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Screening Strawberry Clones for Anthracnose Disease Resistance Using Traditional Techniques and Molecular Markers

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SCREENING STRAWBERRY CLONES FOR ANTHRACNOSE DISEASE RESISTANCE USING TRADITIONAL TECHNIQUES AND MOLECULAR MARKERS

by

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ABSTRACT

SCREENING STRAWBERRY CLONES FOR ANTHRACNOSE DISEASE RESISTANCE USING TRADITIONAL TECHNIQUES AND MOLECULAR MARKERS

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May 2016

Cultivated strawberry, *Fragaria* x *ananassa* Duchesne, is host for many pathogens. One of the most destructive diseases of strawberry is anthracnose, whose symptoms include fruit rot, leaf and petiole lesions, crown rot, wilt, and death. Three species of *Colletotrichum* are considered causative agents of anthracnose diseases of strawberry. *Colletotrichum acutatum* causes anthracnose fruit rot, has a broad host range, and occurs in most areas of the world where strawberries are grown. *Colletotrichum fragariae*, the primary causal fungus of anthracnose crown rot, may infect all above ground parts of the strawberry plant, and has restricted host and geographic ranges. *Colletotrichum gloeosporioides* causes symptoms indistinguishable from those caused by *C. fragariae* but has much broader host and geographic ranges.

Plant breeding programs generally require many years to produce commercially acceptable disease resistant cultivars; however, molecular tools may be used to identify genes that convey disease resistance and decrease the overall time required to develop new disease resistant cultivars. Two disease resistance genes have been identified in strawberry through controlled crosses: *Rpf1* for resistance to *Phytophthora fragariae* and *Rca2*, a dominant gene that has been suggested to provide resistance to pathogenicity group 2 (q.v.) of *C. acutatum* (Van de Weg et al., 1997; Denoyes-Rothan et al., 2005).

Two sequence characterized amplified region (SCAR) markers for the *Rca2* resistance allele were found in several European and U.S. cultivars and, although there was not a perfect association with anthracnose resistance, these SCAR markers provide a unique set of tools to use in screening for anthracnose-resistant genotypes in strawberry breeding programs.

The primary goal of this research was to establish the resistance or susceptibility of 81 strawberry germplasm lines to all three anthracnose-causing *Colletotrichum* species and to determine the degree to which an association exists between the resistance or susceptibility of these plants to the presence or absence of the *Rca2* resistant allele SCAR markers. A secondary goal of this research was to determine if a detached leaf assay could be used to define the resistance or susceptibility of strawberry plants to anthracnose, thus providing an efficient, non-destructive method to screen strawberry germplasm for anthracnose resistance.

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DEDICATION

This dissertation is dedicated to my loving husband, Jody, for his patience, and his belief that I could accomplish this personal goal.

This dissertation is also dedicated to my family, especially my Mom, Dorothy, my siblings, Susan, Frank, and Robert, my friends, PJ, Lavonne, Jenna, Rosemary, Estelle, Monika, Sarah, and Sean, who have helped with the research or offered moral support and encouragement and to Mrs. Wanda Elliott for her friendship, may she find the peace in death that eluded her in life.

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Fungal Component of the Disease — Infection Process

Anthracnose is a term applied to fungal diseases that are characterized by dark spots or sunken lesions with slightly raised edges that appear on the leaves or stems of a host plant. Typically these lesions are described as a layer of host tissue that overlies disintegrating host cells. The center of the lesion usually contains conidia produced within a black, cushion-shaped hyphal structure called an acervulus, which develops in the host's epidermal and subepidermal tissue and is erumpent through the epidermis. Conidia develop in a moist, hydrophilic, mucilaginous matrix composed mostly of polysaccharides and glycoproteins. This matrix may prevent conidial germination while the conidia are still within the acervulus and also aid in maintaining the conidia when surroundings are dry (Louis and Cooke, 1985; Nicholson and Moraes, 1980; Louis et al., 1988). Conidia are released from mature acervuli in matrix droplets that are pigmented depending on the color of the conidia, which may be white, cream, pink, orange, black, or other colors (Alexopoulos and Mims, 1979). Conidia from young acervuli are generally dispersed by water droplets; but if environmental conditions are dry, the matrix can form a crusty deposit that will bind several conidia together, and these conidia may be wind dispersed (Bailey et al., 1992).

The first step to successful pathogenesis is the attachment of a conidium to the surface of a plant. The conidium germinates producing a germ tube, the tip of which differentiates into an appressorium. Appressorial formation is often accompanied by the development of a mucilaginous material that surrounds the appressorium and appears to

be involved in adhesion of the appressorium to the plant surface. A penetration hypha (infection peg) develops from the appressorium and penetrates the plant tissue through natural openings, wounds, or direct penetration of the cuticle. Two mechanisms of penetration have been proposed which are not necessarily mutually exclusive: secretion of cutinases (enzymes that degrade cutin) (Manandhar et al., 1985; O'Connell et al., 1985) and mechanical force (Mercer et al., 1975; O'Connell et al., 1985). Melanin, a brown pigment in the appressorial wall, may provide protection from solar radiation and also plays a role by strengthening the appressorial wall to support the internal hydrostatic pressure necessary for penetration. Hyphae developing from the penetration peg begin to grow inside host cells, within host cell walls, and in the intercellular spaces, infecting and colonizing the plant tissues (Bailey et al., 1992).

The success of *Colletotrichum* species as pathogens may depend on their initial infection and colonization strategies. Bailey et al. (1992) noted *Colletotrichum* species primarily have two infection strategies: intracellular hemibiotrophic and subcuticular intramural. Both strategies begin with a symptomless phase followed by a destructive, necrotrophic phase in which host cells are killed before the fungus enters them. Species that exhibit the intracellular hemibiotrophic infection strategy begin with a biotrophic infection which is symptomless as the fungus establishes itself by penetrating cell walls and growing within the cell lumina without killing host cells. Histopathology studies by Curry et al. (2002) determined that two *Colletotrichum* species, *C. acutatum* and *C. fragariae*, have a very brief biotrophic phase of less than 12 hours on strawberry host plants. Arroyo et al. (2005) also found a brief biotrophic phase between 12 and 24 hours following inoculation of strawberry petioles with *C. acutatum*. *Colletotrichum* species

displaying a subcuticular intramural infection strategy grow beneath the cuticle and within the periclinal walls of the epidermal cells, but do not enter the cell lumina; therefore, the cuticle is not destroyed. The necrotrophic phase begins after the plant tissues are colonized and the pathogen begins to produce enzymes to break down structural components of the host cell walls. Two frequently occurring types of enzymes are produced by the pathogen: polygalacturonases that degrade pectin dissolving cell walls and cutinases that hydrolyze the plant cuticle. Some *Colletotrichum* species also produce low molecular weight phytotoxins which may kill cells in advance of the hyphal invasion (Bailey et al., 1992). The necrotrophic phase is visibly destructive to the plant and is manifested as anthracnose and blight symptoms.

Plant Component of the Disease — Defense Responses

Plants are continually exposed to pathogens in their environment, and their structure offers pathogens a wide diversity of habitats, e.g., aerial region (phyllosphere), root zone (rhizosphere), and internal transport or vascular system (endosphere). Fortunately, most pathogens are capable of causing disease only on a limited range of plants. Plants in which the pathogen is unable to induce disease are considered to be nonhost plants. Plants do not have an animal-like immune system, but do have a number of efficient defense mechanisms. Nonhost and host plant defenses are triggered by different mechanisms and have specific defense responses when attacked.

Nonhost resistance is the most common form of disease resistance in plants. Mysore and Ryu (2004) proposed that nonhost resistance consists of two types of reactions which depend on the plant/pathogen interaction. Type I nonhost resistance has no visible disease symptoms. The pathogen cannot overcome the mechanical and

chemical barriers of the plant that provide nonspecific protection against a wide range of organisms, e.g., waxy cuticles, cell wall components, enzyme inactivators, and antimicrobial and toxic compounds. The pathogen is also unable to overcome inducible resistance. Type II nonhost resistance is characterized by limited necrosis and appears to be associated with the penetration process of the pathogen and detection of nonspecific pathogen-elicited molecular signals. Type II is always associated with the hypersensitive response (HR), a highly specific cell suicide at the penetration point. A pathogen may overcome the preformed mechanical and chemical barriers of defense and directly penetrate cells. The plant recognizes pathogen-derived molecules within the cells and produces the HR. The HR is a spatially confined rapid reaction killing the plant cells at the spot of infection and confining the pathogen to the necrotic tissue. A nonhost plant may display type I resistance against one pathogen and type II against another. Either of these two types of response will stop the pathogen from invading additional plant tissues.

Host resistance consists of constitutive and inducible defense mechanisms. Constitutive defenses are constantly available and considered passive defenses. The waxy surface of the cuticle can repel water and without water many conidia are unable to germinate. Thick cuticles or thick epidermal cell walls may increase resistance against direct penetration of some pathogens. Many pathogenic fungi enter through open stomata, and some plants have the ability to open their stomata only when the chance of infection is low, such as in the case of stem rust of wheat (*Puccinia graminis*) and some wheat varieties, which open their stomata late in the day. The stem rust spores germinate in the night dew, but the germ tubes desiccate due to evaporation before the stomata begin to open (Hart, 1929). Some constitutive defenses that inhibit fungal growth are

preexisting chemicals on the surface (fungitoxic exudates) or within the cells of the plant, such as phytoanticipins which are constitutive phytoalexins synthesized at a constant rate within the cell. Constitutive defenses in plants are basically the same for pathogens and nonpathogens (Agrios, 2005). Inducible defense mechanisms become active upon pathogen recognition and are also considered active defenses. A plant is capable of recognizing and responding to stimuli produced by an invading pathogen in the early stages of the invasion. Pathogen recognition by the plant gives the idea of a surveillance system to detect pathogen-generated stimuli (Hutcheson, 1998).

Qualitative and quantitative terms are used to distinguish types of disease resistance or to characterize trait expressions. Qualitative resistance does not occur in degrees. The plant is either resistant or susceptible based on one or a few tightly linked genes which form the basis of Mendelian ratios. Phenotypic plasticity may influence the trait but not enough to mistake resistance for susceptibility. Quantitative resistance occurs in degrees and there are no distinct categories of phenotypes. Phenotypic variation is obvious across populations and blurs the distinction between resistance and susceptibility (Pataky and Carson, 2008).

Resistance is a phenotypic expression of the genotype. Genotypic systems may be either monogenic, oligogenic, or polygenic, depending on the number of nuclear genes involved in the inheritance of a single characteristic. A monogenic system is where a single character is tied to a single gene. Such a system can be placed in discrete categories because the phenotype occurs in genetically segregated populations and is considered to have qualitative resistance at the phenotypic level. Oligogenic systems are characterized by multiple genes that code for a single phenotypic trait. If the trait can be

placed into a discrete category, it is considered qualitative, just as a monogenetic system. If the trait cannot be placed in a discrete category, it is considered quantitative. Polygenic systems are characterized by multiple genes that code for multiple, closely related phenotypic traits that cannot be placed in discrete categories. They are considered a quantitative system just as with an oligogenic quantitative system.

The monogenic gene-for-gene theory was introduced by Flor (1955) specifying that host plant resistant (R) genes mediate the recognition of the products of the plant pathogens' avirulence (avr) genes. Avirulence gene proteins (elicitors) are recognized by R gene proteins (receptors) present in resistant host plants. Plants with the complementary R gene (receptor) use the avr gene (elicitor) as a recognition device. The plant mounts a defense when a pathogen has an elicitor and the plant has a receptor for the elicitor. The avr gene products are needed by the pathogen for unknown functions associated with virulence and are highly conserved in the genome of the pathogen (Ferreira et al., 2008). Virulence is the pathogen's ability to cause a compatible reaction on a host cultivar with genetic resistance. Resistance genes in plants evolved in response to avr genes or their products. Detection of specific molecular signals is required to resist pathogens that are capable of overcoming constitutive barriers. The gene-for-gene theory refers to avr genes in the pathogen that correspond to genes for specific resistance in the host. Specific resistance is effective against some races of a pathogen (avirulence), but ineffective against virulent races. Therefore, if the host has more than one gene conveying specific resistance, there will be a number of potential races of the pathogen. The number of races increases exponentially, 2^N, where N equals the number of host resistance genes. Host-pathogen interactions may be compatible or incompatible as

conveniently shown by the quadratic check (Table 1). A compatible reaction is when disease occurs because the host lacks the R gene, or when the host has the R gene, but the pathogen lacks the corresponding avr gene. An incompatible reaction occurs when the host is resistant owing to a specific R gene and the pathogen carries the corresponding avr gene.

Many pathogenic fungi release substances that act as nonspecific or general elicitors recognized by the host plant. General elicitors can be any molecule (proteins, glycoproteins, peptides, carbohydrates, and lipids) capable of triggering a plant defense reaction and are often grouped collectively as pathogen-associated molecular patterns (PAMPs). The initial elicitor(s) starts signal transduction pathways and the end products of the pathways induce defenses of the plant leading to disease resistance (Agrios, 2005). Receptors in the host plant may be extracellular, in the cell membrane, or intracellular. Recognition by the plant of signals from a plant pathogen activates host cell proteins and nuclear genes. When activated, they elicit the production of pathogen inhibitory substances and mobilize defensive chemical reactions and structural changes at the location of the pathogen attack (Figure 1). The extremely rapid response which causes localized host cell death is known as the hypersensitive response (HR). The HR kills the invaded host cells, the surrounding host cells, and the invading pathogen cells. The HR reaction often triggers a transduction pathway which signals nonspecific resistance throughout the plant. The plant may synthesize phytoalexins (antimicrobial products produced in response to a stimulus) and reactive oxygen species. The alarm is often transmitted to cells adjacent to the one being attacked and is also transmitted systemically

to the rest of the plant. The systemic transmission of pathogen resistance is known as systemic acquired resistance.

General elicitors may be endogenous host components released or modified by the pathogen (Figure 1, blue arrows). Some plant pathogens produce cell wall degrading enzymes that break down molecules in the plant's surface cells, e.g., cutinases may be released by the pathogen to break down polysaccharides in plant cell walls and glucanases may be released to break down oligosaccharides. The breakdown of polysaccharides and oligosaccharides creates carbon and energy sources for the fungus and elicitors for the host plant defenses. Some host plants release pathogen cell wall degrading enzymes, glucanases and chitinases that fragment fungal cell walls, creating oligosaccharides which act as elicitors, inducing defense responses. The elicitors are detected by receptors in the host and predictably inform the plant's surveillance system of the invader.

General elicitors may also be exogenous of pathogen origin (Figure 1, red arrows). Plant pathogen elicitors are often constitutive, essential for the pathogen, highly conserved in the genome, and not found in plant hosts. Fungal plant pathogens may produce endopolygalacturonases (EPGs) that cleave pectin in plant cell walls, producing oligogalacturonides (OGAs), which are elicitors of plant defenses. The EPGs quickly convert OGAs to smaller inactive fragments, but not before some can elicit the production of polygalacturonase inhibiting proteins (PGIPs) within the plant. The PGIPs form reversible complexes with fungal EPGs, increasing the elicitor active OGAs. Fungal pathogens release glucanase-inhibiting proteins (GIPs) to inhibit cell wall degrading enzymes from the plant and limit the plants' awareness (Ferreira et al., 2008).

Specific elicitors are also released by some pathogens, triggering cultivar-specific responses (Figure 1, yellow arrow). The specific elicitors are produced by the avirulence (avr) genes of the pathogen and are recognized by the complimentary resistance (R) gene (gene-for-gene). The avr gene products elicit plant defenses by way of the R gene products. This event can be likened to radar, the R gene products looking for a ping from the avr gene products. When a signal (ping or elicitor) is received by the R gene products, defense responses are triggered.

Distribution of Anthracnose Diseases

Anthracnose diseases are incited by fungi within the genus *Colletotrichum* that are favored by high temperatures and humidity and are found in tropical zones where they are frequently isolated from both healthy and diseased tissues. Diseases caused by *Colletotrichum* species may affect the shoots, leaves, flowers, and fruit. Sometimes fruit infections will remain latent until after the fruit has reached the consumer. Tropical crops can be decimated by anthracnose diseases, causing significant problems for farmers as well as consumers in areas lacking refrigeration. A few *Colletotrichum* species cause anthracnose diseases on almost all tropical and subtropical crops, including avocado, yam, cassava, banana, papaya, citrus, and mango. Several species of *Colletotrichum* infect ripening coffee berries, but *Colletotrichum coffeanum* causes twig blight, infection of the leaves and berries, as well as fruit drop and defoliation. Temperate zones, such as the Gulf Coast area of the United States, often experience warm, humid conditions, and anthracnose can be severe on annual crops in these areas. Legumes and cucurbits (e.g., watermelon, cantaloupe, and cucumber) are subject to anthracnose at all stages of growth. Ripe rot of tomato, eggplant, and pepper incited by *Colletotrichum* species can cause

severe crop loss. Onions are also susceptible to an anthracnose disease, called smudge, which is caused by *Colletotrichum circinans*. Anthracnose is an important disease of strawberries, caused by a complex of three *Colletotrichum* species, which may infect the leaves, stolons, fruit, and crown, causing plant death. Many of the world's most important cereal crops (corn, wheat, barley, and rice), ornamental plants, and turf grasses may host anthracnose disease organisms. By contrast, anthracnose is insignificant as a plant disease in arid and semi-arid areas (Waller, 1992).

Colletotrichum Nomenclature and Identification

Fungi are unique in their dual nomenclature with a name for the teleomorph (sexual state) and a distinctly different name for the anamorph (asexual state). The term holomorph refers to both states, sexual and asexual, collectively. Many pathogenic fungi were discovered on plant hosts in their asexual state and given documented names before the connection between sexual and asexual states was unequivocally determined.

Sexual states. Fungi that incite anthracnose diseases normally are found in nature as the anamorph, but some do occur as the teleomorph, including the ascomycetous fungi in the genera *Diplocarpon*, *Discula*, *Elsinoe*, *Gnomonia*, and *Glomerella*. These fungi may attack and destroy the foliage, stem, flower, or fruit of the host plant. *Diplocarpon rosae* is destructive to roses and, although it is an anthracnose disease, it is better known as black spot of rose whose symptoms are black lesions on the leaves that prematurely drop and purple-red lesions on immature canes. *Discula destructiva* is the causal agent of anthracnose of dogwood, a relatively new disease first reported in the 1970s. The genus *Elsinoe* is associated with anthracnose of grape and raspberry, and *Gnomonia* is associated with anthracnose of walnut, forest trees, and shade trees (Agrios, 2005).

Glomerella is another teleomorph of *Colletotrichum,* but it is rarely associated with anthracnose. The anamorph *Colletotrichum* has been identified causing anthracnose diseases on many different host plants, but the teleomorph *Glomerella* has not been identified for all of these hosts. *Colletotrichum* species produce conidia in an acervulus, and *Glomerella* species produce ascospores in asci that develop within a perithecium. Past and current literature can be confusing when authors report the sexual name when only the asexual state has been found in nature. Some of these fungi can be manipulated in the laboratory or greenhouse to produce the sexual state. Kendrick and Di Cosmo (1979) suggested many of these anamorphs were identified by association with the teleomorph and should be considered with a degree of skepticism. Referring to a pathogen by its teleomorph name may not be prudent for a fungus that is well known by its anamorph, except in rare cases where the teleomorph is recovered from an infected plant.

The teleomorph of *C. acutatum* was first reported in the U.S. from apple (Guerber and Correll, 1997) and then characterized as *Glomerella acutata* in 2001 (Guerber and Correll, 2001). The teleomorph of *C. fragariae* has not been described as of this writing. The teleomorph of *C. gloeosporioide*s was first identified as *Glomerella cingulata* by Von Schrenk and Spaulding (1903). *Glomerella cingulata* has been isolated from blueberry, grape, strawberry, banana, coffee, apple, buckwheat, mango, *Taxus mairei* (yew), *Hevea brasiliensis* (rubber tree), *Catostemma fragrans* (Central American timber tree), *Mora excelsa* (Central American timber tree), and *Chlorocardium rodiei* (South American timber tree) (Maas and Howard, 1985; Cannon et al., 2008). Bitter rot of apple fruit and bitter rot cankers on the trunk and branches of apple trees are caused by the

teleomorph, *G. cingulata*. Ripe rot of grape, pears, and peaches are also caused by *Glomerella* species, but more often the fungus is found as the anamorph producing acervuli in which conidia are formed (Agrios, 2005). *Colletotrichum gloeosporioides* isolated from avocado will produce the teleomorph, *G. cingulata*, in culture, but has thus far not been found in nature (Freeman, 2000).

Asexual states (Colletotrichum species). Anthracnose diseases of many plants are caused by the anamorph *Colletotrichum* species. Sutton (1992) reported about 900 species that had been referred to as *Colletotrichum* species.

Traditional methods for identification of *Colletotrichum* plant pathogens have relied on the morphological features of colony color, size and shape of conidia, optimal growth temperature, growth rate on specific agars, presence or absence of setae, and the existence of a teleomorph. Identification can be difficult due to frequent subculture of the pathogen, type of storage, and environmental influences. New molecular tools have been introduced to allow more reliable identification of the many plant pathogens based on deoxyribonucleic acid (DNA) analysis. Nuclear DNA polymorphisms, arbitrarily primed PCR (apPCR), ribosomal DNA (rDNA), mitochondrial DNA (mtDNA), and DNA sequence analysis are some of the techniques being utilized to discriminate among populations of *Colletotrichum* species (Freeman, 2000).

Colletotrichum gloeosporioides was described by Penzig and Saccardo (1884). It is a cosmopolitan plant pathogen with a broad host range, having been associated with approximately 470 different host genera (Sutton, 1992). *Colletotrichum gloeosporioides* is considered a species complex with a wide variation in morphology on different host plant species. The anthracnose disease symptoms caused by *C. gloeosporioides* on

strawberry are not distinguishable from those caused by *C. fragariae*. Isolates are gray to olive gray with dark olive to dark gray in reverse. Conidia are cylindrical with both ends rounded and shorter than the conidia of *C. acutatum* and *C. fragariae* (Smith and Black, 1990; Sutton, 1980; Baxter et al., 1983; Gunnell and Gubler, 1992). Formation of setae on strawberry leaf agar was reported by Gunnell and Gubler (1992). Conidia of *C. gloeosporioides* do not survive very long in the natural environment. Ureña-Padilla et al. (2001) determined that the inoculum of *C. gloeosporioides* does not survive between strawberry growing seasons in Florida. They buried strawberry crown tissue infected with *C. gloeosporioides* and found the fungus was stable for 2–3 weeks, but could not detect the fungus after 56 and 98 days in 1998 and 1999, respectively.

Colletotrichum fragariae, identified as the causal organism of anthracnose on strawberry in Florida by Brooks (1931), can cause anthracnose disease on all aerial parts of the strawberry plant. Von Arx (1957) and Lenné (1977) considered *C. fragariae* to be synonymous with *C. gloeosporioides*. The fungal organism causing anthracnose on strawberry in Florida was identified by morphological features as the *C. fragariae* type of *C. gloeosporioides* and was continually isolated for fifteen years before December, 1982, at which time *G. cingulata* (=*C. gloeosporioides)* was isolated from strawberry in Florida for the first time (Howard and Albregts, 1983). The authors noted that the morphological features of *C. fragariae* remained the same over 15 years of isolations, differing from *C. gloeosporioides* in the color of conidia and abundance of aerial conidia. Several researchers (Howard and Albregts, 1983, 1984; Maas and Howard, 1985) either alluded to, or suggested that, *C. fragariae* was possibly a separate species from *C. gloeosporioides*. Smith and Black (1987) suggested the name anthracnose crown rot to

distinguish disease caused by *C. fragariae* from disease caused by other *Colletotrichum* species. *Colletotrichum fragariae* isolates exhibit beige to olive to dark gray color in culture with dark olive to dark gray in reverse. Conidia are cylindrical with one end pointed and setae are produced in culture (Smith and Black, 1990). Gunnell and Gubler (1992) found the conidia to be narrowly obovate and setae formation was observed. Horn and Carver (1968) reported that *C. fragariae* did not survive in the soil in Louisiana between plantings (over summer), but was found living in an inactive state in the crowns of apparently healthy plants through the winter months and served as a primary inoculum the following spring. Howard et al. (1992) found pathogenic *Colletotrichum* species do not survive from year to year in the soil in Florida or Louisiana. *Colletotrichum fragaria*e had been considered host specific to strawberry, but more recent research confirms it is a pathogen on other hosts. Isolates repeatedly found on silver date palm (*Phoenix sylvestris*) and cyclamen (*Cyclamen persicum*) plants in Florida were morphologically identified as *C. fragariae* and used to inoculate strawberry plants (MacKenzie et al., 2008). The isolates from the silver date palm were not pathogenic on strawberry. The isolates from cyclamen were pathogenic on strawberry and, after DNA testing, appeared to be from the same *C. fragariae* group that infects strawberry. Survival of *C. fragariae* on *Senna obtusifolia* (coffee weed) was determined by Howard and Albregts (1983). The ability of *C. fragariae* to survive on hosts other than strawberry possibly creates a reservoir for inoculum.

Colletotrichum acutatum was treated as a morphological variant of *C. gloeosporioides* until Simmonds (1965) described it as a distinct species in Queensland, Australia, in a pathogen survey of fruit rot. Smith and Black (1986) first reported *C.*

acutatum on strawberry in the U.S. They identified isolates from strawberry originating from California, Florida, Mississippi, and Missouri. *Colletotrichum acutatum* may cause anthracnose disease on the runners, petioles, leaflets, and fruit of strawberry (Howard et al., 1992). Colonies of *C. acutatum* grown in the laboratory are generally white to light gray, becoming covered with pink to orange conidial masses, and are salmon color in reverse. The conidia are primarily produced in acervuli and are ellipsoid and fusiform (Gunnell and Gubler, 1992; Smith and Black, 1990). Setae were not observed in the isolates grown on potato-dextrose agar (PDA) or oatmeal:potato dextrose agar (OMA:PDA; 1:1, v/v) (Smith and Black, 1990), but were observed on isolates grown on strawberry leaf agar (SLA) (Gunnell and Gubler, 1992). Leandro et al. (2001) found the production of secondary conidia by *C. acutatum* increased the overall total conidia threefold on asymptomatic strawberry plants. Secondary conidia can remain latent for an undetermined amount of time and may become pathogenic under specific environmental conditions. Eastburn and Gubler (1990) found *C. acutatum* survived for at least nine months in the soil within plant debris in California. *Colletotrichum acutatum* had a 100% recovery rate from infested mummified fruit which overwintered both on the soil surface and 5–8 cm below in a two-year field study in Ohio (Wilson et al., 1992). Freeman et al. (2001) found *C. acutatum* exhibited epiphytic and endophytic lifestyles on various plant species without causing visible disease symptoms.

Plant Breeding Programs (History)

Classical plant breeding is the discipline where sexual crosses of individuals are made to introduce traits from one variety or line into another variety or line. Plant breeders often use local plants to cross sexually with wild species or plants from different

localities to introduce new genes into the germplasm. Strawberry plant breeders perform sexual crosses between pairs of strawberry plants, and the resulting seeds are collected and germinated. The seedlings are tested for desirable traits, such as disease resistance, better plant and fruit quality, and increased yield. Gene pool diversity may become a concern when many cultivars have been produced from the same parent gene pool over the years. This has happened with strawberries where the intense selection for specific traits has decreased the germplasm variation and (parent) base. The inbreeding of cultivated strawberries may result in loss of vigor, yield, and fruit size. Breeders are working to increase the genetic diversity of the cultivated strawberry by using wild strawberries in their programs (Degani et al., 2001; Hancock and Luby, 1993).

Strawberry plants may be diploid, tetraploid, hexaploid (mainly in Europe and Asia), or octoploid (North and South America). The woodland strawberry, *Fragaria vesca*, is the only diploid native to North America. The octoploid nature of the cultivated strawberry has hindered breeding programs to some extent due to various traits of discontinuous and continuous inheritance patterns within the same plant (Galletta and Maas, 1990; Hancock, 1999). Some studies show the octoploid strawberry exhibits diploid inheritance patterns for some traits. Two disease resistance genes have been identified in strawberry through controlled crosses: *Rpf1* for resistance to *Phytophthora fragariae* and *Rca2*, a dominant gene that has been suggested to provide resistance to pathogenicity group 2 (q.v.) of *C. acutatum* (Van de Weg et al., 1997; Denoyes-Rothan et al., 2005). Valuable traits used in breeding strawberries are disease and insect resistance, yield, plant vigor, flowering date, runner density, fruit set, and fruit appearance and flavor. Many lower ploidy species exhibit traits (cold tolerance, heat and drought

resistance, high aroma, and disease resistance) that could be valuable in the cultivated species. Most commercial strawberries have been selected to be self-pollinating.

Strawberries have been cultivated in Europe since the 14th century, maybe even earlier. Today the most popular cultivated strawberry is *Fragaria* x *ananassa*, an accidental hybrid of *F. chiloensis* from Chile and *F. virginiana* from the eastern U.S. *Fragaria virginiana* was introduced to Europe in approximately 1629 and the hybridization occurred sometime after *F. chiloensis* was introduced to France in 1714 by French Army Lieutenant Colonel Frezier (Darrow, 1966). The name 'ananassa' is for the perfume of the strawberry fruit, which smells like *Ananas* or pineapple (Hancock et al., 2008).

Many strawberry plants exhibit some resistance to anthracnose disease based on controlled inoculations and field trial studies (Brooks, 1931; Delp and Milholland, 1980, 1981; Smith and Black, 1987; Gupton and Smith, 1991). Commercial strawberry growers, state and federal breeders, and plant pathologists have worked to develop anthracnose resistant strawberry plants for many years. The USDA, Agricultural Research Service, established a strawberry breeding program in the 1980s at the Small Fruit Research Station, Poplarville, MS, to develop strawberry germplasm resistant to *C. fragariae* (anthracnose crown rot) and adapted to the southeastern U.S. (Smith, 2006). A total of 1515 anthracnose-resistant selections were identified following greenhouse screening for anthracnose resistance and field testing for horticultural traits. These clones were designated as MSUS to denote that they were from the USDA breeding program in Mississippi. One of the MSUS clones was released as the anthracnose resistant cultivar Pelican (Smith et al., 1998), and four others were released as breeding lines, i.e., US70,

US159, US292, and US438 (Galletta et al., 1993). These strawberry clones have been used as parent lines in other breeding programs and continue to be clonally propagated at the USDA, ARS, Poplarville, MS research station along with approximately 66 unreleased MSUS selections.

Strawberries are commercially propagated by cloning, a type of asexual propagation. The daughter plants (runners) are allowed to establish a root system, removed from the "mother" plants, and established in the greenhouse or field. Cloning is also used for the rapid multiplication of a promising cross for further study or testing without affecting the plant's genome.

Tissue culture, another form of asexual propagation, is valuable for producing disease-free plants. A meristem-tip, shoot-tip, or bud can be established *in vitro* on artificial medium. These *in vitro* plants are used for asexual multiplication or micropropagation on medium containing specific growth hormones. After the plants are clonally increased in number on multiplication medium, they are separated, and each plant may be placed on various media such as holding medium for maintaining the plant or rooting medium to stimulate the growth of roots. Once roots are established, the plant can be established in a potting medium to prepare the plant to be moved to the greenhouse. *In vitro* asexual multiplication is a quick method for increasing selections for field evaluations. Tissue culture can also be used to preserve the germplasm of plants such as strawberry which have a short regeneration time for whole plants (Zimmerman, 1983).

Anthracnose Disease Control Methods

Commercial strawberry growers rely on various types of disease control measures including disease-free plants, sanitation practices, pesticides, cultural controls, and disease resistant cultivars. Disease-free plants may be initiated from tissue culture and can be multiplied quickly. They are then propagated clonally in field plots following strict sanitation protocols before being transplanted into production fields. Establishing fields with disease-free plants decreases the possibility of introducing plant pathogens from other locales into the grower's field. Sanitation practices are used to destroy fungal pathogens on equipment and in the soil but do not stop latent infections in plants that appear healthy.

Fungal pathogens are often controlled by the use of fungicides; however, fungicides rarely eradicate the pathogens. Frequent use of fungicides by strawberry growers has resulted in fungicide resistant pathogen populations and the failure of fungicides to control anthracnose epidemics (Smith and Black, 1993). The use of ineffective fungicides adds unnecessary expense when applied to several hectares of strawberries or other crops. Although *Colletotrichum* species are not considered soil pathogens, it is important to note that *C. acutatum* and *C. fragariae* have both been found to overwinter in soil and crowns in some areas. Many soil pathogens and weeds are controlled by fumigating the soil with methyl bromide plus chloropicrin before planting, but the use of methyl bromide is being phased out because it has been found to cause damage to the ozone layer of the stratosphere. There is ongoing research of other methods of sterilization of the soil, including placing plastic sheets over the soil on hot sunny days (solarization) to cause the soil temperature to increase sufficiently to kill

many soil-borne pathogens and weed seeds near the surface thus reducing pathogen inoculum and weed populations. Soil may be heat sterilized with steam or hot water in greenhouses (Agrios, 2005). Samtani et al. (2012) tested the efficacy of pre-plant soil steam and solarization treatments for control of soil pests and weeds in commercial strawberry fields in California. Solarization alone was an inexpensive treatment, but the fruit yield was 16% less than with the methyl bromide plus chloropicrin treatment. Steam applied with spikes effectively controlled pests, was comparable to methyl bromide plus chloropicrin in cost of weed control, and increased the gross harvest revenue by $10,500 per hectare, but due to high application costs ($25,212/ha) , the net revenue was decreased by $11,500 per hectare.

Mulch is an important cultural control used to protect the strawberry fruit from soil pathogens, decrease weeds, and maintain soil moisture. The concept of mulch encompasses a wide variety of materials, including black plastic, wheat straw, and chopped corn stalks. The use of straw mulch reduces within-field spread of anthracnose by reducing conidial dispersal by splashing water. The use of drip irrigation in place of overhead irrigation also decreases the spread of conidia due to water splash and the amount of water required.

Delp and Milholland (1980, 1981) found that strawberry cultivars resistant to *C. fragariae* can decrease the rate of disease spread in a field. The best disease control is planting disease resistant strawberry cultivars (Agrios, 2005).

Screening for Anthracnose Resistance

Strawberry plants found to be disease resistant in the greenhouse are usually transplanted to the field to evaluate the plants for horticultural characteristics and disease

response based on natural infections. The efficacy of field testing for disease resistance presents problems due to the inconsistency of disease pressure and variable environmental conditions, and it is time consuming.

Plants are often screened for disease resistance in greenhouses where the inoculum and environment can be better controlled. A set of standard cultivars with known disease reactions is often included to help compare results. Thousands of strawberry seedlings can also be screened for anthracnose disease resistance efficiently in the greenhouse. Whole plants of standard cultivars and seedlings are inoculated with a conidial suspension of a pathogenic *Colletotrichum* species and evaluated for resistance or susceptibility. High humidity in greenhouses offers an ideal environment for the development of fungal diseases. The use of more aggressive pathogen isolates in inoculation studies separates germplasm reactions more effectively than less aggressive isolates. Resistant gene expression depends on the interaction of the plant with the pathogen in a suitable environment for disease development. Screening for disease resistance using detached strawberry leaves is a possible alternative to greenhouse screening using whole plants. Inoculating detached leaves with *Colletotrichum* species may provide an accurate, rapid, non-destructive method of identifying anthracnose resistant germplasm. Howard and Albregts (1983) determined that the black leaf spot phase of anthracnose is very often the first symptom of anthracnose in the strawberry nursery and can serve as an early warning that anthracnose is present.

Molecular markers also can be used to identify resistance in plants. Many organisms are diploid with pairs of alleles with slight differences (polymorphisms) in their DNA sequences. These differences may cause one allele to be dominant over the

other, thus determining the phenotypic expression. Polymorphisms can be tracked as molecular markers which are short fragments of DNA associated with a specific location within the genome. The markers can be used to identify desirable genes as long as they are inherited together (linkage disequilibrium).

Most commercially grown strawberry cultivars are octoploid, and this causes difficulty in studying their genetics. Some traits demonstrate disomic inheritance which opens the possibility that some traits of interest can be found with dominant and recessive traits (Lerceteau-Köhler et al., 2003; Folta and Davis, 2006). Denoyes and Baudry (1995) recognized two pathogenicity groups for *C. acutatum* isolates during a pathogenicity study of *Colletotrichum* isolates from strawberry. The grouping was based on pathogenicity of the isolates on five strawberry cultivars with known susceptibility to these isolates. Another study (Denoyes-Rothan et al., 2003) using cluster analysis performed on random amplified polymorphic DNA (RAPD) and ITS sequence data from 95 representative isolates of *C. acutatum* and *C. gloeosporioides* provided evidence of at least two genetic groups of *C. acutatum*. The two genetic groups were termed CA-clonal with 54 isolates exclusively from strawberry and CA-variable with 24 isolates from hosts other than strawberry. *Colletotrichum gloeosporioides* clustered separately from *C. acutatum* in the cluster analysis. The genetic subgroups did not correlate with the pathogenicity groups. Denoyes-Rothan et al. (2005) reported the inheritance of a dominant gene (*Rca2*) controlling strawberry resistance to pathogenicity group 2 of *C. acutatum*. Four amplified fragment length polymorphism (AFLP) markers were found linked to the *Rca2* gene. Two were converted to sequence characterized amplified region

(SCAR) markers and used to screen European and U.S. strawberry genotypes for the *Rca2* gene (Lerceteau-Köhler et al., 2005).

Dissertation Research

The goal of this research was to screen strawberry germplasm lines (consisting of 50 MSUS clones and 31 named cultivars) for the two *Rca2* gene SCAR markers and to establish the degree of host resistance/susceptibility to anthracnose incited by three *Colletotrichum* species known to be pathogenic on strawberry. The resistance/susceptibility of the germplasm lines was determined by using a disease severity rating (DSR) assigned to individual plants inoculated with *Colletotrichum* isolates. The same germplasm lines were also screened for the two SCAR markers using polymerase chain reaction (PCR) with specific primers. This information was used to establish the degree of association between the resistance/susceptibility of the strawberry germplasm to anthracnose and the presence/absence of the SCAR markers.

A secondary goal was to determine if a detached leaf assay could be used in future studies to establish a plant's anthracnose resistance/susceptibility without sacrificing the whole plant. Detached strawberry leaves were inoculated with the same *Colletotrichum* isolates used in the whole plant inoculation study in order to establish the degree of association and agreement between the two rating methods.

Table 1

Quadratic check.

Pathogen	Host Gene	
Gene	R	S
avr	I	C
no avr	C	C

Note: Four possible combinations of host-pathogen interactions may occur among pathogens with or without an avirulent gene (avr) and hosts with a resistance gene (R) or without a resistance gene (S). A single incompatible (I) reaction occurs when the host has the R gene and the pathogen has the corresponding avr gene. Compatible (C) reactions occur when the host has the R gene and the pathogen lacks the corresponding avr gene or when the host does not have the R gene and the pathogen has or does not have the avr gene.

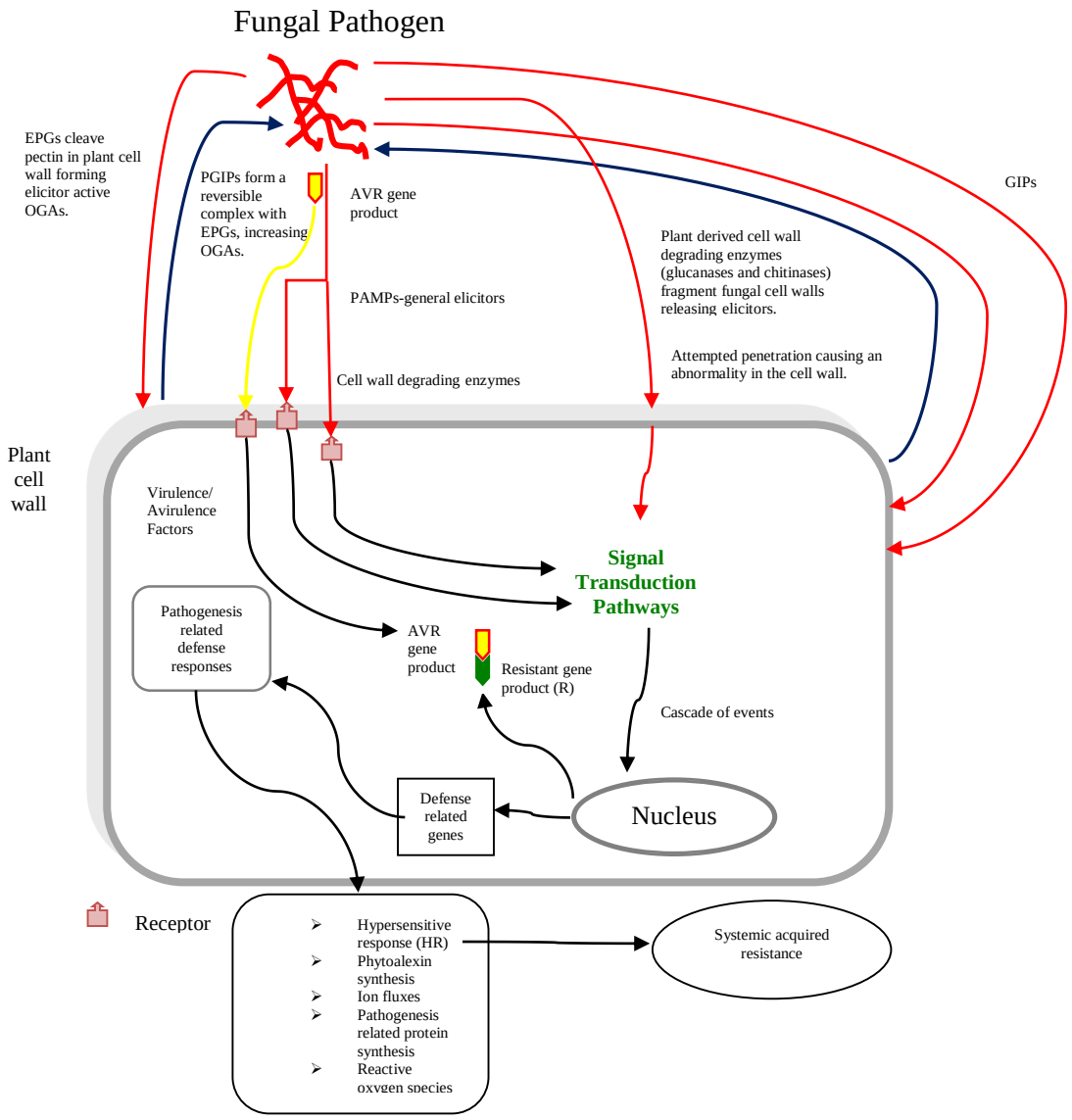

Figure 1. Host cell/pathogen interaction.

The sequence of events between a plant host cell and a fungal plant pathogen begins with perception of the initial signal by the host cell, then the signal transduction pathways, leading to defense responses by the plant. Red arrows indicate general elicitors of exogenous origin, blue arrows indicate general elicitors of endogenous origin, and yellow are specific elicitors. (EPGs-endopoligalacturonases, OGAs-oligogalacturonides, PGIPs-polygalacturonase inhibiting proteins, PAMPs-pathogen associated molecular patterns, GIPs-glucanase inhibiting proteins, AVR-avirulent gene.).

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CHAPTER II - SCREENING WHOLE STRAWBERRY PLANTS FOR ANTHRACNOSE RESISTANCE AND FOR TWO SCAR MARKERS

Introduction

Anthracnose is a destructive disease of commercial strawberries grown in the warm and humid climate of the southeastern United States. Three *Colletotrichum* species, *C. acutatum*, *C. fragariae*, and *C. gloeosporioides*, are the primary causal agents of anthracnose disease on strawberries. *Colletotrichum acutatum* incites anthracnose disease on the runners, petioles, leaves, roots, and fruit of strawberry (Howard et al., 1992; Freeman and Katan, 1997) and causes disease on many other crops (Freeman et al., 1998). It can remain latent on symptomless strawberry plants and has been found to overwinter in plant debris in the soil in some areas of the U.S. (Eastburn and Gubler, 1990; Wilson et al., 1992). *Colletotrichum acutatum* may live on a number of other plant species without causing visible disease symptoms (Freeman et al., 2001). Both *C. fragariae* and *C. gloeosporioides* may cause anthracnose disease on all aerial parts of the strawberry plant, including a devastating crown rot. *Colletotrichum fragariae* was thought to only occur on strawberry, but Howard and Albregts (1983) found that *C. fragariae* survived on *Senna obtusifolia* (coffee weed), a common weed often found surrounding strawberry fields. *Colletotrichum gloeosporioides* is a cosmopolitan plant pathogen with a broad host range.

Fungicides are regularly used to control anthracnose disease on strawberries, but fungicides seldom eradicate the pathogen. Frequent use of the same fungicides has resulted in failure of the fungicides to control anthracnose epidemics due to pathogen resistance to the fungicides (Smith and Black, 1993). The development of resistance to

fungicides in pathogen populations requires researchers to create stronger, more effective fungicides. Disease resistant cultivars would eliminate the need for fungicides.

Commercial strawberry growers, breeders, and plant pathologists are working to develop anthracnose resistant strawberry germplasm. A strawberry breeding program was established in the 1980s by the Agricultural Research Service (ARS), an agency of the United States Department of Agriculture (USDA) at Poplarville, Mississippi (Smith, 2006) with the goal of developing strawberry germplasm adapted to the southeastern U.S. resistant to anthracnose crown rot caused by *C. fragariae*. A total of 1515 anthracnose-resistant selections were identified through greenhouse screening for anthracnose resistance and field tests for agronomic traits. These selections were given the prefix MSUS and numbered to denote they were from the USDA-ARS breeding program in Mississippi. One of the MSUS clones was released as the anthracnose resistant cultivar Pelican (Smith et al., 1998), and four others were released as breeding lines, US70, US159, US292, and US438 (Galletta et al., 1993). Many of the clones and the strawberry releases continue to be clonally propagated at the Poplarville research location.

Screening for disease resistance in strawberry germplasm commonly requires inoculating whole plants of each seedling. A breeder may produce thousands of seedlings and choose to screen all the seedlings first to determine the most resistant germplasm, thus limiting the quantity of seedlings that require further evaluation for other desirable traits. Whole plants of seedlings are inoculated with a conidial suspension of a pathogen, grown in a greenhouse for at least 30 days, and then evaluated for resistance or susceptibility. The high humidity in a greenhouse offers an ideal

environment for the development of fungal diseases. Cultivars with known disease reactions are typically included in the screening process for comparison.

Field studies are also used to determine disease resistance by subjecting the plants to natural infection. Strawberry plants found to be resistant in the greenhouse are transplanted to the field to expose them to natural infection under field conditions. Field testing for disease resistance presents problems due to the inconsistency of inoculum and environmental conditions, and is labor intensive and time consuming.

The expression of disease resistance in a plant depends on the interaction of the plant with the pathogen in an environment conducive for disease development. The use of an aggressive pathogen in inoculation studies increases disease incidence and separates germplasm reactions more effectively. Greenhouse screening is preferable to field screening because the researcher can control the environment within the greenhouse and can choose the isolates to use as inoculum, whereas the researcher is at the mercy of the weather and the population of natural occurring pathogens in the field, which may differ from year to year.

Diploid organisms contain two complete sets of homologous chromosomes, and each gene or allele on a chromosome may have slight differences from their homolog in their DNA sequences, called polymorphisms, which can be tracked as molecular markers. These markers can be used to identify desirable genes if the marker and gene are linked. Many disease resistance genes in plants have been identified and molecular markers are being used in many plant breeding programs to search for these genes. The breeder's ability to select for anthracnose-resistant strawberry germplasm would be greatly enhanced with the identification of the genetic control of resistance.

Denoyes-Rothan et al. (2003) investigated genetic polymorphism and pathogenicity of *C. acutatum* isolates from European bred strawberry cultivars using random amplified polymorphic DNA (RAPD) analysis and internal transcribed spacer (ITS) sequencing. Cluster analysis of the RAPD data and the ITS sequence data provided genetic evidence of at least two genetic groups of *C. acutatum* isolates, termed CA-clonal or CA-variable. The CA-clonal group only contained isolates from strawberry with a low level of genetic polymorphism and includes one of the isolates used in this research, Goff. The CA-variable group contained isolates from strawberry and other hosts. The researchers then conducted pathogenicity tests using a subset of 81 European *C. acutatum* isolates plus the American *C. acutatum* isolate, Goff, to assign each isolate to pathogenicity group 1 or 2. These pathogenicity groups had been established previously in an inoculation study in which *C. acutatum* isolates were grouped based on their pathogenicity on five named cultivars: Elsanta, Valeta, Addie, Sequoia, and Dover (Denoyes and Baudry, 1995). The pathogenicity tests were conducted on two named strawberry: Belrubi (resistant to group 2 and susceptible to group 1) and Elsanta (susceptible to both groups) (Denoyes-Rothan et al., 2003). Subsequently, the *C. acutatum* isolates were grouped based on these tests. Both group 1 and group 2 isolates were found in the CA-clonal genetic group and one group 1 isolate was found in the CA-variable genetic group.

Most commercial strawberries are octoploid, which presents difficulties in determining their genetics in classical breeding programs (Hancock et al., 2008). Disomic inheritance in strawberry was demonstrated by some researchers (Lerceteau-Köhler et al., 2003; Folta and Davis, 2006) and allows breeders to determine dominant

and recessive traits. Researchers examining the inheritance of high and intermediate level plant resistance to *C. acutatum* isolates of the pathogenicity group 2 found the inheritance of a dominant gene (*Rca2*) controlling strawberry resistance to that group (Denoyes-Rothan et al., 2005). Two sequence characterized amplified region (SCAR) markers were identified and used to screen European and U.S. strawberry genotypes for the *Rca2* gene (Lerceteau-Köhler et al., 2005). Lerceteau-Köhler et al. (2005) considered these results indicative of monogenic control of resistance to *C. acutatum* pathogenicity group 2.

The purpose of this research was to screen strawberry germplasm, represented by 31 named cultivars and 50 USDA breeding lines (MSUS clones) from Mississippi, to establish the degree of host resistance/susceptibility to anthracnose caused by the three *Colletotrichum* species and to screen the same germplasm for the two SCAR markers linked to the *Rca2* gene to determine whether or not the allele associated with resistance is present at the *Rca2* gene. The knowledge of the presence or absence of the *Rca2* gene and the germplasm lines' resistance should improve breeding decisions on strawberry germplasm to be incorporated into breeding programs.

Materials and Methods

Production of Plant Material

Strawberry plants were either purchased, obtained from the National Clonal Germplasm Repository in Corvallis, Oregon, or grown in-house (MSUS clones). Plants were established in 10-cm plastic pots in a 1:1 mixture of Jiffy-Mix (JPA, West Chicago, IL, U.S.) and sand and propagated by rooting young runner plants in the Jiffy-Mix:sand potting mixture. The plants were maintained in a greenhouse at 28 ºC ±10 ºC with a 16-

hour photoperiod. The 31 named cultivars and MSUS clones (Table 2) used in this research will be collectively referred to as germplasm lines.

Nine of the 31 named cultivars, received as *in vitro* plantlets from the National Clonal Germplasm Repository, were rooted *in vitro* before transplanting to Jiffy®-7 (Jiffy International AS, Kristiansand, Norway) peat pellets and grown for 7–10 days in the laboratory in a seedling tray with a clear plastic cover at 100% relative humidity and natural light. The relative humidity was gradually reduced until the plantlets were established. The plantlets were then transferred to 10-cm plastic pots and grown in the Jiffy-Mix:sand mixture in a controlled environment room before being moved to the greenhouse.

Growth of Fungal Isolates and Preparation of Inoculum

Six *Colletotrichum* isolates were used in these studies: two isolates of *C. acutatum* [Goff (Smith and Black, 1990) and Ca-1 (Chang and Smith, 2007)], two isolates of *C. fragariae* [Cf-63 and Cf-75 (Smith and Black, 1990; Chang and Smith, 2007)], and two isolates of *C. gloeosporioides* [Ark-P1 and Cg-162 (Smith and Black, 1990)]. Each isolate was initiated from silica gel cultures maintained at the USDA-ARS in Poplarville, MS, and grown on 1:1 oatmeal potato dextrose agar (OMA:PDA) at 20–28 °C under fluorescent lights with a 12-hour photoperiod. Inoculum was prepared as a conidial suspension from 7- to 14-day-old cultures by flooding each culture plate with sterile deionized water and gently scraping the agar surface with a glass rod to remove conidia. The resulting conidial suspension was filtered through one layer of bandage gauze (Johnson and Johnson, New Brunswick, NJ, U.S.) and adjusted to a concentration of 1.5×10^6 conidia/mL by diluting with sterile deionized water with Tween-20 (Sigma

Chemical Co., St. Louis, MO, U.S.) added as a surfactant at the rate of one drop per 1 L of water.

Whole Plant Inoculations: Resistance to Colletotrichum Species

Whole plant inoculations were conducted three times over two years (summer 2010, winter 2010, and summer 2011), which will hereafter be referred to as three "seasons". Not all clones were inoculated with all isolates for all three seasons partly because of the small starting population of many of the MSUS clones and because some named cultivars and MSUS clones died after the first inoculation. Older leaves, runners, and flowers were removed from plants 3–4 days before inoculation, leaving 4–5 young leaves on the plant at inoculation. Whole strawberry plants were initially inoculated with two isolates of *C. acutatum* (Goff and Ca-1), two isolates of *C. fragariae* (Cf-63 and Cf-75), and two isolates of *C. gloeosporioides* (Ark-P1 and Cg-162); however, isolate Ark-P1 exhibited no disease symptoms on any plants and was removed from the study. Plant inoculation was accomplished by uniformly misting each plant with a conidial suspension to the point of runoff. A set of standard named cultivars with known disease reactions was included for comparison: Elsanta for susceptibility to *C. acutatum*, Chandler for susceptibility to *C. fragariae* and *C. gloeosporioides*, and Pelican for resistance to *C. fragariae* and *C. gloeosporioides*. Plants used for non-inoculated controls were misted with deionized water instead of the conidial suspension. Inoculated and non-inoculated plants were immediately placed in a dew chamber at 100% relative humidity (RH), 30 ºC, incubated in the dark for 48 hours, and then transferred to a greenhouse for the remainder of the study. Disease development was assessed on the plants at 10 and 30 days after inoculation (DAI) using a disease severity rating (DSR) scale, which utilizes lesions on

petioles as part of the rating scale, adapted from Smith and Black (1987). This rating scale has been used for almost 30 years to determine resistant strawberry germplasm. The 10 DAI rating was included to allow observation of any early disease symptoms that may no longer be visible at the 30 DAI. The highest disease rating at either the 10 DAI or 30 DAI was used for data analysis. The whole plant DSR rating scale was: 0 = healthy plant with no visible lesions, 1 = < 3 mm long petiole lesion, 2 = 3–10 mm long petiole lesion, 3 = > 10–20 mm long petiole lesion, 4 = > 20 mm long petiole lesion, 5 = youngest leaf wilted, and 6 = dead plant. Plants with a DSR $\leq$ 2.0 were classified as resistant, plants with a DSR of > 2.0 or < 4.0 were classified as intermediate, and plants with a DSR $\geq$ 4.0 were classified as susceptible. Disease development was also assessed by rating three leaflets (collectively) from a single leaf (without the petiole) with the greatest disease symptoms at the same time that the whole plants were rated. The 0–5 DSR scale used for rating the leaflets was 0 = no visible disease symptom on any leaflet, 1 through 4 increasing disease symptom severity, and 5 = total area of leaflets necrotic. Each plant was visually assessed and assigned a DSR for the whole plant and a DSR for the leaflets by two independent raters. DSR means for the whole plant and DSR means for the leaflets were calculated for each combination of rater, germplasm line, and *Colletotrichum* isolate.

DNA Extractions and Allele Identification (2009–2013)

Fresh strawberry leaves were collected, frozen with liquid nitrogen, and ground to a fine powder with a mortar and pestle. DNA was extracted from 50–90 mg of the ground tissue using a DNeasy Plant Mini Kit from Qiagen (Valencia, CA, U.S.) and quantified with a Nanodrop 7 ND-1000 spectrophotometer (Thermo Fisher Scientific,

Inc., Wilmington, DE, U.S.). The extracted DNA was used in a polymerase chain reaction (PCR) to amplify the SCAR markers STS-Rca2_240 and STS-Rca2_417 (Lerceteau-Köhler et al., 2005).

The amplification procedure, adapted from Lerceteau-Köhler et al. (2005), for the STS-Rca2_240 SCAR marker was carried out in a total volume of 25 µL with 1× reaction buffer, diluted from 10× PCR buffer (P2192, Sigma-Aldrich, Inc., St. Louis, Mo, U.S.), 0.8 units JumpStart Taq DNA Polymerase (D9307, Sigma-Aldrich, Inc.), 0.2 mM deoxynucleotide (dNTP) Mix (D7295, Sigma-Aldrich, Inc.), 0.2 µM of each primer, CAC_240_2F and CAC_240_2Rb, 0.08 µM of each control primer, EMFv020_F and EMFv020_R (Sigma-Aldrich, Inc.), and 20–40 ng DNA template. PCR conditions consisted of a 3-min denaturation at 95 ºC, 35 cycles of 50 s at 95 ºC, 50 s at 64 ºC, and 1-min at 72 ºC, with a final extension step of 5-min at 72 ºC. The amplification product was separated on a 1% agarose gel. Electrophoresis gels were photographed with a Kodak EDAS 290 (Carestream Health, Rochester, NY, U.S.) on a Spectroline UV Transilluminator (Spectroline, Westbury, NY, U.S.) and visualized with Kodak 1D Software (Scientific Imaging Systems, New Haven, CT, U.S.). Individual strawberry germplasm lines were classified according to the presence or absence of the marker associated with the resistance allele.

The amplification procedure for the STS-Rca2_417 marker, adapted from Lerceteau-Köhler et al. (2005), was carried out in a total volume of 25 µL with 1× reaction buffer, diluted from 10× PCR buffer (P2192, Sigma-Aldrich, Inc.), 0.8 units JumpStart Taq DNA Polymerase (D9307, Sigma-Aldrich, Inc.), 0.2 mM dNTP Mix (D7295, Sigma-Aldrich, Inc.), 0.2 µM of the M13 tailed CAC_417_3F primer (Boutin-

Ganache et al., 2001), 0.2 µM of the CAC_417_3R primer (Sigma-Aldrich), 0.1 µM of the M13 labeled primer (LI-COR, Lincoln, NE, U.S.), and 20–40 ng of template DNA. PCR conditions were the same as for the STS-Rca2_240 marker with the exception of the annealing temperature of 62 ºC. The amplification product was visualized using a LI-COR 4300 DNA sequencer with a 50–700bp size standard (LI-COR) and scored using Gene Image IR v. 3.55 (LI-COR Biosciences, Lincoln, NE, U.S.).

Statistical Analysis

Plants were chosen at random from the population of that particular germplasm line for each of the three seasons, and the DSR for each of the individual plants within a germplasm line from each of the three inoculations were used to calculate the whole plant DSR mean for the germplasm line. Leaflets' DSR for each plant in each of the germplasm lines from each of the three inoculations were used to calculate the leaflets DSR mean for each germplasm line. Whole plant DSR means and leaflets DSR means were analyzed for association and agreement between the two independent raters using Pearson's product-moment correlation coefficient (r_p) and weighted Kappa coefficient (k_w), respectively, using the FREQ procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Pearson's correlation coefficient statistically measures the degree of linear association between two sets of variables. The whole plant DSR mean data and leaflets DSR mean data were analyzed with analysis of variance (ANOVA) using the GLM procedure of SAS with germplasm line, season, and germplasm line × season for each isolate as independent variables and whole plant DSR mean as the dependent variable. Initial analysis found the interaction for germplasm line × season for all inoculation isolates to be significant; therefore, subsequent analyses were conducted after removing

the data of any isolates not used in all three seasons, and again the germplasm line × season interaction was significant. The whole plant DSR data were analyzed for association (Pearson's product-moment correlation coefficient) and agreement (weighted Kappa coefficient) between pairs of seasons for the isolates Cf-75 and Cg-162 using the FREQ procedure of SAS. The data were then separated by origin of the germplasm (named cultivar and MSUS clones) and analyzed with ANOVA with named cultivar, season, and named cultivars × season as independent variables and the whole plant DSR means as the dependent variable, and then the analysis was repeated with MSUS clone, season, and MSUS clone × season as independent variables and the whole plant DSR means as the dependent variable.

The whole plant DSR means were calculated for each inoculation isolate and season and analyzed with ANOVA with season, isolate, and season × isolate for the origin as independent variables and whole plant DSR mean as the dependent variable. The leaflets DSR means were calculated for each inoculation isolate and season and analyzed with ANOVA with season, isolate, and season × isolate for the origin as independent variables and leaflets DSR mean as the dependent variable. The Cochran-Mantel-Haenszel statistic in the FREQ procedure of SAS was used to evaluate the degree of an association between the resistance category (resistant, intermediate, or susceptible) and whole plant DSRs. Generalized linear models (binary distribution and logit link function) were run using the GLIMMIX procedure of SAS to model the probability of the germplasm line's resistance with the presence/absence of the STS-Rca2_240 marker, the presence/absence of STS-Rca2_417 marker, and their interaction for each *Colletotrichum*

species as the independent variables and the resistance category (resistant or not resistant) as the dependent variable, with separate analysis.

Results

Whole Plant Inoculations: Resistance to Colletotrichum Species

Whole plants of 31 named cultivars and 50 MSUS clones were inoculated with *C. fragariae* isolate Cf-75 and *C. gloeosporioides* isolate Cg-162 in all three seasons, but *C. acutatum* isolates Ca-1 and Goff and *C. fragariae* isolate Cf-63 were used in only two of the three seasons. There were 3754 total observations including 3230 inoculated whole plants and 524 non-inoculated whole plant controls. The control plants in the three seasons had a whole plant DSR mean of 0.31 and a leaflets DSR mean of 0.35. This indicates the plants used did not have disease symptoms prior to inoculation with *Colletotrichum* isolates, and the conditions of the experiment alone did not cause anthracnose-like symptoms; therefore, the control plant data were excluded from further statistical analyses.

There was a strong, positive correlation and substantial agreement association between the raters' whole plant DSRs (n =3230, r_p =0.89, k_w =0.89) and between the raters' leaflets DSRs (n =3230, r_p =0.87, k_w =0.87) (Gwet, 2012), and the inter-rater agreement was "almost perfect" based on the Kappa statistic interpretation by Viera and Garrett (2005); therefore, the two visual raters' DSRs for each plant were averaged for the remainder of the statistical analyses and will be referred to as the whole plant DSR (WP-DSRs) means and the leaflets DSR (L-DSRs) means.

Analysis found significant clone × season interactions for the WP-DSRs for all inoculation isolates (ranging from p =<0.0001 to p =0.04). The differences in WP-DSRs

disease development between pairs of seasons was considered to be due to some germplasm lines not being inoculated with all *Colletotrichum* isolates in all three seasons, as mentioned previously. Therefore, the data from each of the three seasons that included all germplasm lines inoculated with the same *Colletotrichum* isolates (Cf-75 and Cg-162) were analyzed with ANOVA using the GLM procedure of SAS with germplasm line, season, and germplasm line × season for each isolate as independent variables and WP-DSRs as the dependent variable, with separate analyses for each isolate. The germplasm line × season interaction was significant for isolate Cf-75 (n =654, p =<0.0001) and isolate Cg-162 (n =620, p =<0.0001); therefore, the WP-DSRs were tested between seasons for association (Pearson) and agreement (weighted Kappa) for all germplasm lines for each of the two isolates. There was a positive correlation between pairs of seasons for both isolates, ranging from r_p =0.59 to 0.75. Not all rating values of the WP-DSR scale were obtained in all seasons for all isolates; thus, without a balanced table (columns equal rows), the agreement statistic, weighted Kappa, could not be determined for all isolates and seasons. Isolate Cf-75 had the strongest positive association and moderate agreement between summer 2010 and summer 2011 (r_p =0.71, k_w = 0.54) and isolate Cg-162 had the strongest positive association and substantial agreement between winter 2010 and summer 2011 (r_p =0.75; k_w =0.71).

The significant germplasm line × season interaction was then considered to be due to the MSUS clones originally being selected for resistance to *C. fragariae* (Galletta et al., 1993; Smith et al., 1998) and the named cultivars, although selected for many positive agronomic attributes, were not selected for resistance to *C. fragariae*; therefore, the data were analyzed with ANOVA for the null hypothesis of no difference in the WP-DSRs

response of named cultivars and MSUS clones to inoculation with isolates, Cf-75 and Cg-162. A *p* value of <0.0001 indicated a difference in the WP-DSRs response of named cultivars and MSUS clones, therefore, the null hypothesis was rejected. The data were then analyzed by origin with ANOVA with named cultivar, season, and named cultivar × season and MSUS clone, season, and MSUS clone × season for each isolate as independent variables and the WP-DSRs as the dependent variable (Table 3). The named cultivar × season interaction was significant (p <0.0001) for named cultivars inoculated with isolate Cf-75 and not significant (p =0.398) for named cultivars inoculated with isolate Cg-162. The MSUS clone × season interaction was found marginally significant (p =0.089) for MSUS clones inoculated with isolate Cf-75 and significant (p =0.03) for MSUS clones inoculated with isolate Cg-162.

The WP-DSRs for each inoculation isolate and inoculation season collectively, (Figure 2) were analyzed with ANOVA with season, isolate, and season × isolate as independent variables and WP-DSR as the dependent variable with separate analyses for each origin. The season × isolate interaction was not significant for named cultivars or MSUS clones. Generally, named cultivars had higher WP-DSRs than MSUS clones for each isolate. Both named cultivars and MSUS clones inoculated with *C. fragariae* isolate Cf-75 and *C. gloeosporioides* isolate Cg-162 received higher WP-DSRs the first summer season than the second summer season.

L-DSRs data were analyzed the same as the WP-DSRs data. The L-DSRs data from each of the three seasons, which included all germplasm lines inoculated with the same *Colletotrichum* isolates (Cf-75 and Cg-162), were analyzed by origin with ANOVA with named cultivar, season, and named cultivar × season and MSUS clone, season, and

MSUS clone × season for each isolate as independent variables and L-DSRs as the dependent variable. The named cultivar × season interaction was moderately significant for named cultivars (p =0.06) and MSUS clones (p =0.06) inoculated with *C. fragariae* isolate Cf-75, and was not significant for named cultivars (p =0.48) and MSUS clones (p =0.24) inoculated with *C. gloeosporioides* isolate Cg-162.

The L-DSRs for each inoculation isolate and inoculation season, collectively (Figure 3) were analyzed with ANOVA with season, isolate, and season × isolate as the origin as independent variables and L-DSRs as the dependent variable. The season × isolate interaction was not significant for named cultivars or MSUS clones. The same pattern of resistance by season was found with the L-DSRs as the WP-DSRs. Generally, named cultivars had higher L-DSRs than MSUS clones for all isolates. Both named cultivars and MSUS clones inoculated with *C. fragariae* isolate Cf-75 and *C. gloeosporioides* isolate Cg-162 received higher L-DSRs the first summer season than the second summer season.

The WP-DSRs for each germplasm line inoculated with *C. fragariae* isolate Cf-75 and *C. gloeosporioides* isolate Cg-162 among all three seasons were used to classify the germplasm lines into resistance categories: WP-DSR $\leq$ 2.0 = resistant, WP-DSR > 2.0 or < 4.0 = intermediate, and WP-DSR $\geq$ 4.0 = susceptible. Among named cultivars (17) inoculated with *C. fragariae* isolate Cf-75, 94%, 24%, and 59% were susceptible in all three seasons: summer 2010, winter 2010, and summer 2011, respectively. Among the MSUS clones (39) inoculated with Cf-75, 23%, 79%, and 62% were resistant in all three seasons: summer 2010, winter 2010, summer 2011, respectively. Among the named cultivars (23) inoculated with *C. gloeosporioides* isolate Cg-162, 96%, 70%, and 65%

were susceptible in all three seasons: summer 2010, winter 2010, summer 2011, respectively. Among the MSUS clones (30) inoculated with Cg-162, 0%, 70%, and 57% were resistant in all three seasons: summer 2010, winter 2010, summer 2011, respectively. The Cochran-Mantel-Haenszel statistic in the FREQ procedure of SAS was used to test the null hypothesis of no association between the category (resistant, intermediate, or susceptible) and WP-DSRs. The probability of no association was significant (p <0.0001).

Of the standard named cultivars used for comparison, Elsanta was susceptible to all isolates. Chandler varied in response to the *C. acutatum* isolate Goff, being susceptible in summer 2010, intermediate in summer 2011, and resistant in winter 2010. Chandler was susceptible to the *C. acutatum* isolate Ca-1 in summers 2010 and 2011. Chandler was susceptible to isolate Cf-63 in summer 2010 and resistant in winter 2010. Chandler was susceptible to the *C. fragariae* isolate Cf-75 in summers 2010 and 2011 and intermediate in winter 2010. Chandler was susceptible to the *C. gloeosporioides* isolate Cg-162 in all three seasons: summers 2010 and 2011, and winter 2010. Pelican was resistant to the *C. acutatum* isolate Ca-1 in both summer seasons, 2010 and 2011, and resistant to the *C. acutatum* isolate Goff in summer 2010 and winter 2010. Pelican was resistant to the *C. fragariae* isolate Cf-63 in summer 2010 and winter 2010, but was intermediate to the *C. fragariae* isolate Cf-75 both summer seasons, 2010 and 2011, and winter 2010. Pelican was intermediate to the *C. gloeosporioides* isolate in both summer seasons, 2010 and 2011, and resistant in winter 2010.

Allele Identification

DNA was extracted from the same 81 strawberry germplasm lines used in the whole plant inoculations. Of the 31 named cultivars tested for the STS-Rca2_240 marker, 19 were positive for the marker. Twenty-one named cultivars were also tested for the STS-Rca2_417 marker. Both the STS-Rca2_240 and STS-Rca2_417 markers were present in ten named cultivars, and the STS-Rca2_417 marker was present without the STS-Rca2_240 marker in five named cultivars. Six named cultivars tested negative for the STS-Rca2_417 marker. Fifty MSUS clones were tested for the two markers, including US70, US159, US292, and US438, released breeding lines. Results were not obtained from MSUS1352 for the STS-Rca2_417 marker. The STS-Rca2_240 marker was found in 42 MSUS clones, both the STS-Rca2_240 and STS-Rca2_417 markers were present in 26 MSUS clones, and two MSUS clones had the STS-Rca2_417 marker without the STS-Rca2_240.

During identification of the STS-Rca2_417 marker, other alleles measuring 421, 415, and 397 base pairs were noted. The 421bp allele was found in 20 named cultivars and 45 MSUS clones. The 415bp allele was not found in any named cultivar germplasm line tested, but was found in 6 MSUS clones, and the 397bp allele was found in 15 named cultivars and 40 MSUS clones. Table 5 lists the named cultivars used in both my research and the Lerceteau-Köhler et al. (2005) study that were tested for resistance and the presence of the STS-Rca2_240 and STS-Rca2_417 SCAR markers.

Association of SCAR Markers and Plant Resistance

The WP-DSRs were calculated for the *Colletotrichum* isolates combined as species (*C. acutatum* species = isolates Ca-1 and Goff WP-DSRs combined, *C. fragariae*

species = isolates Cf-63 and Cf-75 WP-DSRs combined, and *C. gloeosporioides* = isolate Cg-162 WP-DSRs) and used to classify a germplasm line's disease response category (resistant, intermediate, or susceptible) for the *Colletotrichum* species. The SCAR marker data were separated by presence or absence of the STS-Rca2_240 and STS-Rca2_417 SCAR markers. Then the WP-DSRs for each *Colletotrichum* species, categorized as resistant or not resistant (intermediate and susceptible categories combined), were analyzed for comparison with the SCAR marker data using the binary distribution and logit link function in the GLIMMIX procedure of SAS to model the probability of the germplasm line's resistance to each *Colletotrichum* species with the presence/absence of the STS-Rca2_240 marker, the presence/absence of STS-Rca2_417 marker, and their interaction as the independent variables and the resistance category (resistant or not resistant) as the dependent variable (Table 4). The effect of the interaction between the two SCAR markers on resistance was not significant for any of the three *Colletotrichum* species. The effect of the STS-Rca2_240 marker was significant (p =0.01) for resistance to *C. fragariae*, marginally significant (p =0.09) for resistance to *C. acutatum*, and not significant (p =0.33) for resistance to *C. gloeosporioides*. The effect of the STS-Rca2_417 marker was marginally significant (p =0.06) for resistance to *C. acutatum* and not significant for resistance to *C. fragariae* (p =0.44) and *C. gloeosporioides* (p =0.30).

Discussion

Whole Plant Resistance to Colletotrichum Species

Strawberry is a high value crop grown in most countries, but it is often host to pathogens that ruin the fruit and/or destroy the plant. Strawberry plant breeders' and

plant pathologists' 'holy grail' is development of a disease resistant cultivar that will produce abundant, tasty, quality fruit. The goal of this research was to determine the degree of resistance or susceptibility of strawberry germplasm to anthracnose disease caused by three *Colletotrichum* species and to establish the degree of association between the host's reaction and the presence or absence of the two *Rca2* gene SCAR markers found in the European study by Lerceteau-Köhler et al. (2005).

Generally, the MSUS clones exhibited greater anthracnose resistance to each of the three *Colletotrichum* species than the named cultivars. This was expected because the MSUS clones were initially selected for resistance to *C. fragariae*. The WP-DSRs of the germplasm lines varied by season. Many of the MSUS clones exhibited an increased resistance in the winter compared to summer. Explanations could be sought along two lines of reasoning. Either the plant or the pathogen reacts differently between seasons. The phenomenon of plants showing greater resistance during cooler months than warmer months was reported by Mangandi et al. (2015) for strawberry in two different years during a field trial in Florida where the strawberry plants had been artificially inoculated with *C. gloeosporioides*. They found a significant genotype × season interaction where some genotypes had higher resistance as measured by decreased anthracnose symptoms in the second year with cooler temperatures than the first warmer year. Lewers et al. (2007) conducted an inoculation study of native strawberry germplasm for resistance to anthracnose crown rot disease using the same protocol and isolates as the current research. The named cultivars Pelican and Chandler were included in two of their three years of inoculations. Their study showed a significant genotype × year effect, but closer observation of their article indicates a possible seasonal effect. Following inoculation of

Pelican and Chandler with two *C. fragariae* and one *C. gloeosporioides* isolates, higher WP-DSRs were reported for the late summer 2003 inoculation than in the early spring 2005 inoculation, with the exception that Chandler inoculated with Cf-75 had a higher WP-DSR in 2005 than in 2003. The research conducted by Mangandi et al. (2015) and Lewers et al. (2007) agrees with the results of my observations of what appears to be a seasonal phenomenon in degree of plant resistance or fungal pathogenicity. The second line of reasoning is that the pathogen is less aggressive during the cool, dry months than during warm, humid months. The environment in greenhouses is controlled, but still tends to be warm and humid in the summer and relatively cool and dry in the winter. Leandro et al. (2001) conducted a *C. acutatum* conidial germination and sporulation study on inoculated strawberry at 26 °C, under continual wetness (100% relative humidity). This was the first report of secondary conidial production by *C. acutatum* on symptomless strawberry leaves. Secondary conidiation is also known as microcyclic conidiation, which occurs directly after conidial germination with little or no mycelial growth. Leandro et al. (2001) demonstrated that secondary conidiation was responsible for a threefold increase in conidia on the inoculated strawberry leaves, suggesting *C. acutatum* can survive and multiply on symptomless strawberry leaves. Generally, *C. acutatum* conidia germinate and form appressoria or germ tubes, but it has been shown that primary conidia can also germinate by forming specialized structures (conidial phialides) that produce secondary conidia without forming a mycelium (Parberry et al., 1978). Leandro et al. (2003a) found temperature and moisture influenced conidial germination, secondary conidiation, and appressorial development of *C. acutatum*. They included temperatures ranging from 10 to 35 °C, with continual wetness plus six

intermittent wetness parameters at 25 °C. Samples of the inoculated leaf tissue were obtained from 6 to 132 hours after inoculation. The optimum temperature and moisture for conidial germination was 23–27.7 °C and ≥8 hours of wetness per day. The optimum temperature for secondary conidiation and appressorial development was 21.3–32.7 °C and 17.6–26.5 °C, respectively. Both secondary conidiation and appressorial development were augmented with increasing wetness duration, but greater than four hours wetness was required for secondary conidiation. Wilson et al. (1990) found a positive correlation between increased wetness duration and increased disease incidence of *C. acutatum* on strawberry fruit at the optimum temperature of 25 to 30 °C. King et al. (1997) conducted a sporulation study with the same three *Colletotrichum* species used in this study and found the latent period (time from infection to first sporulation) was dependent on temperatures ranging from 2–3 days at 25 °C up to 6–17 days at 5 °C. *Colletotrichum acutatum* had the shortest latent period of the three species at 5 and 10 °C and also produced more conidia at the lower temperatures than *C. fragariae* or *C. gloeosporioides*. The latent periods were similar for all species at higher temperatures. Another interesting observation that involves both the plant and the pathogen concerns the phenomenon of flowering that occurs during the warm temperatures and high humidity of summer. Leandro et al. (2003b) found that leaves inoculated with *C. acutatum* showed increased secondary (microcyclic) conidial formation when the leaves were treated with flower extracts than when treated with leaf extracts, suggesting inoculum levels may increase during flowering.

The plants in my study inoculated with the *C. fragariae* isolates received higher WP-DSRs in the summer than in the winter, and the Cf-75 isolate was more aggressive

than the Cf-63 isolate. The *C. gloeosporioides*-inoculated plants received higher WP-DSRs in the summer seasons, also. The WP-DSRs for the plants inoculated with *C. fragariae* and *C. gloeosporioides* decreased between the first and second summer seasons. MacKenzie et al. (2006) found pathogen populations vary in aggressiveness, and disease incidence may increase in a particular season. Both *C. fragariae* isolates and the *C. gloeosporioides* isolate in my study were more aggressive than the *C. acutatum* isolates. Curry et al. (2002) found *C. acutatum* and *C. fragariae* have similar infection processes, but *C. fragariae* invaded the strawberry tissue more rapidly than did *C. acutatum*.

The seasonal effect of *Colletotrichum* isolates on strawberry plants, and the aggressiveness of an isolate should be considered when planning an inoculation study. The *Colletotrichum* species causing anthracnose on strawberry have been shown to vary in aggressiveness from season to season, and performing repetitive inoculations in different seasons will help in the identification of resistant germplasm. The isolates used in this study may not be representative of all the genetic variability among *Colletotrichum* species, but they have been used successfully for almost 30 years to identify anthracnose resistance in strawberry (Smith and Black, 1987, 1990; Lewers et al., 2007).

Association of SCAR Markers and Plant Resistance

The categories of resistant, intermediate, and susceptible were assigned to the germplasm based on the whole plant disease severity ratings. The majority of named cultivars were classified as either intermediate or susceptible to all the *Colletotrichum* species used in this study. The MSUS clones were classified as mainly resistant to *C.*

acutatum and *C. fragariae* and resistant or intermediate to *C. gloeosporioides*. Germplasm lines with both the STS-Rca2_240 and STS-Rca2_417 markers, or the STS-Rca2_417 marker alone were predicted to be resistant at best to *C. acutatum* based on findings by Lerceteau-Köhler et al. (2005) that the two alleles were linked to a dominant gene, *Rca2*, for resistance to anthracnose disease caused by *C. acutatum* group 2 isolates. Their study included 43 named cultivars of which 28 were resistant to *C. acutatum* group 2. These 28 resistant named cultivars carried either both the STS-Rca2_240 and STS-Rca2_417 markers or the STS-Rca2_417 marker alone. They reported that 13 of 28 resistant named cultivars contained both the STS-Rca2_240 and STS-Rca2_417 markers and eight carried the STS-Rca2_417 markers only; this would correctly predict 75% (21/28) of the named cultivars as resistant. The results of my research with *C. acutatum*-inoculated named cultivars showed 15 of 21 (71%) named cultivars were predicted to be resistant with 10 having both the STS-Rca2_240 and STS-Rca2_417 markers and five with the STS-Rca2_417 marker only; however, only four named cultivars (19%) were resistant and one of these had neither marker. Lerceteau-Köhler et al. (2005) reported 14 of the 22 named cultivars predicted to be susceptible by the absence of both markers were actually susceptible. Only three germplasm lines in my study were without either marker which predicts them to be susceptible, but of these three germplasm lines, two were classified as intermediate (Redchief and Treasure) and one was classified as resistant (Tioga). This research positively confirmed the presence or absence of the markers in 10 of the 18 named cultivars used in the European study by Lerceteau-Köhler et al. (2005) (Table 5). Denoyes-Rothan et al. (2003) assigned *C. acutatum* isolates to two pathogenicity groups 1 or 2 based on their pathogenicity on the named cultivars Belrubi

(resistant to group 2 and sensitive to group 1) and Elsanta (susceptible to both groups 1 and 2). Belrubi was not included in the present study because it was unavailable. Elsanta was included in the present study and was rated susceptible (WP-DSR =4) to both *C. acutatum* isolates, Goff and Ca-1, and Elsanta was susceptible to Goff in the European study by Lerceteau-Köhler et al. (2005). Isolate Goff was placed in group 1 in the previous research by Lerceteau-Köhler et al. (2005), which may explain some differences in resistance between this and the European study for *C. acutatum*, because the *Rca2* resistance gene was identified for resistance to anthracnose disease caused by *C. acutatum* group 2, not group 1. Isolate Ca-1 has not been assigned as group 1 or group 2.

This research found the SCAR markers STS-Rca2_240 and STS-Rca2_417, had marginally significant effects (p =0.0893 and p =0.0556, respectively) on disease resistance on germplasm lines when inoculated with *C. acutatum*. While *C. acutatum* isolate Goff was previously classified as a group 1 isolate and the group for Ca-1 is not known, the findings of my research supported the European study that an association exists between the presence or absence of the *Rca2* resistant allele SCAR markers and a positive effect on a strawberry plant's resistance to anthracnose disease, lending credence to the presence of the *Rca2* gene. This knowledge will ultimately help strawberry breeders identify plants in their breeding program that will possibly pass resistance genes to their progeny. The effect of the STS-Rca2_240 maker on anthracnose resistance was significant (p =0.0086) for plants inoculated with *C. fragariae,* while the STS-Rca2_417 marker did not have a significant effect. This finding suggests a possible association in the plant's genetic resistance to anthracnose for multiple *Colletotrichum* species.

Genetic markers create tools that can be used by breeders for selecting favorable germplasm. Active research into genetic marker-assisted detection of resistance genes is ongoing in many plant/pathogen systems from root-knot nematode resistance in sweet potato (Nakayama et al., 2012) to *Phytophthora cactorum* resistance in *Fragaria vesca* (Davik et al., 2015). Davik et al. utilized genetic markers to assist in locating the proposed *Rpc1* resistant gene in *Fragaria vesca*. This gene provides resistance to a crown rot disease caused by *P. cactorum*. The first resistance gene in strawberry to be identified was *Rpf1*, which provides resistance to *Phytophthora fragariae*, the causal agent of red stele root rot (Van de Weg et al., 1997).

Although host pathogen relationships are complex and this complexity may impede a simple solution to finding resistance genes in hosts, as the European study suggested with the SCAR markers (Lerceteau-Köhler et al., 2005), my research has increased our understanding of the host-pathogen relationship.

Table 2

Named cultivars and MSUS clones used in the whole plant inoculations.

Named cultivars				
Aiko	Dover	Jewel	Salinas	Surecrop
Albion	Earliglow	Kent	Scott	Sweet Charlie
Allstar	Elsanta	Ovation	Seascape	Tangi
Aromas	Festival	Pelican[a]	Selva	Tillamook
Camino Real	Gaviota	Portola	Senga	Tioga
Chandler	Honeoye	Redchief	Sequoia	Treasure
Diamante				
MSUS clones				
US70[b]	912	1066	1217	1343
US159[b]	922	1078	1229	1352
US292[b]	927	1093	1230	1356
US438[b]	933	1094	1240	1359
478	944	1105	1265	1362
518	1010	1142	1269	1365
572	1039	1154	1270	1426
574	1049	1180	1271	1432
868	1055	1196	1311	1494
890	1061	1197	1331	1511

[a]MSUS clone released as a named cultivar.

[b]MSUS clones released as breeding lines.

Table 3

Analysis of variance for strawberry plants inoculated with Colletotrichum.

Origin	Isolate	n	Source of variation	df	Whole Plant DSR Mean		Leaflet DSR Mean	
					F	*P* > *F*	*F*	*P* > *F*
Named Cultivar	Cf-75	201	Named cultivar	16	5.08	<0.0001	2.79	0.0006
			season	2	122.85	<0.0001	45.49	<0.0001
			Named cultivar × season	32	2.82	<0.0001	1.5	0.0559
MSUS Clone	Cf-75	453	MSUS clone	38	1.94	0.0012	3.32	<0.0001
			season	2	41.92	<0.0001	63.76	<0.0001
			MSUS clone × season	76	1.26	0.0885	1.3	0.064
Named Cultivar	Cg-162	273	Named cultivar	22	4.84	<0.0001	6.06	<0.0001
			season	2	25.66	<0.0001	15.13	<0.0001
			Named cultivar × season	44	1.05	0.3982	1	0.4768
MSUS Clone	Cg-162	347	MSUS clone	29	2.92	<0.0001	2.81	<0.0001
			season	2	124.48	<0.0001	117.02	<0.0001
			MSUS clone × season	58	1.46	0.0261	1.15	0.2354

Analysis of variance (ANOVA) for three seasons in which named culivars and MSUS clones of strawberry were inoculated with two Colletotrichum isolates (C. fragrance Cf-75 and C. gloeosporioides Cg-162) for the dependent variables of whole plant DSR mean and leaflet DSR mean

Table 4

SCAR markers effect on resistance to anthracnose.

Fixed effects	Num df	Den df	*C. acutatum*		*C. fragariae*		*C. gloeosporioides*	
			F	*P > F*	*F*	*P > F*	*F*	*P > F*
STS-Rca2_240	1	66	2.97	0.0893	7.34	0.0086	0.97	0.3278
STS-Rca2_417	1	66	3.80	0.0556	0.59	0.4447	1.08	0.3028
STS-Rca2_240 × STS-Rca2_417	1	66	1.45	0.2322	0.59	0.4447	0.27	0.6034

F-tests for the effects of the presence/absence of the two SCAR markers STS-Rca2_240 and STS-Rca2_417, and their interaction on the resistance to anthracnose disease on whole strawberry plants inoculated with three Colletotrichum species

Table 5

Strawberry origin and comparison of SCAR markers.

Genotype/Cultivar names	Origin	SCAR Markers							2010 and 2011 Whole Plant Inoculations[d]		
		European[a]		U.S.[b]		Alleles[c]					
		240[e]	417	240	417	421	415	397	*Ca*	*Cf*	*Cg*
Aiko	USA	+	+	+	+	+	-	+	sus[f]	sus	sus
Chandler	USA	+	+	+	+	+	-	+	int	sus	sus
Dover	USA	+	+	+	+	+	-	+	res	sus	sus
Earliglow	USA	-	-	+	+	+	-	+	int	sus	sus
Elsanta	Netherlands	-	-	+	-	-	-	-	sus	sus	sus
Redchief	USA	-	-	+	-	-	-	+	int	sus	sus
Salinas	USA	+	-	-	+	+	-	+	int	sus	sus
Scott	USA	+	+	+	+	+	-	+	int	sus	sus
Seascape	USA	+	+	+	+	+	-	-	res	sus	sus
Selva	USA	+	+	+	+	+	-	+	sus	sus	sus
Senga Sengana	Germany	-	-	-	-	-	-	-	int	int	sus
Sequoia	USA	-	+	+	+	+	-	+	int	sus	sus
Surecrop	USA	-	-	+	-	+	-	+	int	sus	sus
Tioga	USA	+	+	-	-	+	-	-	res	sus	sus
US159	USA	+	+	-	-	+	+	+	res	int	sus
US292	USA	-	-	-	-	+	+	+	res	sus	sus
US438	USA	-	-	-	-	+	-	+	res	res	res
US70	USA	-	+	-	+	+	-	+	res	res	res

Table 5 (continued).

[a] SCAR marker identification in European study (Lerceteau-Köhler et al., 2005).
[b] SCAR marker identification in present study.
[c] Other alleles identified using primers for 417 SCAR marker.
[d] *Colletotrichum* species used in present whole plant inoculation study: *Ca = Colletotrichum acutatum, Cf = C. fragariae, Cg = C. gloeosporioides*.
[e] +=marker or allele present, -=absent.
[f] sus=susceptible, res=resistant, int=intermediate.

Strawberry genotype or cultivar name, origin, comparison of SCAR markers found in a previous European study (Lerceteau-Köhler et al., 2005) and in this study, including the resistance phenotype found in this inoculation study for three *Colletotrichum* species.

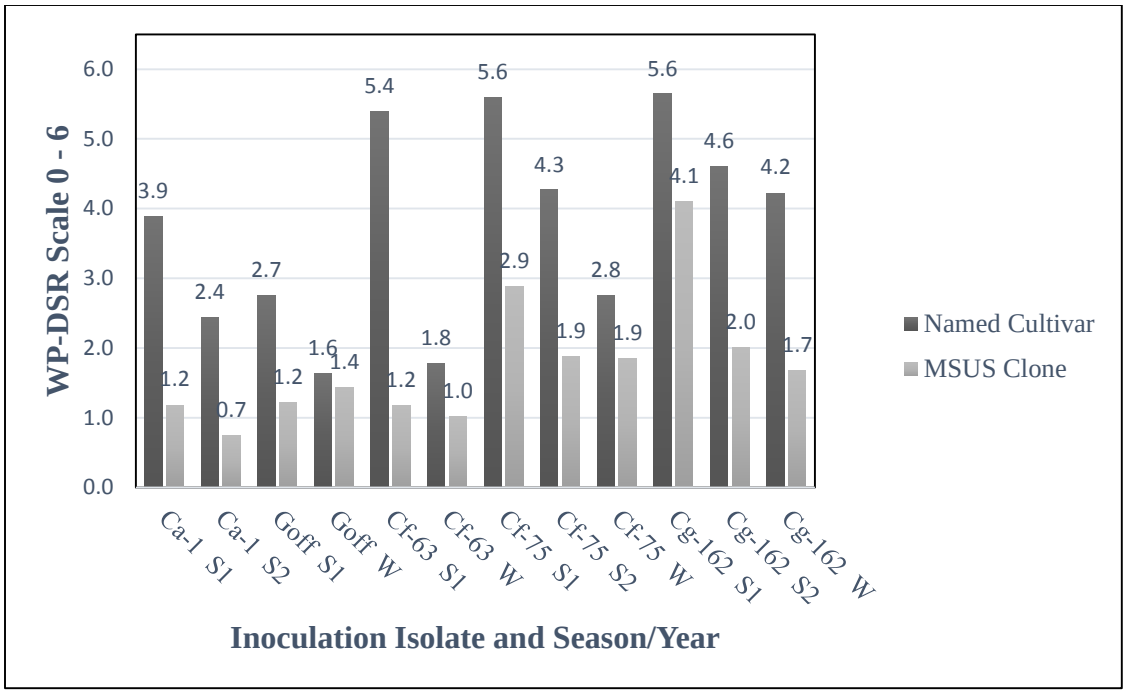

Figure 2. Whole Plant disease severity rating (WP-DSR).

Whole plant disease severity rating (WP-DSR) means for named cultivars and MSUS clones by inoculation isolate (*C. acutatum* = Ca-1 and Goff, *C. fragariae* = Cf-63 and Cf-75, *C. gloeosporioides* = Cg-162) and season and year (S1 and S2 = summer 2010 and summer 2011, respectively, and W = winter 2010).

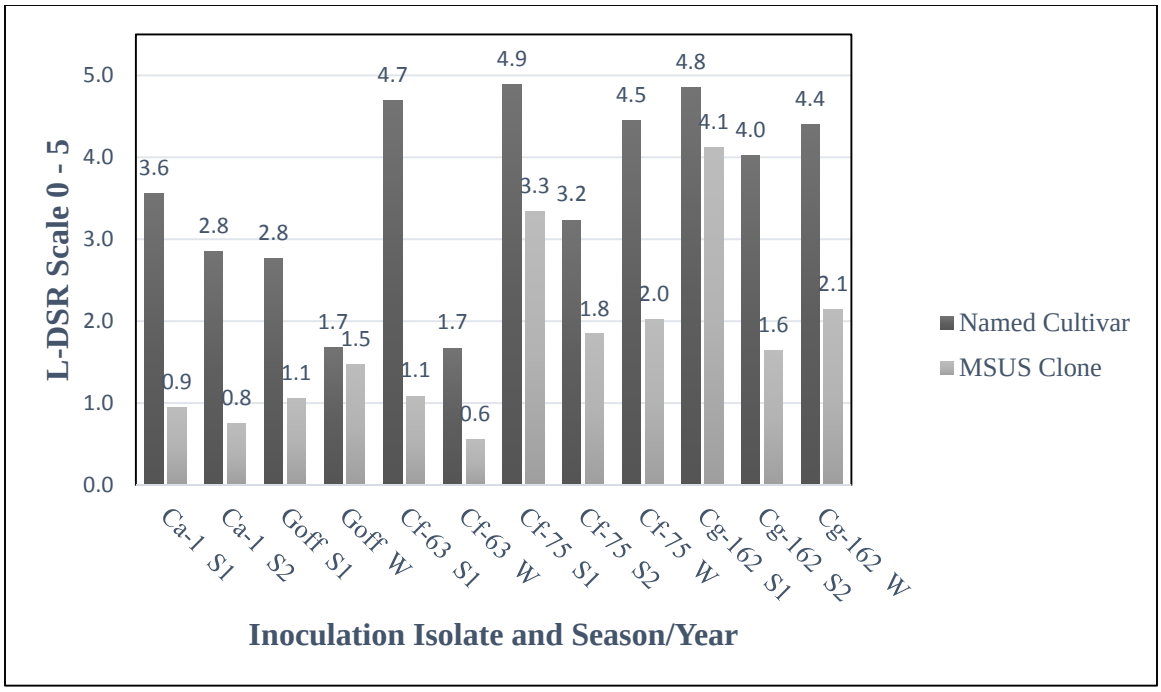

Figure 3. Leaflet disease severity rating (L-DSR).

Leaflet disease severity rating (L-DSR) means for named cultivars and MSUS clones by inoculation isolate (*C. acutatum* = Ca-1 and Goff, *C. fragariae* = Cf-63 and Cf-75, *C. gloeosporioides* = Cg-162) and inoculation season and year (S1 and S2 = summer 2010 and summer 2011, respectively, and W = winter 2010).

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CHAPTER III - COMPARISON OF VISUAL AND ELECTRONIC EVALUATIONS OF DETACHED STRAWBERRY LEAVES INOCULATED WITH *COLLETOTRICHUM* SPECIES

Introduction

Strawberry consumption has continued to increase in the United States since the 1980s, and 7.9 pounds of fresh strawberries were consumed per capita in 2013, setting a new record (Perez and Plattner, 2014). The increased consumption has been attributed to the public becoming more aware of the importance of a healthy diet and the availability of fruit year round. More than 21,800 hectares were planted in strawberries in the U.S. in 2014, and the top two production states, California and Florida, planted 16,800 and 4,400 hectares, respectively (Perez and Plattner, 2014). Unfortunately, diseases cause major losses of strawberries wherever they are grown.

Anthracnose is one of the more destructive plant diseases of strawberry and may be caused by three *Colletotrichum* species (Smith, 1998): *C. acutatum*, *C. fragariae*, and *C. gloeosporioides*. *Colletotrichum acutatum* causes anthracnose fruit rot and irregular leaf spot. Both *C. fragariae* and *C. gloeosporioides* may infect all above-ground parts of the plant, inciting anthracnose crown rot, anthracnose fruit rot, and anthracnose leaf spot (also called black leaf spot). Both *C. acutatum and C. gloeosporioides* have broad host ranges, whereas *C. fragariae* has a narrow host range of strawberry and a few non-crop plants, as well as a limited geographic range of primarily the southeastern U.S. Warm temperatures and high humidity conditions allow these fungal pathogens to produce spores (conidia) rapidly. These spores are easily dispersed throughout a production field

by rain splash, people, animals, insects, and equipment. Anthracnose is an important disease of strawberry in the southeastern U.S. in both production fields and nurseries. California's dry climate helps keep anthracnose at more manageable levels there. Howard and Albregts (1983) described the black leaf spot phase of anthracnose as the first symptom of anthracnose in strawberry nurseries serving as an early warning that the disease is present.

Commercial strawberry growers continually strive to improve their disease control strategies. Planting disease-free plants, good sanitation, cultural controls, and fungicides are all general disease control concepts. Planting disease-free plants decreases the likelihood of introducing pathogens into a field. Sanitation practices help destroy pathogens on equipment or in soil but will not control latent infections. A critical change in cultural controls for strawberry production was the switch from overhead irrigation (which wets plant foliage, helping to spread fungal spores in the field) to use of drip irrigation tubing within the planting bed (which wets the soil, but not the plant). Fungicides help to control pathogens, but rarely eradicate them. The overuse of fungicides has resulted in pathogen resistance and the failure of the fungicides to control anthracnose epidemics (Smith and Black, 1993a, 1993b; LaMondia, 1995). Although disease control practices are important for growers, the ultimate solution for disease control is the development and planting of disease resistant cultivars.

It often takes years for classical breeding programs to develop acceptable strawberry germplasm with the desired plant growth habit, fruit taste and production, and disease and insect resistance. During this developmental stage, thousands of seedlings

are produced. In order to identify disease resistance, whole plants are usually inoculated with the pathogen and rated for disease resistance once symptoms are evident. The disease response of each plant must be assessed with accurate and reproducible techniques because inoculation trials are time consuming, and plants may be destroyed by the disease. This presents a problem to the breeder since the plant may have possessed many other desired horticultural traits which could be utilized in the breeding program as parent material.

Screening strawberry germplasm for disease resistance using detached strawberry leaves is an alternative to inoculating whole plants. Inoculating detached strawberry leaves with a pathogen allows the plant's disease response to be established without destroying the whole plant. This should decrease the time between inoculation and disease assessment, thus reducing the overall time for breeders to identify acceptable germplasm for breeding programs. Another positive outcome of screening using detached leaves is that the pathogen is confined to the laboratory, allowing the breeder to test for pathogens or races of pathogens from other areas. Inoculating detached leaves with *Colletotrichum* species may provide an accurate, rapid, non-destructive method of identifying anthracnose resistant germplasm (Miller-Butler et al., 2013).

Disease severity refers to the amount of plant tissue that is diseased (necrotic) and may be expressed as the percentage of plant area destroyed by a pathogen. Disease assessment scales, such as visual rating scales, are often used for quick assessments of disease severity. These scales may be percentage or numerical. Most percentage scales are adapted from the Horsfall-Barratt (HB) scale (Horsfall and Barratt, 1945) which

contains 12 grades with the percent disease varying disproportionately from 1 to 100 percent. Visual bias can influence accuracy and percentage scales may be difficult to use when relating to plants that exhibit noticeably different amounts of infections such as rusts, powdery mildews, and leaf spots. When there is little disease, the rater's visual focus is drawn to the small amount of necrotic or dark tissue in comparison to the healthy or green tissue. When there is only a small amount of healthy tissue in a very diseased sample, the rater's visual focus can discern the amount of healthy (green) tissue better. Small areas of disease or no disease can be seen and a percentage can be estimated fairly accurately, but when the disease ranges from 10% to 90%, it is much more difficult to give an accurate percentage. More categories in a rating scale may increase rater accuracy, but the increased time required for the ratings eliminates the positive effect of rating a large number of plants quickly. Slopek (1989) compared five variations of a 1–5 visual rating scale for estimating the percent diseased leaf area of barley plants. He determined that two of the five visual rating scales worked well for estimating leaf disease, were as precise as the HB scale, and decreased the time required for disease assessment. Nita et al. (2003) suggested the use of an equal interval scale over the HB scale for assessing disease severity.

Disease symptoms on whole plants or leaves are often evaluated using numbered grade scales which are sometimes referred to as arbitrary, nominal, or ordinal scales. Ordinal scales have some degree of subjective interpretation of the disease by the rater. An ordinal scale of 0 to 5 (0 = no disease, 1 = very slight, 2 = slight, 3 = moderate, 4 = severe, 5 = dead plant) is only interpretable in the arrangement of the order and can only

provide qualitative data. Many disease assessment keys are ordinal and cannot quantitatively measure a difference between the values. Russell (1978) found these scales to be satisfactory when used by experienced observers for rating plants or plots in an order of increasing symptom severity.

When there is more than one visual rater, good agreement and association between the raters is desirable. Understanding the interpretation of results from ordinal data can be explained by the concepts of accuracy (agreement) and precision (association). Accuracy is the raters' ability to rate disease closest to a true value (such as the electronic percent disease measurements) and precision is the repeatability of the scoring. It is unfortunate that accuracy and precision may or may not coincide. Statistical analysis helps determine if the raters are interpreting the disease the same or very close to the same. The Kappa coefficient (*k*) was developed by Cohen (1960) to describe a proportion of agreement (accuracy), correcting for chance agreement, and is scaled to vary from –1 to +1. Viera and Garrett (2005) explain the Kappa coefficient as a "quantitative measure of the magnitude of agreement between observers". Kappa measures the difference between the raters agreement and expected agreement. A negative Kappa coefficient indicates less than chance agreement, zero indicates exactly chance agreement, and a positive value indicates better than chance agreement (+1 would be perfect agreement). The Kappa coefficient can be interpreted as < 0 = less than chance agreement, 0.01–0.20 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and 0.81–0.99 = almost perfect agreement (Viera and Garrett, 2005). Agreement and disagreement are not

mutually exclusive in an ordinal rating scale. If two raters see the disease on a plant as slight disease and moderate disease, they are not in complete agreement, but they are not necessarily in complete disagreement either. Both raters have established that there is disease on the plant. This problem was addressed with the weighted Kappa coefficient by assigning weights to different degrees of disagreement and less weight to agreement as categories are further apart (Cohen 1960, 1968; Fleiss and Cohen, 1973). Pearson's product moment correlation coefficient (r_p) statistically measures the degree of linear association (precision) between two sets of data, such as the two visual raters' disease severity rating. A positive Pearson correlation coefficient designates both sets of data change in the same direction, and a negative Pearson correlation coefficient designates both sets of data change in opposite directions.

Precise quantitative analysis can be performed on images of diseased plant tissues using computer software. Electronic images can be stored indefinitely allowing the researcher to process the images as time permits. Digital imaging and analytical software were used by Wang et al. (2008) to develop a miniaturized strawberry leaf disk bioassay. Their goal was to find the percent disease caused by an isolate used for inoculation of a leaf disk and percent phytotoxicity that may be caused by the antifungal compounds being used on 15-mm excised strawberry leaf disks from the cultivar Chandler in a miniaturized antifungal bioassay (Wang et al., 2008). The leaf disks were dipped in antifungal compounds and then inoculated with the same *Colletotrichum* species being utilized in this research. The analyzing software transformed the images to show healthy parts of the leaf as green, diseased parts as black, and parts exhibiting phytotoxicity as

gray. Photographs of detached strawberry leaves were used by Abril et al. (2009) for visual assessment of disease severity in a study testing the efficacy of natural product-based fungicides. The percent diseased leaf area was assessed with an arbitrary scale of 0–3 (0 = no disease and 3 = most severe disease). Kwack et al. (2005) used digital image analysis to assess the severity of cucumber anthracnose caused by *Colletotrichum orbiculare*. The leaves were also visually assessed for percentage of diseased area. Comparison of visual assessments with image analysis indicated that the visual ratings were significantly higher than the electronic ratings. They noted that processing the images took approximately three minutes each, which would not be challenging for a small number of samples.

The objective of this study was to compare visual assessments with image analysis of anthracnose disease on inoculated detached strawberry leaves to find the degree of agreement and association between the two methods. This research expands upon the Miller-Butler et al. (2013) research by increasing the number of named cultivars and MSUS clones inoculated for assessment.

Materials and Methods

Growth of Fungal Isolates and Preparation of Inoculum

Three *Colletotrichum* isolates were used: two isolates of *C. fragariae*, Cf-63 and Cf-75 (Smith and Black, 1990), and one isolate of *C. gloeosporioides*, Cg-162 (Smith and Black, 1990). Each isolate was initiated from silica gel cultures maintained at the USDA-ARS Thad Cochran Southern Horticultural Laboratory, Poplarville, MS, and grown on 1:1 oatmeal potato dextrose agar (OMA:PDA) at 20 to 28 °C under fluorescent lights

with a 12-hour photoperiod. Inoculum was prepared as a conidial suspension from 7- to 14-day-old cultures by flooding each culture plate with sterile deionized water, and gently scraping the agar surface with a glass rod to remove conidia. The resulting conidial suspension was filtered through one layer of bandage gauze (Johnson & Johnson, New Brunswick, NJ) and adjusted to a concentration of 1.5×10^6 conidia/mL by diluting with sterile deionized water to which Tween-20 (Sigma Chemical Co., St. Louis, MO) had been added as a surfactant at the rate of one drop from an eyedropper per 1 liter of water.

Detached Leaf Inoculations (2009, 2010, 2011)

Young, fully developed, blemish-free leaves, composed of the petiole and three leaflets, were removed from plants no more than four hours before inoculation, rinsed in tap water, and the petiole was inserted into a 10 × 150 mm test tube filled with sterile deionized water. Detached leaves from each of 98 strawberry clones (32 named cultivars and 66 unreleased MSUS clones) were inoculated with each of three isolates: *C. fragariae* (Cf-63 and Cf-75) and *C. gloeosporioides* (Cg-162). *Colletotrichum acutatum* isolates Goff and Ca-1 (Chang and Smith, 2007) were initially used in the detached leaf study, but no disease symptoms developed on the leaflets, so they were removed from the detached leaf inoculations. Each leaf was inoculated with the conidial suspension by misting the adaxial surface of the three leaflets with a hand pump sprayer to the point of runoff. Detached leaves used for non-inoculated controls were misted with deionized water. The inoculated leaves (still in the test tubes) were immediately placed in a dew chamber at 100% RH, 30 ºC, and incubated in the dark for 48 hours. They were then

transferred to sealed, clear plastic containers at 100% RH and 23–25 ºC with continuous fluorescent light for an additional three days before assessing disease symptoms. The leaflets of each detached leaf were visually assessed for disease severity using a 0 to 5 scale: 0 = no visible disease symptom on any leaflet, 1 through 4 increasing disease symptom severity, and 5 = total area of leaflets necrotic. Hereafter, the visual disease ratings obtained in the detached leaf study will be termed the DL-DSR.

After the visual assessment, the petiole was removed and each leaf was separated into individual leaflets, placed on a light box, and photographed either with a DXCB151A color video camera (Hitachi Instruments, Inc., Houston, TX, U.S.) and captured with Bioquant® 98 image analysis software (R&M Biometrics, Inc., Nashville, TN, U.S.) at the University of Southern Mississippi (USM), Hattiesburg, MS, or with a Nikon COOLPIX 5000 digital camera (Nikon Corp., Tokyo, Japan), then uploaded as JPG files at the USDA, ARS Thad Cochran Southern Horticultural Laboratory (TCSHL), Poplarville, MS. Initially the photographs of the leaflets were taken at USM, but due to time constraints after the first inoculation study, leaflets from the detached leaves were photographed at TCSHL. Photographs were enlarged electronically 200% and individually marked and colorized for image analysis with healthy leaf tissue as green and tissue with lesions as black using Corel® Photo-Paint X4 or X5 (Corel Corp., Ottawa, Ontario, Canada). Originally, lesions were counted electronically, but due to the nature of anthracnose symptoms on strawberry leaflets (small lesions coalesce into a larger lesion), these data were not used in separate analyses, but were used to find the total percent lesion area. Total leaf area was calculated as the green (healthy) area plus

the black (lesion) area using Image Pro Plus 7.0 (Media Cybernetics, Bethesda, MD). Percent lesion area was calculated as lesion area divided by total leaf area multiplied by 100. The electronic percent lesion area was paired with the visual raters' DL-DSR and then both the electronic and each rater's DL-DSR were averaged for each clone across all repetitions for each isolate. Hereafter the electronic disease ratings will be termed percent disease.

Experimental Design and Statistical Analysis

Detached strawberry leaves from each of 98 clones were inoculated with each of three *Colletotrichum* isolates each year for three years. The availability of leaves at each collection date determined the number of leaves inoculated. Clones with less than eight leaves inoculated across the three studies were removed. Twelve was the average number of leaves inoculated of the remaining clones. The experimental design was a completely randomized design. Inter-rater association and agreement between the two independent raters were established using Pearson's product-moment correlation coefficient (r_p) and weighted Kappa coefficient (k_w), respectively. Both raters' visual DL-DSRs were then averaged together (rater average) and tested for association and agreement with the percent disease. The rater average for each number on the 0 to 5 rating scale was adjusted to whole numbers.

Results

The total observations (N =4028) were made with an overall average of 4 leaves per clone per isolate per year. Controls inoculated with deionized water were used at each inoculation date and had an overall average DL-DSR for both raters of 0.09 and a

percent disease of 0.40%. This indicates the detached leaves used at the inoculation date did not have disease symptoms prior to inoculation with *Colletotrichum*, therefore, the data from the controls (615 observations) were removed from further statistical evaluations.

The two visual raters' average DL-DSRs (n =3413) were in substantial agreement with a weighted Kappa of 0.80 (95% CI =0.79–0.82). Frequencies of the visual raters' DL-DSR agreement is tabulated in Table 6 and summarized graphically in Figure 4. The visual raters had perfect agreement for 52% of the DL-DSRs. The visual raters had perfect agreement 30% of the time for the DL-DSR of 0; 16% perfect agreement for the DL-DSR of 1; 1% agreement for the DL-DSRs of 2, 3, and 4; and 3% perfect agreement for the DL-DSR of 5. The DL-DSRs of 2, 3, and 4 had the least amount of perfect agreement, but the raters were not in total disagreement for these DL-DSRs. Rater 1 rated 365 inoculated leaves a 2, 269 a 3, and 222 a 4; whereas within these same rater 1 DL-DSRs, rater 2 rated 320 a 1, 135 a 2, and 111 a 3. The DL-DSRs that were more than one disease rating from each other (e.g., one rater rated 2 and the other rated 0) only totaled 203, or 6% of the total 3413 DL-DSRs, with most of this variation occurring for DL-DSRs of 3 (113) and 4 (74). The association of the two raters' average DL-DSRs was good with a Pearson's product moment correlation coefficient of r_p =0.84, and their agreement was substantial with a weighted Kappa of k_w =0.80. The substantial agreement and good association between the two raters' DL-DSRs showed consistency between the visual raters. Therefore, the DL-DSRs of the two raters were averaged and considered the rater average DL-DSR for comparison with the electronic ratings.

Descriptive statistics for the rater average DL-DSR using the percent disease from the electronic ratings as the analysis variable can be seen in Table 7 and means are plotted in Figure 5. The association of the rater average DL-DSR and the percent disease was good with a Pearson's product moment correlation coefficient of r_p =0.79. The weighted Kappa statistic, which expresses the agreement between two sets of variables, requires both sets to contain the same number of scoring values. Due to the precision of the percent disease producing hundreds of different percentage measurement values and the visual rating scale containing six measurement values, a weighted Kappa could not be calculated using the rater average DL-DSR and percent disease.

The rater DL-DSR of 0 was equivalent to 0.3% disease and the DL-DSR of 5 was equivalent to 93.7% disease. The percent disease separates well with the visual rater 0 to 5 DL-DSR scale (Table 10).

The average percent disease and the rater average DL-DSR were calculated for each of the fungal isolates used for the inoculations. These ratings were also separated by the source of the leaves, either named cultivar or MSUS clone, because the MSUS clones were bred to be resistant to *C. fragariae* (Table 8). *Colletotrichum fragariae* isolate Cf-75 was the most aggressive of the isolates with an overall average percent disease of 10% and rater average DL-DSR of 1.4. *Colletotrichum gloeosporioides* isolate Cg-162 was the next most aggressive with ratings of 7.1% disease and average DL-DSR of 1.1; and *C. fragariae* isolate Cf-63 was the least aggressive with an overall 3.4% disease and 0.8 average DL-DSR. When the DL-DSRs and percent disease were compared by source, the isolates remained in the same order of aggressiveness, but the difference between the

ratings of the cultivars and the ratings of the MSUS clones indicates the MSUS clones are more resistant to all of the isolates. The percent disease for two cultivars used for comparison were Chandler for susceptible (Cf-63 =2.2%, Cf-75 =38.8%, and Cg-162 =16.7%) and Pelican for resistant (Cf-63 =0.7%, Cf-75 =0.5%, and Cg-162 =2.4%).

Discussion

There are diverse options when it comes to disease rating methods, e.g. percentage or ordinal rating scales, with or without reference photographs of diseased plant organs with associated ratings to help the visual rater in determining the correct rating. When choosing a disease rating method to use, the researcher must take into consideration the purpose of the research, as well as the time, practicality, cost, and accuracy of the ratings. Visual ratings can be used to quickly assess disease severity and are often utilized for rapid decisions on pesticide use in production fields. Electronic percent disease ratings made from image analysis are more accurate than visual ratings and are often utilized when the research requires more detailed observations for detecting and quantifying plant disease.

Regardless of the rating method, low (<10%) and high (>90%) levels of disease on plant tissue are less difficult to distinguish visually than disease affects 10 to 90% of the plant tissue. The association between the two raters' DL-DSRs in this study was very good (r_p =0.87) with exact agreement on 52% of the DL-DSRs and varying amounts of agreement on the remaining 48% of the DL-DSRs. The mid-range ratings had the least amount of agreement. This variation may be explained by visual bias, which is supported

by previous research on the subject (Horsfall and Barratt, 1945; Sherwood et al., 1983; Slopek, 1989).

A rating scale with few categories (as the 0 to 5 scale in this study) often presents the possibility of rater agreement by pure chance. The weighted Kappa is a quantitative measure of the magnitude of the inter-rater agreement between the two raters' DL-DSRs and the value of k_w =0.80 obtained in this study indicates a much better than chance agreement. The raters were in exact agreement on 52% of the total DL-DSRs with 30% agreement at the lowest DL-DSR of 0, 16% agreement at the DL-DSR of 1, and 3% agreement at the DL-DSR of 5. The use of a 0 to 5 ordinal scale is sufficient to distinguish between susceptible and resistant strawberry leaves. An element of training, such as providing the visual raters with photographs representing each category on the rating scale, would improve the rating process, thus leading to a higher level of agreement between raters.

Image analysis is expected to give a very precise disease rating and is appealing as a means of assessing percent lesion area. A disadvantage of image analysis is the considerable time required to prepare and photograph each set of leaflets and to mark electronically each lesion. It can be difficult to make a decision between healthy and diseased tissue when visually rating leaves under laboratory lights without any type of enhancement, especially if the lesions are very small. When the leaves are backlit and photographed, the visibility of the lesions is improved (Miller-Butler et al., 2013). Visually assessing disease severity of a set of leaflets using a photograph on a computer monitor often takes less time and is easier than assessing the actual leaflets in the

laboratory. Initial screening of large seedling populations for anthracnose resistance could be accomplished with preliminary screening completed by visual raters. Before releasing a new named cultivar, final decisions of anthracnose resistance or susceptibility could then be based on electronic imaging. The choice of a disease rating method is dependent upon the researcher's requirement for precision.

Colletotrichum fragariae isolate Cf-75 was the most aggressive of the three isolates used in this study with an overall average percent disease of 10% and rater average DL-DSR of 1.4. *Colletotrichum gloeosporioides* isolate Cg-162 was the next most aggressive isolate, and *C. fragariae* isolate Cf-63 was the least aggressive of the three isolates. The use of a more aggressive isolate in an inoculation study helps to separate efficiently the more susceptible germplasm from the more resistant germplasm. Since the MSUS clones were bred to be resistant to *C. fragariae*, the data were separated by source to confirm that the isolates were of the same order of aggressiveness on the MSUS clones as on the cultivars. When the ratings were separated by source, the isolates remained in the same order of aggressiveness. The difference in the ratings of the cultivars and MSUS clones indicates the MSUS clones are more resistant than the cultivars to each of these isolates.

Detached leaf inoculations can be used as a rapid preliminary screen to separate anthracnose susceptible from anthracnose resistant germplasm in large populations within breeding programs. The rating of the germplasm (whether visual or electronic) can be fine-tuned higher or lower to separate susceptible from resistant germplasm by adjusting the scale to a more or less severe rating. Image analysis is more accurate and precise than

visual ratings and was used as a "yardstick" in this study to compare percent lesion area obtained from the image analysis to the visual disease ratings. Comparison of visual assessments with image analysis of anthracnose disease on inoculated detached strawberry leaves was the main objective of this study. The 0 to 5 disease rating scale was more than sufficient to separate susceptible from resistant strawberry leaves. The degree of agreement between the two methods was positively related (r_p =0.79) and supports a preliminary study (Miller-Butler et al., 2013) that also found a strong correlation between image analysis and visual disease ratings. The results of this research confirm that a detached leaf assay can reliably be used to distinguish between anthracnose-resistant and susceptible germplasm. The detached leaf assay was compared to a whole plant assay using the same isolates and disease severity rating scale that has been used for almost 30 years to identify anthracnose resistant strawberry germplasm (Chapter IV, this dissertation).

Table 6

Frequency of two visual raters' DL-DSR.

Rater 1 DL-DSR	Rater 2 DL-DSR					
	0	1	2	3	4	5
0	**1015**	792	2	0	0	0
1	43	**550**	10	0	0	0
2	3	320	**42**	0	0	0
3	0	113	135	**19**	2	0
4	0	9	65	111	**37**	3
5	0	0	0	11	32	**99**

Frequency of the two visual raters' detached leaf disease severity rating (DL-DSR) based on a 0 to 5 visual rating scale for 3413 detached strawberry leaves inoculated with three isolates of Colletotrichum species.

Table 7

Summary statistics for detached leaves inoculated with Colletotrichum[a] species.

Rater DL-DSR[b]	Percent Disease					
	Average	Std Dev	Median	Minimum	Maximum	N
0	0.3	0.4	0.1	0.0	3.5	1309
1	1.2	1.5	0.7	0.0	15.3	1166
2	5.1	4.0	4.0	0.1	29.4	488
3	16.9	8.7	15.8	2.2	48.6	204
4	45.5	20.8	45.5	8.6	91.9	137
5	93.7	10.6	98.9	44.6	100.0	109

[a]Two isolates of *Colletotrichum fragaria* and one isolate of *C. gloeosporioides* were used to inoculate the detached strawberry leaves. Isolates were not all applied on the same leaf.

[b]The DL-DSRs are based on the average of two visual raters.

Summary statistics for the percent disease of 3413 detached strawberry leaves following inoculation with three isolates of Colletotrichum[z] species, corresponding to the rater average disease severity rating (DL-DSR) on the same leaves. Percent disease was calculated from computer image analysis of photographed leaves.Average percent disease and average DL-DSR for Colletotrichum isolates.

Table 8

Disease Severity Ratings and Percent Disease for Detached Strawberry Leaves.

	Overall		Cultivar		MSUS	
Colletotrichum Isolate	Disease (%)	DL-DSR	Disease (%)	DL-DSR	Disease (%)	DL-DSR
C. fragariae Cf-63	3.4	0.8	6.9	1.3	1.7	0.6
C. fragariae Cf-75	10.0	1.4	19.1	2.3	5.7	0.9
C. gloeosporioides Cg-162	7.1	1.1	13.3	1.8	4.2	0.8

Average percent disease and average DL-DSR of 3413 detached strawberry leaves following inoculation with three isolates of Colletotrichum species. Ratings are separated by Colletotrichum isolates, average percent disease and average DL-DSRs, and leaf source (named cultivar or MSUS clone).

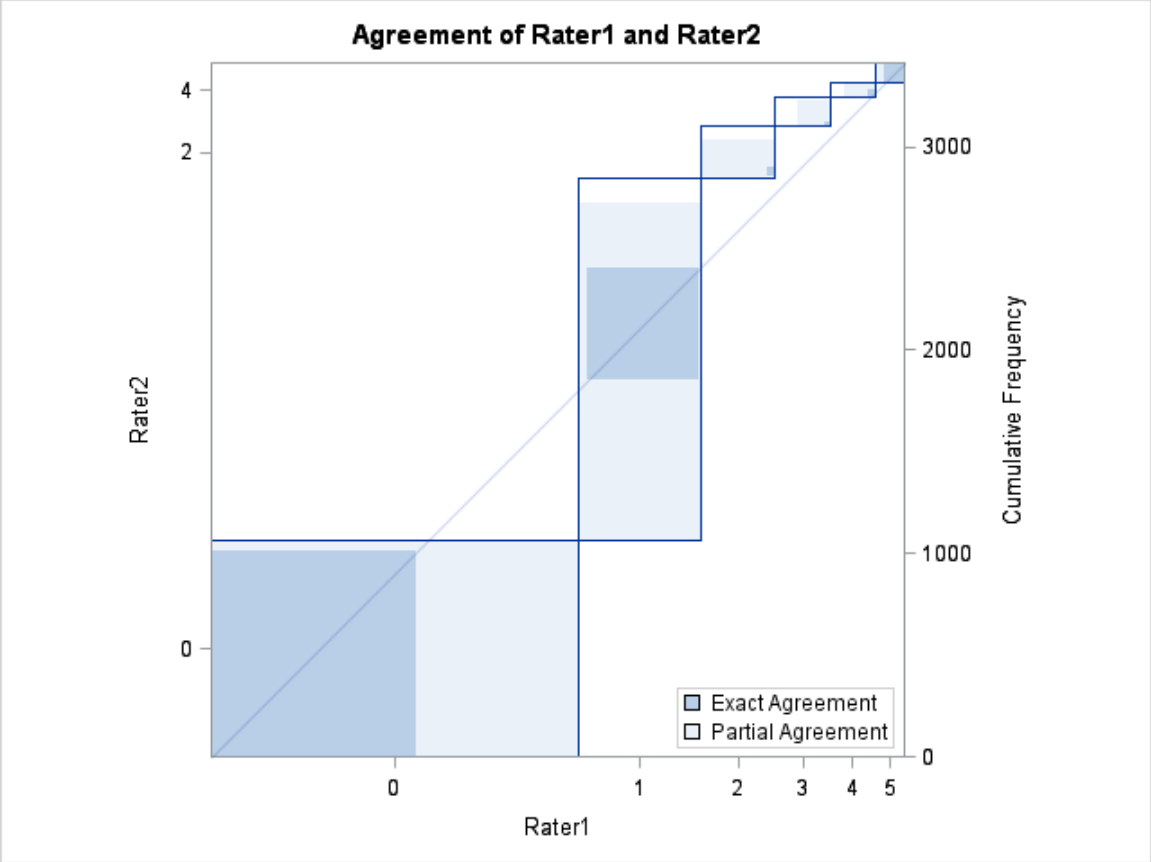

Figure 4. Agreement of visual raters.

Agreement between the two visual raters' DL-DSRs (k_w =0.80, 95% CI 0.79–0.82), was better at the lower end (0 and 1) and the higher end (5) of the scale. The ratings of 2, 3, and 4 had minimal perfect agreement.

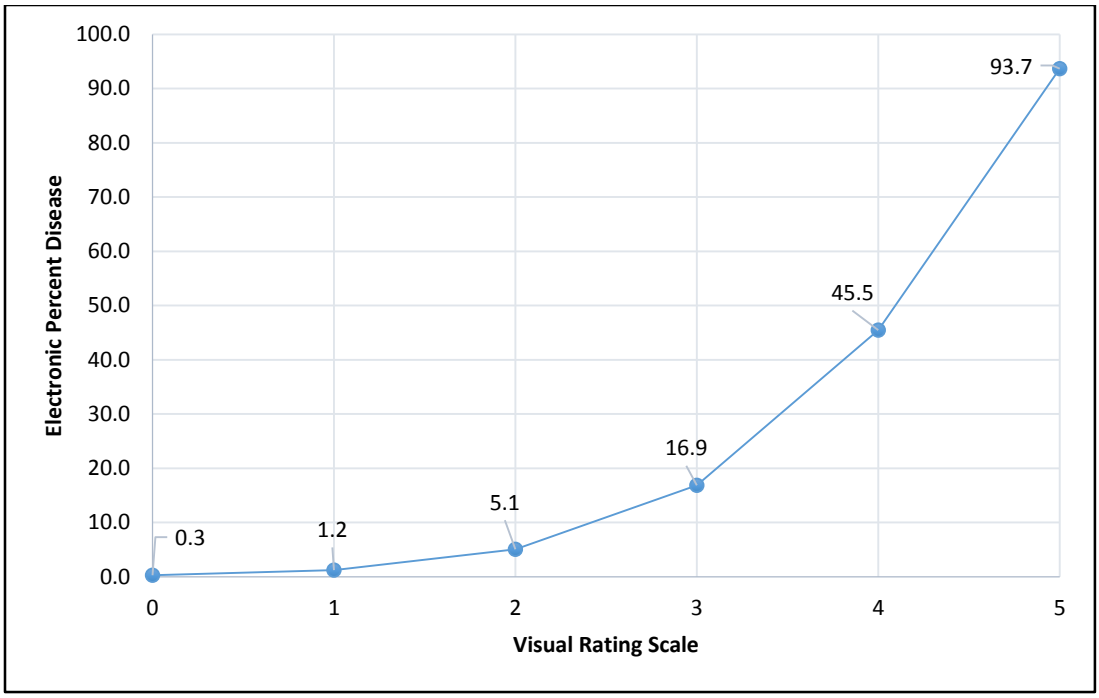

Figure 5. Percent disease vs. visual rater average DL-DSR.

Percent disease vs. the visual rater average DL-DSR of 3413 detached strawberry leaves following inoculation with three isolates of *Colletotrichum* species. Percent disease was calculated from computer image analysis of photographed leaves. Rater average DL-DSR are based on the average of two visual raters.

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CHAPTER IV – COMPARISON OF SCREENING TECHNIQUES (WHOLE PLANT AND DETACHED LEAF) FOR IDENTIFYING ANTHRACNOSE RESISTANCE IN STRAWBERRY PLANTS

Introduction

The dessert strawberry (*Fragaria* × *ananassa*) is a popular fruit grown in most arable regions of the world. In the United States, California and Florida are the top strawberry producing states. California produces approximately 85% of the strawberries grown in the U.S. (USDA-NASS, 2014) and, as their production tapers off in the fall, the Florida growing season begins and runs through the winter months. Strawberry is host to many pathogens that cause major losses in strawberry production, wherever they are grown. Anthracnose is a destructive disease of strawberry caused by three *Colletotrichum* species: *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* (Smith 1998). *Colletotrichum acutatum* was first reported on strawberry in the U.S. by Smith and Black (1986) and may cause anthracnose disease on the runners, petioles, leaves, and fruit (Howard et al., 1992). Both *C. fragariae* and *C. gloeosporioides* can infect all aboveground parts of the plant and may cause anthracnose crown rot and anthracnose leaf spot (also called black leaf spot).

Commercial strawberry growers continually strive to improve their disease control measures. Integrated Pest Management (IPM) programs help growers combine strategies that focus on long-term prevention of disease and pests utilizing such management practices as planting disease-free plants, good sanitation practices, cultural controls, and the use of pesticides if necessary. Disease-free plants are critical to a successful crop and also decrease the chances of introducing pathogens from other

locales. Good sanitation practices help destroy pathogens on equipment or in the soil, but cannot control latent infections. Cultural controls, such as drip irrigation instead of overhead irrigation, decrease the movement of fungal conidia through water splash. Pesticides, applied correctly, help control many pests but rarely eradicate them. The overuse of some pesticides, such as fungicides, has resulted in pathogen resistance and failure of the fungicides to control anthracnose epidemics (Smith and Black, 1993a, 1993b; LaMondia, 1995). These disease control practices are important tools for growers, but the ultimate solution for controlling plant disease is developing and planting disease resistant cultivars.

Strawberry breeding programs attempt to produce disease resistant germplasm, while maintaining desirable fruit and horticultural traits. Commercial strawberry growers, breeders, and plant pathologists work together in teams to develop anthracnose resistant strawberry plants. The release of a new strawberry cultivar entails classical plant breeding (cross pollination), germinating the seed (progeny), testing for disease resistance, increasing the selected population by clonal propagation (daughter plants on stolons or through *in vitro* tissue culture), and field testing under natural conditions for plant quality, quality and quantity of the fruit, and desirable horticultural characteristics. This process generally requires many years. The Agricultural Research Service (ARS), a branch of the United States Department of Agriculture (USDA), established a strawberry breeding program in Poplarville, MS in the 1980s (Smith, 2006) with the goal of developing strawberry germplasm resistant to anthracnose crown rot caused by *C. fragariae* and adapted to the southeastern U.S. Anthracnose-resistant selections were identified following greenhouse screening for anthracnose resistance and field testing for

horticultural traits. These selections were numbered and given the prefix MSUS to denote they were from the USDA breeding program in Mississippi. One of the MSUS clones was released as the anthracnose resistant cultivar 'Pelican' (Smith et al., 1998), and four others were released as breeding lines, i.e., US70, US159, US292, and US438 (Galletta et al., 1993). Approximately 100 of the clones and the strawberry releases continue to be clonally propagated at the Poplarville research location.

Screening for disease resistance in strawberry germplasm commonly requires inoculating whole plants with isolates of the pathogens to determine their degree of resistance. Resistance depends on the plant-pathogen interaction in an environment suitable for disease development. Greenhouse screening is preferable to field screening because the researcher can control the environment within the greenhouse and choose the isolates to use as inoculum, whereas field research is at the mercy of the weather and pathogen populations which may differ from year to year.

The use of an aggressive isolate of the pathogen in a disease screening program increases disease incidence and separates germplasm reactions more effectively. The disease response must be assessed with accurate and reproducible techniques. Inoculation trials are time-consuming, and plants may be destroyed by the disease which can present a problem for the breeder because the germplasm may have possessed other desired horticultural traits. Screening for disease resistance using detached strawberry leaves is an alternative to inoculating whole plants and can possibly eliminate the destruction of desirable germplasm.

Disease severity refers to the amount of plant tissue that is diseased and is usually expressed as the percentage of plant area visibly affected by a pathogen. Disease

assessment scales, such as visual rating scales which may be percentage or numerical, have been in use for many years and are generally employed for a quick assessment of disease severity (Horsfall and Barratt, 1945; Slopek, 1989). Visual disease ratings rely on some degree of subjective interpretation by the rater. In previous research with inoculated detached strawberry leaves, image analysis was shown to be more precise than visual disease ratings (Miller-Butler et al., 2013, Chapter III, this dissertation). Image analysis was used as a "yardstick" to compare the percent lesion area of the detached leaves to visual disease assessment and showed a high positive correlation between the image analysis and visual disease assessment, lending support to using a visual rating scale for evaluating disease severity on detached strawberry leaves.

Development of a whole strawberry plant inoculation protocol for resistance to *C. fragariae* began at the USDA-ARS Poplarville location in the 1970s (Smith and Spears, 1982). Smith and Black (1987) investigated the environmental conditions necessary for disease development when assessing strawberry germplasm for resistance to *C. fragariae*. Their efforts produced the inoculation assay still in use today. Plants to be assessed for resistance are inoculated by misting the foliage with a conidia suspension of a fungal isolate and held at 35 °C and 100% relative humidity (RH) for 48 hours in a humidity chamber. The plants are then moved to a 32 °C greenhouse and assessed for disease at 30 days after inoculation (DAI) using a 0 to 6 disease severity rating (DSR) scale. The resistance or susceptibility of the germplasm is then categorized using the DSR where plants with a rating $\leq$ 2.0 are classified as resistant, those with a rating > 2.0 to < 4.0 are classified as intermediate, and those with a rating $\geq$ 4.0 are classified as susceptible.

Research for the development of a detached strawberry leaf inoculation protocol has been ongoing at the USDA-ARS in Poplarville, MS. The detached leaf protocol is similar to the whole plant protocol, with the exception being after 48 hours in a dew chamber the detached leaves are placed in a sealed clear plastic container and held at room temperature and 100% RH. The leaves are assessed for disease severity five DAI using a 0 to 5 disease severity rating scale. A reliable anthracnose screening protocol based on a detached leaf assay would cut the time between inoculation and disease assessment, thus reducing the overall time for breeders to identify anthracnose resistant germplasm for their breeding programs. It would also preserve valuable germplasm since plants of susceptible breeding lines would not be killed, and would allow for evaluation using isolates of the pathogen from other areas without the risk of those isolates infecting local plants.

I reasoned that the resistance or susceptibility of strawberry plants could be reliably established by inoculating detached leaves with isolates of the anthracnose pathogens. This alternative to an assay using whole plants should be faster and just as accurate. This plan could be tested by inoculating whole strawberry plants and detached strawberry leaves from the same plant population with isolates of the *Colletotrichum* species known to cause anthracnose disease on whole strawberry plants. The anthracnose symptoms would be visually rated on both whole plants and detached leaves and the ratings compared between the two inoculation techniques.

Materials and Methods

Production of Plant Material

Strawberry plants were established in 10-cm plastic pots in a 1:1 mixture of Jiffy-Mix (JPA, West Chicago, IL, U.S.) and sand. Named cultivars and MSUS clones (Table 9) were propagated by rooting young runner plants in the Jiffy-Mix:sand potting mixture. Plants were maintained in a greenhouse at 28 ºC ±10 ºC with a 16-hour photoperiod. Named cultivars and MSUS clones of strawberries used in this research will be collectively referred to as germplasm lines.

Growth of Fungal Isolates and Preparation of Inoculum

Three *Colletotrichum* isolates were used: two isolates of *C. fragariae*: Cf-63 and Cf-75 (Smith and Black, 1990; Chang and Smith, 2007), and one isolate of *C. gloeosporioides*: Cg-162 (Smith and Black, 1990). Each isolate was initiated from silica gel cultures and grown on 1:1 oatmeal potato dextrose agar (OMA:PDA) at 20–28 °C under fluorescent lights with a 12-hour photoperiod. Inoculum was prepared as a conidial suspension from 7- to 14-day-old cultures by flooding each culture plate with sterile deionized water and gently scraping the agar surface with a glass rod to remove conidia. The resulting conidial suspension was filtered through one layer of bandage gauze (Johnson and Johnson, New Brunswick, NJ) and adjusted to a concentration of 1.5×10^6 conidia/mL by diluting with sterile deionized water with Tween-20 (Sigma Chemical Co., St. Louis, MO) added as a surfactant at the rate of one drop per 1 liter of water.

Whole Plant Inoculations

Older leaves, runners, and flowers were removed from plants 3 to 4 days before inoculation leaving 4 to 5 young leaves on each plant at inoculation. Plants were inoculated with a conidial suspension by misting with a hand pump sprayer to the point of runoff. Plants used for non-inoculated controls were misted with deionized water. Inoculated plants were immediately placed in a dew chamber at 100% relative humidity (RH), 30 ºC, and incubated in the dark for 48 hours. They were then transferred to a greenhouse (28 ºC ±10 ºC) for the remainder of the study. Disease development was assessed on the whole plants (n = 2194, minus the non-inoculated controls) at 10 and 30 DAI using a disease severity rating (DSR) scale adapted from Smith and Black (1987). Whole plant rating categories were 0 = healthy plant with no visible lesions, 1 = < 3 mm long petiole lesion, 2 = 3 to 10 mm long petiole lesion, 3 = > 10–20 mm long petiole lesion, 4 = > 20 mm long petiole lesion, 5 = youngest leaf wilted, and 6 = plant dead. The highest DSR from the either the 10-day or 30-day evaluations was used for data analysis. Plants with a DSR $\leq$ 2.0 were considered resistant, those with a DSR > 2.0 to < 4.0 were considered intermediate, and those with a DSR $\geq$ 4.0 were considered susceptible. This rating scale has been used successfully for almost 30 years to identify anthracnose resistant strawberry germplasm.

The decision was made also to rate the leaf blade (composed of three leaflets without the petiole) on each plant with the greatest disease symptoms at the same time as rating the whole plant. This rating would be used to classify the anthracnose response of leaflets from the whole plant and correlate it with both the whole plant DSR and the detached leaf disease severity rating. The disease severity rating scale used to evaluate

the leaflets was: 0 = no visible disease symptom on any leaflet, 1 through 4 = increasing disease symptom severity, and 5 = total area of leaflets necrotic. Plants inoculated in the whole plant assay were not returned to the original greenhouse after inoculation but were kept in a separate greenhouse.

Detached Leaf Inoculations

Young, fully developed, blemish-free leaves, composed of the petiole and three leaflets, were removed from plants no more than four hours before inoculation, rinsed in tap water, and the petiole was inserted into a 10 × 150 mm test tubes filled with sterile deionized water. Each detached leaf was inoculated with the conidial suspension by misting the adaxial surface of the three leaflets with a hand pump sprayer to the point of runoff. Detached leaves used for non-inoculated controls were misted with deionized water. The inoculated leaves (still in the test tubes) were immediately placed in a dew chamber at 100% RH, 30 ºC, and incubated in the dark for 48 hours. They were then transferred to sealed, clear plastic containers at 100% RH and 23–25 ºC with continuous fluorescent light for an additional three days before assessing disease symptoms. The leaflets of each detached leaf were visually assessed for disease severity using the same scale described for leaflets in the whole plant inoculations: 0 = no visible disease symptom on any leaflet, 1 through 4 increasing disease symptom severity, and 5 = total area of leaflets necrotic. Hereafter, the whole plant DSR will be known as the WP-DSR, leaflet DSR from the whole plant inoculations will be known as the L-DSR, and the detached leaf DSR will be known as the DL-DSR.

Experimental Design and Statistical Analysis

The experimental design for both the whole plant and the detached leaf studies was a completely randomized design within each germplasm line, meaning the plants and leaves were chosen at random from the population of that particular germplasm line. The whole strawberry plant inoculations were conducted three times over two years (2010 and 2011) and the WP-DSR for each of the individual plants within a germplasm line from each of the three inoculation studies were used to calculate the mean WP-DSR for the germplasm line. The L-DSR for each of the germplasm lines from each of the three whole plant inoculation studies were used to calculate the mean L-DSR for each germplasm line. The detached leaf inoculations were conducted three times over three years (2009, 2010, and 2011) and the DL-DSR for each of the individual leaves within a germplasm line from each of the three inoculation studies were used to calculate the mean DL-DSR for the germplasm line. The non-inoculated controls in the whole plant experiments had an overall WP-DSR mean of 0.31 and an overall L-DSR mean of 0.35. The non-inoculated controls from the detached leaf experiments had an overall DL-DSR mean of 0.09. This indicates the whole plants and detached leaves used for the inoculation studies were not infected with *Colletotrichum* species prior to the studies; therefore, the data from the non-inoculated controls were removed from further statistical evaluations. The mean WP-DSR, L-DSR, and DL-DSR data from the two inoculation assays were analyzed for association using Pearson's correlation coefficient. The mean DSR data for the whole plant (WP-DSR and L-DSR) inoculations and the mean DSR data for the detached leaf (DL-DSR) inoculations, based on the categories (resistant, intermediate, or susceptible) were analyzed with analysis of variance (ANOVA). If

significant differences were found with ANOVA, a post hoc Tukey's test was performed. All data were analyzed using SAS (version 9.4; SAS Institute Inc., Cary, NC).

Results

Due to the difference in rating scales (whole plant: 0 to 6 and leaf: 0 to 5), the twelve germplasm lines with a WP-DSR mean rating greater than 5.5 (rounded up to a rating of 6 = plant dead) were amended to equal 5 (youngest leaf wilted) for statistical analysis between the whole plant ratings and the leaf ratings. This allowed all of the data from the two DSR scales to be analyzed using the same scale. A WP-DSR of 5 or 6 is comparable to a category of susceptible when determining the resistance of the germplasm.

The data were analyzed for association (precision) using the CORR procedure of SAS and the WP-DSR and L-DSR were found to have a very high degree of association (r_p =0.96). The association between the L-DSR and DL-DSR (r_p =0.70) was positive. The association between the WP-DSR and DL-DSR was also positive (r_p =0.66), slightly less than the association between the L-DSR and DL-DSR, but moderately less than the association between the WP-DSR and L-DSR from the whole plant inoculations. This is not unexpected since the detached leaves were rated for disease five DAI compared to the 30-day whole plant disease rating that allowed the attached leaves a longer time to develop disease.

The WP-DSR for all the plants in each germplasm line was averaged across all inoculation isolates, and this average was used to assign the resistance categories (R = resistant, I = intermediate, S = susceptible) to each germplasm line. The WP-DSR means for each of the categories were analyzed with ANOVA and found to be significantly

different (F =809.3, p <0.0001). A post hoc Tukey's test showed that the WP-DSR means of each category differed significantly from each other at p <0.05. The categories for the WP-DSR means were used to establish the corresponding leaflet (from the same plant) L-DSR means and detached leaf (from the same plant population) DL-DSR means. The L-DSR means for each of the categories were analyzed with ANOVA and found to be significantly different (F =565.0, p <0.0001). A post hoc Tukey's test showed that the L-DSR means differed significantly from each other at the p <0.05. The DL- DSR means for each of the categories were also analyzed with ANOVA and found to be significantly different (F =87.4, p <0.0001). A post hoc Tukey's test showed that the DL-DSR means differed significantly from each other at the p <0.05 (Table 10).

Descriptive statistics for each of the mean DSRs within each germplasm line based on the resistance category can be found in Table 10. The mean WP-DSR (R =1.3, I =2.9, S =4.8) and mean L-DSR (R =1.2, I =2.9, S =4.5) corresponding to the same categories were equal for the intermediate category and within 0.1 and 0.3 for the resistant and susceptible categories, respectively. The WP-DSR means for each category (R =1.3, I =2.9, S =4.8) were compared to the DL-DSR mean for each category (R =0.7, I =1.1, S =1.9) and found not to be as closely related as the L-DSR means with the WP-DSR means, both from the same plant. Based on these results, the DL-DSR means can be used to establish categories to be used in future detached leaf studies.

Discussion

Developing and releasing a new strawberry cultivar generally takes many years, so any part of the process that can be shortened is a plus for the breeder, grower, and the public who benefit from the availability of strawberry fruit. The germplasm must be

clonally propagated to move forward with screening research, whether for disease resistance or agronomic traits. Clonally replicating the germplasm adds a considerable amount of time to the overall process of cultivar development. Destroying possible breeding germplasm with preliminary disease screening using whole plant inoculations further slows a program since screening the germplasm for disease resistance may require years of greenhouse and field testing. Inoculating detached strawberry leaves with the anthracnose pathogen allows assessment for disease resistance without destroying whole plants and also lessens the time between inoculation and disease assessment (Miller-Butler et al., 2013 and Chapter III this dissertation). Utilizing the detached leaf assay may provide a rapid, non-destructive method of reliably identifying anthracnose resistant germplasm, thus moving the screening process forward with enhanced efficiency. My research also suggests that there can be a seasonal effect on disease development on strawberry plants held in a greenhouse after inoculation (Chapter II). Inoculation studies performed in a laboratory permit the environment (temperature, humidity, and light) to be controlled more completely than in a greenhouse, requires less space, and confines the pathogen to the laboratory.

My research supports previous research using disease severity ratings obtained from whole plant inoculations to separate strawberry germplasm into three resistance categories: resistant, intermediate, and susceptible (Smith and Black, 1987, 1990; Lewers et al., 2007). The category means for the WP-DSR and L-DSR were almost equivalent as would have been expected, but were unknown prior to this research. This demonstrates that the whole plant disease severity rating corresponds well to the leaflet tissue disease severity rating obtained from the same plant. The overall means for each category based

on the WP-DSR, L-DSR, and DL-DSR were found to be significantly different based on Tukey's test. The category mean WP-DSR and DL-DSR were not as closely related mathematically as the WP-DSR and L-DSR; however, the detached leaves did not have as much time to develop disease as the whole plants, so this was not unexpected. The mean DL-DSRs for each category were found to be significantly different from each other, based on Tukey's test, describing the disease in increments that can be separated. The significant difference between the categories for the DL-DSR means adds credibility to using a detached leaf assay for initially separating resistant germplasm from susceptible germplasm.

Previous research indicated that anthracnose resistant germplasm could be distinguished from susceptible germplasm using inoculated detached strawberry leaves and that a 0 to 5 ordinal scale was sufficient for rating the disease severity (Miller-Butler et al., 2013; Chapter III). My research compared the whole plant (benchmark), leaflets, and detached leaf disease severity ratings, and all three were able to distinguish significant differences among the resistance categories (Table 10). My research indicates that a strawberry detached leaf assay can be used reliably and quickly to determine the resistance or susceptibility of strawberry germplasm to anthracnose.

Table 9

Disease severity ratings for whole plants and detached leaf inoculations.

	C. fragariae Cf-63						*C. fragariae* Cf-75						*C. gloeosporioides* Cg-162					
	Detached Leaf		Whole Plant				Detached Leaf		Whole Plant				Detached Leaf		Whole Plant			
Germplasm	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S) [a]	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)
US 70							16	0.3	6	2.6	2.0	I	12	0.3	4	1.4	1.5	R
US 159	14	0.6	8	1.9	1.7	R	17	0.9	11	3.0	3.0	I	19	1.1	12	2.8	3.0	I
US 292	9	1.1	8	2.8	2.1	I	13	1.1	11	2.7	2.3	I	20	0.7	12	2.9	2.7	I
US 438	13	0.7	3	1.3	0.3	R	17	1.2	6	2.0	1.7	R						
MSUS 478	15	0.5	8	0.6	0.5	R	13	1.6	11	2.4	3.5	I	16	1.4	10	2.6	3.1	I
MSUS 518	13	0.5	8	1.1	0.5	R	14	0.9	12	2.1	2.1	I	15	0.4	12	2.0	2.2	I
MSUS 572	10	0.8	8	0.6	0.6	R	12	1.7	12	2.1	2.7	I	9	1.7	11	3.0	3.4	I
MSUS 574	10	0.1	8	1.4	1.0	R	16	1.5	12	2.0	2.5	R	15	1.1	11	2.9	2.7	I
MSUS 868	12	0.4	8	0.4	0.6	R	16	0.7	12	1.7	1.5	R	14	0.5	12	1.3	1.4	R
MSUS 890	11	0.7	4	1.1	1.0	R	15	2.4	3	2.5	1.8	I						
MSUS 912	8	0.4	4	0.5	0.5	R	12	0.9	7	1.1	1.9	R	13	0.8	7	1.5	1.6	R
MSUS 922	12	0.8	8	1.1	1.1	R	15	1.0	13	1.3	1.8	R	14	1.6	12	1.5	1.8	R
MSUS 927	9	0.8	8	0.8	1.1	R	12	1.4	12	2.3	2.4	I	11	0.3	12	3.9	3.7	I
MSUS 933	12	0.7	8	0.6	0.6	R	16	1.7	12	2.4	2.5	I	15	1.3	11	3.7	4.0	I
MSUS 944	10	0.4	8	1.0	0.7	R	15	0.7	12	2.6	2.2	I	13	0.7	12	2.2	2.7	I
MSUS 1010	9	0.6	8	1.1	0.7	R	11	0.7	12	1.7	1.7	R	10	0.8	12	3.1	3.4	I
MSUS 1039	12	0.7	5	1.3	0.2	R	14	0.9	10	2.3	2.9	I	15	0.5	8	2.5	2.4	I
MSUS 1049	9	0.5	8	0.7	0.6	R	12	1.5	12	1.9	2.3	R	11	1.6	12	1.8	1.8	R

	C. fragariae Cf-63						*C. fragariae* Cf-75						*C. gloeosporioides* Cg-162					
	Detached Leaf		Whole Plant				Detached Leaf		Whole Plant				Detached Leaf		Whole Plant			
Germplasm	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S) [a]	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)
MSUS 1055	9	0.7	8	1.3	1.1	R	12	0.7	12	2.2	2.6	I	11	0.8	15	1.6	2.0	R
MSUS 1061	19	0.4	6	1.7	1.4	R	22	1.0	11	1.8	2.0	R	18	0.8	9	1.2	1.8	R
MSUS 1066	9	0.5	8	0.6	0.5	R	15	1.0	12	1.7	0.8	R	16	0.7	11	2.2	2.1	I
MSUS 1078	13	1.0	8	0.5	0.2	R	16	0.9	12	1.6	1.0	R	15	1.5	11	3.0	2.3	I
MSUS 1093	11	0.4	8	1.6	0.9	R	14	0.6	12	1.8	1.2	R	15	0.4	11	3.1	2.7	I
MSUS 1094	10	0.6	5	1.1	0.2	R	15	1.3	9	1.8	2.1	R	12	0.8	8	1.6	1.7	R
MSUS 1105	14	1.2	7	2.0	2.0	R							16	1.6	11	3.8	3.8	I
MSUS 1142	10	0.8	8	1.1	0.6	R	15	1.2	12	2.8	3.3	I	17	1.3	11	2.4	2.5	I
MSUS 1145													14	0.7	4	3.8	3.5	I
MSUS 1154	11	0.7	8	1.5	0.3	R	13	0.6	12	1.9	2.4	R	9	0.1	10	2.0	1.5	R
MSUS 1180	13	0.3	8	1.4	0.9	R	17	0.5	12	2.3	1.4	I	17	0.3	12	2.7	2.4	I
MSUS 1196	14	1.0	8	1.1	1.0	R	17	1.0	12	3.3	3.6	I	14	1.4	11	3.5	3.6	I
MSUS 1197	17	0.4	6	0.6	0.9	R	20	1.8	10	2.3	3.3	I	14	0.7	9	2.4	2.7	I
MSUS 1217	6	0.9	8	1.1	0.7	R	12	2.0	12	2.4	2.7	I	11	0.7	12	2.6	2.6	I
MSUS 1229	13	0.9	6	1.2	0.6	R	18	0.9	12	2.8	2.7	I	15	0.7	10	3.7	3.5	I

	C. fragariae Cf-63						*C. fragariae* Cf-75						*C. gloeosporioides* Cg-162					
	Detached Leaf		Whole Plant				Detached Leaf		Whole Plant				Detached Leaf		Whole Plant			
Germplasm	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S) [a]	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)
MSUS 1230	16	1.2	7	2.7	2.2	I	21	1.5	12	2.3	2.6	I	15	1.3	11	2.3	2.4	I
MSUS 1240	10	0.5	8	0.8	0.7	R	14	2.5	12	1.8	3.1	R	14	1.1	12	2.9	3.1	I
MSUS 1265	9	0.7	4	1.6	0.7	R	16	1.1	9	1.7	1.7	R	10	0.5	4	1.4	0.6	R
MSUS 1269	7	0.3	8	1.1	0.8	R	14	0.8	12	2.2	2.7	I	11	0.9	12	2.8	2.6	I
MSUS 1270	17	0.7	8	1.4	0.7	R	19	1.3	12	2.1	2.4	I	17	0.6	12	2.5	2.8	I
MSUS 1271	10	0.2	8	0.8	0.8	R	13	1.2	12	2.5	3.1	I	12	0.4	12	2.5	2.5	I
MSUS 1311	9	0.8	7	0.5	0.5	R	15	0.8	8	1.8	2.0	R	13	1.1	8	1.2	1.1	R
MSUS 1331							11	0.5	6	2.4	2.7	I	10	0.3	3	1.3	1.1	R
MSUS 1343	6	0.1	7	0.6	0.4	R	11	0.4	12	1.3	2.1	R	10	0.7	12	2.5	2.5	I
MSUS 1352	15	0.3	8	1.0	0.6	R	17	1.0	12	2.6	2.5	I	15	0.2	12	2.6	2.5	I
MSUS 1356	10	0.5	8	0.5	0.4	R	13	1.5	10	2.7	2.6	I	10	0.6	11	1.0	1.0	R
MSUS 1359	10	0.4	6	0.8	0.5	R	12	1.3	10	1.9	1.9	R	12	0.5	11	1.9	1.9	R
MSUS 1362	14	0.5	8	0.5	0.4	R	17	0.5	13	1.9	2.0	R	16	0.5	12	2.0	1.5	R
MSUS 1365	13	1.3	8	1.3	1.3	R	16	1.9	10	2.2	2.0	I	14	0.9	11	2.0	1.9	R
MSUS 1367							12	1.1	3	1.3	2.3	R						

	C. fragariae Cf-63						*C. fragariae* Cf-75						*C. gloeosporioides* Cg-162					
	Detached Leaf		Whole Plant				Detached Leaf		Whole Plant				Detached Leaf		Whole Plant			
Germplasm	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S) [a]	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)
MSUS 1426							15	1.0	12	2.1	2.1	I	14	0.7	13	1.5	0.9	R
MSUS 1432							14	1.0	4	1.0	0.9	R	7	0.5	4	1.8	2.3	R
MSUS 1494							5	0.3	4	2.6	2.5	I						
MSUS 1511	21	0.2	8	1.1	0.4	R	16	1.3	8	2.8	2.9	I	16	1.5	10	2.2	2.3	I
Named Cultivar																		
Aiko	5	0.5	8	3.5	3.0	I	5	2.6	8	4.7	4.6	S	12	2.5	7	5.0	4.9	S
Albion	9	0.9	8	3.7	3.2	I	9	2.4	12	4.7	4.4	S	12	2.1	12	5.0	5.0	S
Allstar	16	1.2	8	3.4	2.9	I	16	1.9	12	3.9	4.0	I	18	1.1	14	4.4	3.9	S
Aromas	9	1.5	8	3.9	3.8	I	10	2.4	12	5.0	4.7	S	12	2.1	12	5.0	4.8	S
Camino Real	9	1.6	8	4.1	3.5	S	10	3.3	8	4.4	4.3	S	12	2.1	10	5.0	4.7	S
Chandler	20	1.1	12	4.2	4.1	S	24	3.0	19	4.2	4.0	S	27	1.7	20	4.2	4.3	S
Diamante	8	1.3	8	3.2	3.2	I	10	2.2	11	4.0	4.2	S	12	1.4	12	5.0	4.7	S
Dover	5	0.3	8	1.4	1.0	R	5	1.5	8	2.2	2.2	I	12	1.8	9	3.9	4.0	I
Earliglow	16	0.9	7	3.9	3.4	I	18	1.6	12	4.5	4.2	S	17	1.1	10	5.2	4.8	S
Elsanta	9	0.5	8	3.9	3.0	I	8	2.0	12	4.7	4.5	S	12	2.3	12	5.2	4.8	S
Festival	9	0.7	8	4.3	3.5	S	10	2.1	12	4.0	4.1	I	12	1.7	12	5.3	4.7	S
Gaviota	9	1.6	8	5.3	4.7	S	10	3.2	10	5.4	5.0	S	12	1.6	11	5.0	4.9	S
Honeoye	15	1.8	6	2.7	2.6	I	18	2.7	12	4.5	4.6	S	17	2.3	12	5.4	4.8	S
Jewel	14	2.0	4	5.0	5.0	S	17	2.0	8	4.9	4.7	S	15	2.0	8	4.9	4.9	S
Kent	13	1.8	7	4.1	4.0	S	15	2.6	11	3.9	4.2	I	15	1.3	12	4.2	4.3	S

	C. fragariae Cf-63						*C. fragariae* Cf-75						*C. gloeosporioides* Cg-162					
	Detached Leaf		Whole Plant				Detached Leaf		Whole Plant				Detached Leaf		Whole Plant			
Germplasm	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S) [a]	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)	N	DL-DSR	N	WP-DSR	L-DSR	Category (R,I,S)
Ovation	13	1.5	7	3.1	2.4	I	17	1.5	12	5.1	4.8	S	14	1.7	12	5.0	4.8	S
Pelican	17	0.4	8	0.8	0.3	R	15	0.4	12	2.9	3.2	I	20	0.5	12	1.9	1.6	R
Portola	5	0.6	8	3.7	3.3	I	6	2.6	6	4.8	4.7	S	8	1.6	7	5.0	5.0	S
Redchief	8	0.7	4	5.0	5.0	S	12	1.7	6	4.6	4.0	S	10	0.8	4	5.0	5.0	S
Salinas	9	0.8	8	3.6	3.0	I	8	2.0	10	4.3	4.5	S	12	2.6	11	5.2	4.6	S
Scott	9	2.2	8	4.3	3.7	S	9	2.3	8	4.3	4.4	S	12	2.0	12	5.0	4.8	S
Seascape	14	1.5	8	3.5	3.8	I	15	1.8	10	5.0	4.8	S	18	1.5	11	4.2	4.1	S
Selva	8	1.5	6	4.4	4.3	S	12	2.7	7	4.5	4.4	S	11	1.3	8	4.9	4.5	S
Senga	8	1.6	8	1.9	2.3	R	8	1.3	8	2.5	2.6	I	11	2.0	11	4.1	4.1	S
Sequoia	10	2.0	8	3.3	3.0	I	8	2.5	8	3.5	3.7	I	12	1.5	12	5.1	4.7	S
Surecrop							15	1.8	4	4.9	4.8	S	11	1.5	8	5.0	5.0	S
Sweet Charlie	8	0.4	8	3.3	2.9	I	10	2.2	10	3.6	3.7	I	12	1.7	12	4.7	4.4	S
Tangi	9	1.1	8	3.7	3.4	I	8	2.3	8	5.4	4.9	S	12	1.4	9	3.8	3.9	I
Tillamook							8	2.0	6	5.1	4.6	S	12	0.9	7	5.4	4.5	S
Tioga	9	1.0	8	1.8	1.8	R	9	2.2	11	3.4	3.3	I	12	2.3	9	4.6	4.4	S
Treasure	9	0.4	8	3.6	2.8	I	10	1.9	12	3.3	4.0	I	13	1.8	13	3.8	4.0	I

[a] Category: R = resistant, I = intermediate, S = susceptible. Based on WP-DSR scale: 0 = healthy plant with no visible lesions, 1 = < 3 mm long petiole lesion, 2 = 3–10 mm long petiole lesion, 3 = > 10–20 mm long petiole lesion, 4 = > 20 mm long petiole lesion, 5 = youngest leaf wilted, and 6 = plant dead. Plants with a WP-DSR $\leq$ 2.0 were considered resistant, those with a WP-DSR > 2.0 and < 4.0 were considered intermediate, and those with a WP-DSR $\geq$ 4.0 were considered susceptible.

Germplasm lines inoculated with three Colletotrichum isolates, number (N) inoculated, average disease severity rating (DSR) for detached leaf (DL-DSR) and whole plant (WP-DSR) and leaf (L-DSR) for each germplasm line, and resistance category based on WP-DSR.

Table 10

Descriptive statistics for anthracnose disease severity ratings on strawberry.

Tissue	Category	N	Mean [a]	Std Dev	Median	Minimum	Maximum
Whole Plant (WP-DSR)	Resistant	87	1.3 c	0.5	1.3	0.4	2.0
	Intermediate	90	2.9 b	0.6	2.8	2.0	4.0
	Susceptible	57	4.8 a	0.4	4.9	4.0	5.4
Whole Plant Leaf (L-DSR)	Resistant	87	1.2 c	0.7	1.0	0.2	3.1
	Intermediate	90	2.9 b	0.6	2.8	1.4	4.2
	Susceptible	57	4.5 a	0.4	4.6	3.5	5.0
Detached Leaf (DL-DSR)	Resistant	87	0.7 c	0.4	0.7	0.1	2.5
	Intermediate	90	1.1 b	0.6	1.1	0.2	2.6
	Susceptible	57	1.9 a	0.6	2.0	0.7	3.3

[a] Means for each category within a tissue type followed by the same letter are not significantly different from each other according to Tukey's test (p <0.05).

Descriptive statistics of the anthracnose disease severity ratings for whole strawberry plants inoculated with three Colletotrichum isolates and assessed for disease using a 0 to 6 disease severity rating (WP-DSR) scale. The strawberry germplasm was then categorized using the WP-DSR $\leq$ 2.0 were considered resistant, those with a WP-DSR > 2.0 and < 4.0 were considered intermediate, and those with a WP-DSR $\geq$ 4.0 were considered susceptible. The categories assigned from the WP-DSRs were used for the descriptive statistics for the corresponding whole plant leaf and detached leaf disease severity ratings. Below are the descriptive statistics for the DSRs for all strawberry tissues that were inoculated

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CHAPTER V – CONCLUSIONS

The development and release of a commercially acceptable disease resistant strawberry cultivar can take many years because of the time required to grow the germplasm, increase the number of germplasm plants, and then test it for good agronomic traits and disease resistance. Decreasing the time component of any part of this process is advantageous for the breeder and grower. Molecular tools are increasingly being used to identify genes that may be associated with disease resistance and can decrease the overall time required for selective breeding for anthracnose resistance in strawberry. Two sequence characterized amplified region (SCAR) markers STS-Rca2_240 or STS-Rca2_417 for the *Colletotrichum acutatum* resistant gene, *Rca2*, were developed in previous research. Establishment of the presence or absence of these SCAR markers in selected strawberry germplasm lines and the anthracnose resistance of the same germplasm lines would help breeders in choosing parent breeding lines more quickly. The selection time for anthracnose disease resistant germplasm can also be decreased substantially by inoculating detached strawberry leaves versus whole plants. These studies were conducted to increase our knowledge on the strawberry-*Colletotrichum* host-pathogen system and discover possible key areas in testing for disease resistant germplasm where the time component can be decreased.

The primary goal of this research was to establish the degree to which a relationship exists between the two SCAR markers for the *Colletotrichum acutatum* resistant gene, *Rca2*, and the resistance or susceptibility to anthracnose caused by three *Colletotrichum* species (*C. acutatum*, *C. fragariae*, and *C. gloeosporioides*) in 81

strawberry germplasm lines. The germplasm lines comprised named commercial cultivars and unreleased clones (MSUS) from USDA-ARS, Mississippi bred for *C. fragariae* resistance. Identification of anthracnose resistant germplasm containing the *Rca2* gene will aid strawberry breeders in choosing parent lines with a better likelihood of passing the resistant gene to their progeny.

In my first study, whole strawberry plants were inoculated with isolates of *Colletotrichum acutatum, C. fragariae*, and *C. gloeosporioides* used in previous studies to separate anthracnose resistant germplasm from susceptible germplasm. The plants were assessed for disease severity based on symptom development on whole plants and on leaflets. Each germplasm line was then categorized as resistant, intermediate, or susceptible based on the mean whole plant disease severity rating. Many of the germplasm lines exhibited increased resistance in the winter months compared to the summer months, which may be explained by a seasonal effect by either the isolates and/or the strawberry plants. The finding of a significant seasonal component in the strawberry-*Colletotrichum* system warrant consideration of its effect in future inoculation studies.

The germplasm lines used in the inoculation study also were screened for the two SCAR markers. The presence of the 240 and 417 SCAR marker alleles together or the 417 marker alone predicted that those lines would be resistant to *C. acutatum*. I found that the presence of both SCAR markers together did not have a significant effect on disease caused by any of the three *Colletotrichum* species. The presence of either of the two SCAR markers had a moderately significant effect (240 allele p =0.09, 417 allele p

=0.06) on disease caused by the two *C. acutatum* isolates, the presence of the 240 marker alone had a significant effect (p =0.01) on disease caused by *C. fragariae*, and no significant effect was found on disease caused by *C. gloeosporioides*. These findings suggest there is a connection between the SCAR markers and that a gene, *RCa2*, indicates resistance to anthracnose caused by more than one *Colletotrichum* species.

A secondary goal of this research was to establish the degree of association and agreement between disease severity ratings of detached strawberry leaves and whole plants inoculated with the same *Colletotrichum* isolates (Chapter II). The detached leaf disease severity ratings were made five DAI, whereas, the whole plant and leaflet ratings were made 10 to 30 DAI. The 'benchmark' whole plant disease severity ratings were used to place the strawberry germplasm into three categories (resistant, intermediate, or susceptible). The mean leaflet disease severity ratings and the mean detached leaf disease severity ratings were calculated for each germplasm line and separated using the whole plant resistance categories. The whole plant and leaflet disease severity ratings had a high degree of association (r_p =0.96), and the whole plant and detached leaf disease severity ratings had a good association (r_p =0.66). The leaves in the detached leaf assay did not have thirty days to develop disease symptoms as those in the whole plant assay. Therefore, the decreased association between the whole plant and detached leaf disease severity ratings was not unexpected. There was a significantly different numerical separation between the mean disease severity ratings for each tissue type in the categories (resistant, intermediate, and susceptible). The significant difference among categories

indicates a detached leaf assay can be used reliably and quickly to separate anthracnose resistant strawberry germplasm from susceptible germplasm.

Science moves forward in small steps by making tentative discoveries that have to be verified by repeated experimentation and through different methodologies. Variability is found within all biological systems, whether between species, among individuals within a species, or among different tissues on the same individual. The chances of obtaining every possible outcome when performing research with biological systems are rather low but, with many observations, the chances of arriving closer to the correct answer are greater. This research has increased our knowledge of strawberry-*Colletotrichum* host-pathogen interactions and will help in future research to find genetic markers that can be used for rapid evaluation of potentially promising germplasm.