60% average relative humidity. In Experiment 2, disease chambers maintained $28 \pm 2^\circ\text{C}$ and humidity averaged 82%. Foggers maintained constant leaf wetness and the 65 micron water particles minimized splashing, runoff, and loss of inoculum. Visible symptoms were not observed on *C. acutatum*-inoculated plants in either experiment. However, *C. gloeosporioides*-inoculated leaves developed classic ‘ink spot’ or black leaf spot symptoms within 12 hours of inoculation in both experiments (Figure 3.7). Leaf spots appeared during the 48 hour incubation period but no new spots appeared after this time. A few plants developed symptoms of ACR late in the experiment and collapsed due to this disease. No sporulation was detected in samples collected prior to inoculation. Sporulation was seen in a few samples from untreated controls but was confined to one or two small lesions, likely resulting from accidental transfer of conidia during inoculation.

A linear relationship was observed between ImageJ data and visual estimation of sporulating leaf area (Figure 3.1). The correlation between data collection methods was high ($r=0.91$, $P<.0001$) but visual estimates generally underestimated the actual disease severity

calculated by ImageJ, especially at higher infection severities.

There were significant interactions between experiment and *Colletotrichum* species ($P=0.0071$) as well as experiment and inoculum concentration ($P=0.0001$) (Table 3.1). The interaction between experiment and *Colletotrichum* species is due to a change in species rank between Experiments 1 and 2 (Figure 3.3). Both *C. acutatum* and *C. gloeosporioides* produced significantly different responses between Experiments 1 and 2 (Figure 3.3). *C. acutatum* had an average PSLA of 6.09% in Experiment 1 compared to 27.46% in Experiment 2. *C. gloeosporioides* had an average PSLA of 0.76% in Experiment 1 versus 36.16% in Experiment 2. Average PSLA scores between *Colletotrichum* species were not significantly different within Experiment 2 (Figure 3.3). Inoculum concentration performed as expected in Experiment 2 with significant differences in PSLA produced between each tenfold increase in inoculum concentration. However, the same was not true for Experiment 1, which had no significant differences between inoculum concentrations (Table 3.2, Figure 3.4). Experiment 1 PSLA scores were lowest at 1.0×10^4 conidia·mL⁻¹ and highest at 1.0×10^5 with 1.0×10^6 falling between these two (Figure 3.5). Within-experiment effects of *Colletotrichum* species and concentration are shown for Experiment 1 (Figure 3.5) and Experiment 2 (figure 3.6).

The main effect of concentration was significant ($P<0.0001$) (Table 3.1) and PSLA increased with increasing inoculum concentration (Figure 3.2). *Colletotrichum* species did not have a significant effect ($P=0.2128$) nor did its interaction with inoculum concentration ($P=0.2841$). The effect of experiment was significant ($P<0.0001$), with an overall PSLA average of just 2.78% for Experiment 1 versus 31.66% for Experiment 2. There was a significant effect of DAI ($P=0.0012$) and a significant interaction of DAI and *Colletotrichum*

species (Table 3.1). However, no general time trends were observed which explained this main effect or interaction and results were averaged over DAI.

Discussion

A comparison of visual estimates and ImageJ analysis of PSLA found that the two measures were highly correlated ($r=0.91$, $P<.0001$). However, visual estimation had a tendency to underestimate PSLA in cases of higher infection severity, as indicated by a deviation from 1.0 in the slope of the fitted line in Figure 3.1. This trend was investigated by selecting data points where visual estimates underestimated the actual disease severity and observing the corresponding images. Underestimation primarily occurred in samples where leaves had small but widespread lesions over all leaf surfaces. Visual estimation of this pattern of sporulation is difficult and falls short of image-based measurements. In contrast, leaves with only a few large blotches were overestimated by visual ratings (Figure 3.9).

While a significant main effect of *Colletotrichum* species was not found, there was a significant interaction between *Colletotrichum* species and experiment ($P=0.0071$) (Table 3.1, Figure 3.3). *C. gloeosporioides* and *C. acutatum* responded very differently in Experiment 1. *C. acutatum* was more successful at establishing foliar infections than *C. gloeosporioides* across all inoculum concentrations and there were no significant differences in concentration across *Colletotrichum* species (Figure 3.5). On the other hand, *C. gloeosporioides* and *C. acutatum* responded similarly across concentrations in Experiment 2, especially at the highest concentration (1.0×10^6) (Figure 3.6). The uppermost level of

inoculum concentration uniformly produced high levels of disease severity between *C. gloeosporioides* (55.80%) and *C. acutatum* (56.66%) in the cultivar ‘Chandler’. This supports standardized inoculations at 1.0×10^6 to best evaluate severity of hemibiotrophic infections across both *Colletotrichum* species. The elevated PSLA scores produced by 1.0×10^6 conidia·mL⁻¹ in the susceptible cultivar ‘Chandler’ should provide adequate inoculum density to evaluate a range of resistance phenotypes in future studies.

A significant main effect was found for experiment ($P < 0.0001$) (Table 3.1) with disease severity of Experiment 1 averaging 2.78%, only a fraction of the 31.67% average severity of Experiment 2. Although these experiments were conducted in the same greenhouse and measures taken to control environmental conditions, differences in plant physiology between the dates of Experiments 1 and 2 may have played a role in the amount of disease observed. Despite growing in a warm environment ($25 \pm 5^\circ\text{C}$) for one month prior to inoculation, plants in Experiment 1 appeared dormant with a low growth habit and small, leathery leaves. Dormancy in strawberries is a period of rest controlled by both temperature and day length, with the majority of leaves produced during long days (≥ 14 hours of day length) (Darrow, 1936; Hancock, 1999). Experiment 1 was inoculated on March 22, 2013 when day length in Kannapolis, NC was 12 hours and 12 minutes. Although day length was increasing rapidly during this time of year, plants were only just beginning to break dormancy. Leaves of dormant strawberry plants were produced slowly and were leathery, smaller in size, and borne on short petioles in comparison to the leaves of actively-growing plants. Plants in Experiment 2 were inoculated on May 17, 2013 when day length was 14 hours and 5 minutes and plants were actively growing with large leaves borne on tall, upright petioles. The dramatic difference in average PSLA scores between experiments was likely

due to differences in the physiological state of strawberry leaf tissue and its susceptibility to infection by *C. gloeosporioides* and *C. acutatum* during incubation. Alternatively, differences in leaf physiology may have influenced the programmed differentiation and infection strategy of germinating conidia. Topographical features and chemical signals of host plant surfaces are known to play a role in determining the fate of germinating *Colletotrichum* conidia (Latunde-Dada, 2001; Prusky et al., 2013).

There was a significant main effect of inoculum concentration ($P < 0.0001$) (Table 3.1) and PSLA was seen to increase incrementally between 1.0×10^4 and 1.0×10^6 when averaged over all other factors (Figure 3.2). PSLA response to inoculum concentration was very different between experiments and produced a significant experiment by inoculum concentration interaction ($P = 0.0001$) (Table 3.2, Figure 3.4). This interaction was largely due to a lack of means separation from the limited disease development in Experiment 1 versus distinct separation of inoculum concentration means in Experiment 2 (Table 3.4). Separation of inoculum concentration means in Experiment 2 suggested that environmental conditions present during this experiment are desired for differentiating levels of hemibiotrophic infection severity.

Results from this study underline the importance of the physiological state of leaf tissue in conducting resistance studies, as dormant leaves are colonized at a much lower level than leaves from actively-growing plants. Separation of inoculum concentration means in Experiment 2 suggest that differences in leaf colonization are more easily distinguished in leaf tissue from actively-growing plants. Within Experiment 2, the 1.0×10^6 level of inoculum concentration produced similar average PSLA scores across *Colletotrichum* species, indicating that this concentration may be used to evaluate a range of resistance

phenotypes to both *Colletotrichum* species. Both temperature and day length are important in breaking dormancy and should be considered when preparing plant material in the future. A comparison of visual estimation and image-based calculation of PSLA found the two measurements to be highly correlated but the accuracy of visual estimates was reduced at higher disease severities. The underestimation of disease at higher severities appeared dependent on the pattern of sporulation. Visual estimation of PSLA is faster than the current method of analysis with ImageJ and could be used to evaluate larger studies with clonal replication without significant loss of accuracy. However, the increased accuracy and precision of image-based analysis is advantageous when evaluating smaller trials or segregating populations where clonal replication is not possible.

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Table 3.1. Analysis of variance of the effects of experiment, sample date, *Colletotrichum* species and inoculum concentration on severity of foliar hemibiotrophic infections in cultivar ‘Chandler’. Severity was evaluated via image-based analysis as the percentage of total leaf area with sporulation.

Source of variation	df	<i>F</i>	<i>P</i> > <i>F</i>
Species	1	1.94	0.2128
Concentration	2	22.34	<0.0001
Species x Concentration	2	1.33	0.2841
DAI ^a	7	3.55	0.0012
Species x DAI	7	3.80	0.0006
Concentration x DAI	14	1.00	0.4511
Species x Concentration x DAI	14	0.71	0.7677
Experiment	1	143.86	<0.0001
Experiment x Species	1	16.05	0.0071
Experiment x Concentration	2	13.05	0.0001
Experiment x Species x Concentration	2	1.44	0.2571

^aDAI = Days After Inoculation.

Table 3.2. t-test letter grouping of least-squares means for inoculum concentration over Experiments 1 and 2. PSLA scores represent the average percent sporulating leaf area for each level of experiment and inoculum concentration. Least-squares means with the same letter are not significantly different ($\alpha = 0.05$).

Inoculum concentration	Experiment 1		Experiment 2	
	PSLA ^a estimate	Letter groups	PSLA estimate	Letter groups
1.0 x 10 ⁶	3.17	D	56.23	A
1.0 x 10 ⁵	3.82	D	31.50	B
1.0 x 10 ⁴	1.62	D	14.21	C

^aPSLA = percent sporulating leaf area.

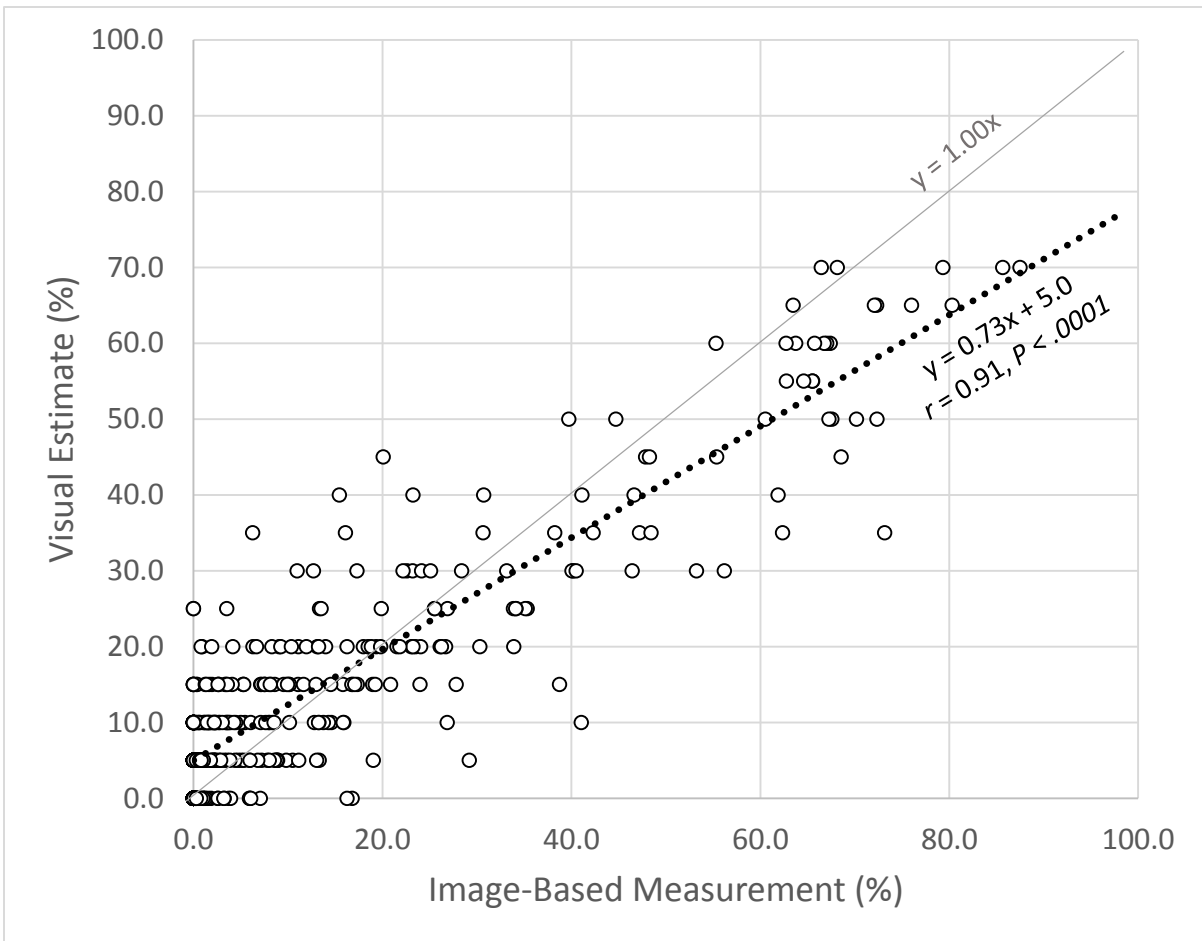


Figure 3.1. The relationship between actual disease severity (image-based measurement) and direct visual estimates. Actual disease severity is calculated by the image-processing software ImageJ to deliver percent sporulating leaf area (PSLA) measurements. The two measurements are highly correlated but visual estimates underestimate actual disease severity at higher levels of infection. This deviation is seen in the figure above as the deviation from a slope of 1.00.

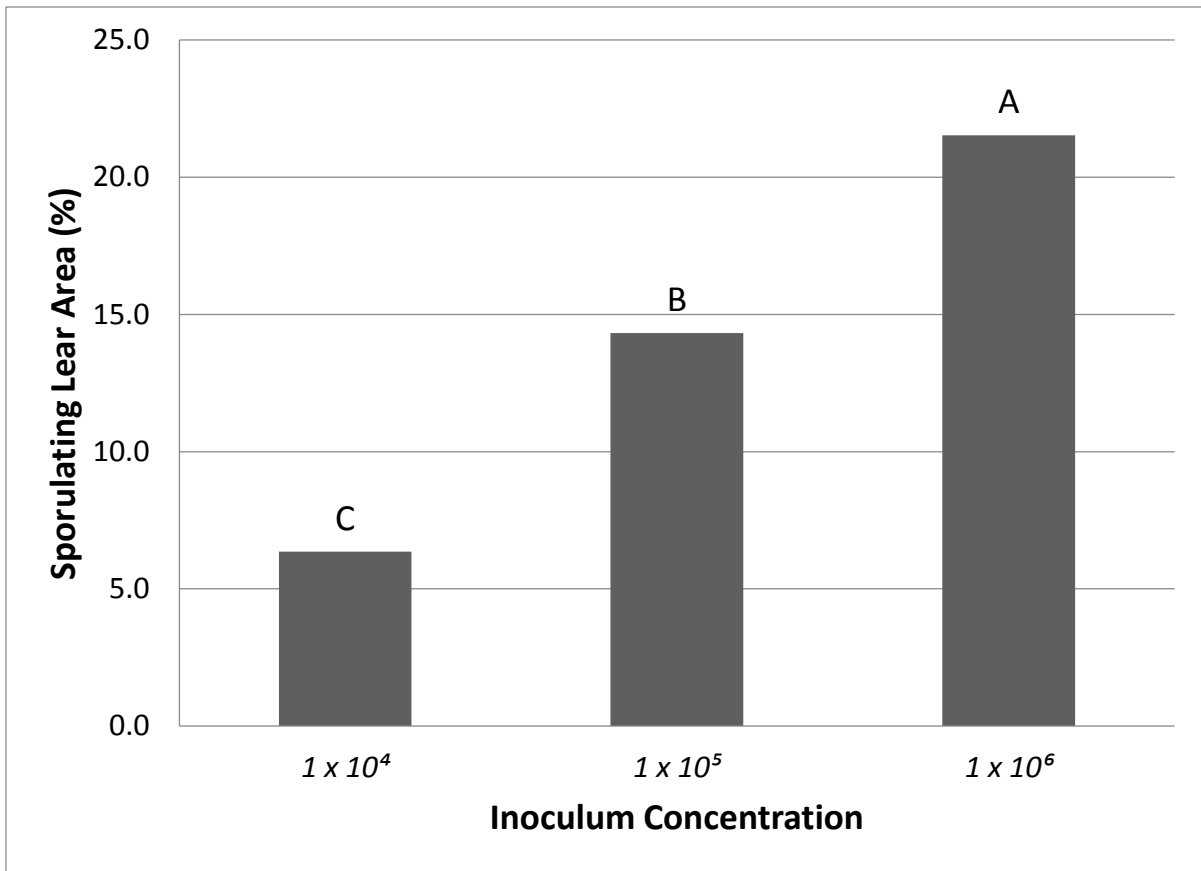


Figure 3.2. Percent sporulating leaf area scores showing the effect of inoculum concentration, averaged over all other factors. Treatments with the same letter are not significantly different ($\alpha = 0.05$).

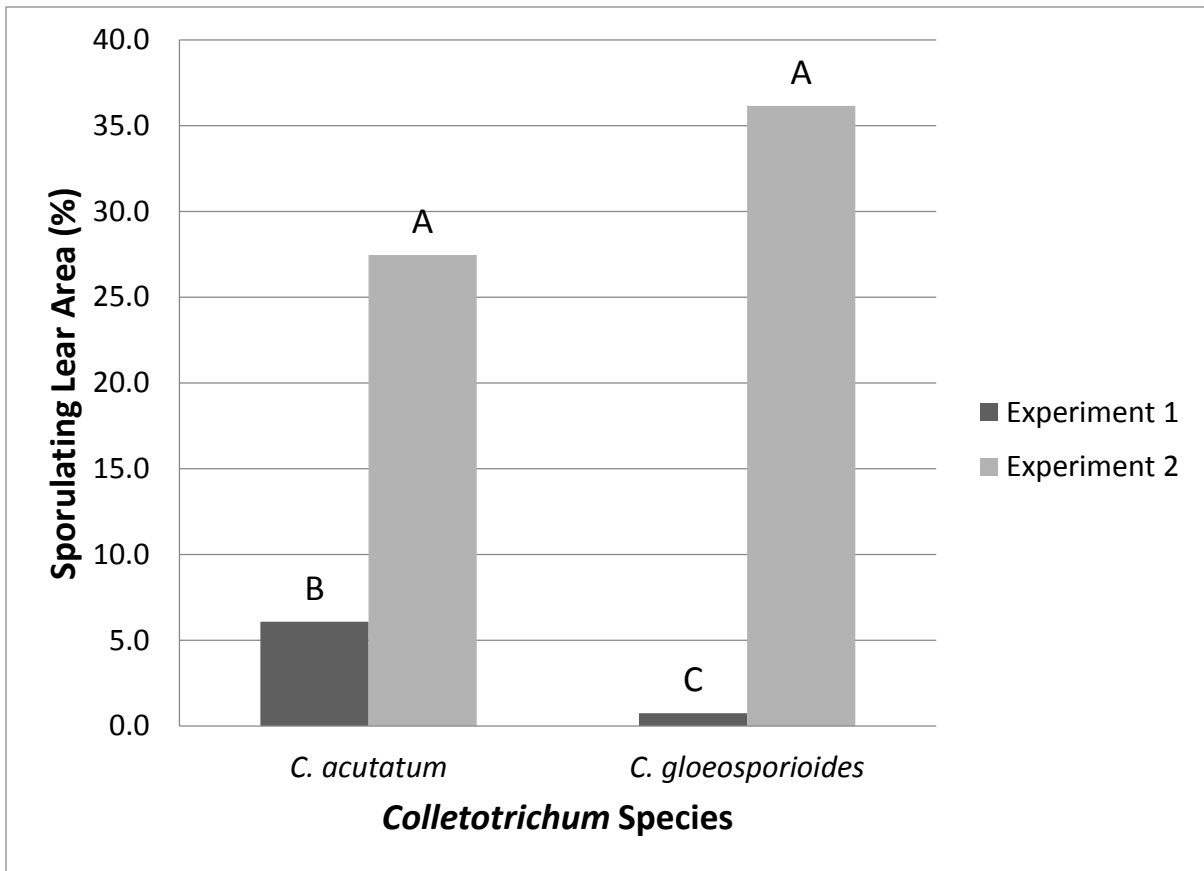


Figure 3.3. Percent sporulating leaf area scores showing the interaction between season and *Colletotrichum* species, averaged over all sample dates and concentrations. Treatments with the same letter are not significantly different ($\alpha = 0.05$).

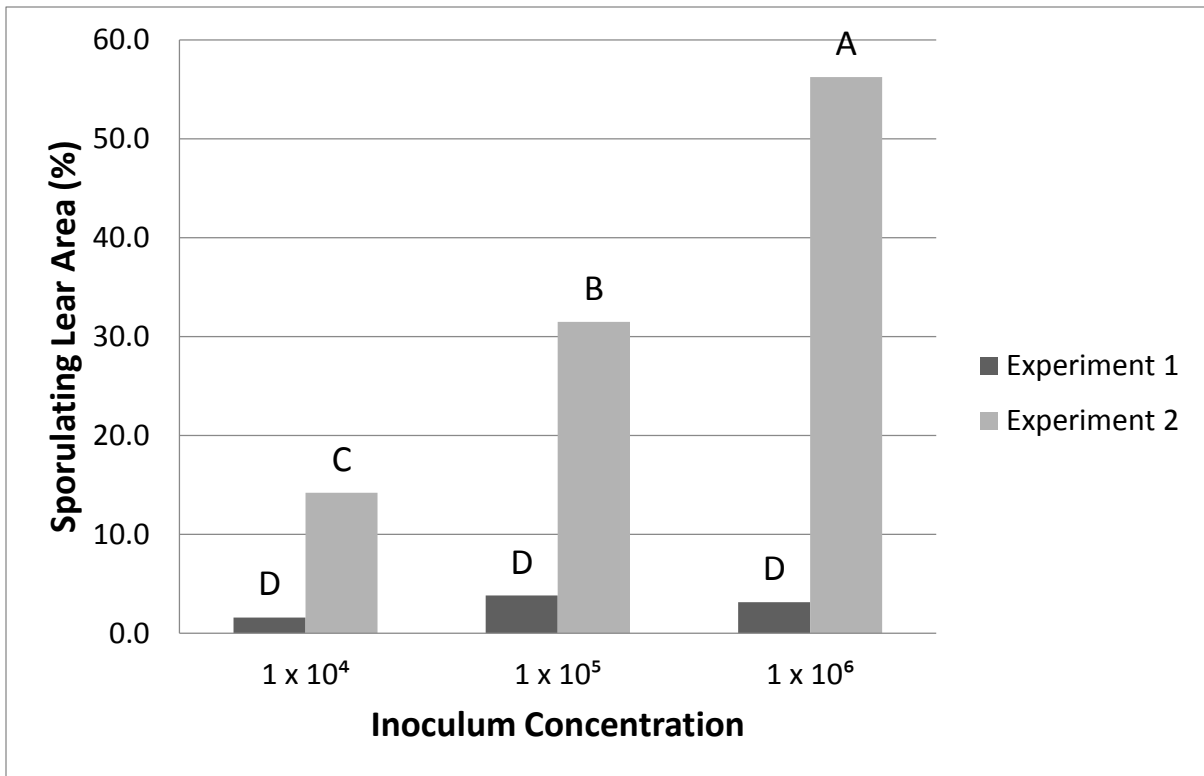


Figure 3.4. Percent sporulating leaf area scores showing the interaction between season and inoculum concentration, averaged over all sample dates and *Colletotrichum* species. Treatments with the same letter are not significantly different ($\alpha = 0.05$).

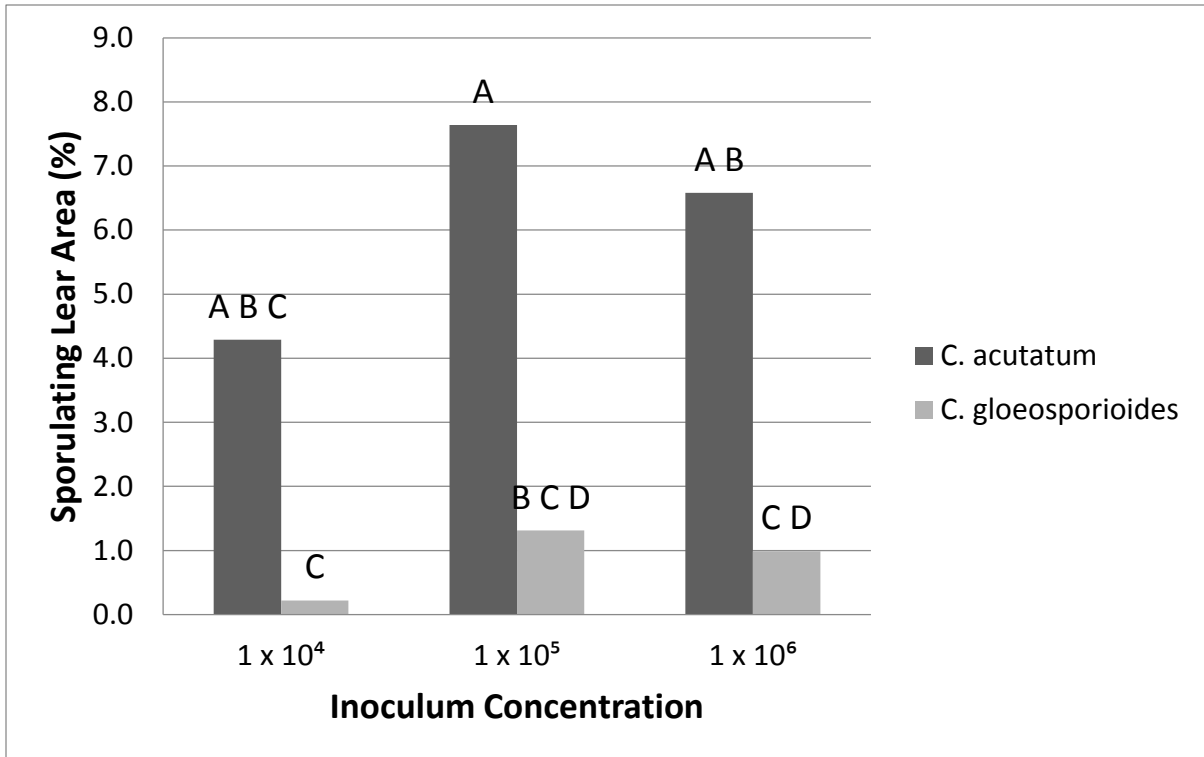


Figure 3.5. Season 1 response to three inoculum concentrations and two *Colletotrichum* species, averaged over all sample dates. Treatments with the same letter are not significantly different ($\alpha = 0.05$). *Colletotrichum* species were significantly different at 1×10^5 and 1×10^6 concentrations, but inoculum concentrations were not significantly different from one another for either *Colletotrichum* species.

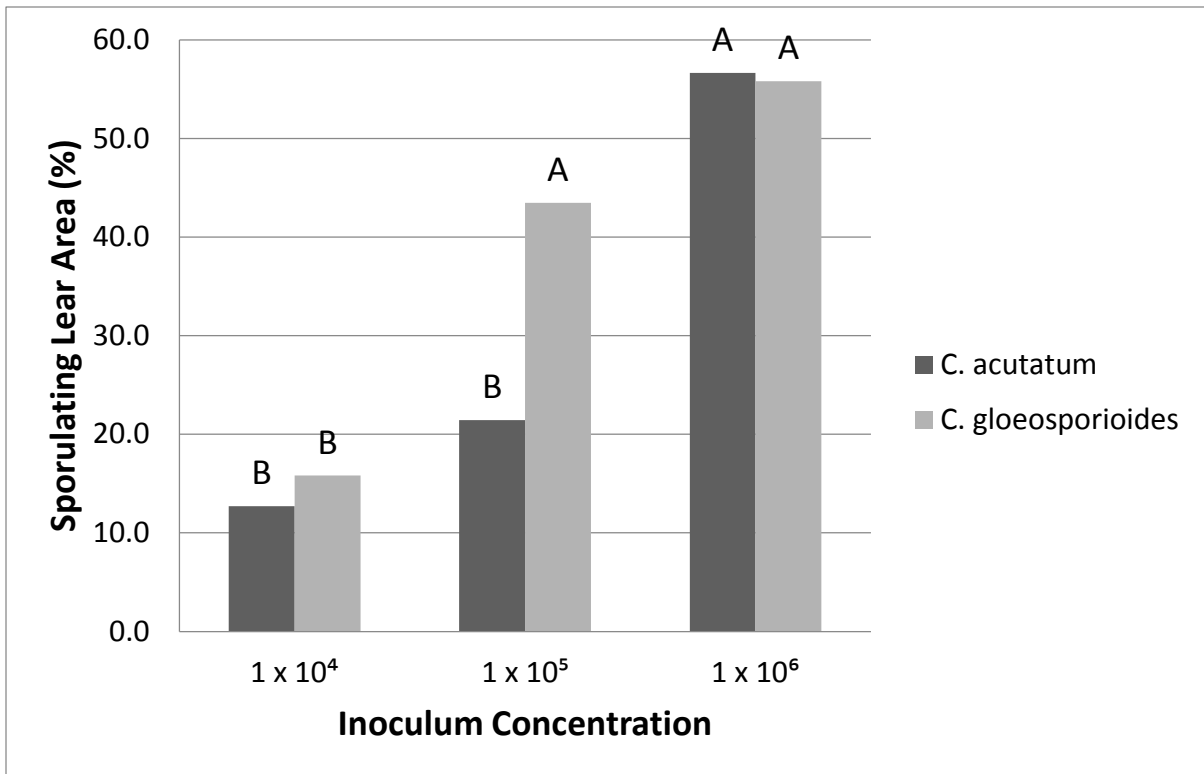


Figure 3.6. Season 2 response to three inoculum concentrations and two *Colletotrichum* species, averaged over all sample dates. Treatments with the same letter are not significantly different ($\alpha = 0.05$). *Colletotrichum* species were not significantly different from one another and responded similarly at all concentrations except 1×10^5 . Inoculum concentration had a significant effect and produced higher PSLA scores at increasing inoculum concentrations.



Figure 3.7. Symptoms of anthracnose leaf spot which developed between 12 and 48 hours after inoculation with *Colletotrichum gloeosporioides*. Similar symptoms did not develop on *C. acutatum*-inoculated plants.



Figure 3.8. (A) Zipper bags used to incubate paraquat-treated leaves. Bags were left unsealed to allow gas exchange. (B) Growth room were maintained at 25°C and 40% relative humidity. 24 hours of strong supplemental lighting were provided followed by 72 hours of ambient room lighting.

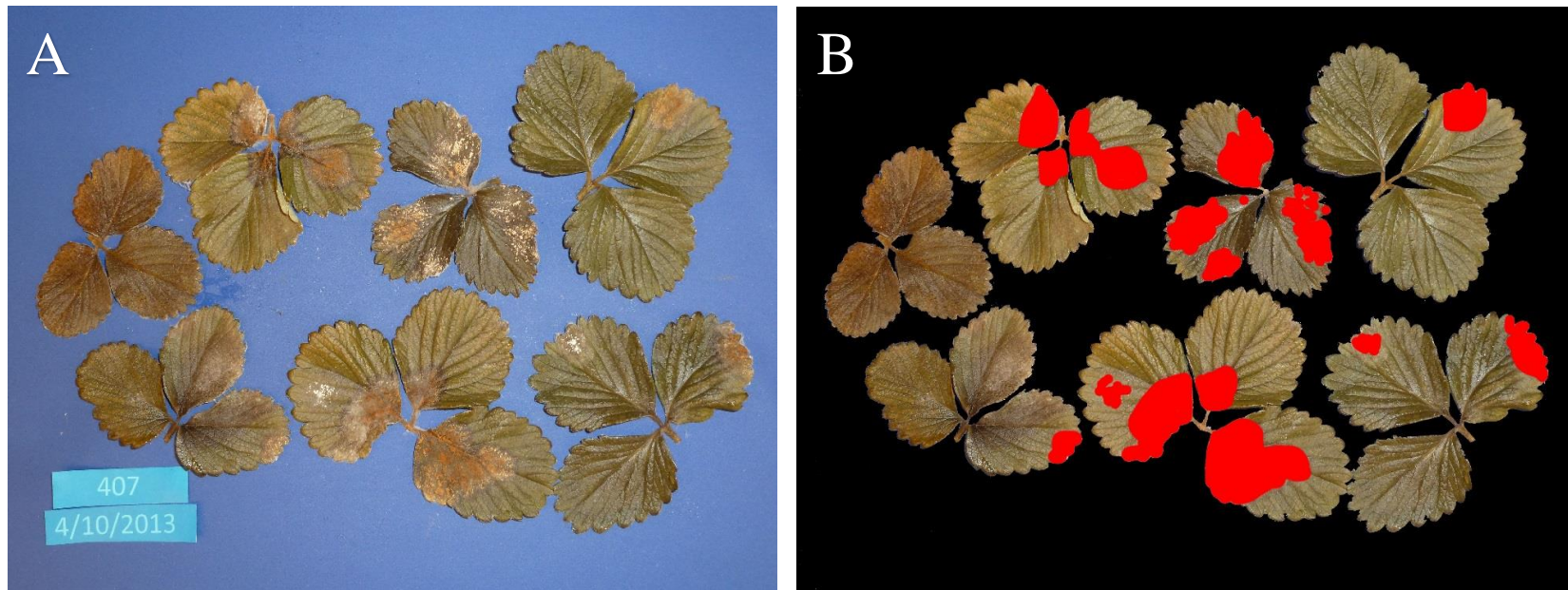


Figure 3.9. (A) Paraquat-treated strawberry leaves showing senescence of leaf tissue and induced sporulation of quiescent fungal colonists. This sample was inoculated with *C. acutatum* at 1.0×10^6 conidia·ml⁻¹. Visual estimation of PSLA was 25.0% for this sample. (B) Image-based analysis of percent sporulating leaf area (PSLA) using the Phenotype Quant macro and ImageJ image-processing software. Image-based calculation indicated a PSLA score of 19.32% for this sample.

Chapter Four

RESISTANCE OF STRAWBERRY CULTIVARS AND SELECTIONS TO HEMIBIOTROPHIC FOLIAR INFECTIONS AND ANTHRACNOSE CROWN ROT OF *COLLETOTRICHUM* SPP.

Abstract

A greenhouse study was conducted to assess variation in resistance to hemibiotrophic foliar infections of *Colletotrichum gloeosporioides* and *C. acutatum* in a panel of strawberry cultivars and advanced selections. Eighteen strawberry genotypes were selected based on field observations of response to anthracnose infection and previous data on resistance to hemibiotrophic infections (HBI). Apparent disease-free clonal plants of each genotype were propagated and grown to maturity in a greenhouse. Supplemental lighting was provided throughout the experiment to increase day length and maintain active vegetative growth. Plants were inoculated with water or with 1.0×10^6 conidia·mL⁻¹ suspensions of *C. gloeosporioides* or *C. acutatum* inoculum. Leaf samples were collected 7, 14, 21, and 28 days after inoculation. Percent sporulating leaf area (PSLA) of paraquat-treated leaves was measured using the macro ‘Phenotype Quant’ and the image processing-software ‘ImageJ’. Analysis of PSLA data found significant differences between genotypes. The most resistant genotypes tested were NCS 10-147 and NCS 10-080 with mean PSLAs of 8.5% and 10.3%, respectively. ‘Treasure’ had the highest overall mean PSLA at 26.5% and ‘Chandler’ scored

21.2%. Plant crowns were inoculated with a 1.0×10^6 conidia·mL⁻¹ suspension of *C. gloeosporioides* conidia after all foliar samples were collected to assess genetic variation in resistance to anthracnose crown rot (ACR). Plants were evaluated weekly for wilt symptoms using a six-point disease index. Disease index data were used to calculate relative area under disease progress curve (rAUDPC) scores, which represented a proportion of maximum possible disease severity. Significant differences in disease resistance were found among the 18 strawberry genotypes. Camino Real, Albion, and Chandler were highly susceptible to ACR. This was reflected in their high rAUDPC scores (between 0.93 and 0.88) scoring near the maximum potential disease severity for the duration of these experiments. Treasure and Sweet Charlie scored 0.53 and 0.45 respectively, a result that corroborates other reports of moderate resistance to ACR for these cultivars. Pelican, which is known to be highly resistant to ACR, was the most resistant genotype tested (0.05). This study found moderate variation in resistance to *Colletotrichum* HBI and determined that resistance to HBI was moderately correlated ($r=0.4251$) between *Colletotrichum* species. Significant variability in resistance was observed for ACR, which was weakly correlated ($r=0.2430$) with resistance to *C. gloeosporioides* HBI.

These results provide anthracnose resistance profiles for a panel of NC State and commercial germplasm. Commercial germplasm ranked among the most susceptible for HBI and ACR traits and underline the need for development of resistant varieties. Data from this work suggest resistance to *C. acutatum* and *C. gloeosporioides* may be shared in strawberry foliage, permitting simultaneous selection of resistant lines across multiple *Colletotrichum* species. However, resistance to HBI and ACR were only weakly correlated and would require independent evaluations in these different tissue types.

Introduction

Anthracnose crown rot (ACR) and anthracnose fruit rot (AFR) are two of the most challenging diseases of strawberry production around the world and are a limiting factor to strawberry production in North Carolina (Poling, 2008). Symptoms of ACR and AFR can be incited by any of three *Colletotrichum* species, though two of these are predominantly responsible for anthracnose incidence in North Carolina. ACR is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. This pathogen kills plants by rapidly invading and producing a reddish-brown marbled necrosis of crown tissue, resulting in wilt and eventual collapse of the plant (Rahman et al., 2015). AFR is caused by *C. acutatum* J.H. Simmons and produces dark sunken lesions on developing fruit that render them unmarketable (Freeman and Katan, 1997; Rahman et al., 2013). Losses due to ACR and AFR are not typically experienced in the same location annually. Rather, the location and timing of epidemics depend on the health and distribution of nursery stock and the presence of environmental conditions conducive to disease development.

C. gloeosporioides and *C. acutatum* are hemibiotrophic pathogens that employ a two-phase infection strategy: a preliminary asymptomatic biotrophic phase followed by a necrotrophic phase in which tissue damage and economic impacts are incurred (Arroyo et al., 2005; Gan et al., 2013; Muench et al., 2008; Perfect et al., 1999). Quiescence contributes to the overall success of a hemibiotrophic infection strategy and is defined as an asymptomatic period between initial host infection and the activation of necrotrophic behavior (Prusky et al., 2013). Germinating conidia may produce disease symptoms immediately but are arguably more destructive when symptom appearance is delayed by the establishment of

quiescent hemibiotrophic infections, permitting the widespread dissemination of asymptomatic transplants to fruiting fields (Leandro et al., 2003; Smith, 2008).

Asymptomatic nursery stock carrying quiescent *Colletotrichum* infections are often the most important source of inoculum in fruiting fields and have been implicated in many anthracnose epidemics in the southeastern United States (Delp and Milholland, 1980; Leandro et al., 2003; MacKenzie et al., 2009; Poling, 2008; Smith, 2008). Once inoculum is introduced to a production field, it can continue to proliferate through secondary conidiation to initiate new hemibiotrophic infections or can survive epiphytically on leaf tissue (Leandro et al., 2001; Legard, 2000).

Overall incidence and severity of ACR and AFR are impacted by several resistance mechanisms including direct resistance of the crown and fruit tissue as well as rate-limiting mechanisms that inhibit proliferative components of the disease cycle such as colonization of foliar tissue or proliferation through secondary conidiation. Previous studies have identified usable genetic variation in resistance to ACR and AFR based on crown and fruit tissue phenotypes (Ballington et al., 2002; Gupton and Smith, 1991; MacKenzie et al., 2006; Osorio et al., 2014; Smith and Spiers, 1982). However, few studies have characterized resistance to hemibiotrophic *Colletotrichum* infections in strawberry foliage, a common and critical host tissue in the disease cycles of both *C. gloeosporioides* and *C. acutatum* (Gan et al., 2013; Rahman et al., 2013).

Foliar resistance is often characterized as rate-limiting (or rate-reducing) resistance. Rate-limiting resistance is a form of partial resistance wherein a host is susceptible to a degree but possesses the capacity to resist or limit the infection, colonization, or subsequent reproduction of a pathogen (Parlevliet, 1979). Rate-limiting resistance is generally a durable

and quantitatively inherited form of resistance that impedes epidemics through reduced pathogen fecundity (Ballington et al., 2002; Ram, 2014; Van der Plank, 1968). Rate-limiting resistance has been described in leaf tissue of other crop species and has been deployed in resistant germplasm to inhibit the spread of inoculum to economically valuable tissues to reduce the risk of disease epidemics (Kirk et al., 2001; Liu and Halterman, 2009; Reddy and Singh, 1993; Simko et al., 2006; Tooley and Grau, 1984). A recent study by Rahman *et al.* (2013) found significant variation among 14 strawberry genotypes evaluated for rate-limiting resistance to hemibiotrophic foliar infections of *C. acutatum*. Similarly, a proof-of-concept trial by Jacobs *et al.* (unpublished) found preliminary evidence of variable resistance to *C. gloeosporioides* foliar infections among advanced selections from the NC State strawberry breeding program. We hypothesized that resistance to *C. gloeosporioides* and *C. acutatum* may be correlated in strawberry foliage, providing a shared rate-limiting mechanism to reduce overall ACR and AFR incidence by restricting the spread and buildup of inoculum in nurseries and fruiting fields.

In addition, we hypothesized that resistance to *C. gloeosporioides* in leaf tissue is not correlated with resistance in crown tissue and that resistance in these tissues is differentially expressed. Many studies investigating the differential expression of host resistance to late blight (*Phytophthora infestans*) in potato foliage and tubers report little to no correlation between foliage and tuber resistance (Kirk et al., 2001; Liu and Halterman, 2009; Simko et al., 2006). Park *et al.* (2005) found that correlation of late blight resistance in foliage and tubers varied between populations and was dependent on the *R* gene(s) involved. Casado-Diaz (2006) investigated gene expression of strawberry fruit and crown tissue inoculated with *C. acutatum* and suggested that different strawberry tissues vary in response and

resistance to infection by the same pathogen species. Rahman *et al.* (2013) found that hemibiotrophic foliar infection severity was not well correlated with severity of AFR, supporting a model where distinct genetic mechanism control resistance to *C. acutatum* in foliage and fruit.

The objectives of this study were to 1) assess genetic variation in resistance to hemibiotrophic foliar infections of *C. gloeosporioides* and *C. acutatum* in a panel of strawberry cultivars and advanced selections; 2) evaluate these genotypes for genetic variation in resistance to ACR caused by *C. gloeosporioides*; and 3) determine if these resistance traits are correlated within genotypes.

Materials and Methods

Plant production. Eighteen genotypes were selected for this study based on field observations of response to anthracnose infection or previous data on resistance to hemibiotrophic *Colletotrichum* infections (Jacobs *et al.*, unpublished). The genotypes included 11 selections from the NC State strawberry breeding program and seven commercially-available cultivars. The cultivar ‘Chandler’ was previously shown to be susceptible to foliar infections of *C. acutatum* and was included as a positive control (Rahman *et al.*, 2013). On August 16, 2013, four plug plants of each genotype were potted into 7.5 liter pots containing Fafard® 3B mix (Sun Gro Horticulture, Agawam, MA) to serve as mother plants for runner production. Mother plants received drip irrigation twice per day and were fertilized with a solution of Jack’s Professional® LX Ca-Mg 15-5-15 fertilizer (JR Peters Inc., Allentown, PA) via a Dosatron® injector system calibrated to dispense 100 ppm

N. Runners were produced in a greenhouse from plants on drip irrigation to minimize free moisture and the spread of conidia to runner tips. Beginning on October 1, 2013, plants received supplemental lighting via EnviroGro T5 fixtures (Hydrofarm Inc., Petaluma, CA) to increase day length to 15 hours for continued runner production. Runners were collected through November, 2013 and stored in a 4°C refrigerator until sufficient numbers were attained. Runner tips were processed to remove all but the youngest expanded leaf to further reduce the risk of quiescent infections in starting material. Cleaned tips were planted in 50-cell plug trays containing Fafard® 3B mix and rooted under periodic mist (30 second duration, 10 minute interval) in a 21 ± 5°C greenhouse for one week until roots developed. After two additional weeks, disease-free (symptomless) rooted plug plants were transplanted into 10 cm pots containing Fafard® 3B potting mix, grown to maturity at 21 ± 5°C with supplemental lighting (15-h photoperiod, 73 μmol·m⁻²·s⁻¹). Plants were hand-watered and fertilized daily with Jack's Professional® LX Ca-Mg 15-5-15 fertilizer.

Experiment design. The experiment was designed as a randomized complete block split-split-plot design with 4 blocks. Each block was divided into 3 main plots to which *Colletotrichum* species (*C. gloeosporioides*, *C. acutatum*, or UTC) were randomly assigned. Main plots were each divided into 18 split-plots to which 3 clonal plants of each genotype were randomly assigned. Leaf samples were collected weekly on four sample dates (7, 14, 21, and 28 DAI). Sample dates were treated as a split-split-plot factor, as these within-plot measurements were independent of one another (different leaves sampled on each date). Foliar experiments were conducted twice, from February 5, 2014 to March 5, 2014 (Experiment 1F) and again from April 16, 2014 to May 14, 2014 (Experiment 2F). Plants screened for foliar resistance to *C. gloeosporioides* were then evaluated for resistance to

ACR by applying *C. gloeosporioides* inoculum directly to the crown. The crown evaluation was conducted twice, from March 12, 2014 to May 21, 2014 (Experiment 1C) and again from June 4, 2014 to August 13, 2014 (Experiment 2C). Individual plants were evaluated weekly for ACR symptoms for ten weeks (7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 DAI). Treatments were randomly assigned to main plots and split-plots separately between Experiments 1 and 2, but were carried over from foliar to crown evaluations of the same experiment (1F→1C and 2F→2C).

Inoculum preparation. Two weeks prior to inoculation of each foliar experiment, a mixed isolate inoculum of *C. gloeosporioides* and *C. acutatum* was prepared to represent diverse strains found in strawberry plantings. Isolates #28, #58, and #84 were selected for *C. gloeosporioides* and isolates #34, #40, and #89 were selected for *C. acutatum* based on confirmation of *Colletotrichum* species level identity in previous work (Jacobs et al., unpublished). The selected isolates were originally collected from infected strawberry tissue in multiple locations across North Carolina and are known to be highly pathogenic on strawberry (M.E. Carnes, personal communication). Ten plates of each isolate were made by transferring mycelia with a sterile probe to fresh plates of half strength PDA and incubating these at 25°C for 10 to 14 days under 12-h fluorescent lighting. Conidia were collected from mature cultures by flooding the plate with double-distilled water (ddH₂O) containing 5 drops of the surfactant Tween 20 per liter of water and disturbing the mycelium with a glass rod to suspend conidia. Conidial suspensions of each isolate were kept separate and filtered through a doubled layer of cheesecloth to remove cellular debris. The concentration of each isolate suspension was measured using a hemocytometer and adjusted to 1.0 x 10⁶ conida·ml⁻¹. Combining 500 mL of each isolate yielded a total volume of 1.5 liters of mixed-isolate

inoculum for each *Colletotrichum* species. Inoculum concentrations were checked by hemocytometer to confirm a final concentration of 1.0×10^6 conida·mL⁻¹.

Two weeks prior to inoculation of each crown experiment new cultures were initiated for each species using *C. gloeosporioides* isolates #28, #58, and #84 and *C. acutatum* isolates #34, #40, and #89 as described above. A total volume of 1.5 liters of inoculum was prepared for each *Colletotrichum* species and final inoculum concentrations were checked by hemocytometer and confirmed to be 1.0×10^6 conida·mL⁻¹.

Foliar Inoculation. Plants were arranged according to the experiment design two weeks prior to inoculation. To prevent cross-contamination from inoculum drift, plastic barriers were constructed and placed between main-plot treatments and greenhouse fans were turned off during inoculation. Experiment 1F plants were inoculated on February 5, 2014 and experiment 2F on April 16, 2014. Inoculations were performed by misting the foliage of each treatment with inoculum of the appropriate species or ddH₂O in the case of UTCs. Inoculum was applied via handheld sprayer (Solo® model 419) just until runoff to ensure even inoculum density across all leaf surfaces. Untreated controls (UTC) were the first treatment applied. The sprayer was completely purged of inoculum, washed with 70% ethanol, and dried between treatment applications. Barriers were removed and greenhouse fans were turned on once inoculation was complete, allowing leaves to dry for 20 minutes prior to initiation of overhead mist to minimize runoff of inoculum. Approximately 45 cm was left between main-plot treatments to avoid cross-contamination of *Colletotrichum* species during incubation and regular plant maintenance.

Inoculated plants were incubated for 48 hours at $28 \pm 2^\circ\text{C}$ with periodic mist (3 sec duration, 5 min interval) provided by 20 liter per hour VibroNet misters (Netafim USA,

Fresno, CA). Mist was applied automatically by a greenhouse controller from 8:00 AM to 8:00 PM to maintain humidity near 100%. Ambient relative humidity was greater than 90% overnight and no additional mist was necessary to maintain leaf wetness. Plants received supplemental lighting (15-h photoperiod, $73 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) during incubation and throughout the experiment in addition to natural daylight. Following incubation, misting was discontinued and day/night temperatures were set to 28/20°C for the remainder of the screen to represent environmental conditions of the North Carolina piedmont during May and June when ACR and AFR are problematic. Temperature and humidity data were collected by a Vantage Pro2™ weather station module (Davis Instruments Corp., Hayward, CA) located in the greenhouse.

Crown Inoculation. The experiment design and randomization of plants in experiments 1F and 2F were maintained in experiments 1C and 2C, respectively. All main-plot treatments (*C. gloeosporioides*, *C. acutatum*, and UTC) were inoculated in experiments 1C and 2C, but *C. acutatum*-inoculated plants were maintained for observation only as minimal ACR symptoms were expected. UTC- and *C. gloeosporioides*-inoculated plants from experiments 1F and 2F were examined prior to inoculation and notes were made of any visible symptoms of ACR. Experiment 1C was inoculated on March 12, 2014 and experiment 2C on June 4, 2014. Inoculations were performed by applying approximately 3 mL of inoculum directly onto the crown of each plant with a handheld sprayer (Solo® model 419). UTC main-plots were inoculated first with ddH₂O, followed by *C. gloeosporioides* and *C. acutatum* main-plots. The sprayer was completely purged of inoculum, washed with 70% ethanol, and dried between treatment applications.

Crown-inoculated plants were incubated for 48 hours following the incubation

parameters outlined for experiments 1F and 2F. Temperature and humidity data were collected by a Vantage Pro2™ weather station module located in the greenhouse.

Foliar disease assessment. Starting plant material was subsampled and screened for pre-existing quiescent infections one week prior to inoculation. Four leaves were arbitrarily collected from each 3-plant split-plot on four sample dates in experiments 1F and 2F (7, 14, 21, and 28 DAI). Leaf samples were placed in labeled plastic bags, and temporarily held in a 4°C refrigerator for analysis.

Leaves were evaluated for severity of fungal colonization using a modification of the paraquat protocol developed by Cerkauskas *et al.* (1982) which has been used to quantify hemibiotrophic infections of *C. acutatum* and *C. gloeosporioides* in strawberry leaf tissue (Rahman *et al.*, 2013). The herbicide paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride) was used to rapidly induce senescence of leaf tissue and trigger the production of orange-colored acervuli from quiescent and otherwise asymptomatic fungal colonists. Leaves were surface sterilized by immersing them in 70% ethanol for 15 seconds, 10% bleach for 60 seconds, and rinsed twice in ddH₂O. Leaflets were then treated with paraquat by immersing them for one minute in a solution of 20 mL Gramoxone® (30.1% paraquat dichloride) / 1 liter ddH₂O and rinsing a final time in ddH₂O. To increase throughput, 26 x 30 cm plastic zipper bags were used in place of incubator boxes (crispers). Bags were labeled and lined with a double layer of 25 x 25 cm paper towel. Paraquat-treated leaves were placed adaxial side up in a single layer inside prepared bags and bags were left unsealed to allow gas exchange. Bags were incubated in a growth room (Harris Environmental Systems Inc., Andover, MA) set to maintain a constant 25°C and 40% relative humidity. Twenty-four hours of strong supplemental lighting (175 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was provided by

EnviroGro T5 lights (Hydrofarm Inc., Petaluma, CA) to encourage rapid and uniform senescence of leaf tissue. T5 lights were turned off after 24-h and samples received an additional 72-h of ambient room lighting for acervular development. Samples were removed from the growth room after 96-h and photographed on a blue background under standardized lighting using a Panasonic Lumix DMC-ZS10 camera (Panasonic Corporation, Osaka, Japan). Conidia were sampled from paraquat-treated leaves of each main-plot treatment and compared to established morphological characteristics of each *Colletotrichum* species.

A protocol for image-based analysis of paraquat-treated leaves was previously developed and determined to more accurately assess the high sample-to-sample variability of percent sporulating leaf area (PSLA) than direct visual estimates (Jacobs et al., unpublished). This protocol was used for evaluation of experiments 1F and 2F as variation between genotypes was expected. A macro plugin called “Phenotype Quant” was developed by Ahmed Abd-El-Haliem (2012) as an unbiased and semi-automated method to quantify visible disease symptoms using the image-processing software “ImageJ”. Phenotype Quant uses filters to quantify the area (in pixels) of diseased leaf tissue and total leaf tissue, distinguishing each by user-defined colors. *Colletotrichum* acervuli (orange to brown) and paraquat-treated leaves (copper to brown) were too similar in color for automatic differentiation by the macro. However, digital enhancement of sample images permitted quantification of the diseased and total leaf area by the program. The program analyzed each image in succession and compiled a list of output values which were used to calculate PSLA.

Crown disease assessment. Because plants received foliar inoculations in experiments 1F and 2F, it is possible that crown infections may have been previously established. Therefore, plants were examined prior to inoculation and notes were made of

any visible symptoms of ACR.

Individual plants were assessed for wilt severity every seven days for 10 sample dates per experiment. Infection severity was assessed using a 6-point disease index: [0 = no wilt, 1 = youngest leaf wilted, 2 = 25% of leaves wilted, 3 = 50% of leaves wilted, 4 = 75% of leaves wilted, 5 = complete collapse]. Plants were hand-watered around 9:00 AM each morning and wilt severity was assessed at noon to ensure that wilt symptoms were caused by disease rather than dry media. Infected crowns were periodically examined by making a cross-section through the crown tissue to observe internal ACR symptoms. If symptoms were non-typical of ACR, diseased crown tissue was plated onto PDA for observation of colony and conidia morphology.

Analysis. PSLA from experiments 1F and 2F were calculated from ImageJ data in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA) as $PSLA = \left(\frac{\text{Diseased Leaf Area}}{\text{Total Leaf Area}} \right) \times 100$. PSLA scores for each genotype by *Colletotrichum* treatment combination were examined using the means procedure (PROC MEANS) in SAS v. 9.4 (SAS Institute, Cary, NC). Outliers were removed ($z=3$) and the univariate procedure (PROC UNIVARIATE) was used for model fitting. Normality of the distribution and homogeneity of variance were evaluated with statistical moments and plotting residual vs. fitted values. To improve normality of the distribution and stabilize variance, a square root transformation was applied with the distribution anchored at 1.0. Data were analyzed using the generalized linear mixed model procedure (PROC GLIMMIX) in SAS to compute type III tests of fixed effects (Table 4.1). Block was considered a random effect, while experiment, treatment, genotype, and DAI were considered fixed effects. Simple effects of significant interactions

were further analyzed using the SLICE and DIFF options of PROC GLIMMIX. Significant effects were compared using t-test letter groupings of least squares means ($\alpha=0.05$), which were back-transformed for comparison. Phenotypic correlation in resistance to *C. gloeosporioides* and *C. acutatum* was examined using the mean of each genotype over replications and experiments. Pearson's correlation coefficient was computed using the correlate procedure (PROC CORR) in SAS to determine the strength of correlation between the two treatments.

Individual plant wilt ratings were used to calculate an average score for each 3-plant split-plot [min – 0, max – 5.0] on each sample date. The area under the disease progress curve (AUDPC) was calculated as described by Shaner and Finney (1977). Relative AUDPC (rAUDPC) was calculated for each split-plot by dividing AUDPC scores by the maximum potential AUDPC for the experiment (Fry, 1978). The maximum potential AUDPC is the AUDPC score that would be reached if disease were assessed at maximum severity (5.0 split-plot avg.) on every sample date. Homogeneity of variance was assessed through diagnostic plots of residual vs. fitted values. Normality and homogeneity of variance of the distribution were improved for statistical analysis through an arcsine square root transformation of rAUDPC data. The formula for the transformation was $\sin^{-1} \sqrt{rAUDPC}$. Transformed rAUDPC values were analyzed using PROC GLIMMIX in SAS. Significant effects were compared using t-test letter groupings of least squares means ($\alpha=0.05$), which were back-transformed for comparison.

Disease severity in leaf and crown tissues inoculated with *C. gloeosporioides* were compared using transformed PSLA and rAUDPC means. Plot averages were calculated for each variable using PROC MEANS in SAS. The relationship between leaf and crown

phenotypes was examined using PROC CORR. Pearson's correlation coefficient was computed to determine the strength of correlation between resistance to *C. gloeosporioides* in leaf and crown tissues. Average PSLA and rAUDPC scores were calculated for each genotype across both experiments and their relationship was plotted in Figure 4.7.

Results

Paraquat screening of starting plant material prior to inoculation indicated that no prior quiescent infections were present in these plants. Periodic checks of *Colletotrichum* species throughout the experiment found no deviations from expected species in samples examined microscopically and/or plated on PDA and examined in culture. Additionally, direct visual identification of species was often possible due to difference in color of *C. gloeosporioides* and *C. acutatum* acervuli and conidia. *C. gloeosporioides* acervuli typically appeared brown with dark borders and *C. acutatum* acervuli were orange with light borders (Figure 4.4).

Mean PSLA scores from experiments 1F and 2F were not significantly different ($P=0.5982$), however, there was a significant effect of DAI ($P<.0001$) and a significant interaction of experiment by DAI ($P<.0001$; Table 4.1). Mean PSLA scores decreased steadily from a high of 25.12% at 7 DAI to a low of 9.28% at 28 DAI. Experiments 1F and 2F showed similar decreases over time (Figure 4.1), but the interaction of experiment by DAI resulted from significant differences in the simple effect of experiment on PSLA means at 7 DAI ($P=0.0541$) and 21 DAI ($P=0.0256$). The overall treatment mean for *C. gloeosporioides* (16.53%) was significantly lower ($P=0.0159$) than that of *C. acutatum* (18.80%). A

significant three-way interaction of experiment, treatment, and DAI was caused by significant differences in the simple effect of experiment on PSLA means for *C. acutatum* at 7 DAI ($P<.0001$) and 28 DAI ($P=0.0015$) and for *C. gloeosporioides* at 21 DAI ($P=0.0058$). *C. acutatum* had significantly higher scores for experiment 2F than experiment 1F at 7 and 28 DAI. *C. gloeosporioides* had higher scores for experiment 1F than experiment 2F at 21 DAI.

There were significant effects of genotype ($P<.0001$) and the interactions of genotype by experiment ($P=0.0021$) and genotype by treatment ($P=0.0040$; Table 4.1). Genotype means (averaged over both *Colletotrichum* species) ranged from 8.5% for NCS 10-147 to 26.5% for Treasure (Figure 4.2). Better separation of genotype means occurred at the lower and higher ends of the PSLA range with less distinct separation of genotypes with mid-range PSLA means. The interactions of genotype by experiment and genotype by treatment (*Colletotrichum* sp.) are examined in Table 4.2, which describes the simple effects of experiment and treatment at each level of genotype. Simple effects of experiment were seen only in the genotype NCS 11-107 ($P=0.0034$), which had a PSLA of 14.98% for experiment 1F versus 23.90% for experiment 2F. Simple effects of treatment were observed for only three of the 18 genotypes tested: Festival ($P=0.0084$), NCS 11-107 ($P=0.0033$), and Treasure ($P<.0001$). All other genotypes were not significantly different between *Colletotrichum* treatments (Figure 4.3). A significant Pearson's correlation coefficient was calculated that indicated a moderate positive correlation ($r=0.4251$, $P<.0001$) between *C. gloeosporioides* and *C. acutatum* foliar treatments within genotypes.

Plants were evaluated for wilt symptoms prior to crown inoculation with *C. gloeosporioides*. Minor wilt was observed in only a very small percentage of plants and no

plants exhibited symptoms greater than a 2-rating on the 0 to 5 disease index. Significant wilt was not observed until 7 days post-crown inoculation and increased steadily from that point forward (Figure 4.5). UTC plants developed no wilt symptoms during the course of either crown experiment. *C. acutatum*-inoculated plants showed symptoms of stunting rather than the distinct wilting seen in *C. gloeosporioides*-inoculated plants.

Averaged over all genotypes, mean wilt severity of split-plots increased from 0.74 at 7 DAI to 3.90 at 70 DAI (Figure 4.5). Analysis of transformed rAUDPC data found a significant major effect of experiment ($P<.0001$) and the interaction of experiment by genotype ($P<.0001$). Average split-plot severity was lower in experiment 1C (1.87) than experiment 2C (3.12), which may be due to higher ambient humidity and nighttime temperatures during experiment 2C. An examination of the simple effect of experiment across levels of genotype indicated that rank changes of several genotypes between experiments 1C and 2C produced this interaction. A significant effect of genotype ($P<.0001$) was examined through t-test letter grouping of genotype least-squares means, which were back-transformed to rAUDPC means for comparison (Figure 4.6). rAUDPC genotype means encompassed nearly the entire range from 0.0 to 1.0 based on the ratio of AUDPC to maximum potential AUDPC. The most susceptible genotypes were Camino Real (0.93), Albion (0.89), and Chandler (0.88). The most resistant genotype was Pelican (0.05). A significant weak positive correlation ($r=0.2430$, $P<.0001$) was observed between foliar and crown tissue resistance to infection by *C. gloeosporioides* within genotypes (Figure 4.7).

Discussion

The significant effect of genotype (Figure 4.2) and the interaction of genotype by *Colletotrichum* treatment (Figure 4.3) are of particular interest to this study. Many genotypes were significantly different in overall hemibiotrophic infection severity, though genotypes in the middle of the range of responses were not different from one another. The most resistant genotype tested was NCS 10-147 with a mean PSLA of 8.5%. Interestingly, NCS 10-147 is the progeny of ‘Treasure’ (mother) x ‘Chandler’ (father), which were two of the most susceptible genotypes screened in this study. ‘Treasure’ had the highest overall mean PSLA at 26.5%, while ‘Chandler’ scored 21.2%. The second most resistant genotype was NCS 10-080 with a mean PSLA of 10.3%. NCS 10-080 is the open-pollinated progeny of NCC 02-63, the genotype reported most resistant to foliar infections of *C. acutatum* of the genotypes screened by Rahman et al. (2013).

A significant moderate correlation was detected between *Colletotrichum* treatments, indicating significant correlation of these two resistance phenotypes within the genotypes tested. No significant differences were found in the treatment responses of 15 of the 18 genotypes (Figure 4.3). However, ‘Festival’, NCS 11-107, and ‘Treasure’ had significantly different responses to *Colletotrichum* treatments. ‘Festival’ had a 7.3% difference in treatment means and was one of the only genotypes to show greater severity of colonization by *C. gloeosporioides* than *C. acutatum*. NCS 11-107 also had a 7.3% difference between treatment means, though *C. acutatum* was more severe than *C. gloeosporioides* for this genotype. The largest difference in treatment means was found for ‘Treasure’, which had 11.9% greater severity of *C. acutatum* HBI than *C. gloeosporioides* HBI. Interestingly, this

observed difference between *Colletotrichum* foliar treatments in ‘Treasure’ is analogous to established knowledge of Treasure’s greater susceptibility to AFR than to ACR. MacKenzie *et al.* (2006) reported the involvement of a major resistance gene in Treasure’s resistance to ACR. It is possible that a major resistance gene could explain the significant difference in foliar treatment means in the current study.

Propagating runners in a greenhouse environment from mother plants on drip irrigation minimized the presence of quiescent infections in starting material for foliar evaluations. This was likely due to limited free moisture on plant surfaces in this environment, a critical component in the germination of *Colletotrichum* conidia. Experiments 1F and 2F showed similar trends of decreasing PSLA at later DAI (Figure 4.1). We suggest that this decrease over time may have been due to environmental requirements for secondary infection to occur and the sampling scheme used in these experiments. Hemibiotrophic infections were successfully established in the tissue to which inoculum was applied due to the temperature and constant leaf wetness during the 48-h incubation period at the beginning of each experiment. This period was the only time when plants received prolonged leaf wetness during these experiments. Previous research has demonstrated that leaf wetness of 4 to 6 h or more is required for the production of secondary conidia (Leandro *et al.*, 2003; Wilson *et al.*, 1990). Despite receiving overhead water each morning, this 4-h requirement was not met post-incubation, as foliage typically dried within 2 h of each watering event, especially on sunny days. Without this increase from secondary infections, the incidence of hemibiotrophic infections in split-plots was diluted at each successive sample date by the sampling of inoculated leaves and the emergence of new non-inoculated leaves. These details are noted as environmental parameters in the current study may place

greater emphasis on resistance to infection rather than resistance to post-penetrative invasion or subsequent reproduction. Distinct components of resistance mechanisms such as these have been explored in a previous study and were found to be independently inherited (Iwano et al., 1997).

Evaluation of resistance to ACR caused by *C. gloeosporioides* identified significant differences in disease resistance among the 18 strawberry genotypes tested (Figure 4.6). This trial characterized ACR resistance in many NC State selections that had not been previously evaluated and enhanced our knowledge of ACR resistance in seven commercial cultivars. Camino Real, Albion, and Chandler were highly susceptible to ACR. This was reflected in their high rAUDPC scores (between 0.93 and 0.88) scoring near the maximum potential disease severity for the duration of these experiments. Treasure and Sweet Charlie scored 0.53 and 0.45 respectively, a result that corroborates other reports of their moderate resistance to ACR (Chandler et al., 1997; MacKenzie et al., 2007). Pelican, which is known to be highly resistant to ACR, was the most resistant genotype tested (0.05) (Smith et al., 1998). Other resistant genotypes included NCH 11-304 (0.13), NCH 09-068 (0.14), and NCH 11-319 (0.15), which were all selected under high anthracnose disease pressure in Castle Hayne, NC.

Though it is conceivable that the appearance of wilt symptoms in crown-inoculated plants could be due to the previous foliar inoculation, only minor wilt symptoms were noted in very few plants prior to crown inoculations with *C. gloeosporioides* and no total collapse was observed. Given that plant death occurred relatively synchronously after inoculation of each crown trial, it appears that observed disease symptoms during experiments 1C and 2C were caused by the crown-targeted inoculation.

Correlation of the crown and foliar data detected a weak positive though significant correlation between these two traits. While this somewhat inconclusive result indicates neither a strong correlation nor lack thereof, it is important to recognize that this correlation describes only the genotypes in the current study. Correlation between crown and foliar resistance phenotypes are likely genotype-dependent and stronger or weaker correlations may exist in different subsets of the strawberry germplasm. Similar studies on inheritance of resistance in potato foliage and tubers found that resistance was controlled by major and minor genes and correlation of resistance between tissues depended on the *R*-genes (and genotypes) in each population (Park et al., 2005).

These data characterized the resistance of 18 strawberry genotypes against two *Colletotrichum* species and contributes to our limited knowledge of rate-limiting resistance mechanisms in strawberry foliage. We have found moderate variation in resistance to hemibiotrophic foliar infections of *C. gloeosporioides* and *C. acutatum*. Foliar resistance between *Colletotrichum* species was found to be moderately correlated and the majority of genotypes tested had no significant difference between *Colletotrichum* treatments. The accompanying crown study contributed additional knowledge about resistance in common vs. divergent tissues. We have found significant variation in resistance to ACR caused by *C. gloeosporioides* and detected a weak correlation between this phenotype and resistance to *C. gloeosporioides* foliar infections.

These results provide anthracnose resistance data for several NC State selections and demonstrate the overall susceptibility of both crown and foliar tissues in a sample of commercial cultivars. This study underlines the importance of separate evaluations of tissues that may be under the control of different resistance mechanisms. Understanding how

resistance is inherited in these tissues may provide insight into mechanisms controlling resistance in different tissue types of strawberry and other crops. Future work studying segregating populations designed to investigate these resistance phenotypes will further elucidate genetic parameters governing inheritance of anthracnose resistance and will aid breeding programs in developing and deploying such resistance in new cultivars.

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Table 4.1. Analysis of variance of the effects of experiment, treatment (*Colletotrichum* species), genotype, and days after inoculation on the severity of hemibiotrophic foliar infections. Severity was evaluated via image-based analysis as percent sporulating leaf area.

Source of variation	df	<i>F</i>	<i>P</i> > <i>F</i>
Experiment	1	0.31	0.5982
Treatment	1	11.07	0.0159*
Experiment x Treatment	1	3.41	0.1145
Genotype	17	16.14	<.0001***
Experiment x Genotype	17	2.40	0.0021**
Treatment x Genotype	17	2.26	0.0040**
Experiment x Treatment x Genotype	17	1.03	0.4321
DAI ^a	3	180.75	<.0001***
Experiment x DAI	3	10.43	<.0001***
Treatment x DAI	3	31.15	<.0001***
Experiment x Treatment x DAI	3	16.82	<.0001***
Genotype x DAI	51	1.48	0.0187*
Experiment x Genotype x DAI	51	1.18	0.1917
Treatment x Genotype x DAI	51	0.74	0.9130
Experiment x Treatment x Genotype x DAI	51	0.81	0.8275

^aDAI = Days After Inoculation.

* significant at $P < 0.05$; ** significant at $P < 0.005$; *** significant at $P < 0.001$.

Table 4.2. Test of the simple effects of experiment and treatment on PSLA^a means at levels of genotype. Treatments consisted of either *C. acutatum* or *C. gloeosporioides* conidia applied to strawberry foliage. Means are significantly different at $P < 0.05$.

Genotype	Experiment		Treatment	
	<i>F</i> value ^b	<i>P</i> > <i>F</i>	<i>F</i> value ^b	<i>P</i> > <i>F</i>
Albion	0.59	0.4451	0.63	0.4281
Camino Real	0.65	0.4227	1.84	0.1762
Chandler	1.44	0.2308	0.05	0.8266
Festival	0.32	0.5714	7.09	0.0084*
NCH 09-068	1.85	0.1752	0.47	0.4924
NCH 11-304	1.14	0.2862	0.33	0.5649
NCH 11-309	0.61	0.4374	1.29	0.258
NCH 11-319	0.04	0.8399	2.12	0.1471
NCL 11-185	0.01	0.9031	0.05	0.8192
NCL 11-208	3.01	0.0841	3.84	0.0515
NCS 10-080	3.43	0.0654	2.59	0.1092
NCS 10-147	0.16	0.6865	1.00	0.3192
NCS 10-193	0.21	0.6451	0.68	0.4116
NCS 11-107	8.76	0.0034**	8.85	0.0033**
NCS 11-113	0.10	0.7516	1.47	0.2264
Pelican	0.58	0.4466	1.73	0.1895
Sweet Charlie	2.84	0.0937	2.61	0.1078
Treasure	2.75	0.0985	17.05	<.0001***

^aPSLA = percent sporulating leaf area.

^b*F* values for the test of simple effects of experiment and treatment were calculated using 204 degrees of freedom.

* significant at $P < 0.05$; ** significant at $P < 0.005$; *** significant at $P < 0.001$.

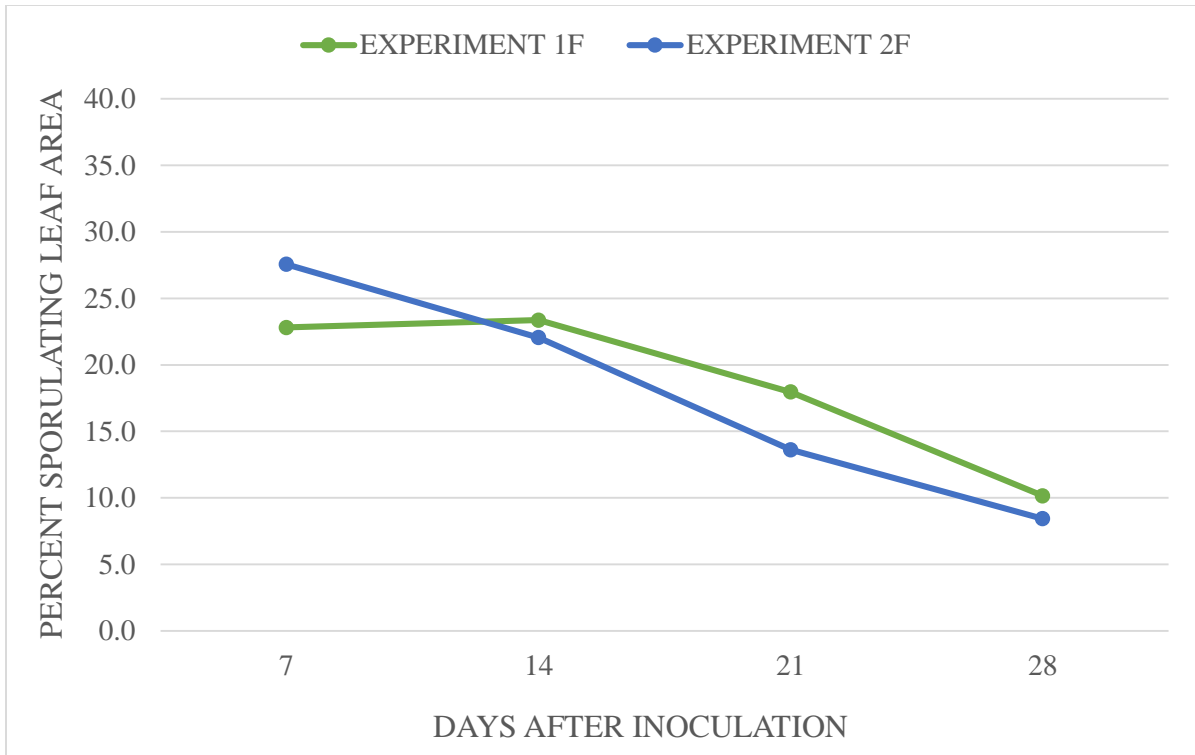


Figure 4.1. Mean percent sporulating leaf area (PSLA) scores for experiments 1F and 2F on four sample dates (days after inoculation).

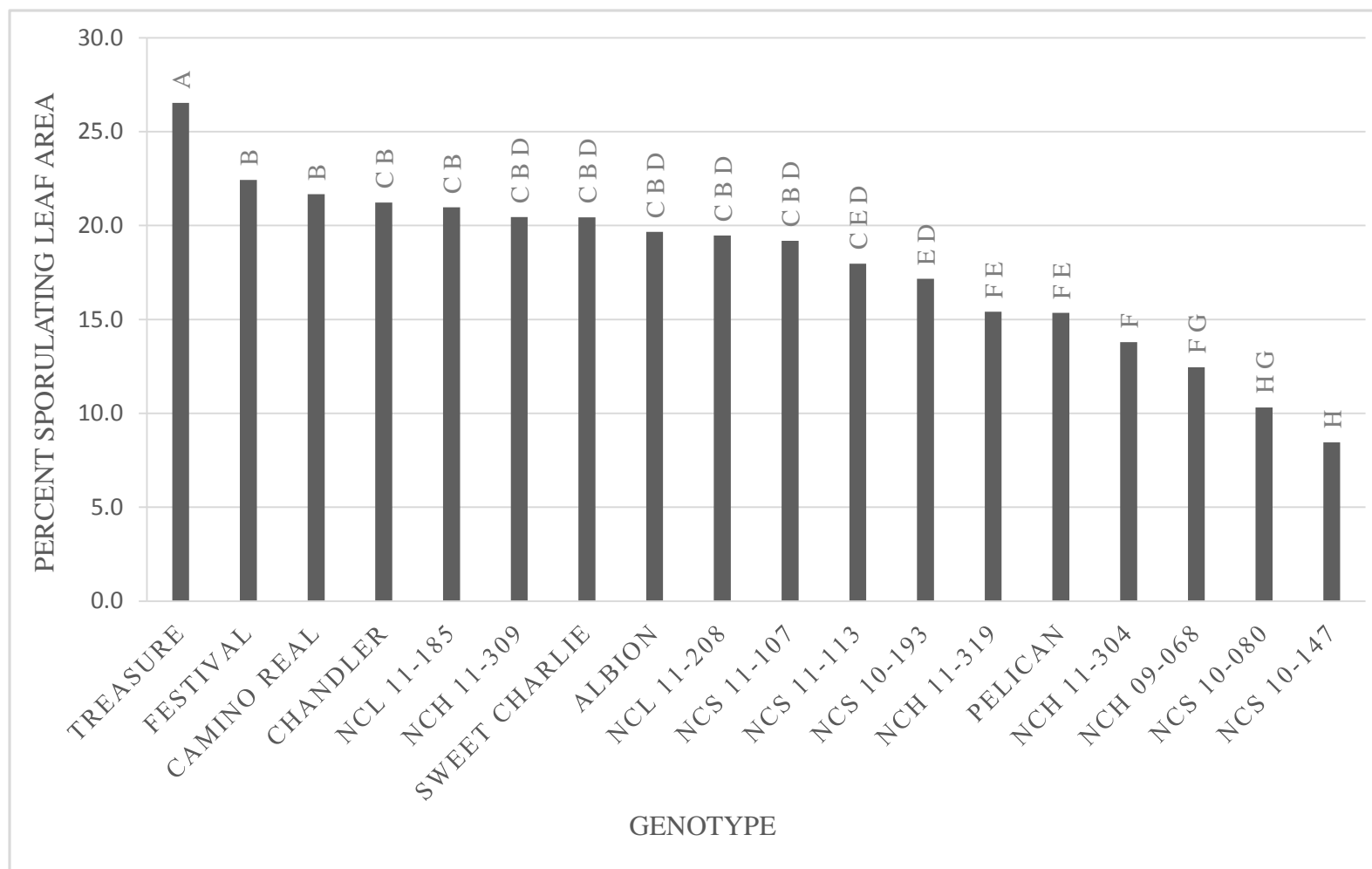


Figure 4.2. Mean PSLA scores for 18 genotypes averaged over both *C. gloeosporioides* and *C. acutatum* treatments. t-test letter groupings appear above each genotype. Genotypes with the same letter are not significantly different ($P=0.05$).

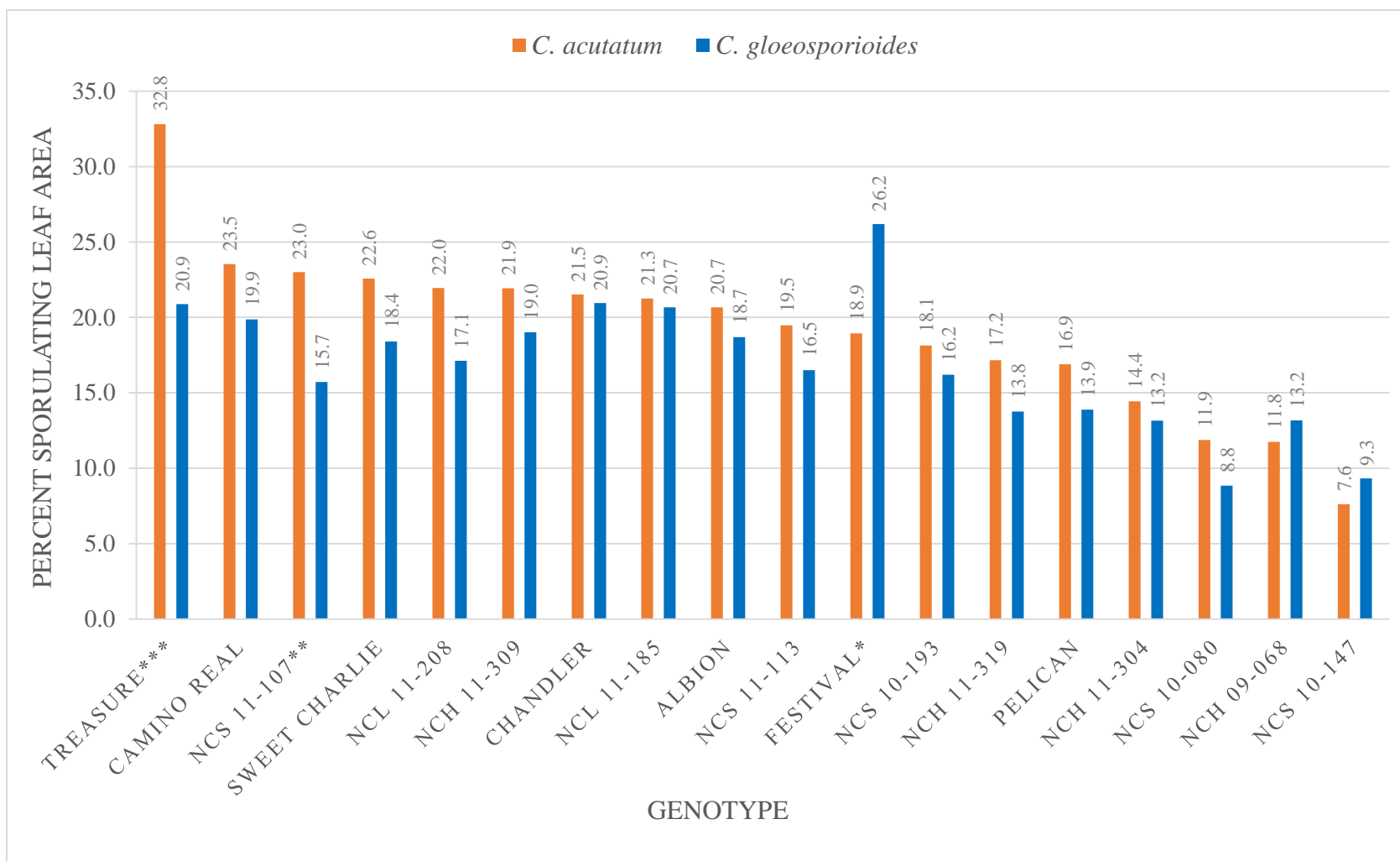


Figure 4.3. *C. gloeosporioides* and *C. acutatum* treatment means for PSLA at each level of genotype. Genotypes with significant differences in treatment response are denoted by: * significant at $P < 0.05$; ** significant at $P < 0.005$; *** significant at $P < 0.001$.

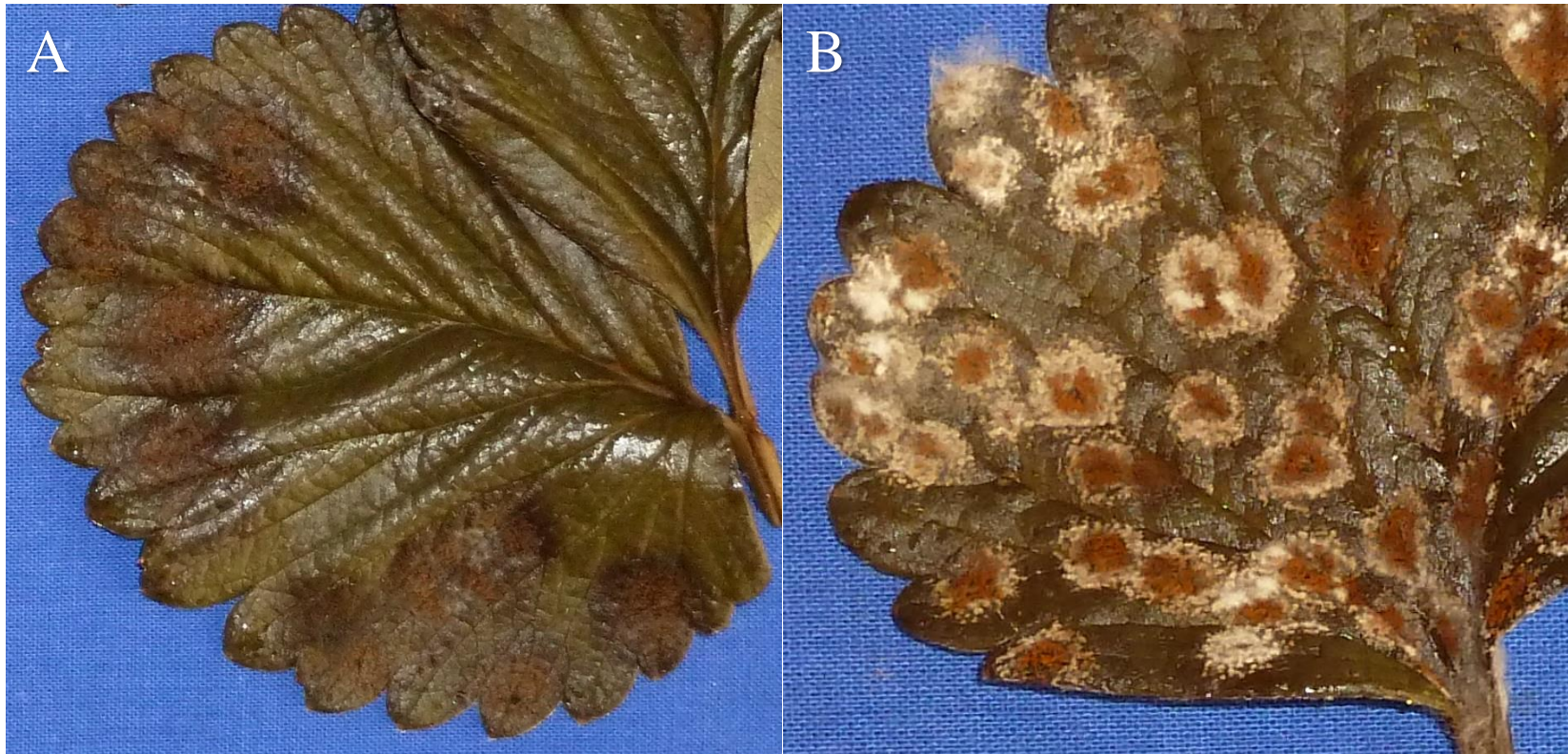


Figure 4.4. Appearance of *Colletotrichum* acervuli in paraquat-treated strawberry leaves. (A) Acervuli and conidia of *C. gloeosporioides* on leaf of NC State advanced selection NCS 10-107, appearing brown with dark borders. (B) Acervuli and conidia of *C. acutatum* on leaf of cultivar 'NCS 10-107', appearing orange with light borders.

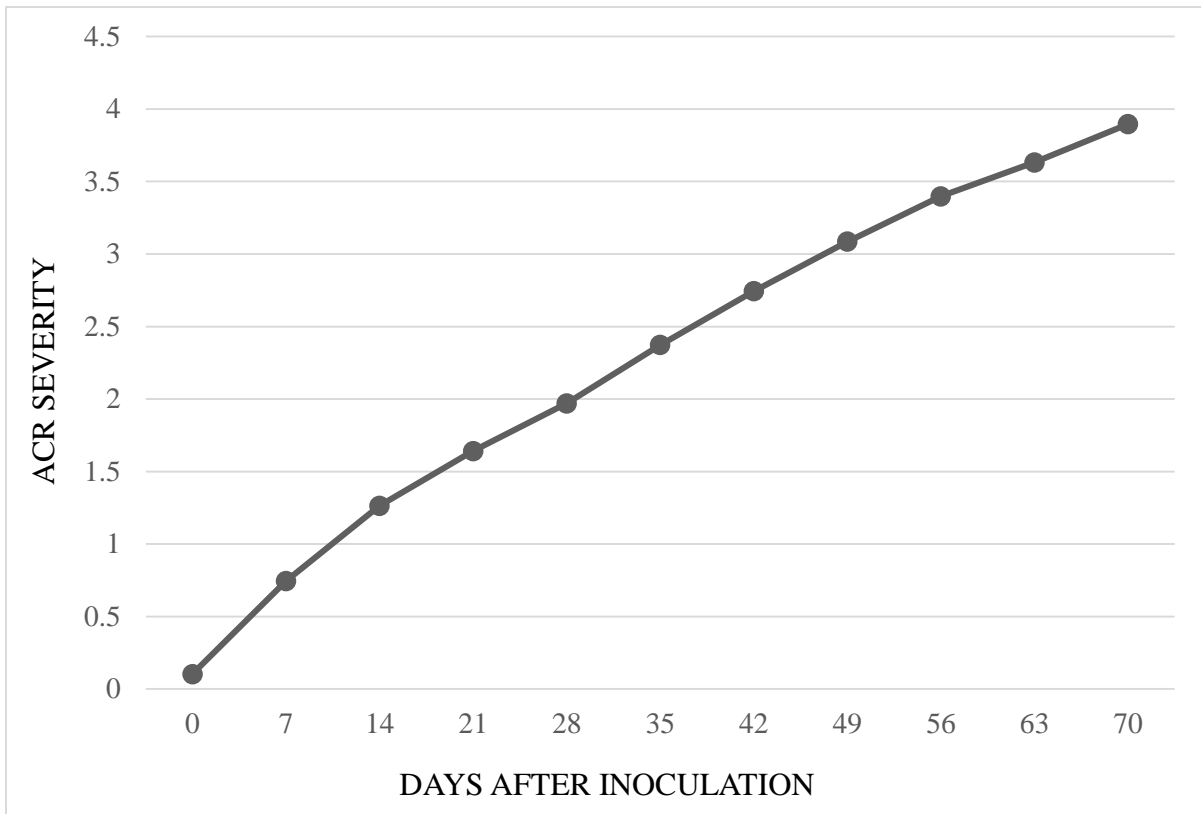


Figure 4.5. Severity of *C. gloeosporioides* crown infections over time averaged over all genotypes and experiments. Individual plants were rated for ACR wilt severity every seven days using a 0 to 5 point disease index and split-plot means were calculated for every three-plant plot. ACR severity was calculated as the mean split-plot rating on each sample date.

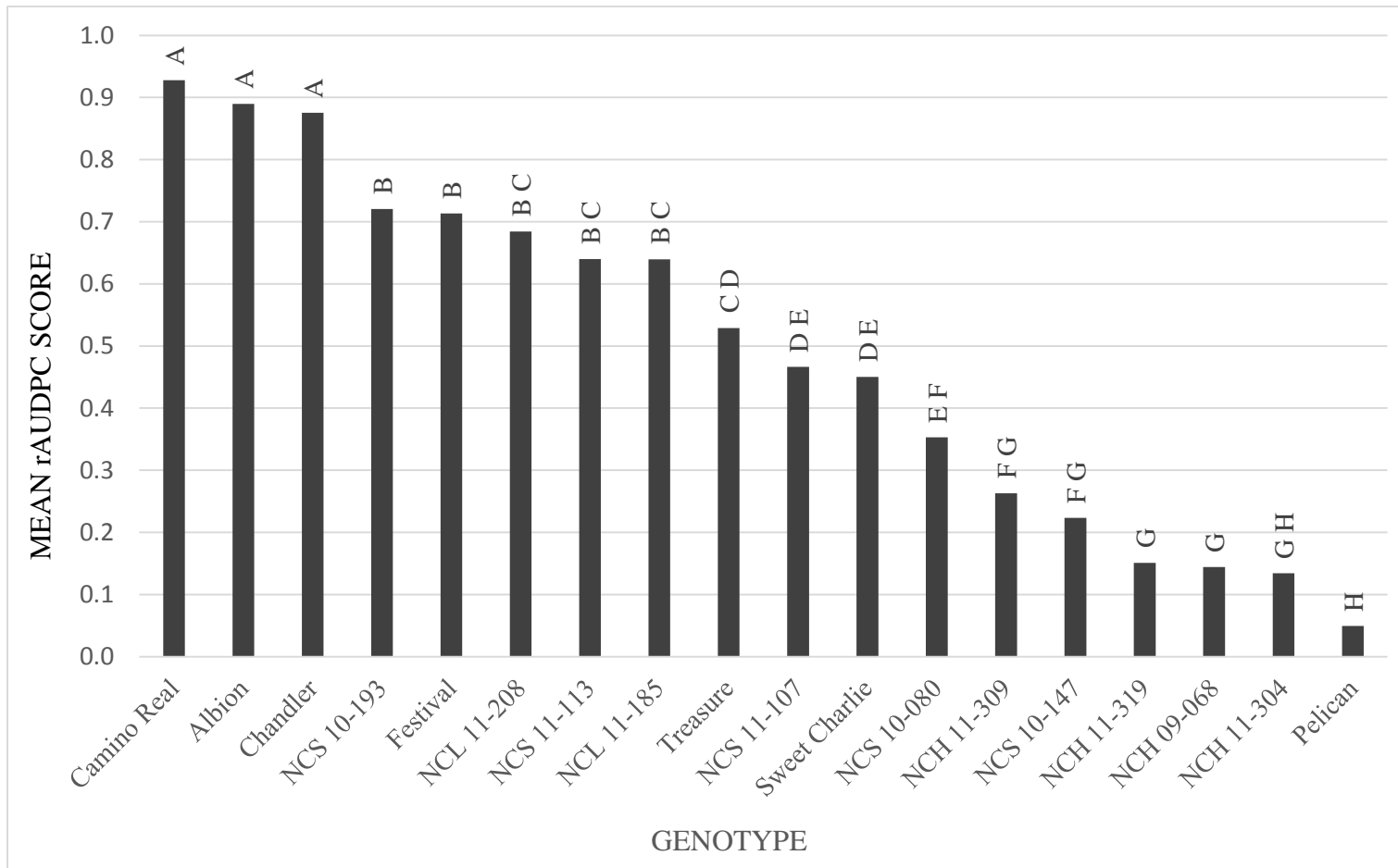


Figure 4.6. Mean relative area under the disease progress curve (rAUDPC) scores for 18 genotypes inoculated with *C. gloeosporioides* and scored for ACR wilt severity. t-test letter groupings appear above each genotype. Genotypes with the same letter are not significantly different ($P=0.05$).

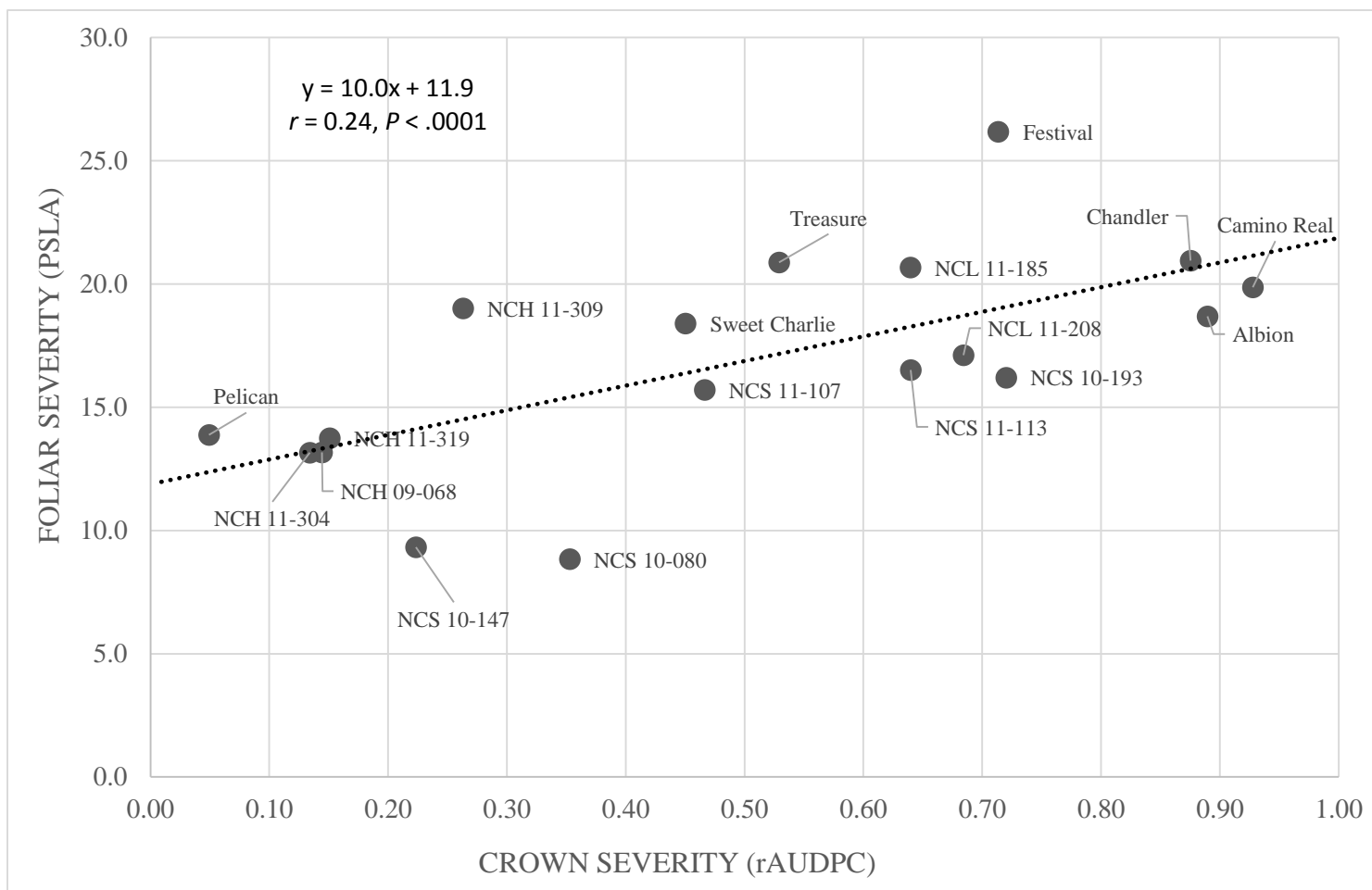


Figure 4.7. Correlation between the average severity of leaf and crown tissue infection of 18 strawberry genotypes by *C. gloeosporioides*. Leaf severity is given as a percent sporulating leaf area (PSLA) measurement and crown severity as the relative area under the disease progress curve (rAUDPC) score.

Chapter Five:

INHERITANCE OF RESISTANCE TO HEMIBIOTROPHIC FOLIAR INFECTIONS AND ANTHRACNOSE CROWN ROT OF *COLLETOTRICHUM* SPP. IN STRAWBERRY

Abstract

A population was constructed to examine the inheritance of rate-limiting resistance to *C. gloeosporioides* and *C. acutatum* hemibiotrophic infections (HBI) in strawberry leaf tissue and to compare this to the inheritance of resistance to anthracnose crown rot (ACR) in crown tissue. Six parental genotypes, varying from resistant to susceptible for HBI and ACR, were selected and crossed in a half diallel mating design to generate 15 half-sib families. Seedlings and clonal plants of parental genotypes were allocated to either the HBI experiment or the ACR experiment. HBI and ACR experiments were conducted concurrently and were inoculated with 1.0×10^6 conidia·mL⁻¹ suspensions of *C. gloeosporioides* or *C. acutatum* applied directly to strawberry leaf or crown tissue, respectively. Leaves were collected from each plant 21 days after inoculation. HBI resistance phenotypes were evaluated by treating leaf samples with the herbicide paraquat dichloride and measuring the percent sporulating leaf area (PSLA) with imaging software. ACR resistance phenotypes were evaluated for wilt symptoms once per week for eight weeks using a six-point disease index. Relative area under the disease progress curve (rAUDPC)

scores were calculated to represent the proportion of maximum potential disease severity for the duration of the screen. Diallel analysis was conducted in SAS to estimate variance components. Low dominance to additive variance ratios for *C. acutatum* HBI (0.13) and *C. gloeosporioides* ACR (0.20) suggested strong additive genetic control of resistance to these traits. *C. gloeosporioides* HBI had a much higher dominance to additive variance ratio (0.91) suggesting that this trait was under nearly equal dominance and additive genetic control. Heritability estimates were relatively low for *C. acutatum* HBI (0.25) and *C. gloeosporioides* HBI (0.16) but were moderate for *C. gloeosporioides* ACR (0.61). Gain from selection was predicted to be high for ACR resistance, moderate for *C. acutatum* HBI resistance, and low for *C. gloeosporioides* HBI resistance. High levels of resistance to ACR could be achieved in as few as two cycles of selection. Progress would be slower for the HBI traits. Approximately three cycles would be required for each 10% decrease in PSLA for *C. acutatum* HBI, but a similar decrease for *C. gloeosporioides* HBI would require six or more cycles. A strong genetic correlation ($r_A = 0.98$) between resistance to *C. acutatum* HBI and *C. gloeosporioides* HBI suggests that resistance to these *Colletotrichum* species is controlled by common genes in strawberry leaf tissue. Selecting for increased resistance to HBI for one *Colletotrichum* species should produce gains in resistance to the other. Negative genetic correlations between ACR and both HBI traits ($r_A = -0.85$ and -0.61) suggest resistance in crown tissue is inherited independently of resistance in leaf tissue in this population. These results recommend independent evaluation and advancement of resistance to HBI and ACR.

Introduction

Anthracnose crown rot (ACR) and anthracnose fruit rot (AFR) are two of the most challenging diseases of strawberry production around the world and are a limiting factor to strawberry production in North Carolina (Poling, 2008). Symptoms of ACR and AFR can be incited by any of three *Colletotrichum* species, though two of these are predominantly responsible for anthracnose incidence in North Carolina. ACR is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and kills plants by rapidly invading and producing a reddish-brown marbled necrosis of crown tissue, resulting in wilt and eventual collapse of plants (Rahman et al., 2015). AFR is caused by *C. acutatum* J.H. Simmons and produces dark sunken lesions on developing fruit that render them unmarketable (Freeman and Katan, 1997; Rahman et al., 2013).

Asymptomatic nursery stock carrying quiescent *Colletotrichum* infections are often the most important source of inoculum in fruiting fields and have been implicated in many anthracnose epidemics in the southeastern United States (Delp and Milholland, 1980; Leandro et al., 2003; MacKenzie et al., 2009; Poling, 2008; Smith, 2008). Once inoculum is introduced to a production field, it can continue to proliferate through secondary conidiation to initiate new hemibiotrophic infections (HBI) or can survive epiphytically on leaf tissue (Leandro et al., 2001; Legard, 2000).

Overall incidence and severity of ACR and AFR are impacted by several resistance mechanisms including direct resistance of the crown and fruit tissue as well as rate-limiting mechanisms that inhibit proliferative components of the disease cycle such as colonization of foliar tissue or proliferation through secondary infections. Previous studies have identified

usable genetic variation in resistance to ACR and AFR based on crown and fruit tissue phenotypes (Ballington et al., 2002; Gupton and Smith, 1991; MacKenzie et al., 2006; Osorio et al., 2014; Smith and Spiers, 1982). However, few studies have characterized resistance to *Colletotrichum* HBI in strawberry foliage, a common and critical host tissue in the disease cycles of both *C. gloeosporioides* and *C. acutatum* (Gan et al., 2013; Rahman et al., 2013). Foliar resistance is often characterized as rate-limiting (or rate-reducing) resistance, a form of partial resistance wherein a host is susceptible to a degree but possesses the capacity to resist or limit the infection, colonization, or subsequent reproduction of a pathogen (Parlevliet, 1979). Rate-limiting resistance is generally a durable and quantitatively inherited form of resistance that impedes epidemics through reduced pathogen fecundity (Ballington et al., 2002; Ram, 2014; Van der Plank, 1968).

A previous study by Rahman *et al.* (2013) evaluated 14 strawberry genotypes for resistance to AFR and foliar HBI caused by *C. acutatum*. This study found significant variation in resistance to AFR and HBI among genotypes, but these traits were only moderately correlated ($r=0.57$, $P<.005$) within genotypes. A similar study was conducted by Jacobs *et al.* (unpublished) to evaluate variation in resistance to foliar HBI of *C. gloeosporioides* and *C. acutatum* as well as resistance to ACR across 18 strawberry cultivars and selections. Resistance to *C. gloeosporioides* HBI was moderately correlated ($r=0.43$, $P<.0001$) to resistance to *C. acutatum* HBI within genotypes. Only three of the 18 genotypes had significantly different responses between *Colletotrichum* foliar treatments. An examination of resistance to ACR and HBI by *C. gloeosporioides* found only a weak positive correlation ($r=0.24$, $P<.0001$) between these traits.

Research based on mating designs is less common today because major agronomic

traits have been thoroughly studied and the variance components controlling these traits are largely understood. However, mating designs still hold great value in less-studied horticultural crops and in quantitatively controlled traits that are of new or developing interest. Establishment of *Colletotrichum* HBI in nurseries and the subsequent distribution of asymptomatic infected plant material has been identified as a causal source of anthracnose epidemics (F.J. Louws, personal observation). Preliminary examinations of this trait suggests that variation may exist in strawberry germplasm which could be deployed as a form of rate-limiting resistance to control the spread of *Colletotrichum* conidia within strawberry nurseries and fruiting fields (Rahman et al., 2013). There have been no published mating studies investigating the genetic components of resistance to *Colletotrichum* HBI in strawberry foliage. Additionally, we know of few publications that compare resistance across strawberry tissue types and none that compare resistance to ACR and HBI in crown and leaf tissue, respectively. This study was undertaken to enhance our knowledge of the inheritance of rate-limiting resistance to *C. gloeosporioides* and *C. acutatum* HBI in strawberry leaf tissue and to compare this to the inheritance of ACR resistance in strawberry crown tissue.

Materials and Methods

Plant production. Six parental genotypes were selected for this study based on their varied resistance responses to HBI and ACR in a previous study (Jacobs et al., unpublished). Selected parents included: Chandler (HBI-susc., ACR-highly susc.), NCH 11-304 (HBI-mod., ACR-highly res.), NCL 11-185 (HBI-susc., ACR-mod.), NCS 10-080 (HBI-res., ACR-

mod. res.), NCS 10-147 (HBI-res., ACR-res.), and Treasure (HBI-highly susc., ACR-mod.). Parents were crossed in a half diallel mating design between May and July, 2014 with a total of $\frac{p(p-1)}{2} = 15$ crosses made for $p = 6$ parents (Figure 5.6). Seed were bulked from both parents to achieve sufficient seed numbers for 14 of the 15 crosses. However, NCS 10-080 is male-sterile and was therefore only used as a female during crossing. Approximately 300 to 400 seed were produced from each cross. All seed from each cross were scarified by immersing them for 10 minutes in 18 M sulfuric acid (98% H₂SO₄) and were then rinsed with double-distilled water (ddH₂O). Scarified seed were stratified by placing them in 50 mL centrifuge tubes filled halfway with slightly moistened vermiculite. Sealed tubes were placed in a 4°C refrigerator for two weeks. Scarified and stratified seed from each cross were sown onto separate 27.8 x 54.5 cm 1020 trays (T.O. Plastics, Inc., Clearwater, MN) partially filled with Fafard® super-fine germinating mix (Sun Gro Horticulture, Agawam, MA) (Figure 5.7A). Seed were covered with a light (< 2mm) layer of super-fine germinating mix, misted with water, and covered with clear plastic humidity domes (T.O. Plastics, Inc., Clearwater, MN). On August 26, 2014, seedling trays were placed in a 21°C growth room under T5 light fixtures (EnviroGro, Hydrofarm Inc., Petaluma, CA) with 14-h day length. Trays were germinated in a clean growth room to avoid seedling loss from anthracnose which can be problematic with greenhouse germination (Figure 5.7B).

Once seedlings reached the two true leaf stage, they were transplanted to 50-cell plug trays containing Fafard® 3B mix and maintained under periodic mist (30 second duration, 10 minute interval) in a 21 ± 5°C greenhouse for three to four days until new roots developed. Apparent disease-free runner tips of parental genotypes were collected from greenhouse-grown mother plants and rooted in 50-cell plug trays under periodic mist. Seedlings and

parents were watered daily and after one month were fertilized daily with Jack's Professional® LX Ca-Mg 15-5-15 fertilizer (JR Peters Inc., Allentown, PA) via a Dosatron® injector system calibrated to dispense 100 ppm N. Flowers and runners were removed weekly from seedlings to encourage new vegetative growth. A total of 120 individuals of each full-sib family and 60 clonal plants of each parental genotype were produced for inclusion in these experiments. Eighty individuals from each family and 40 clonal plants of each parental genotype were allocated to the HBI experiment. Forty individuals from each family and 20 clonal plants of each genotype were allocated to the ACR experiment. Due to poor seed germination, only 61 individuals from the cross of NCS 10-080 x NCH 11-304 were available and all were allocated to the HBI experiment. This family was eliminated from the ACR experiment and its effects were considered equal to zero in the combining ability analysis.

Experiment design. The HBI experiment was designed as a randomized complete block split-plot design with 4 blocks. Each block was divided into two main plots to which *Colletotrichum* species (*C. gloeosporioides* or *C. acutatum*) were randomly assigned. Ten individuals from each family and five clonal plants of each parental genotype were randomly assigned to split-plots within each main plot.

The ACR experiment was designed as a randomized complete block design with 4 blocks. Ten individuals from each family and five clonal plants of each parental genotype were randomly assigned to plots within each block.

Inoculum preparation. Two weeks prior to inoculation, a mixed isolate inoculum was prepared to represent diverse strains of *C. gloeosporioides* and *C. acutatum* found in strawberry plantings. Three isolates were selected for each *Colletotrichum* species based on

confirmation of *Colletotrichum* species level identity in previous work (Jacobs et al., unpublished). The selected isolates were originally collected from infected strawberry tissue in multiple locations across North Carolina and are known to be highly pathogenic on strawberry (M.E. Carnes, personal communication). Twenty plates of each *C. gloeosporioides* isolate and ten plates of each *C. acutatum* isolate were made by transferring mycelia with a sterile probe to fresh plates of half strength PDA and incubating these at 25°C for 10 to 14 days under 12-h fluorescent lighting. Conidia were collected from mature cultures by flooding the plate with double-distilled water (ddH₂O) containing 5 drops of the surfactant Tween 20 per liter of water and disturbing the mycelium with a glass rod to suspend conidia. Conidial suspensions of each isolate were kept separate and were filtered through a doubled layer of cheesecloth to remove cellular debris. The concentration of each isolate suspension was measured using a hemocytometer and adjusted to 1.0 x 10⁶ conida·mL⁻¹. Combining 500 mL of each *C. acutatum* isolate yielded a total volume of 1.5 liters of mixed-isolate inoculum. One liter of each *C. gloeosporioides* isolate was combined to produce 3.0 liters of inoculum to be split between HBI and ACR experiments. Mixed isolate inoculum were checked by hemocytometer to confirm a final concentration of 1.0 x 10⁶ conida·mL⁻¹.

Inoculation. Plants were arranged according to the experiment design two weeks prior to inoculation and were subsampled and screened for pre-existing hemibiotrophic infections. Plants received overhead supplemental lighting (15-h photoperiod, 73 μmol·m⁻²·s⁻¹) in addition to natural daylight to encourage vegetative growth. To prevent cross-contamination from inoculum drift, plastic barriers were placed between main-plot treatments and greenhouse fans were turned off during inoculation. The HBI experiment was

inoculated on October 26, 2014 when plants were approximately two months old. Inoculations were performed by misting the foliage of each treatment with inoculum of the appropriate *Colletotrichum* species. Inoculum was applied via handheld sprayer (Solo® model 419) just until runoff to ensure even inoculum density across all leaf surfaces. The sprayer was completely purged of inoculum, washed with 70% ethanol, and dried between treatment applications. Plants in the ACR experiment were also inoculated on October 26, 2014. Crown inoculations were performed by applying approximately 3 mL of inoculum directly onto the crown of each plant with a handheld sprayer (Solo® model 419). Barriers were removed and greenhouse fans were turned on once inoculation was complete, allowing plants to dry for 20 minutes prior to initiation of overhead mist to minimize runoff of inoculum. A 45 cm gap was left between main-plot treatments to avoid cross-contamination of *Colletotrichum* species during incubation and regular plant maintenance.

Inoculated plants were incubated for 48 hours at $28 \pm 2^\circ\text{C}$ with periodic mist (3 sec duration, 5 min interval) provided by 20 liter per hour VibroNet misters (Netafim USA, Fresno, CA). Mist was applied automatically by a greenhouse controller from 8:00 AM to 8:00 PM to maintain humidity near 100%. Overnight mist was not required to maintain leaf wetness as ambient relative humidity generally climbed above 90% by 10:00 PM. Plants received supplemental lighting (15-h photoperiod, $73 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) during incubation and throughout the experiment in addition to natural daylight to maintain active vegetative growth. Following incubation, misting was discontinued and day/night temperatures were set to 28/20°C for the remainder of the screen to represent environmental conditions of the North Carolina piedmont during May and June when ACR and AFR are problematic. Temperature and humidity data were collected by a Vantage Pro2™ wireless weather station

module (Davis Instruments Corp., Hayward, CA) located in the greenhouse.

HBI disease assessment. Samples were collected from the HBI experiment after 21 days by removing all inoculated leaves from individual plants and placing them in labeled plastic sandwich bags. Leaves were temporarily stored in a 4°C refrigerator while awaiting paraquat analysis. One to two leaflets were removed from each bag, placed into labeled coin envelopes, and frozen for possible subsequent molecular work.

Leaves were evaluated for severity of fungal colonization using a modification of the paraquat protocol developed by Cerkauskas *et al.* (1982) which has been previously used to evaluate variation in HBI of *C. acutatum* and *C. gloeosporioides* in strawberry leaf tissue (Jacobs *et al.*, unpublished. Rahman *et al.*, 2013). The herbicide paraquat dichloride (1,1'-dimethyl-4',4'-bipyridinium dichloride) was used to rapidly induce senescence of leaf tissue and trigger the production of acervuli from quiescent and otherwise asymptomatic fungal colonists. Leaves were surface sterilized by immersing them in 70% ethanol for 15 seconds, 10% bleach for 60 seconds, and rinsed twice in ddH₂O. Leaflets were then treated with paraquat by immersing them for one minute in a solution of 20 mL Gramoxone® (30.1% paraquat dichloride) / 1 liter ddH₂O and rinsing a final time in ddH₂O. Paraquat-treated leaves were placed adaxial side up in a single layer inside plastic bags lined with paper towel and bags were left unsealed to allow water vapor to escape. Bags were incubated in a growth room (Harris Environmental Systems Inc., Andover, MA) set to maintain a constant 25°C and 40% relative humidity. This method was limited only by the size of the growth room which permitted approximately 200 samples to be assayed simultaneously. Twenty-four hours of strong supplemental lighting (175 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was provided by overhead T5 light fixtures (EnviroGro, Hydrofarm Inc., Petaluma, CA) to encourage rapid and uniform

senescence of leaf tissue. T5 lights were turned off after 24-h and samples received an additional 72-h of ambient room lighting for acervular development. Samples were removed from the growth room after 96-h and photographed on a blue background under standardized lighting using a Panasonic Lumix DMC-ZS10 camera (Panasonic Corporation, Osaka, Japan). Conidia were sampled from paraquat-treated leaves of each main-plot treatment for comparison to established morphological characteristics of each *Colletotrichum* species.

A protocol for image-based analysis of paraquat-treated leaves was previously developed to more accurately assess the high sample-to-sample variability of percent sporulating leaf area (PSLA) than direct visual estimates (Jacobs et al., unpublished). This protocol was used for evaluation of HBI sample images. A macro plugin called “Phenotype Quant” was developed by Ahmed Abd-El-Haliem (2012) as an unbiased and semi-automated method to quantify visible disease symptoms using the image-processing software “ImageJ”. Phenotype Quant uses filters to quantify the area (in pixels) of diseased leaf tissue and total leaf tissue, distinguishing each by user-defined colors. *Colletotrichum* acervuli (orange to brown) and paraquat-treated leaves (copper to brown) were too similar in color for automatic differentiation by the macro. However, digital enhancement of sample images permitted quantification of the diseased and total leaf area by the program. Phenotype Quant analyzed each image in succession and compiled a list of output values which were used to calculate PSLA.

ACR disease assessment. Individual plants were assessed for wilt severity every seven days for eight sample dates. Infection severity was assessed using a 6-point disease index: [0 = no wilt, 1 = youngest leaf wilted, 2 = 25% of leaves wilted, 3 = 50% of leaves wilted, 4 = 75% of leaves wilted, 5 = complete collapse]. Plants were hand-watered around

9:00 AM each morning and wilt severity was assessed at noon to ensure that wilt symptoms were caused by disease rather than dry media. Infected crowns were periodically examined by making a cross-section through the crown tissue to observe internal ACR symptoms. If symptoms were non-typical of ACR, diseased crown tissue was plated onto PDA for observation of colony and conidia morphology.

Analysis. PSLA scores from the HBI experiment were calculated from ImageJ data in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA) as $PSLA =$

$\left(\frac{\text{Diseased Leaf Area}}{\text{Total Leaf Area}} \right) \times 100$. PSLA scores for each genotype by *Colletotrichum* treatment combination were examined using the means procedure (PROC MEANS) and univariate procedure (PROC UNIVARIATE) in SAS v. 9.4 (SAS Institute, Cary, NC). Normality of the distribution and homogeneity of variance were evaluated with statistical moments and by plotting residual vs. fitted values. To improve normality of the distribution and stabilize variance, a square root transformation was applied to PSLA data with the distribution anchored at 1.0.

Individual plants from the ACR experiment were scored for degree of wilt based on a six-point disease index. Ratings were made every week for eight weeks. The area under the disease progress curve (AUDPC) was calculated as described by Shaner and Finney (1977). Relative AUDPC (rAUDPC) was calculated for each single-plant plot by dividing AUDPC scores by the maximum potential AUDPC for the experiment (Fry, 1978). The maximum potential AUDPC is the AUDPC score that would be reached if disease were assessed at maximum (5.0) severity on every sample date. Homogeneity of variance was assessed through diagnostic plots of residual vs. fitted values.

Analysis of variance (ANOVA) was performed on HBI split-plot data using the mixed model procedure (PROC MIXED) in SAS and appropriate Type III tests of fixed effects were calculated. Separate analyses based on Griffing's Method II were carried out to estimate the general combining ability (GCA) effects of parents and specific combining ability (SCA) effects of crosses for *C. gloeosporioides* HBI, *C. acutatum* HBI, and ACR. Block and *Colletotrichum* treatment were considered fixed effects while GCA, SCA, and the error term were considered random and were associated with zero mean and variance. GCA estimates were back-transformed for presentation. SAS code developed by F. Isik (2009) for diallel mating designs was used to estimate combining abilities and partition variance components. Gain from selection was estimated based on full-sib family selection of the (10%) most resistant progeny using the formula $G_S = k p c \sigma_A^2 / \sqrt{\sigma_P^2}$ where k = selection differential, p = coefficient of parental control, and c = coefficient of additive covariance of selected relatives. Progress from selection was estimated from genetic gain using the formula $\mu_F = \mu_0 + (\#cycles \times G_S)$, where μ_0 = the initial population mean and μ_F = the final population mean. Correlation between traits was evaluated using the correlate procedure (PROC CORR) in SAS and genetic correlation was calculated using the formula $r_A = \sigma_{Gij}^2 / (\sigma_{Gi}^2 \times \sigma_{Gj}^2)^{1/2}$, where σ_{Gij}^2 = the genetic covariance between traits and σ_{Gi}^2 and σ_{Gj}^2 = the genetic variance components of each trait.

Results

In total, 1,024 strawberry genotypes from 15 half-sib families were evaluated for resistance to *Colletotrichum* HBI in leaf tissue and 560 genotypes from 14 half-sib families were evaluated for resistance to ACR in crown tissue. Summaries of experiments conducted and the mating design utilized in each experiment are given in Tables 5.1 and 5.2, respectively. ANOVA of HBI experimental factors found no significant differences for the fixed effects of block, *Colletotrichum* treatment, and the interaction of genotype by treatment. Significant effects of genotype ($P < .0001$), parent ($P = 0.0141$), and cross ($P = 0.0121$) were detected and supported individual diallel analyses of HBI traits (Table 5.3).

GCA effects for the parents refer to the average performance of a parent across hybrid combinations (Griffing, 1956). In the case of resistance to HBI, negative GCA values indicated that progeny of crosses including a particular parent had reduced PSLA values. Positive GCA values are associated with parents whose progeny had elevated PSLA measurements. Diallel analysis found no significant effects of GCA and SCA for either HBI trait tested at $\alpha = 0.05$ but marginal p-values (Table 5.4) encouraged exploration of GCA and SCA effects (Table 5.5). The lowest p-value for HBI traits ($P = 0.0821$) was associated with the GCA effect of *C. acutatum* HBI. GCA values were small for both *C. acutatum* and *C. gloeosporioides* HBI (less than $\pm 6.0\%$ effect), but three genotypes had significant GCA effects for *C. acutatum* HBI (Table 5.5, Figure 5.1B). NCH 11-304 had a significant positive GCA effect of 5.03 ($P = 0.0328$). ‘Chandler’ and NCS 10-147 had significant negative GCA effects of -4.34 ($P = 0.0431$) and -4.27 ($P = 0.0489$), respectively. GCA estimates for *C. gloeosporioides* were smaller in effect and just one was found to be significant (Table 5.5).

NCS 10-080 had a significant positive GCA of 3.29 ($P=0.0490$) (Table 5.5, Figure 5.1A). No significant SCA effects were detected for any cross included in the HBI experiment.

In the case of ACR, negative GCA estimates were associated with parents whose progeny had overall reductions in ACR incidence while positive GCA estimates indicated parents whose progeny had higher incidence of ACR. Diallel analysis of ACR data found marginal but insignificant effects of GCA ($P=0.0735$) and SCA ($P=0.0982$) at $\alpha=0.05$ (Table 5.4). ‘Chandler’ had a significant positive GCA of 0.18 ($P=0.012$) (Table 5.5), indicating greater average susceptibility of hybrid combinations with ‘Chandler’. NCH 11-304 had a significant negative GCA of -0.28 ($P=0.0004$), suggesting that hybrid combinations including NCH 11-304 were on average more resistant to ACR (Figure 5.2). No significant SCA effects were found for resistance to ACR.

Descriptive statistics were calculated for each parental genotype and family and are given in Table 5.5. Segregation within families was examined through frequency distributions constructed for *C. acutatum* HBI (Figure 5.3), *C. gloeosporioides* HBI (Figure 5.4), and *C. gloeosporioides* ACR (Figure 5.5). For HBI traits, the majority of progeny within each family were normally distributed near the midparent value or between the mean PSLA scores of the two parental genotypes (Figures 5.3 and 5.4). However, there were some exceptions where progeny were skewed toward either extreme and away from parental phenotypes. The cross of ‘Chandler’ x ‘Treasure’ produced this effect for both HBI traits, resulting in family means which were much lower than parental means. Crosses involving the parent ‘NCL 11-185’ had a similar effect for HBI traits, producing progeny with greater average resistance than either parent. For *C. gloeosporioides* HBI, crosses involving the parent NCH 11-304 produced progeny with greater average resistance than either parent.

Frequency distributions for ACR were generally bimodal and weighted toward susceptibility. NCH 11-304 produced the most resistant families tested for resistance to ACR and these distributions were heavily weighted toward resistance (Figure 5.5).

Partitioning of variance components and estimation of heritability were performed separately for the three traits and are presented in Table 5.6. Variance components (VC%) were calculated as the percentage of total variance contributed by a given parameter. Residual variance was high for the two HBI traits and contributed more than 92% of the total variance observed. Residual variance was lower for ACR, contributing 78% of the total variance. The relative importance of additive and dominance effects on total genetic variance were assessed with the ratio of additive to dominance variance (σ_A^2/σ_D^2) for each trait. The closer this ratio was to zero, the greater the additive component of genetic variance. The higher this ratio, the greater the proportion of dominance variance. Ratios of dominance variance to additive variance were similarly low for *C. acutatum* HBI (0.13) and *C. gloeosporioides* ACR (0.20), but were much higher for *C. gloeosporioides* HBI (0.91). Narrow sense heritability estimates were 0.25 for *C. acutatum* HBI and 0.16 for *C. gloeosporioides* HBI, but were much higher for *C. gloeosporioides* ACR (0.61). Estimates of genetic gain from selection were calculated for each trait and are presented in Table 5.6. HBI traits were highly positively correlated ($r_A = 0.98$) but negative correlations were found between *C. acutatum* HBI and ACR ($r_A = -0.85$) as well as between *C. gloeosporioides* HBI and ACR ($r_A = -0.61$) (Table 5.7).

Discussion

Anthracnose crown rot and fruit rot are two of the most problematic diseases in strawberry production and have been a significant financial concern to strawberry growers in North Carolina and the southeast for decades (Giménez and Ballington, 2002; Poling, 2008). Although fungicides are available to assist in controlling these diseases, genetic host resistance is a more effective and less costly option that is more easily implemented by growers. This study provided estimates of genetic parameters governing inheritance of resistance to *Colletotrichum* HBI in strawberry leaf tissue, a novel trait in strawberry breeding that may provide rate-limiting resistance in strawberry nurseries and fruiting fields. Additionally, inheritance of resistance to ACR caused by *C. gloeosporioides* was studied and recommendations were made for advancing HBI and ACR resistance traits in breeding programs.

Analysis of combining abilities for the HBI traits found significant (positive and negative) GCA effects for some parental genotypes included in this mating study. NCS 10-147 was ranked in a previous study by Jacobs *et al.* (unpublished) as the most resistant of 18 genotypes screened for resistance to *C. acutatum* HBI. In line with this result, the current study found that NCS 10-147 had a GCA of -4.27 and conveyed resistance to its progeny. Though NCS 10-147 was identified as resistant in the previous study, both of its parents, ‘Chandler’ and ‘Treasure’ were observed to be susceptible to *Colletotrichum* HBI. The relative susceptibility of ‘Chandler’ and ‘Treasure’ were confirmed by high PSLA scores in the current study, averaging 38.32% and 48.73%, respectively. The family mean of ‘Chandler’ by ‘Treasure’ was 25.04% for *C. acutatum* HBI, much lower than the midparent

value of 43.53%. Resistance of NCS 10-147 and hybrids of ‘Chandler’ by ‘Treasure’ support the significant negative GCA of ‘Chandler’ and its capacity to produce resistant progeny, despite its own susceptibility. This is an example of transgressive segregation where progeny exhibit more extreme phenotypes than the phenotypes of the parents. Transgressive segregation is common in plants and is often due to recombination of additive alleles that can accumulate and contribute toward these extreme phenotypes. Transgressive segregation also occurred for HBI traits in families including the parents NCL 11-185 and NCH 11-304. Both of these parents produced several families with mean PSLA scores much lower than their parental means.

Environmental influence, differences in plant physiology, inoculation efficiency, and laboratory-based assays represented sources of experimental error affecting the accuracy with which we are able to evaluate this phenotype. Similar observations have been made by other groups attempting to evaluate resistance to anthracnose diseases in strawberry. Smith and Black (1987) found that strong genotype by environmental interactions affected the expression of resistance to these pathogens, noting that apparent field resistance may not be representative of true genetic resistance. Image-based analysis was employed in a previous study to improve the accuracy and precision of PSLA measurements of HBI resistance (Jacobs et al., unpublished). Despite high (92.4% and 92.2%) residual variance estimates reflecting the inherent variability of hemibiotrophic quiescent foliar infections in the current study, observable genetic variance was estimated for both HBI traits. *C. acutatum* HBI had a GCA variance of 0.1812 (6.7%) and SCA variance of 0.0246 (0.9%). A low dominance to additive variance ratio (0.13) for this trait confirms strong additive genetic control for resistance to *C. acutatum* HBI. *C. gloeosporioides* HBI had a GCA variance of 0.0670

(4.1%) and SCA of 0.0607 (3.7%). This resulted in a much higher dominance to additive variance ratio (0.91) than estimated for *C. acutatum* HBI, which suggested that this trait was under nearly equal dominance and additive genetic control.

Combining ability analysis for ACR found significant GCA effects for two of the parental genotypes tested. ‘Chandler’ had a significant positive GCA (0.18), indicating its hybrid combinations averaged an 18% increase in rAUDPC score and greater susceptibility to ACR. NCH 11-304 had a significant negative GCA (-0.28) and conveyed a 28% average decrease in rAUDPC to its progeny. NCH 11-304 is the progeny of a cross of Winter Dawn by NCH 09-068, a genotype found by Jacobs *et al.* (unpublished) to be one of the most resistant of 18 genotypes screened for resistance to ACR. Additionally, Osorio *et al.* (2014) included NCH 09-068 as a parent in their study of inheritance of resistance to ACR. This study found that NCH 09-068 ranked just behind ‘Pelican’ in terms of resistance conferred and was one of the best parents conveying resistance to ACR. NCH 11-304 also produced transgressive segregation in resistance to *C. gloeosporioides* HBI and its progeny means were much lower than parental means in families in which it was a parent. This result may suggest some shared resistance to *C. gloeosporioides* between crown and leaf tissue and could be a contributing factor in the higher dominance to additive genetic ratio of *C. gloeosporioides* HBI.

Residual variance represented a smaller portion of total variance for ACR (78.5%) than for the HBI traits. SCA variance (0.0053) contributed 3.6% of the total variance, similar to the 3.7% SCA variance found for *C. gloeosporioides* HBI. GCA variance was 0.0265 and represented a much larger variance component percentage (17.9%) than other genetic variance components observed in this study. Similar to *C. acutatum* HBI, the ACR trait

produced a low dominance to additive variance ratio (0.20) which indicates strong additive genetic control of resistance to ACR in this population. This result agrees with findings from other inheritance studies of this trait. Osorio *et al.* (2014) found that dominance variance was roughly one-third of the additive variance for the population screened in North Carolina. This was similar to the current study's finding of dominance variance representing roughly one-fifth the additive variance component. However, this similarity was not surprising due to significant overlap in the pedigrees of genotypes between the two studies.

Genetic improvement of quantitative traits requires reliable estimates of heritability to structure an effective breeding program. Moderately low narrow-sense heritabilities of 0.25 and 0.16 were found for *C. acutatum* and *C. gloeosporioides* HBI, respectively.

Heritabilities in this range are not uncommon for quantitative disease resistance traits or for traits linked to the fitness of individuals. This association was confirmed in a previous study that found low heritabilities were caused by consistently high residual variance rather than low additive variance (Merilä and Sheldon, 2000). It is unclear whether the low percentage of total variance components accurately described the genetic control of HBI traits or if genetic variance was obscured by higher environmental variance. A moderate heritability estimate of 0.61 for ACR suggested that this trait should respond well to selection of resistant genotypes.

Estimates of gain from selection were used to estimate selection progress for each trait. The greatest relative gains from selection would be seen for *C. gloeosporioides* ACR. High levels of resistance (similar to levels seen in 'Pelican') could be achieved in two cycles of selection. Progress would be much slower for the HBI traits. Approximately three cycles would be required for each 10% decrease in PSLA for *C. acutatum* HBI, but a similar

decrease for *C. gloeosporioides* HBI would require six or more cycles. Therefore, gain from selection was predicted to be high for ACR resistance, moderate for *C. acutatum* HBI resistance, and low for *C. gloeosporioides* HBI resistance.

It would be desirable to simultaneously improve populations for multiple anthracnose resistance traits. A strong genetic correlation ($r_A = 0.98$) between resistance to *C. acutatum* HBI and *C. gloeosporioides* HBI suggests that resistance to these two *Colletotrichum* species is controlled by common genes in strawberry leaf tissue. Selecting for increased resistance to HBI for one *Colletotrichum* species should produce gains in resistance to the other. On the other hand, negative genetic correlations between ACR and both HBI traits suggest that resistance in crown tissue is inherited independently of resistance in leaf tissue in the examined population. Genetic correlation was most highly negative across tissue types and between species, with $r_A = -0.85$ for *C. acutatum* HBI and *C. gloeosporioides* ACR. Genetic correlation was moderately negative between tissue types for *C. gloeosporioides*, with $r_A = -0.61$ for *C. gloeosporioides* HBI and ACR. While there is no direct evidence that selecting for resistance to ACR would increase susceptibility to HBI, or vice versa, negative genetic correlations indicates that simultaneously selecting for gains in resistance to HBI and ACR would be slow. These data are more pronounced but in line with findings of a moderate correlation ($r = 0.43$) between HBI traits and a weak correlation ($r = 0.24$) between *C. gloeosporioides* HBI and ACR traits in the previous study (Jacobs et al, unpublished). However, these differences are expected as the previous study was conducted on 18 unrelated genotypes while the current study focuses on a population of related individuals derived from just six parental genotypes.

This study provides data on the inheritance of resistance to *Colletotrichum* species in

two strawberry tissue types. These results recommend independent evaluation and advancement of resistance to HBI and ACR. Though resistance to HBI for both *C. acutatum* and *C. gloeosporioides* may be advanced simultaneously, the high genetic correlation of these traits suggest that independent evaluation of each species may not be necessary. Selecting for resistance to HBI of one *Colletotrichum* species should also convey resistance to the other. Future inheritance studies exploring resistance to *Colletotrichum* HBI are recommended to identify the best sources of resistance to this trait through screening a large panel of diverse strawberry germplasm. Populations developed from parents with only the most divergent HBI resistance phenotypes would optimize estimates of inheritance parameters. Environmental replication incorporated into future studies will permit estimates of environmental variance as a component of non-additive genetic variance. Enhanced breeding methodologies that control the large residual variance associated with resistance to HBI will improve the rate of gain and success of breeding for these traits. Field observations of parental genotypes suggest that results from this greenhouse-based study are correlated to field-level resistance. However, further work will be required to confirm the performance of these genotypes in a natural environment where they may be exposed to different varieties and intensities of biotic stress than were applied in this study.

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Table 5.1. Summary of experiments by *Colletotrichum* species and genotypes included.

Experiment	Pathogen	Half-sib families	Genotypes	Sample dates	Observations
Hemibiotrophic infection (HBI)	<i>C. acutatum</i>	15	548	1	548
	<i>C. gloeosporioides</i>	15	476	1	547
Anthracnose crown rot (ACR)	<i>C. gloeosporioides</i>	14 ^a	560	8	4480

^aProgeny of the cross NCS 10-080 x NCH 11-304 were included only in HBI experiments due to poor seed germination.

Table 5.2. Half diallel mating design for six parents shared between two experiments. Parents were selected for variable response to HBI and ACR infection in a previous screen and include two commercial cultivars and four selections from the NC State strawberry breeding program^{a,b}.

Parent	HBI experiment						ACR experiment					
	‘TR’	‘CH’	‘147’	‘185’	‘304’	‘080’	‘TR’	‘CH’	‘147’	‘185’	‘304’	‘080’
‘Treasure’ (TR)	---	---	---	---	---	---	---	---	---	---	---	---
‘Chandler’(CH)	33 ^c /32 ^d	---	---	---	---	---	40	---	---	---	---	---
NCS 10-147 (147)	39/34	36/30	---	---	---	---	40	40	---	---	---	---
NCL 11-185 (185)	38/32	35/25	35/30	---	---	---	40	40	40	---	---	---
NCH 11-304 (304)	40/38	37/33	40/37	35/31	---	---	40	40	40	40	---	---
NCS 10-080 (080)	38/34	38/33	35/33	39/29	30/25 ^e	---	40	40	40	40	---	---

^aNumber of individual genotypes tested per full-sib family.

^bDeviations from original 40 individuals per family were due to plant death before sample collection 21 days after inoculation.

^cNumber of individuals included for the *C. acutatum* HBI experiment.

^dNumber of individuals included for the *C. gloeosporioides* HBI experiment.

^eNCS 10-080 x NCH 11-304 was only included in the HBI experiment due to insufficient plant numbers.

Table 5.3. Analysis of variance for resistance to hemibiotrophic infections of *Colletotrichum acutatum* and *C. gloeosporioides* in strawberry foliage. Treatments consisted of either *C. acutatum* or *C. gloeosporioides* inoculum applied to strawberry foliage.

Source of variation	df	Mean square
Block	3	2.54
Treatment (<i>Colletotrichum</i> spp.)	1	0.15
Block x Treatment	3	1.91
Genotype	20	14.85***
Parent	5	6.73*
Cross	14	6.00*
Treatment x Genotype	20	2.36
Treatment x Parent	5	1.91
Treatment x Cross	14	1.57
Block x Genotype	60	2.40
Block x Treatment x Genotype	60	2.15

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5.4. Analysis of variance of the general combining ability (GCA) and specific combining ability (SCA) of crosses screened for resistance to hemibiotrophic foliar infections of *C. acutatum* and *C. gloeosporioides* and anthracnose crown rot of *C. gloeosporioides*.

Source of variation	df	<i>C. acutatum</i> HBI		<i>C. gloeosporioides</i> HBI		<i>C. gloeosporioides</i> ACR ^a	
		Estimate	Pr>Z	Estimate	Pr>Z	Estimate	Pr>Z
GCA	5	0.1812	0.0821	0.0670	0.1355	0.0265	0.0735
SCA	14 ^a	0.0246	0.2920	0.0607	0.1204	0.0053	0.0982
Residual error	20	2.5176	<.0001	1.4994	<.0001	0.1164	<.0001

^aDiallel analysis of *C. gloeosporioides* ACR based on 13 SCA degrees of freedom due to insufficient plant numbers for the family NCS 10-080 x NCH 11-304.

Table 5.5. Parental and family means with standard deviations (SD), general combining abilities (GCA), and specific combining abilities (SCA) for resistance to hemibiotrophic foliar infections of *C. acutatum* and *C. gloeosporioides* and anthracnose crown rot of *C. gloeosporioides* for 6 parental genotypes and 15 families in a half diallel mating design.

Parents	<i>C. acutatum</i> HBI				<i>C. gloeosporioides</i> HBI				<i>C. gloeosporioides</i> ACR			
	PSLA ^a	SD	Range	GCA	PSLA	SD	Range	GCA	rAUDPC ^b	SD	Range	GCA
Chandler	38.32	17.9	9.4 – 84.6	-4.34*	34.36	15.2	14.5 – 62.2	-1.42	0.42	0.26	0 – 0.76	0.18*
NCH 11-304	38.71	18.4	12.9 – 75.0	5.03*	41.21	18.5	10.9 – 73.0	1.19	0.04	0.07	0 – 0.36	-0.28***
NCL 11-185	33.44	14.3	14.2 – 65.2	-2.33	38.15	17.4	24.2 – 82.8	-1.63	0.36	0.27	0 – 0.88	0.05
NCS 10-080	29.83	18.2	14.7 – 71.2	3.37	29.82	16.4	20.1 – 72.6	3.29*	0.38	0.29	0 – 0.90	0.08
NCS 10-147	28.64	18.5	9.3 – 78.7	-4.27*	30.19	16.6	21.7 – 84.6	-2.25	0.16	0.23	0 – 0.76	-0.01
Treasure	48.73	14.0	25.1 – 76.8	2.54	50.95	20.4	19.9 – 74.6	0.84	0.19	0.18	0 – 0.68	-0.03
Family	PSLA	SD	Range	SCA	PSLA	SD	Range	SCA	rAUDPC	SD	Range	SCA
080 x 147	28.31	14.1	6.0 – 60.1	-1.14	29.12	15.1	9.4 – 57.9	-1.18	0.52	0.39	0 – 1.0	-0.02
080 x 185	34.37	20.4	4.4 – 79.0	2.02	32.15	15.4	5.2 – 64.2	2.20	0.58	0.38	0 – 1.0	-0.02
080 x 304	38.92	18.1	14.5 – 77.7	0.67	39.46	15.1	9.7 – 67.2	4.66*	---	---	---	---
080 x Chan	29.05	12.9	4.3 – 52.6	-0.37	28.25	11.8	10.3 – 48.7	-1.63	0.77	0.25	0 – 1.0	0.02
080 x Treas	34.55	19.4	8.7 – 68.4	-0.14	31.45	12.1	11.9 – 59.0	0.27	0.57	0.38	0 – 1.0	0.03
147 x Chan	24.15	14.9	6.3 – 57.2	-0.56	29.93	16.5	9.4 – 67.9	1.14	0.71	0.28	0 – 1.0	0.04
147 x Treas	30.21	18.3	5.7 – 69.1	0.01	26.76	9.3	12.4 – 44.6	-1.18	0.37	0.37	0 – 0.9	-0.04
185 x 147	25.36	10.8	5.6 – 44.9	0.01	22.30	9.1	8.5 – 39.1	-2.37	0.58	0.39	0 – 1.0	0.04
185 x Chan	22.61	13.8	5.2 – 57.9	-0.73	21.37	8.7	6.8 – 44.8	-2.68	0.81	0.25	0 – 1.0	0.07
185 x Treas	31.38	15.3	8.4 – 66.5	1.04	30.01	15.9	5.1 – 62.9	2.49	0.43	0.40	0 – 1.0	-0.04
304 x 147	33.43	18.6	4.4 – 75.9	0.35	29.83	12.3	10.1 – 56.4	0.62	0.14	0.24	0 – 0.8	-0.03
304 x 185	24.57	11.0	8.2 – 47.0	-3.07	23.78	7.4	8.7 – 41.3	-1.79	0.17	0.28	0 – 0.9	-0.05
304 x Chan	35.97	20.1	6.2 – 74.1	2.02	28.62	13.7	9.1 – 54.3	-0.07	0.27	0.33	0 – 0.9	-0.06
304 x Treas	39.86	23.2	1.3 – 88.1	1.58	26.41	13.4	4.6 – 55.0	-1.87	0.29	0.39	0 – 1.0	-0.08
Chan x Treas	25.04	14.0	5.8 – 54.2	-1.71	30.65	12.7	9.4 – 54.3	1.38	0.57	0.39	0 – 1.0	-0.04

^aPSLA = mean percent sporulating leaf area, back-transformed for presentation.

^brAUDPC = mean relative area under the disease progress curve.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5.6. Variance components and heritabilities for resistance to hemibiotrophic foliar infections of *C. acutatum* (*C. a.* HBI) and *C. gloeosporioides* (*C. g.* HBI) and anthracnose crown rot of *C. gloeosporioides* (*C. g.* ACR).

Variance component ^a	<i>C. a.</i> HBI		<i>C. g.</i> HBI		<i>C. g.</i> ACR	
	Estimate	VC(%) ^b	Estimate	VC(%)	Estimate	VC(%)
σ_{GCA}^2	0.1812	6.7	0.0670	4.1	0.0265	17.9
σ_{SCA}^2	0.0246	0.9	0.0607	3.7	0.0053	3.6
σ_P^2	2.9046		1.6941		0.1747	
σ_A^2	0.7248		0.2680		0.1062	
σ_D^2	0.0984		0.2428		0.0214	
σ_e^2	2.5176	92.4	1.4994	92.2	0.1164	78.5
h^2	0.25		0.16		0.61	
H^2	0.28		0.30		0.73	
σ_D^2/σ_A^2	0.13		0.91		0.20	
G_S	0.37		0.18		0.22	

^aEstimates of variance components: σ_{GCA}^2 = general combining ability (GCA) variance, σ_{SCA}^2 = specific combining ability (SCA) variance, σ_P^2 = phenotypic variance, σ_A^2 = additive genetic variance, σ_D^2 = dominance genetic variance, σ_e^2 = residual error, h^2 = narrow-sense heritability, H^2 = broad-sense heritability, σ_D^2/σ_A^2 = ratio of dominance to additive variance, and G_S = gain (10%) based on full-sib family selection.

^bVC(%) = the percentage of total observed variance accounted for by each variance component.

Table 5.7. Genetic correlations^a between resistance to *C. acutatum* HBI, *C. gloeosporioides* HBI, and *C. gloeosporioides* ACR in a strawberry population tested by half-sib analysis.

Trait	<i>C. a.</i> HBI	<i>C. g.</i> HBI	<i>C. g.</i> ACR
<i>C. a.</i> HBI	---	0.98	-0.85
<i>C. g.</i> HBI	---	---	-0.61
<i>C. g.</i> ACR	---	---	---

^aGenetic correlations calculated as $r_A = \sigma_{Gij}^2 / (\sigma_{Gi}^2 \times \sigma_{Gj}^2)^{1/2}$, where σ_{Gij}^2 = the genetic covariance between traits and σ_{Gi}^2 and σ_{Gj}^2 = the genetic variance components of each trait.

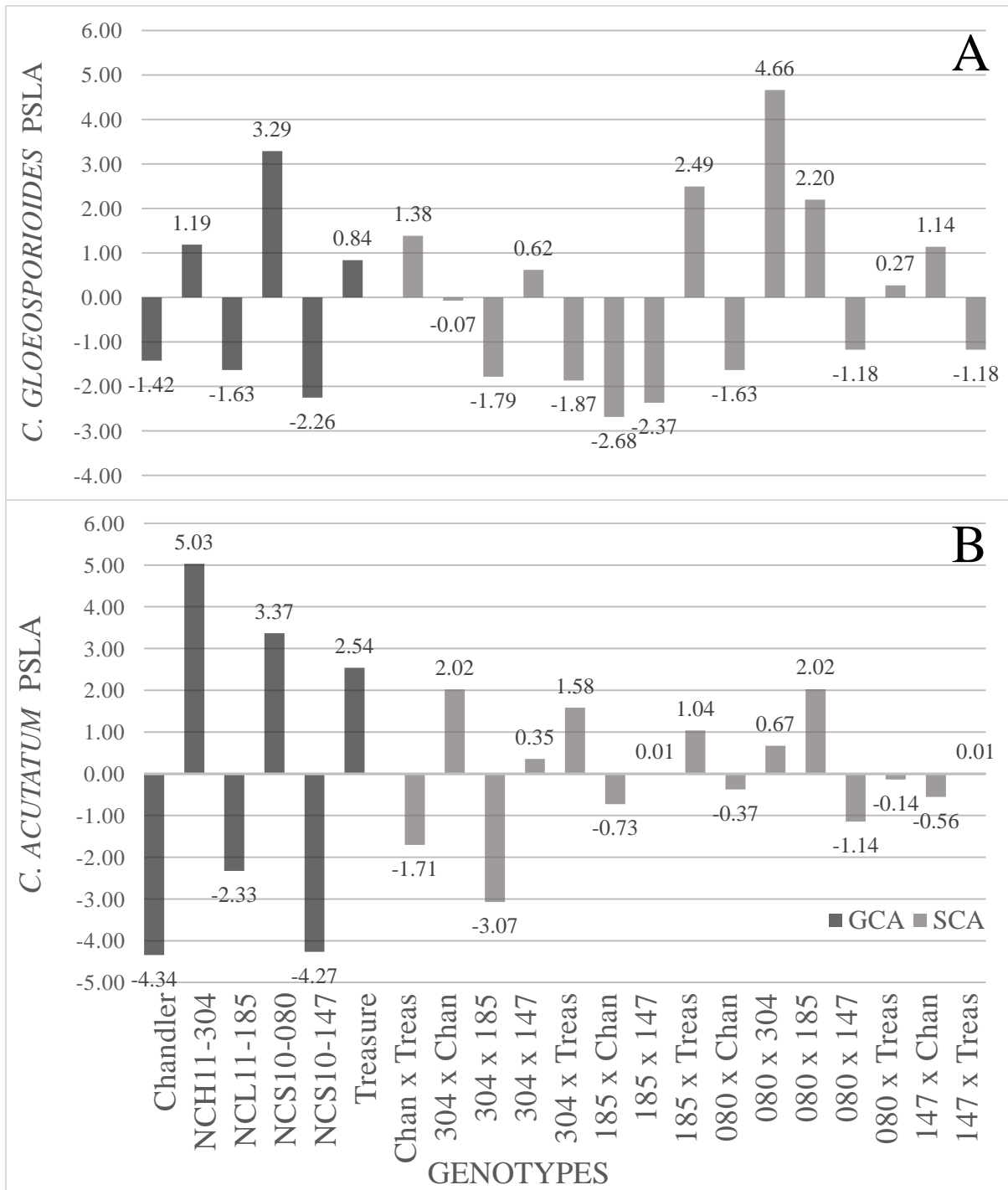


Figure 5.1. General combining abilities (GCA) of parents and specific combining abilities (SCA) of crosses screened for resistance to (A) *C. gloeosporioides* and (B) *C. acutatum* hemibiotrophic infections. GCA and SCA response measured in percent sporulating leaf area (PSLA).

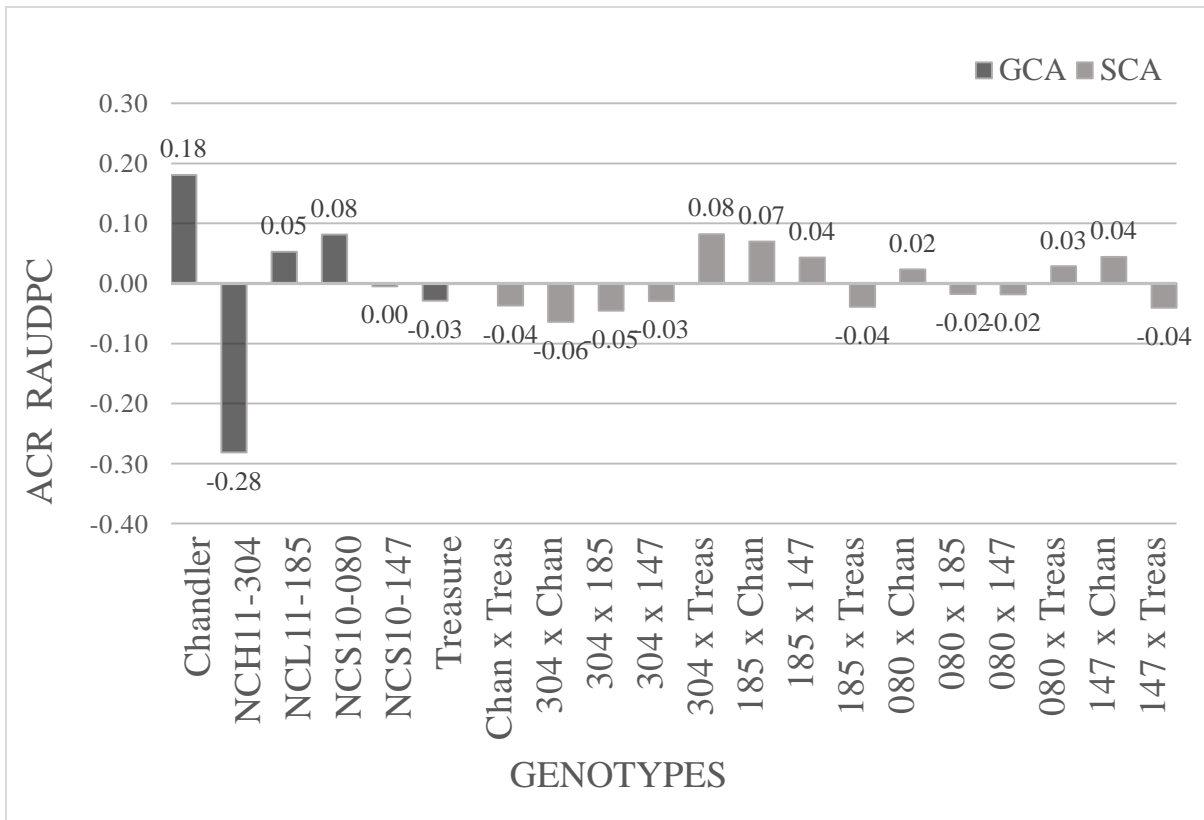


Figure 5.2. General combining abilities (GCA) of parents and specific combining abilities (SCA) of crosses screened for resistance to anthracnose crown rot (ACR) caused by *C. gloeosporioides*. GCA and SCA response measured as the relative area under the disease progress curve (rAUDPC), a proportion of observed to maximum potential disease over time.

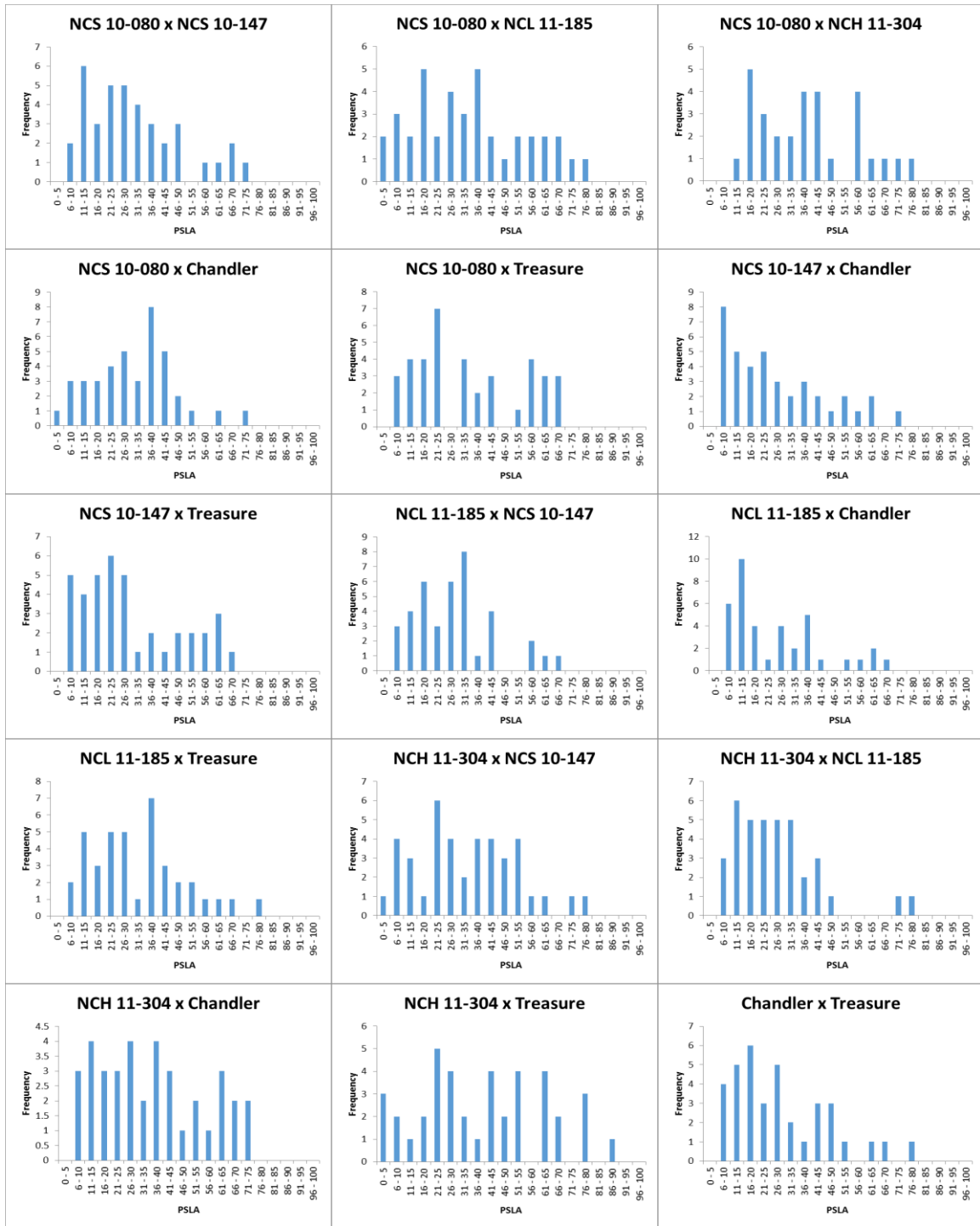


Figure 5.3: Frequency distributions of segregation within families for resistance to *C. acutatum* HBI, measured as percent sporulating leaf area (PSLA).

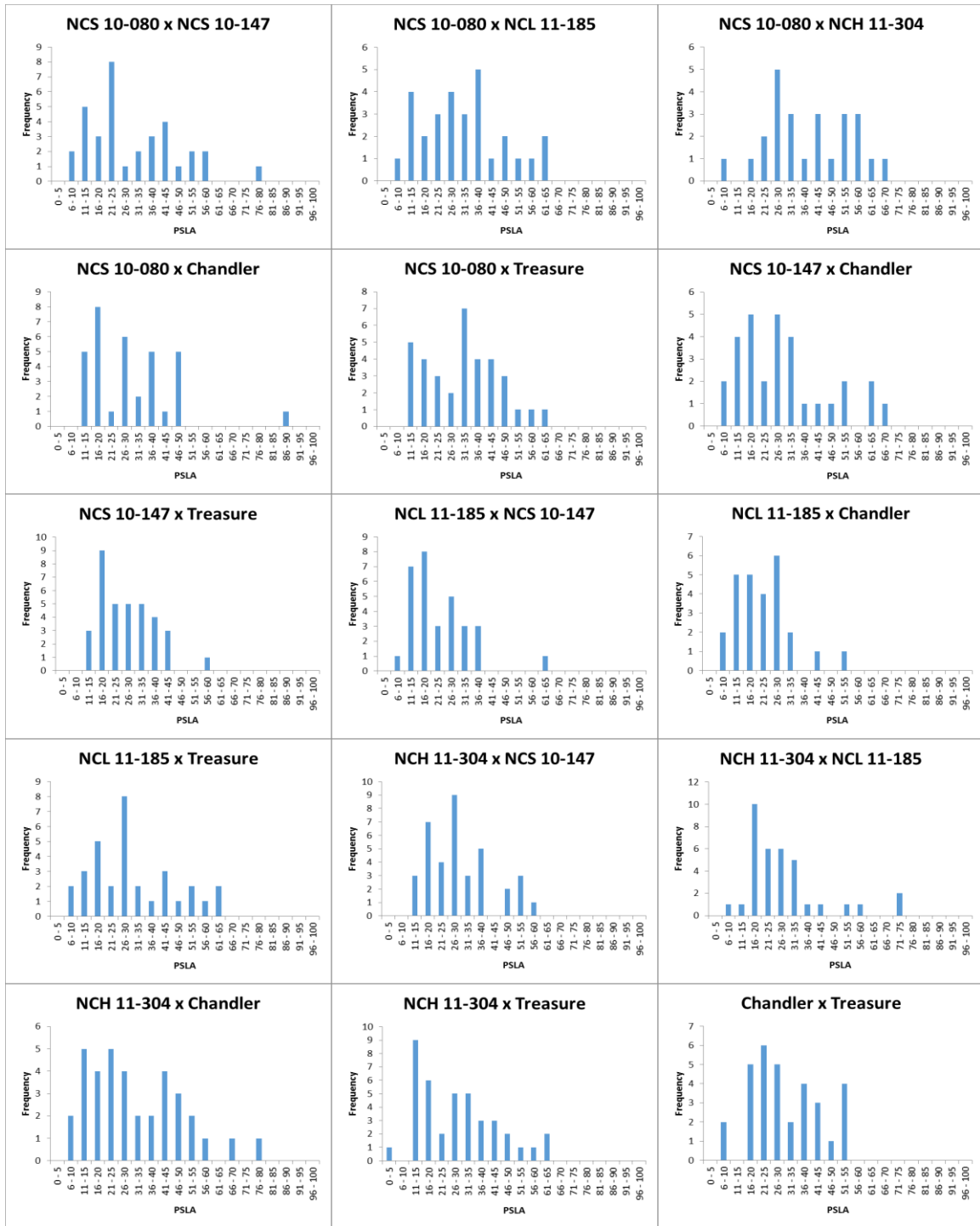


Figure 5.4: Frequency distributions of segregation within families for resistance to *C. gloeosporioides* HBI, measured as percent sporulating leaf area (PSLA).

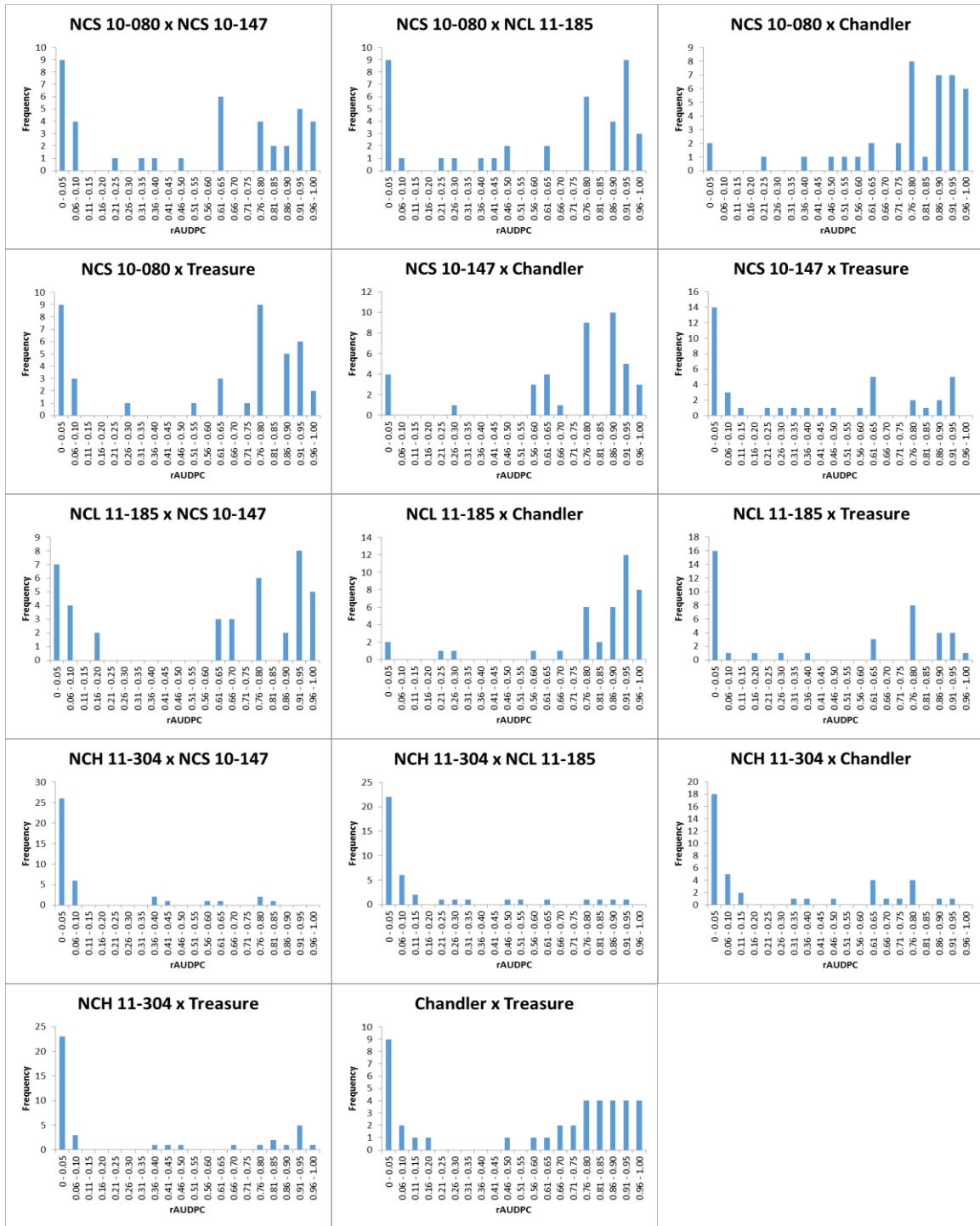


Figure 5.5: Frequency distributions of segregation within families for resistance to *C. acutatum* ACR, measured as relative area under the disease progress curve (rAUDPC).



Figure 5.6. Fruit setting on mother plants in the greenhouse after controlled crosses have been made. Seed were isolated from ripe fruit and were scarified and stratified before germination.



Figure 5.7. (A) Growth room used for seedling germination to prevent premature *Colletotrichum* infection. Growth rooms were maintained at 21°C with 14 hour day lengths. (B) At the two true leaf stage, seedlings were removed from the growth room and transplanted to 50-cell plug trays in the greenhouse.

Chapter Six

GENERAL CONCLUSIONS

Colletotrichum gloeosporioides and *C. acutatum* are important pathogens that cause anthracnose crown rot (ACR) and anthracnose fruit rot (AFR) in the southeastern United States and in most strawberry-growing regions of the world. Previous studies have characterized anthracnose resistance in seedlings, crown tissue (often called whole-plant resistance), fruit, and runners. A general consensus characterizing the inheritance of anthracnose resistance traits is beginning to form for some of the more studied host tissues – namely fruit and crown tissue. Deployment of genetic resistance to ACR and AFR has been met with mixed success due the inferior agronomic or fruit quality characteristics of many resistant cultivars and the tendency for anthracnose epidemics to occur irregularly. Growers are unlikely to adopt resistant cultivars with less desirable qualities in situations where anthracnose is infrequently a problem. Anthracnose epidemics continue to occur despite the many successes of best management practices developed and implemented by IPM programs over the years. Resistance must be incorporated into cultivars with superior agronomic and fruit quality traits to gain favor among growers.

Investigatory work and follow-up studies surrounding anthracnose epidemics in the last decade have implicated asymptomatic nursery transplants harboring quiescent hemibiotrophic *Colletotrichum* infections in their foliage. The hemibiotrophic infection (HBI) strategies of *C. gloeosporioides* and *C. acutatum* allow them to spread

asymptomatically within nursery plantings. The greatest losses due to anthracnose are incurred when large portions of transplants contain hemibiotrophic foliar infections and infected plant material is disseminated and planted in fruit production fields. Strawberry leaf tissue plays a major role in the life cycles of *C. gloeosporioides* and *C. acutatum* and is a major component in the development of anthracnose epidemics. However, very little research has been conducted to characterize resistance to hemibiotrophic infections in strawberry foliage. The research objectives presented in this dissertation aimed to evaluate resistance to HBI in strawberry germplasm and to conduct a mating study to better understand how resistance is inherited. Parallel studies were conducted to evaluate resistance to ACR and to permit a comparison of resistance in different host tissues.

The research conducted has provided valuable insight into a poorly understood but critical trait in the strawberry-*Colletotrichum* pathosystem. Methods were established to evaluate the HBI phenotype, which cannot be observed directly. An ImageJ macro was adapted for use in this study that allowed quantitative image-based measurements to be made and increased the accuracy of data collection. Evaluation of commercial cultivars and selections from the NC State strawberry breeding program found significant variation in resistance to HBI. In general, genotypes responded very similarly in resistance to *C. gloeosporioides* and *C. acutatum*, with very few genotype by treatment interactions observed. Commercial cultivars tended to rank among the most susceptible, while cultivars selected under high disease pressure in North Carolina tended to rank among the most resistant. Significant variation in resistance to ACR was observed among these genotypes, but resistance did not correlate well between HBI and ACR traits. Moderate phenotypic correlation was observed between HBI resistance traits but required further examination

through a formal inheritance study.

Inheritance of *C. gloeosporioides* and *C. acutatum* HBI as well as *C. gloeosporioides* ACR were investigated through a half diallel mating design to permit calculation of variance components and provide greater insight into modes of inheritance for these traits. Ratios of dominance to additive variance were low for *C. acutatum* HBI and *C. gloeosporioides* ACR, indicating strong additive genetic control of these resistance traits. Surprisingly, the dominance to additive variance ratio was much different for *C. gloeosporioides* HBI and indicated nearly equal dominance and additive genetic control of this trait. However, the accuracy of this result should be questioned due to the low total genetic variance observed for *C. gloeosporioides* HBI.

Heritabilities were fairly low for both HBI traits, but this is not atypical for quantitatively-inherited resistance traits. ACR resistance had moderate heritability and suggested strong response to selection. A strong genetic correlation between *C. gloeosporioides* HBI and *C. acutatum* HBI suggested that resistance to hemibiotrophic *Colletotrichum* infections is controlled by common genes in strawberry leaf tissue. On the other hand, negative genetic correlations between ACR and both HBI traits may indicate that resistance to *C. gloeosporioides* is inherited independently in crown and leaf tissue.

Increased resolution and insight into the genetic parameters controlling resistance to *Colletotrichum* HBI may be achieved in part through the identification of greater sources of resistance than those used in the current study. Additionally, environmental replication and enhanced breeding methodologies to control the large residual variance associated with HBI traits will improve the success of future breeding attempts. Populations developed from highly divergent (resistant and susceptible) genotypes evaluated under uniform conditions

will maximize observations of genetic variance components. Identification of greater HBI resistance sources and enhanced breeding methodologies may make the HBI traits ideal candidates for marker assisted selection because these phenotypes cannot be observed directly and high-throughput phenotyping is difficult to achieve.

The work presented in this dissertation has enhanced our understanding of resistance to hemibiotrophic *Colletotrichum* infections in strawberry foliage and provided insight into the tissue-specific resistance mechanisms active in strawberry leaf and crown tissue. This research may be valuable to extension specialists, plant pathologists, and plant breeders interested in the strawberry-*Colletotrichum* or similar pathosystems wherein host foliage plays a critical role in the life cycle of a pathogen.