EFFECT OF INOCULUM CONCENTRATION, TEMPERATURE AND WETNESS DURATION ON ANTHRACNOSE FRUIT ROT DEVELOPMENT ON DIFFERENT STRAWBERRY CULTIVARS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2013

To my family

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Dr. Natalia Peres for giving me the opportunity to study under her guidance. Dr. Peres has taught me to become a better scientist and researcher and to explore my thoughts and ideas about plant pathology. I also thank the staff of the University of Florida – GCREC Strawberry Plant Pathology lab for all their help and support. Thanks to my committee members, Dr. Megan Dewdney and Dr. Jim Marois for guiding my research with valuable comments. A special thanks to my friend Matt Mattia and boyfriend Tyler Mayo for their words of encouragement and for never denying my requests for assistance during their free time. Most importantly, I would like to thank my family, without their unconditional love and investment in my education I could not have accomplished my master's degree. Also I thank my father for being my role model. His passion for plant pathology inspires me to be outstanding in all I accomplish. Thank you all!

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December 2013

Chair: Natalia A. Peres
Major: Plant Pathology

Florida is the largest producer of winter strawberries in the world. Anthracnose Fruit Rot (AFR), caused by *Colletotrichum acutatum*, can greatly affect production if not controlled. The use of fungicides in addition to cultural practices such as the use of certified pathogen-free plants and less susceptible cultivars are important tools for AFR control. The purpose of this study was to evaluate the effects of temperature, Wetness Duration (WD), and inoculum concentration on the development of AFR on strawberry cultivars with different levels of susceptibility to the disease that are commonly grown in Florida. Disease incidence generally increased with increasing inoculum concentration, temperature, and WD for 'Camarosa', Strawberry Festival', and 'Treasure'. In field trials, 'Camarosa' and 'Treasure' were more susceptible than 'Strawberry Festival' and flowers were more susceptible than immature fruit for most cultivars. Detached fruit and field trials were generally correlated. In in vitro studies, mycelial growth occurred from 10 to 30^0C and *C. acutatum* infection on detached immature fruit was observed from 15 to 30^0C for 'Strawberry Festival' and 'Camarosa'. In growth chamber studies, 'Strawberry Festival' and 'Camarosa' had similar disease development curves at 15, 20, and 25^0C

and from 0 to 48 h of WD. Disease incidence was higher for ‘Camarosa’ than for ‘Strawberry Festival’. The results of this project will be used to adapt models in a disease forecasting system to predict AFR outbreaks in Florida strawberry production fields.

CHAPTER 1
LITERATURE REVIEW

Strawberry Production in Florida and the World

Strawberry (*Fragaria* x *ananassa* Duch.) is a perennial plant grown in many subtropical and temperate countries such as the USA, Turkey, Spain, Egypt, Mexico, Japan, Poland, Korea, and Israel (FAOSTAT, 2012). The USA, where cultivation started in the mid 1800's in California, is responsible for most of the strawberry production worldwide. In the past, berries from California were transported to the rest of the country in refrigerated trucks (O'Dell, 2003; Darnell, 2003). Nowadays, American berries are produced in many other states and shipped all over the world thanks to advances in strawberry research (O'Dell, 2003). Florida is the second largest strawberry producer in the USA, following California, and the largest winter producer in the world. Production is centered around Plant City which is considered "The Winter Strawberry Capital of the World" (Latitude: 28^0 N) (USDA, 2013; Brown, 2003). In the 2012-2013 season in Florida, 8,700 acres of strawberries were harvested with a yield of 21,000 pounds per acre (USDA, 2013).

Strawberry is an aggregate fruit that can be vegetatively or sexually reproduced. The real fruit are the brown-black achenes located on the outside of the receptacle (the edible part) (Darnell, 2003; Strand, 2008). Even though the strawberry is considered a perennial plant, it is cultivated as an annual crop in Florida and many other locations (Brown, 2003; Strand, 2008). In Florida, the strawberry season is during the winter, different from other US states that have perennial production and harvest during the summer (Brown, 2003). Strawberry production in Florida starts with site preparation and cultivar selection. The sandy soils with optimal drainage and cultivars that produce high

quality fruit are ideal to achieve high yields (Strand, 2008; Chandler and Legard, 2003). The use of less susceptible cultivars and/or disease-free plants is extremely important and thus transplants are grown in nurseries located in northern latitudes (Nova Scotia, Ontario, and Quebec, Canada) or at higher elevations (California and North Carolina) where climatic conditions are not favorable for pest and pathogen survival (Mertely et al., 2005; Brown, 2003).

Field preparation starts in September, with bedding, plastic laying, establishment of drip tape, and overhead irrigation. Beds are fumigated and the row middles are sprayed with herbicide to reduce nematode and fungal communities and weeds, respectively (Brown, 2003; Strand, 2008). In October, when temperatures are usually around 30^0C, freshly dug transplants are manually planted and overhead irrigated for establishment (Brown, 2003). Since Florida's strawberry production is during the winter, most of the cultivars planted are short-day cultivars which are highly stimulated to flower by photoperiod of ≤14 hours of light (Darnell, 2003; Strand, 2008). Even though temperature is a secondary factor in strawberry flowering, it can determine if stolons or inflorescences will be produced. Warm temperatures of ≥ 15^0C favor the development of stolons and cooler temperatures below 15^0C favor the development of inflorescences (Strand, 2008). When temperatures reach the freezing point, the use of overhead sprinklers is common for frost protection because flowers and immature fruit are extremely sensitive to frost injury. Therefore, when air temperature reaches 0^0C, overhead irrigation is turned on to protect flowers and fruit from frost damage (Fiola, 2003).

From mid-November to early March, strawberry fruit is manually harvested on average 2 to 3 times a week when berries have a red color throughout the fruit. However, different cultivars have different maturity patterns (Brown, 2003; Strand, 2008). After harvest, berries are placed in plastic containers (clamshells) and sent to a central cooling facility with low temperatures (1^0C) to slow fruit deterioration and fungal growth. Finally, strawberries are transported in refrigerated trucks to their market destination (Brown, 2003).

The majority of the strawberries planted in Florida and other US states is grown using conventional production practices. The organic strawberry production industry is growing in states with dry climates like California. From 1997 to 2009, the number of acres of organic strawberries grew from 134 to 2,185. This growth is due to consumers' demand and innovations in fertilization and integrated pest management that are currently permitted by organic production policies (Koike et al., 2012). However, in states with warm humid conditions like Florida, organic production has not been adopted, since weather is extremely favorable for pests and diseases.

Anthracnose Fruit Rot of Strawberry

Anthracnose Fruit Rot (AFR), most often caused by *Colletotrichum acutatum* Simmonds, is a major strawberry disease that can greatly impact yield in production fields in Florida and worldwide if not controlled (Mertely et al., 2005; Legard, 2002). The disease causes yield loss in many strawberry-producing regions like North and South America and Europe (Mertely et al., 2005; Freeman et al., 1997; Kososki et al., 2001; Domingues et al., 2001). In the USA, AFR has been observed in states like Florida, California, New York, Ohio, Pennsylvania, as well as others (Mertely et al., 2005; Strand, 2008; Turechek et al., 2006; Wilson et al., 1990). In production areas where

climatic conditions are extremely favorable for AFR development, infected fruit become unmarketable (Legard et al., 2003). For example, during the 2001-2002 season, Legard and MacKenzie, 2003) reported an AFR disease incidence higher than 70%.

Although AFR is most commonly caused by *C. acutatum*, other *Colletotrichum* species such as *C. gloeosporioides* and *C. fragariae* are capable of causing AFR symptoms on strawberry plants, but with lower economic losses (Maas, 1998). Besides fruit, *C. acutatum* can infect leaves, petioles, flowers, crowns and roots (Mertely et al., 2005). During favorable conditions, lesions may expand and completely cover the surface of the fruit, especially on highly susceptible cultivars (Maas, 1998; Seijo et al., 2008).

AFR was first observed in Florida in 1984, however the disease is not indigenous to Florida (Howard et al., 1992). The primary source of AFR inoculum in annual strawberry commercial fields is the transplants originating from production nurseries, since the inoculum does not survive the high soil temperatures in Florida (Howard et al., 1992). The use of overhead irrigation is common in nurseries and favors spore dispersal and AFR development (Strand, 2008). In annual production systems like those in Florida, new transplants are established every season. Therefore, if infected, these plants are responsible for the introduction of the pathogen into strawberry fields (Legard, 2002). However, in strawberry producing states in the northern USA, where strawberry is cultivated in the summer as a perennial crop, AFR development is caused by *C. acutatum* inoculum that survives in the soil or attached to plant parts (Strand, 2008; Hokanson and Finn, 2000).

Etiology

The anamorphic stage of *Colletotrichum acutatum* is the major causal agent of AFR of strawberry, since the telemorph, *Glomerella acutata*, which belongs to the phylum Ascomycota, has only been observed under laboratory conditions (Damm et al., 2012; Guerber and Correll, 2001; Agrios, 2005). *C. acutatum* is considered a cosmopolitan plant pathogen and can cause yield loss of many tropical crops such as mango, banana, avocado, papaya, coffee, and citrus as well as temperate crops such as apple and grape. However, symptoms and crop loss can vary according to the host (Agrios, 2005).

Simmonds (1965) differentiated *C. acutatum* from other *Colletotrichum* species based on ecological and morphological characteristics. Its small conidia with pointed ends differed from those of *C. gloeosporioides*. However, in recent studies, Damm et al. (2012) using molecular tools and ecological and morphological features, described *C. acutatum* as a species complex that is evolving rapidly. Many *Colletotrichum* species that were considered morphologically similar to *C. acutatum* are now known to be closely related and belong to the *C. acutatum* species complex.

Shape and size of conidia and mycelial growth rate of *C. acutatum* is different and usually distinguishable from other *Colletotrichum* species (Maas, 1998; Grahovac et al., 2012; Damm et al., 2012). However, since different *Colletotrichum* species can cause similar symptoms on strawberry, visual differentiation of symptoms is not always reliable. Therefore, the use of molecular tools, such as Polymerase Chain Reaction (PCR), should be incorporated when trying to distinguish *Colletotrichum* species (Grahovac et al., 2012; Ureña-Padilla et al., 2002).

Colletotrichum acutatum conidia are hyaline, smooth-walled, and cylindrical to fusiform with both ends acute (12.6 ± 1.8 x 3.9 ± 0.3 µm) and are produced in salmon-pink or orange masses (Maas, 1998; Damm et al., 2012). The pathogen produces acervuli which is the asexual fruiting body responsible for conidia production (Maas, 1998), and production of setae on acervuli has not been observed (Damm et al., 2012). Colonies are usually hyaline-white and then turn pink or orange when sporulating. When older, colonies turn pale gray (Maas, 1998; Grahovac et al., 2012; Damm et al., 2012). The rate of *C. acutatum* colony growth on potato-dextrose agar at 27^0C averages 8 to 9 mm/day and can be used to morphologically differentiate *Colletotrichum* species affecting strawberries, since it is slower than *C. fragariae* and *C. gloeosporioides* (Maas, 1998). Temperatures around 20^0C and relative humidity near 100% are ideal for *C. acutatum* spore production, germination, and infection of strawberry (Maas, 1998).

Tanaka and Passos (2002) observed that *C. acutatum* was more aggressive on strawberry fruit and flowers, whereas *C. fragariae* was more aggressive to crown and petioles. However, *C. fragariae* was capable of infecting flowers and fruit, but at a lower level. Similarly, *C. acutatum* can infect and cause lesions on strawberry petioles. Ureña-Padilla et al. (2002) confirmed that *C. acutatum* was more capable of infecting strawberry fruit than crowns. Although *C. acutatum* can cause plant decline and wilt, it differs from the rapid decline caused by *C. gloeosporioides*.

Colletotrichum acutatum affects many other hosts and anthracnose symptoms range from localized lesions (strawberry) to the loss of an entire crop (bitter rot of apple) (Agrios, 2005). In all of the diseases caused by *C. acutatum*, the common conditions that favor disase development are warm and humid weather (Agrios, 2005). In Israel,

Freeman et al. (2001) observed cross-infection between *C. acutatum* isolates from weeds such as *Vicia* spp. or *Conyza* spp. and strawberry. Isolates recovered from these weeds were inoculated on strawberry plants and were highly pathogenic. Contrarily, disease symptoms were not observed when crops such as eggplant, tomato and pepper were inoculated with strawberry isolates because apparently, *C. acutatum* has an epiphytic nonpathogenic, symbiotic lifestyle on Solenaceous crops (Freeman et al., 2001). This is in agreement with cross-inoculation studies of MacKenzie et al. (2009) with *C. acutatum* isolates from strawberry, blueberry, fern and key lime. Isolates only caused disease symptoms on the host and tissue from which they were collected and did not cause an epidemic on other crops (MacKenzie et al., 2009). However, according to Damm et al. (2012) there was no cross-infection in the MacKenzie et al. (2009) study because the isolates collected from each host were from different species in the *Colletotrichum acutatum* species complex and were not *C. acutatum*. Damm et al. (2012) suggests that the *Colletotrichum* species that infects strawberry is *C. nymphaeae*, the blueberry pathogen, *C. fioriniae*, and the key lime pathogen, *C. limetticola*. The fern isolates were not included in the study (Damm et al., 2012).

Symptoms

Colletotrichum acutatum is considered to be specific to the tissue it infects on each host. However, for strawberry, isolates are less specialized and can attack petioles, leaves, flowers, crown, and roots, with or without showing symptoms (Mertely et al., 2005; Peres et al., 2005). On strawberry, the most characteristic symptoms are flower blight and fruit rot (Maas, 1998; Peres et al., 2005) (Figure 1-1), however fruit rot is the most detrimental symptom to crop yield since infected fruit are non-marketable (Peres et al., 2005). Strawberry transplants can come from production nurseries with

quiescent infections, and once established under warm and wet conditions, lesions on petioles may be observed.

Flowers are highly susceptible to *C. acutatum* and the pathogen can infect any part of the flower tissue such as buds, pedicels, and peduncles and result in fruit abortion or flower death (Maas, 1998). Infected petals turn brown and sepals develop burnt tips, later drying but remaining attached to the plant. These symptoms are similar to blighted inflorescences infected by *Botrytis cinerea* (Maas 1998; Mertely et al., 2005; Legard et al., 2003). On immature fruit, AFR symptoms start as hard black or dark brown lesions that expand to slightly sunken lesions (Maas, 1998) initially averaging 1.5 mm across (Mertely et al., 2005; Legard et al., 2003). Infected black achenes, also called black seeds, can be observed on infected immature fruit and can lead to misshapen fruit (Legard et al., 2003). Lesions then expand quickly on ripening fruit and go from light brown, water-soaked to black-brown firm spots measuring from 3 to 12 mm (Maas, 1998; Mertely et al., 2005; Legard et al., 2003). In the center of the lesions, visible masses of orange-pink conidia are produced in acervuli under humid conditions and serve as an inoculum source for further infections (Maas, 1998; Legard et al., 2003). Other organisms can also easily infect the fruit tissue colonized by *C. acutatum* and cause other symptoms. However, if only infected by *C. acutatum*, lesions can merge and cover the entire fruit (Maas, 1998; Mertely et al., 2005; Peres et al., 2005).

During a severe epidemic in strawberry nurseries or commercial fruit fields, wilt and plant death from strawberry crown and root rots can be caused by *C. acutatum*. Infected roots usually develop brown lesions and have a few feeder roots. Such infected plants can slowly decline and die (Legard et al., 2003; Mertely and Peres, 2005).

Symptoms of basal crown rot can be observed under field and greenhouse conditions when plants do not establish well and consequently are stunted and die (Mertely and Peres, 2005). Peres et al. (2005) suggested that root and crown infections can be a consequence of conidia or appressoria splashed from the foliage, flowers and fruit down to the lower parts of the plant. During warm humid conditions and when *C. acutatum* infection levels are high, petiole lesions covered with conidia can also be observed (Legard et al., 2003).

Epidemiology and Life Cycle

In annual production fields such as those in Florida, *C. acutatum* inoculum originates from symptomless infected transplants from nurseries where the use of overhead irrigation can lead to conidial dispersal (Strand, 2008). When transplants with infected petioles and runners are established in the field under warm humid weather, secondary conidia are produced (Maas, 1998; Mertely et al., 2005; Leandro et al., 2003b), and disseminated to flowers and fruit by splashing water, contaminated soil on farming equipment, and harvesting operations, and consequently starting the symptomatic infection process (Mertely et al., 2005; Leandro et al., 2003b; Strand, 2008) (Figure 1-2). *C. acutatum* was thought to be a hemibiotrophic pathogen, with a biotrophic phase of feeding on living cells and a necrotrophic phase of killing cells to complete its life cycle. However, on strawberry, the biotrophic phase is very short (less than 12 hours) and for that reason, the pathogen can be considered a necrotroph (Curry et al., 2002).

AFR development is affected greatly by temperature and leaf wetness duration (Wilson et al., 1990). Wilson et al. (1990) used a regression model to describe the effects of leaf wetness and temperature on the development of AFR on immature and

mature fruit of the strawberry cultivar Midway. At same temperature and wetness periods, mature fruit were found to be more susceptible than immature. In addition, disease incidence on fruit increased with an increase of temperature (from 6 to 25^{0}C), when the wetness period remained the same. When exposed to wetness durations of 0.5 to 51 hours and temperatures of 4 or 35^{0}C, immature fruit did not show symptoms, however at 25^{0}C and after 25 hours of leaf wetness disease incidence reached 100%. For mature fruit, disease incidence reached 97% after only 13 hours at 25^{0}C, and infected fruit were observed at 35^{0}C when wetness durations were shorter than 2 hours. Minimum length of wetness period required to start disease development was different for mature and immature fruit. For immature fruit, infected strawberries were observed after exposure to 5 hours of wetness, whereas for mature, only 1 hour was required. In addition, when fruit were exposed to wetness duration of 50 hours and low temperature (6^{0}C), 13% of immature fruit and 25% of mature fruit showed disease symptoms (Wilson et al., 1990). The temperature and wetness duration relationship directly affected AFR development on strawberry fruit and with higher temperatures, shorter wetness periods were required for immature and mature fruit infection.

Leandro et al. (2003a) observed that germination, secondary conidiation and appressorial development of *C. acutatum* on symptomless strawberry leaves of the cultivar Tristar are critically affected by wetness duration and temperature. Temperatures from 17.6 to 27.7^{0}C with continuous wetness periods were optimal for germination and appressorial development. Temperatures from 21.3 to 32.7^{0}C and wetness periods of more than 4 hours were required for secondary conidiation. Leandro et al. (2003b) also observed that *C. acutatum* secondary conidiation was stimulated

more by strawberry flower extracts than moisture alone. This was shown when secondary conidia were produced on coverslips where *C. acutatum* was exposed to dryness and then treated with flower extracts. Zulfiqar et al. (1996) also observed that production of conidia of *C. acutatum* on citrus leaves was stimulated by flower extracts. Conidial production was seen in treatments when water was applied, however less numerous than when exposed to flower extracts. Thus, presence of flowers extracts can increase inoculum for further fruit and flower infections.

Diéguez-Uribeondo et al. (2011) also tested the effect of temperature and wetness duration on almond cultivars with different anthracnose susceptibility levels. The results were in agreement with those of Wilson et al. (1990): periods of moisture are necessary for disease development and the duration is highly dependent on temperature. Between 15 and 20^0C, anthracnose symptoms can develop with wetness periods as short as 3 hours (Diéguez-Uribeondo et al., 2011).

In addition to *C. acutatum* conidiation and AFR development, temperature influences sporulation and the length of the incubation period of *Colletotrichum* species on strawberry plants (King et al., 1997). King et al. (1997) observed maximum sporulation at 25^0C for 4 days after conidial production. The temperature-incubation time relationship is similar to that of the temperature and wetness period, since with the increase of temperature, shorter incubation periods are necessary for sporulation. Sporulation does not require free moisture and it is suggested that *Colletotrichum* conidia obtain moisture from the fruit to produce spores (King et al., 1997). Nonetheless, when compared to *C. gloeosporioides* and *C. fragariae*, *C. acutatum* had the shortest latent period at low temperatures (5 and 10^0C). Thus, *C. acutatum* can be

favored in comparison to other *Colletotrichum* species in places like Florida, where the strawberry season is during the winter when temperatures are cool (King et al., 1997).

Initial disease symptom development on strawberry plants depends on concentration of inoculum present as quiescent infections. Knowledge of the required inoculum concentration for initial disease development is useful especially in the case of AFR of strawberry, since in commercial fields the disease starts with infected transplants coming from the nurseries (Howard et al., 1992). The weather conditions during plant establishment (temperatures from 20 to 25ºC and 8 hours of overhead irrigation for 7 days) are extremely favorable for AFR development. Therefore, if transplants are already infected, it is difficult to control the disease and the inoculum dispersal to healthy plants. The minimum inoculum concentration required for symptom development on cultivars with different levels of susceptibility is important, since growers plant cultivars that may be highly and less susceptible to anthracnose in close proximity and this information can be useful when deciding the control methods to use and the timing of fungicide applications (Diéguez-Uribeondo et al., 2011). More susceptible cultivars may require a lower inoculum concentration for disease development than less susceptible cultivars. Anthracnose development on almond cultivars was critically affected by inoculum concentration and a concentration of at least 10^4 conidia/ml was required for symptom development for cultivars NePlus Ultra (susceptible to anthracnose) and Nonpareil (less susceptible to anthracnose) (Diéguez-Uribeondo et al., 2011).

Trapero-Casas and Kaiser (1992) studied the effect of different inoculum concentrations (4×10^4, 2×10^5, 1×10^6 and 1×10^7 conidia/ml) on the infection and

development of Ascochyta blight of chickpea cultivars with different susceptibility levels (highly susceptible, moderately susceptible, and resistant) to standardize inoculum concentration for artificial inoculations with *Ascochyta rabiei*. Susceptibility levels of chickpea cultivars was fundamental when assessing the minimum inoculum concentration necessary for symptom development because disease severity of the susceptible cultivars was higher at all inoculum concentrations when compared to the resistant cultivar. When the concentration increased from 4×10^4 to 1×10^7 conidia/ml, disease severity of the cultivar Pedrosillano (moderately susceptible) increased from 32.9 to 83.5%. However, at the highest inoculum concentration of 1×10^7 conidia/ml, disease severity was only 12.7% for the resistant cultivar, whereas for the highly susceptible cultivars at all inoculum concentrations, disease severity did not increase significantly with increasing inoculum concentration and was greater than 50% with all treatments. In summary, inoculum concentration can highly influence disease development on cultivars with different levels of susceptibility and it is important to assess cultivar susceptibility with other epidemiological factors (Trapero-Casas and Kaiser, 1992). The effect of inoculum concentration on AFR development on strawberry cultivars with different levels of susceptibility is currently unknown.

Disease forecast systems have been developed to assist growers by providing information about the risk for disease development and offer an alternative method to control outbreaks with a significant reduction in fungicide applications. Forecast systems for anthracnose diseases are usually built on models based on the effects of temperature and leaf wetness duration on disease progress (MacKenzie and Peres, 2012a; Diéguez-Uribeondo et al., 2011). Several models have been developed to

predict diseases caused by *C. acutatum* and other pathogens such as *Botrytis cinerea*, *Phytophthora cactorum*, and *Mycosphaerella fragariae* on strawberry (Wilson et al., 1990; Carisse et al., 2000; Grove et al., 1985; Sosa-Alvarez et al., 1995; Bulger et al., 1987). Wilson et al. (1990) modeled AFR disease incidence of strawberry as a function of temperature and leaf wetness duration. MacKenzie and Peres (2012a), used the equation published by Wilson et al. (1990) to test its effectiveness for timing fungicide applications compared to the calendar application schedule (weekly) on cultivars with different levels of susceptibility ('Camarosa' and 'Strawberry Festival', highly and moderately susceptible, respectively). The use of a disease threshold INF ≥0.15 (proportion of infected fruit) was ideal for application of captan (Captan 80WDG; Micro Flo Company LLC, Memphis, TN) and INF ≥0.5 for pyraclostrobin (Cabrio 20EG; BASF Corporation, Research Triangle Park, NC). These treatments provided efficient AFR control on both cultivars, reducing fungicide applications without reducing yields compared to weekly applications. Bulger et al. (1987) developed a model to predict flower and fruit infection by *B. cinerea* also based on temperature and leaf wetness duration. Similar model evaluations were conducted by MacKenzie and Peres (2012b) for BFR. The information acquired from the model evaluations to predict *C. acutatum* and *B. cinerea* infection on strawberry fruit and flowers was used to build the "Strawberry Advisory System" (SAS) (http://www.agroclimate.org/tools/strawberry), a disease forecasting system that provides recommendations on timing fungicide applications for AFR and BFR control for Florida growers (Pavan et al., 2011).

Sosa-Alvarez et al. (1995) used a logarithmic polynomial model to describe the effects of length of wetness periods and temperature on sporulation of *B. cinerea* on

dead strawberry leaves. To estimate the potential risk of infection of *Mycosphaerella fragariae* on strawberry leaves, Carisse et al. (2000), developed a model based on the response of *M. fragariae* to temperature and leaf wetness durations. Grove et al. (1985) used the same strawberry cultivar (Midway) as Wilson et al. (1990) for forecasting *P. cactorum* infections on immature fruit under natural conditions. In agreement with the other studies described above, *P. cactorum* infection is also highly influenced by temperature and wetness (Grove et al., 1985). However, there are no models developed to describe these effects on AFR development on strawberry cultivars with different levels of susceptibility that are commonly grown in Florida, such as Strawberry Festival, Camarosa and Treasure. This information would help to adapt the forecasting system currently available for the Florida strawberry production.

The use of polyethylene plastic mulch has been a common practice for many decades in strawberry production fields in Florida and California (Madden et al., 1996). Weed suppression, maintenance of warm soils, and prolonged harvest seasons, are some of the many advantages of the use of plastic mulch as a ground cover (Ag Answers, 2012). For those reasons, the long used system of matted row with straw mulch in strawberry-producing states in the Northern USA is being replaced by the plasticulture system (Madden et al., 1993; Hokanson and Finn, 2000). When compared to soil and straw cover, plastic mulch has a higher impact on AFR development, since its smooth material favors conidial splash dispersal. Studies have found highest number of re-splashing spores hitting the surface of the mulch and greatest number of *C. acutatum* colonies present on the plastic mulch (Yang et al., 1990a; Yang et al., 1990b; Madden et al., 1993). Contrarily, when using soil and straw cover, more spores are

trapped in the straw and consequently dissemination of inoculum can be suppressed. Additionally, straw cover has shown to be the most effective ground cover for control of conidial splash-dispersal because it has the highest rate of loss of spores through surface infiltration (Yang et al., 1990a; Yang et al., 1990b; Madden et al., 1993). Coelho et al. (2008) also observed a significant reduction in flower blight incidence caused by *C. acutatum* when using grass or straw mulch compared to plastic mulch (Coelho et al., 2008). However, the number of growers replacing straw cover with plastic mulch has increased in the past years, because straw and grass mulch have become not feasible for growers and the fact that plastic mulch can maintain the soil warmer and extend the harvest period is a great advantage for growers (Hokanson and Finn, 2000; Ag Answers, 2012).

Rain intensity and duration interact with ground cover to affect *C. acutatum* conidial dispersal (Yang et al., 1990a; Yang et al., 1990b; Madden et al., 1993; Madden et al., 1996). *Colletotrichum acutatum* conidia can spread from 30 to 60 cm from an inoculum source and cause 100% infection on strawberry fruit when exposed to rain intensities of 15 and 30 mm/h with the use of plastic mulch ground cover (Yang et al., 1990b). When the effects of soil and straw covers were tested, with 60 min of rain with an intensity of 30 mm/h disease incidence was decreased significantly with an increase in distance from 30 to 60 cm (Yang et al., 1990b). Furthermore, Yang et al. (1990b), Madden et al. (1996), and Yang et al. (1990a) observed that at high rain intensities, there was an increase in spore wash-off on the fruit, in the volume of droplets splashed, and the number of conidia transported (Yang et al., 1990a; Madden et al., 1996). Rain intensity and duration are environmental factors that growers cannot manipulate,

however when using plastic mulch as a ground cover, growers can rely on cultural practices such as higher plant density to reduce disease spread.

Inoculum dispersal can be reduced with increased plant density (Madden and Boudreau, 1997). Yang et al. (1990a), observed that plant rows with high leaf area index (LAI) (4.9 over the canopy crown), had fewer fungal colonies than in rows with 2.72 LAI. With a higher plant density, water-splashed conidia do not penetrate the plant canopy as much as when plants are further apart. In addition, Madden and Boudreau (1997) found a decrease in re-splashing spores across the plants with an increase in plant density.

Even though temperatures between 20 and 25°C with high relative humidity are optimum for *C. acutatum* spore production, germination, and strawberry infection (Maas, 1998), the pathogen is able to survive under dry conditions and low temperatures (Leandro et al., 2003a). In a greenhouse study, Leandro et al. (2003a) observed that *C. acutatum* survived up to 8 weeks on strawberry leaves as appressoria, and that 48 hours after inoculation, fungal hyphae and primary conidia died, while melanized appressoria, secondary conidia and melanized hyphal segments survived on symptomless leaf surfaces (Leandro et al., 2001). Secondary conidiation on symptomless leaves can increase inoculum levels until susceptible tissue is available.

Howard et al. (1992) suggests that the high temperatures and moist soil conditions in Florida are not favorable for *C. acutatum* survival from year to year. A study by Freeman et al. (2002) evaluated *C. acutatum* survival in natural, autoclaved, and methyl-bromide fumigated soils. They observed a rapid decline in *C. acutatum* conidial viability within 2 to 6 days after placement on natural soil; whereas in sterilized

soils *C. acutatum* and *C. gloeosporioides* conidia survived at up to 1 year. This may be due to low microbial competition in sterilized soils compared to natural soils. Furthermore, in soils fumigated with methyl bromide, conidia survived up to 2 to 4 months at 11% soil moisture. At field capacity (22% soil moisture content), the fungal population declined rapidly within 6 to 7 days after placement in fumigated soils (Freeman et al., 2002). In American strawberry fields, the use of methyl-bromide has been banned and other fumigants such as the mixture of dichloropropene and chloropicrin are used to reduce nematodes and the fungal community in the soil.

Climatic factors and the type of production system used in commercial strawberry fields can influence the survival and dispersal of *C. acutatum* on strawberry plants and in the soil. Thus, understanding the life cycle of *C. acutatum* and the conditions adequate for disease development are useful when managing AFR.

Disease Management

Anthracnose Fruit Rot (AFR) can be managed in nurseries and commercial fields with a combination of cultural, chemical, and biological control.

Cultural and biological control

AFR control should start prior to strawberry planting (Maas, 1998). The best AFR control strategy is to avoid introduction of the pathogen into commercial fields, by planting anthracnose-free transplants. Unfortunately, that is difficult to achieve, since infected plants may not show symptoms (quiescent infections) at planting (Mertely et al., 2005). In the few anthracnose-free areas where strawberries are grown, quarantine regulations can be an effective preventive measure (Maas, 1998).

In Florida, where weather conditions are favorable for AFR, growers rely in part on planting less susceptible cultivars to suppress disease outbreaks and reduce

inoculum dissemination (Chandler et al., 2006). Chandler et al. (2006) observed in three strawberry seasons that ‘Sweet Charlie’, ‘Carmine’ and ‘Earlibrite’ were the least susceptible cultivars, followed by ‘Strawberry Festival’ (intermediate in susceptibility), whereas ‘Treasure’ and ‘Camarosa’ were the most susceptible. Unfortunately, one of the most planted cultivars in Florida, ‘Florida Radiance’, is less susceptible to AFR but highly susceptible to Botrytis Fruit Rot (BFR), another major strawberry disease. However, the opposite was observed for ‘Camarosa’, which is less susceptible to BFR, but highly susceptible to AFR, and ‘Treasure’ which is susceptible to both AFR and BFR (Mertely and Peres, 2006). Choosing strawberry cultivars that are less susceptible to major strawberry diseases is an important cultural control strategy, since it can reduce the need for fungicide applications (Chandler et al., 2006). However, the difficulties of planting only cultivars with low susceptibility levels are that many of these cultivars have low fruit quality and yield. Also, because cultivars have different fruiting periods, growers plant a variety of cultivars to have fruit available all season long.

Drip irrigation is also an important cultural control method since conidia are water splashed dispersed (Maas, 1998). Coelho et al. (2008) observed that flower blight appeared earlier on strawberry plants when overhead irrigation was used than on drip irrigated plants. Another advantage of drip irrigation is that fertilization can also be done simultaneously. However, irrigation and fertilization should be well controlled since nutrients may favor disease development. Maas (1998) reported that high levels of nitrogen favor AFR development, whereas calcium applications in the form of calcium sulfate ($CaSO_4$) and calcium chloride ($CaCl_2$) may delay infection of greenhouse-grown strawberries (Smith and Gupton, 1993).

Heat treatments by submerging bare-root transplants in 49^0C water for 5 minutes have been tested to eliminate quiescent infections. However, the long-term effects of heat treatments on plant vigor and development may be detrimental to the plant and deserves more study (Freeman et al., 1997).

In addition to the cultural control methods cited above, growers should scout their fields after prolonged leaf wetness periods looking for AFR symptoms, such as flower blight or immature fruit with blackened achenes (Mertely et al., 2005) to monitor disease outbreaks and remove symptomatic fruit and flowers from the field to prevent inoculum build-up and infection (Maas, 1998).

The concern about fungicide-resistance, environmental safety and sustainable production has triggered researchers to study biological AFR control methods. Freeman et al. (2004) found that the fungus *Trichoderma* at high concentrations (0.8%) was effective as a biocontrol agent for control of AFR and BFR in strawberries in Israel. Thorpe et al. (2003) tested the suppressive ability of several strawberry fungi and bacteria to *C. acutatum*. Five fungal isolates of the genera *Cephalosporium*, *Myrioconium* and *Paecilomyces* and two bacterial isolates from the Enterobacteriaceae inhibited in vitro fungal growth and reduced the number of appressoria and conidia on strawberry leaves. Unfortunately, there are not many more positive results in AFR biocontrol on strawberries. Thus, more studies about the efficacy of biocontrol agents on AFR on strawberries are needed and if successful, will provide growers alternatives to chemical control.

Chemical control

The use of fungicides to control AFR and other strawberry diseases is a popular practice among growers and is usually based on a calendar schedule of weekly

applications (Mertely et al., 2005; Turecheck et al., 2006). This practice can cause some negative environmental impact, increase the possibility of fungicide resistance, and the cost of production.

Early in the season, from November to December, inoculum levels are low, temperatures in Florida are cool and usually not favorable for *C. acutatum*, and consequently, infected plants do not show symptoms. At this time, low label rates of broad-spectrum protectant fungicides like captan are sprayed to prevent disease outbreaks (Mertely et al., 2005). From January to March, inoculum levels increase and climatic conditions approach the optimum temperature and humidity for AFR development, and the weekly use of higher label rates of broad-spectrum fungicides is essential to control conidial production and dissemination from diseased to healthy plants (Mertely et al., 2005).

In the USA, many fungicides are labeled and effective in controlling AFR of strawberry such as captan (Captan™; Arysta LifeScience North Amercia, Cary, NC), cyprodinil + fluodioxonil (Switch®; Syngenta Crop Protection, Greensboro, NC), pyraclostrobin (Cabrio, BASF Corporation, Research Triangle Park, NC), and azoxystrobin (Abound®, Syngenta Crop Protection) (Mertely et al., 2005). However, some of these fungicides are only registered for strawberry but not labeled for AFR such as the mixture of fluodioxonil and cyprodinil, or thiophanate-methyl (Topsin® M 70WP, UPI, King of Prussia, PA). The active ingredients pyraclostrobin and azoxystrobin are registered for strawberry and labeled for AFR. They have curative and protective properties, thereby provide a high level of control when sprayed pre- and post-infection (Turechek et al., 2006). Turechek et al. (2006) observed that when pyraclostrobin was

sprayed within 3 hours and up to 8 hours after wetting events of 24 hours or less, it controlled AFR on strawberry plants under experimental conditions. However, pyraclostrobin was more effective when sprayed as a protectant (Turechek et al., 2006). When using moderately susceptible strawberry cultivars, like Strawberry Festival, growers can take more risks and pyraclostrobin applications can be made up to 24 hours after a wetting event. The advantage of spraying fungicides when weather conditions are conducive for AFR development instead of following a calendar-based spraying schedule is that the number of applications can be reduced and thus preventing the risk of fungicide-resistance development (Turechek et al., 2006).

When the disease is detected in the field, single-site fungicides are recommended along with the usual multi-site products. Tank mixing a single-site with a multi-site can help to prevent fungicide-resistance (Mertely et al., 2005). In plants where *C. acutatum* infection is already established, the use of specific single-site fungicides such as azoxystrobin, boscalid + pyraclostrobin, and cyprodinil + fludioxonil is a good control method to reduce disease incidence (Daugovish et al., 2009, FRAC, 2013). Furthermore, an advantage of the use of some single-site fungicides is that they also control BFR, another major strawberry disease in Florida (Mertely et al., 2005).

Other active ingredients have been shown to control AFR of strawberry worldwide. As in the USA, Brazilian strawberry production suffers from AFR. Kososki et al. (2001) evaluated different active ingredients and observed that, under controlled conditions, prochloraz at 100 µg/ml and tebuconazole at 50 µg/ml reduced *C. acutatum* mycelial growth. Moreover, prochloraz and tebuconazole were also the most effective in controlling conidial germination when compared to iprodione, thiophanate methyl,

propiconazole, mancozeb, folpet and copper sulfate. In field trials, prochloraz showed fewer blighted flowers than benomyl, a standard treatment in controlling AFR in Brazil, before it was eliminated from the market in the early 2000s. Domingues et al. (2001) and Freeman et al. (1997) also confirmed the efficacy of prochloraz in controlling *C. acutatum* in field and in vitro studies. Freeman et al. (1997) observed that prochloraz-Mn, prochloraz-Zn and the combination of prochloraz-Zn + folpet had the highest in vitro inhibition of *C. acutatum*, compared to captan, propiconazole, prochloraz and difenoconazole. Unfortunately, prochloraz is not registered for strawberries in the USA or Brazil (Kososki et al., 2001).

The use of fungicides by itself is not enough to control AFR in strawberries. Timing fungicide applications is also of extreme importance. With the objective of reducing the number of fungicide applications during a strawberry season, MacKenzie and Peres (2012a) tested the effectiveness of timing fungicide applications based on temperature and leaf wetness duration adequate for AFR development. This information was used to build the “Strawberry Advisory System” (SAS), a disease forecasting system based on temperature and leaf wetness duration to forecast disease outbreaks and advise strawberry growers on timing fungicide applications (Pavan et al., 2011).

Objectives

Strawberry cultivars planted in Florida differ in susceptibility to *C. acutatum*, however not enough information is known about the effects of inoculum concentration, temperature and wetness duration on AFR development on these cultivars. Therefore, the objectives of this project were to evaluate the effects of inoculum concentration, temperature and wetness duration on the development of AFR on flowers and immature fruit of strawberry cultivars with different susceptibility levels under field and laboratory

conditions. The information collected will be used to adapt models used in a disease forecasting system to predict disease outbreaks in Florida commercial fields.

Figure 1-1. Anthracnose fruit rot symptoms on strawberry. A) Symptomatic flower. B) Symptomatic immature fruit. C) Symptomatic mature fruit. Credits: University of Florida, GCREC Strawberry Plant Pathology. Photos courtesy of University of Florida UF/ IFAS GCREC.

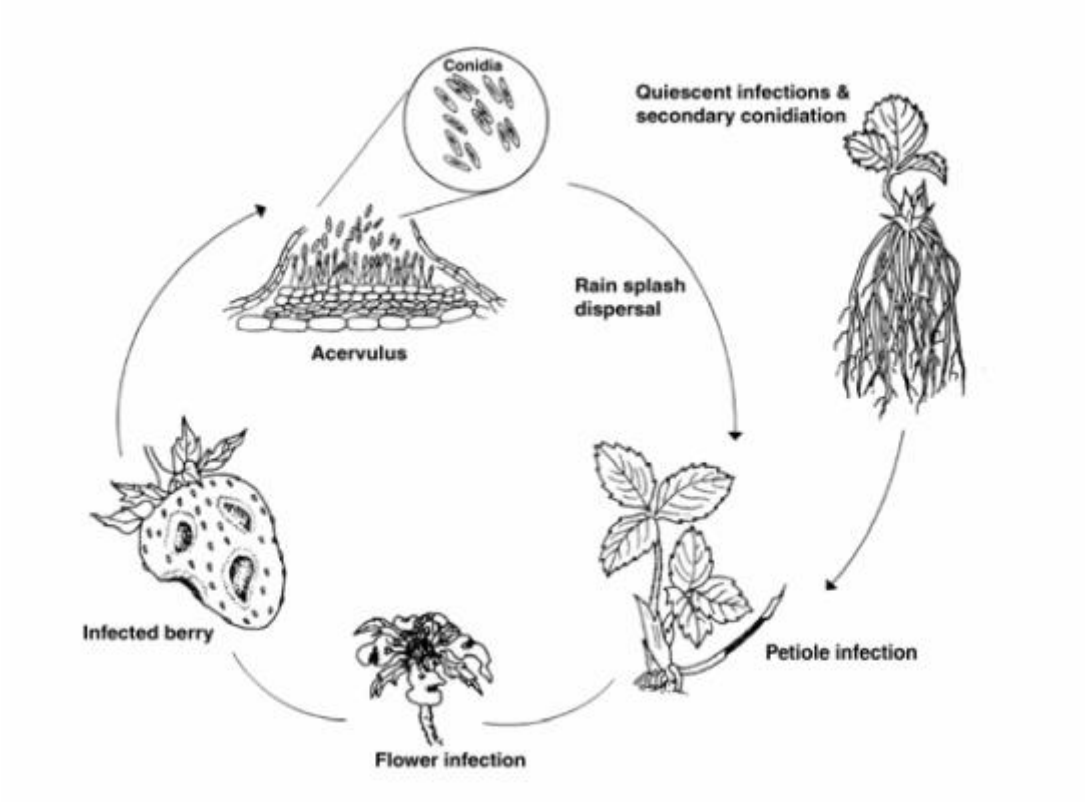

Figure 1-2. Life cycle of *Colletotrichum acutatum*, the causal agent of Anthracnose Fruit Rot of strawberry. Figure by Peres et al., in: Lifestyles of *Colletotrichum acutatum*, 2005.

CHAPTER 2
EFFECT OF INOCULUM CONCENTRATION, TEMPERATURE AND WETNESS DURATION ON ANTHRACNOSE FRUIT ROT DEVELOPMENT ON DIFFERENT STRAWBERRY CULTIVARS

The United States of America is the major producer of strawberries (*Fragaria* x *ananassa* Duch) in the world, followed by Turkey, Spain, Egypt and Mexico (FAOSTAT, 2012). In the USA, California is the largest strawberry producer during the summer (USDA, 2013) and Florida is the main producer and distributor of fresh winter strawberries. During the 2012-2013 season, Florida's yield was 182.7 billion pounds and 8,700 acres of strawberries were harvested (USDA, 2013).

AFR of strawberry, most commonly caused by the fungus *Colletotrichum acutatum* Simmonds, is a major disease in Florida and worldwide (Maas, 1998; Legard, 2002). Transplants infected with *C. acutatum* are responsible for introducing the pathogen in annual strawberry production commercial fields (Strand, 2008; Mertely et al., 2005). Common symptoms of AFR are flower blight and fruit rot, however during severe epidemics crown and root rots as well as petiole lesions may be observed (Legard et al., 2003; Mertely and Peres, 2005; Peres et al., 2005). Additionally, AFR development is highly dependent on strawberry cultivar susceptibility. 'Strawberry Festival' and 'Florida Radiance', the most common cultivars grown in Florida, are considered moderately susceptible, whereas 'Treasure' and 'Camarosa' are highly susceptible to AFR (Chandler et al., 2006; Seijo et al., 2008). Since infected transplants are the main source of inoculum, the amount of quiescent infections on transplants may influence initial AFR symptom appearance in commercial fields depending on cultivar susceptibility, since more susceptible cultivars may require lower inoculum concentrations for symptom development.

Colletotrichum acutatum, the anamorph stage of *Glomerella acutata*, produces conidia that are responsible for plant infection (Damm et al., 2012; Guerber and Correll, 2001; Agrios, 2005). The fungus is a hemibiotrophic pathogen and on strawberries the necrotrophic phase is predominant (Curry et al., 2002). Conidial dispersal occurs mainly through splashing water, harvesting operations and contaminated soil on machinery and farming equipment (Mertely et al., 2005, Leandro et al., 2003b; Strand, 2008). Secondary conidial production, germination, appressorial formation, and fruit infection of *C. acutatum* are highly affected by temperature and wetness duration (WD) (Maas, 1998; Leandro et al., 2003a). Secondary conidial germination and appressorial development on symptomless leaves may occur with continuous wetness and temperatures from 17 to 27^{0}C (Leandro et al., 2003a). Secondary conidiation is stimulated more by the presence of strawberry flower extracts than by water, and thus the presence of flowers may increase inoculum on strawberry plants for subsequent organ infections. In addition, AFR incidence on immature and mature strawberry fruit increases with increasing temperatures from 6 to 25^{0}C and WD from 1 to 51 hours. (Maas, 1998; Wilson et al., 1990).

The effects of temperature and WD on AFR development have been used to build the “Strawberry Advisory System” (SAS). The SAS provides growers with information about weather conditions that are favorable for AFR and BFR development and recommendations on timing fungicide applications. The main advantage of using such system is the reduction of fungicide applications during the season without reducing plant yield (MacKenzie and Peres, 2012a). This is extremely important in Florida where humid conditions favor disease development and the use of preventive

fungicide applications is a common practice among strawberry growers. However, environmental safety and the increase in fungicide resistance is a concern among growers and researchers.

To improve and adapt the SAS, more information was needed about the effects of temperature, WD and inoculum concentrations on commonly grown strawberry cultivars in Florida and plant organs with different levels of susceptibility.

Materials and Methods

Fungal Isolates and Culture

Four isolates of *C. acutatum*, 2-163, 2-179, 3-32 and 98-324 that were sequenced (*G3PD*, *GS*, and *ITS* regions) in a previous study (except for 98-324) (MacKenzie et al., 2009) were used for plant inoculation and cultural characterization. The GenBank accession numbers for sequences from the isolates are for the *ITS*, *G3PD*, and *GS* regions respectively, EU647302, EU647315, and EU647328 for the 2-163, EU647303, EU647316, and EU647329 for 2-179, and EU647304, EU647317, and EU647330 for 3-32 (MacKenzie et al., 2009). Strains were isolated from strawberry petiole (2-163), fruit (2-179 and 98-324) and crown (3-32) and maintained on filter paper in a sterile envelope in airtight container with Drierite (W.A. Hammond Drierite Company, LTD) at - 20^{0}C. To revive the isolates, a small piece of the filter paper (≈ 4 mm^2) containing the isolate was placed on 90 mm plates with potato dextrose agar (BD Difco™ PDA) and grown for 8 days at 22^{0}C.

Effect of Inoculum Concentration on Anthracnose Fruit Rot Development

To determine the effect of different inoculum concentrations on AFR development on different strawberry cultivars, a field and a detached fruit experiment were carried out. The field experiment was conducted during the 2011-2012 and 2012-

2013 strawberry seasons, while the detached fruit experiment was done during the 2012-2013 season.

Field Trial

Bare root strawberry transplants of the cultivars Camarosa, Strawberry Festival and Treasure were established in the field in mid-October, 2011 at the University of Florida Gulf Coast Research and Education Center. In mid-October 2012, transplants of 'Camarosa' and 'Strawberry Festival' were established in the same location. Before transplant, plastic-mulch covered beds were fumigated with *1,3-dichloropropene and chloropicrin* (Telone® C-35, Dow AgroSciences, Indianapolis, IN) in both seasons. The beds were 91.4 m long, 71 cm wide and 15 cm high at the edges and were 18 cm high at the center. Beds were 1.2 m apart, measuring from their centers. Transplants were placed 30 cm apart in two rows. For plant establishment, transplants were irrigated with overhead sprinklers for 10 to 12 days and for the rest of the season plants were irrigated and fertilized daily through drip irrigation. For four days during the 2011-2012 season (January 4, 5 and 15 and February 13), temperatures were below 0^0C and the use of overhead irrigation was necessary for freeze protection. Overhead irrigation was not necessary for freeze protection during the 2012-2013 season.

Four inoculum concentrations (10^3, 10^4, 10^5 and 10^6 conidia/ml) of *C. acutatum* and a control (deionized water) were chosen as the treatments for the 2011-2012 season. In the 2012-2013 season, the inoculum concentration 10^2 conidia/ml was added. Flowers, immature, pink, and mature fruit were inoculated to compare organ susceptibility. For standardization at the time of inoculation, flowers had to be open, have intact petals, and fresh yellow pollen; immature fruit had chlorophyll and were not starting to turn white; pink fruit had lost chlorophyll and started to turn pink; and mature

fruit were beginning to turn red. During the 2011-2012 season, flowers, immature, pink, and mature fruit of ‘Treasure’, ‘Camarosa’ and ‘Strawberry Festival’ were inoculated, whereas in the 2012-2013, only flowers and immature fruit of ‘Camarosa’ were inoculated.

Isolates 2-179, 3-32 and 98-324 were revived as previously described and grown for eight days, until fungal mycelium covered 2/3 of the plate. Colonies were scraped using sterile water and a glass rod to obtain conidial suspensions. The conidial suspension of each isolate was poured into an Erlenmeyer flask through a double layer of cheesecloth. Conidial suspensions were counted with a hemocytometer (Bright-Line, Hausser Scientific) and then adjusted to 10^6 conidia/ml for each isolate. Then, all isolates of the same conidial concentration were mixed. The final conidial concentrations were achieved through serial dilution and refrigerated at 5^0C until inoculation time. All inoculum suspensions were prepared on the same day.

In the 2011-2012 season, ten plants per treatment were chosen arbitrarily and flagged. A total of 50 plants per cultivar were used (10 plants x 5 treatments). No AFR symptoms were observed prior to inoculation. On the days of inoculation, January 21 and February 14, all the flowers, immature, pink, and mature fruit present on the selected plants were tagged with different tape colors to identify the plant organs that would be inoculated (flowers = yellow; immature fruit = green; pink fruit = orange and mature fruit = red) (Figure 2-1A). Mean temperature at the time of inoculation and mean daily temperature were 21.5 and 16.5^0C (January 21) and 23.2 and 16.4^0C (February 14, 2012). During the 2012-2013 season, the experimental unit were flowers and fruit instead of plants like the previous season. Four replications of 10 flowers and immature

fruit per inoculum concentration were inoculated on March 22. The mean temperature at time and day of inoculation were 21.1 and 15.6^{0}C, respectively. On inoculation days, tagged plant organs were mist sprayed with approximately 250 µl of the conidial suspensions at the designated concentration with an atomizer (Spra-tool, Crown). Immediately after inoculation, plants were covered with 46 x 61 cm plastic bags (Uline) containing a small amount of deionized water to maintain humidity (Figure 2-1B). Sixteen hours after inoculation, plastic bags were removed and plants were allowed to dry. Tagged plant organs were evaluated for disease incidence over 21 days starting five days after inoculation during the 2011-2012, season and over 10 days starting five days after inoculation in the 2012-2013 season. The experiment was conducted twice for each cultivar in the first season and once for 'Camarosa' during the second season. In the experiment conducted in the second season, 'Camarosa' flowers were inoculated on plants under a high tunnel (Figure 2-1C), whereas immature fruit were from plants in the open field. The experiment was a completely randomized design.

Detached Fruit Trial

Five inoculum concentrations (10^2, 10^3, 10^4, 10^5 and 10^6 conidia/ml) of the *C. acutatum* isolates 2-163, 2-179, and 3-32, plus a control (deionized water) were tested in the detached fruit trial. Cultivars selected for this experiment were 'Camarosa' and 'Strawberry Festival'. The experimental design was a randomized complete block design consisting of four replications of 4 immature fruit/treatment (16 fruit/treatment). During the 2012-2013 season, immature fruit of 'Camarosa' and 'Strawberry Festival' that were on average 2 to 3 cm^2 and had receptacle with chlorophyll were harvested from the field. In the laboratory, fruit went through a second triage (Figure 2-2A) for better standardization and were surface sterilized using 0.7% sodium hypochlorite for

six minutes and then rinsed four times with sterile water. Four plastic boxes measuring 31.5 x 25 x 10 cm and eight egg cartons (one dozen wells) per cultivar were sprayed with alcohol and sterilized inside a fume hood with ultraviolet light for 20 minutes. Then, fruit were placed in the egg cartons inside the plastic boxes according to replication and treatment (Figure 2-2B) and were surface dried inside the hood for 20 minutes. To maintain humidity inside the boxes, 75 ml of deionized water were placed under the egg cartons.

Inoculum was prepared as described for the field experiment. Fruit were inoculated with a 5 µl droplet of the inoculum suspension at the top of the fruit for standardization and to facilitate disease evaluation. Plastic boxes were covered and maintained at room temperature (23^0C ± 1) for 9 days. Fruit were evaluated for disease incidence and severity for 9 days starting five days after inoculation. The experiment was repeated four times for each cultivar. In addition to this experiment, another trial was conducted for 'Strawberry Festival' where immature fruit were selected by age (days after flowering). Open flowers with fresh pollen were tagged and fruit harvested 8 or 12 days afterwards were used as the experimental unit. This trial was repeated twice for each fruit age.

Effect of Different Temperatures on Mycelial Growth of *C. acutatum* isolates

A mycelial growth assay was conducted to test the influence of temperature on the growth rate of *C. acutatum* isolates 2-163, 2-179 and 3-32. Six-millimeter-diameter mycelial plugs of each of the three isolates where cut from the margin of an 8 day-old colony (when 2/3 of the plate was covered by mycelia) and placed on PDA. Plates were closed with parafilm and placed in growth chambers set at 5, 10, 15, 20, 25, 30 and 35^0C and in the dark. After 7 days, *C. acutatum* colonies were measured in one

direction. The size of the plug (6cm) was subtracted from the measurement to give the final colony size. The experiment was conducted as a split plot design with five plates per isolate (subplots) per temperature (whole plots) and was repeated three times.

Effect of Temperature on Anthracnose Fruit Rot Development on Detached Fruit

A growth chamber study using detached immature fruit was conducted to determine the effect of temperature on AFR development on 'Camarosa' and 'Strawberry Festival' during the 2012-2013 strawberry season. A total of seven temperatures (5, 10, 15, 20, 25, 30 and 35^0C) were evaluated. Isolates 2-163, 2-179 and 3-32 were selected for fruit inoculation and inoculum was prepared as described above except that the inoculum concentration selected in this assay was 10^5 conidia/ml. Nine fruit (6 inoculated and 3 controls) per temperature per cultivar were harvested from the field according to size and color (as described above) and brought to the laboratory for additional triage. Fruit were surface sterilized using 0.7% sodium hypochlorite for six minutes and then rinsed four times with sterile water. Seven boxes and 14 egg cartons (1 egg carton per cultivar/temperature) were sterilized with ultraviolet light. Boxes were considered as the replications and there was 1 box per temperature. Fruit were placed in egg cartons (9 per egg carton) inside boxes and left to air dry inside a fume hood for 20 minutes. Each box contained both cultivars (1 egg carton/cultivar). Seventy-five ml of water was added to boxes to simulate a humid chamber as described in the previous experiment.

A 5 µl droplet of the conidial suspension was placed on the upper surface of the fruit and boxes were closed to prevent droplet movement and were only moved to growth chambers 5 minutes after inoculation. One growth chamber was set to each temperature. AFR incidence on inoculated fruit was evaluated over 9 days, starting on

the fifth day after inoculation. For the lower temperatures (5, 10 and 15^{0}C), the evaluation period was extended to 19 days after inoculation, because at lower temperatures disease progress is slower. This experiment was conducted in a split plot design where the different temperatures were the whole plots and the cultivars were the subplots. This experiment was repeated four times and in the fourth experiment, the number of fruit used per treatment increased to 7.

Effect of Wetness Duration and Temperature on Anthracnose Fruit Rot Development

To determine the effect of Wetness Duration (WD) and temperature on AFR development, an environmental growth chamber study was conducted during the 2011-2012 and 2012-2013 strawberry seasons. For that, growth chamber temperatures 15, 20 and 25^{0}C and wetness periods of 0, 3, 6, 12, 24 and 48 hours plus a control (water treatment + 48 hours of WD) were evaluated.

During the 2011-2012 season, two hundred bare-root transplants of ‘Camarosa’, ‘Treasure’ and ‘Strawberry Festival’ originating from Nova Scotia, Canada were planted in 15 x 15 x 16 cm plastic pots filled with soil containing Canadian sphagnum peat (65%), perlite and vermiculite (Fafard®), in October of 2011. Plants were fertilized with Osmocote® Plus granular slow release (Scotts, 15-9-12), irrigated daily and remained in a greenhouse until they were ready for inoculation. In the 2012-2013 season, transplants of ‘Camarosa’ and ‘Strawberry Festival’ were planted in October 2012 and January 2013, following the same steps as in the previous season. However, plants were then fertilized with Miracle Gro® Water Soluble All Purpose Plant Food (24-8-16) every 10 days.

During the 2011-2012 season, strawberry flowers, immature, pink, and mature fruit were inoculated and during the 2012-2013 season only immature fruit. In the 2011-2012 season, four plants/ WD/temperature/cultivar (84 plants per cultivar) were identified according to treatment and plant organs were tagged (flower = yellow; immature fruit = green; pink fruit = orange and mature fruit = red). During the 2012-2013 season, only immature fruit were used for inoculation and ten immature fruit/WD/temperature/cultivar were tagged and considered as a replication. However, for the third experiment conducted with 'Camarosa', there was limited number of immature fruit, therefore only 4 fruit/WD/temperature combinations were used. One environmental growth chamber was set for each temperature with a 12h light/12h dark photoperiod (2011-2012) or 24h dark (2012-2013), and two growth rooms were adjusted to 25^0C with 12h light/12h dark photoperiod. Data loggers (WatchDog A-Series, Spectrum) were placed inside growth chambers and rooms to monitor temperature.

Eight-day-old cultures of *C. acutatum* isolates 2-163, 2-179 and 3-32 were scraped with sterile water using a glass rod as described above. Conidial suspensions of each isolate were filtered through double-layer cheesecloth and concentration adjusted to 10^6 conidia/ml with the use of a hemocytometer. Subsequently, conidial suspension of the three isolates were mixed and stored in a 236ml container for inoculation. Tagged plant organs were mist-sprayed with a 150µl suspension or sterile water (control) using an atomizer (Spra-tool, Crown). Plants were carefully bagged and closed with a zip tie. To maintain humidity inside the plastic bags, two moist cotton balls sprayed with deionized water were placed on the bottom of each bag. Bagged plants were then transferred to growth chambers according to temperature and WD (Figure 2-

3A), except for the 0 hour of WD treatment. These were inoculated and immediately placed in front of fans (Figure 2-3B) until plant organs were visually dry (approximately 30 minutes). Thirty minutes prior to the end of the wetness period, plants were removed from the bags, dried as described above, and transferred to growth rooms at 25^0C for 21 days in the first season and for 9 days in the second season. Plants were irrigated daily by adding water directly on the soil to avoid leaf, flower or fruit wetness. Starting five days after plant inoculation, tagged organs were evaluated daily for disease incidence. Trials during the 2011-2012 season were conducted at the same time for the three cultivars, however during the 2012-2013 season, cultivars Camarosa and Strawberry Festival were inoculated on different dates since growth chamber space was limitated. In the 2011-2012 season, plant organ were evaluated up to 21 days after inoculation, whereas in the latter season, they were evaluated up to 9 days after inoculation. The experiment was conducted twice for each cultivar in the 2011-2012 season and three times in the 2012-2013 with a split plot design, where the temperatures were the whole plots and the wetness periods the subplots.

Statistical Analysis

Arcsine transformation was used for incidence values because there were many dot points with 0 and 100% disease incidence. For the statistical analysis both disease incidence and arcsine transformed values were used. An Analysis of Variance (ANOVA) was conducted to test the effects of experiment, inoculum concentration, cultivar, temperature, wetness duration, and their interactions (SAS, version 9.3; SAS Institute, Cary, NC). Data of homogenous trials for each cultivar were combined and used to analyze treatment effects and plant organ susceptibility using the Least Significant Difference (LSD) or Tukey (SAS). The correlation between field and detached fruit

assays and cultivars were analyzed using analysis of covariance (SAS) for the inoculum concentration and temperature x WD assays, respectively. There were many missing values in the 2011-2012 inoculum concentration field trials, because of the absence of flowers or fruit on the inoculated plants. Therefore, data from the 4 plants that had the most flowers/fruit instead of 10 plants/treatment were used. In addition, for the statistical analysis of that experiment, disease incidence at 10 days after inoculation was used.

Figure 2-1.Field trial of inoculum concentration experiment. A) Tagged strawberry flowers, immature, pink and mature fruit. B) Inoculated strawberry plants covered with plastic bags to maintain humidity. C) Overview of field plot under the tunnel. All treatments and replications were arranged in a single bed. Photos courtesy of Bruna B. Forcelini.

Figure 2-2. Detached fruit trial of inoculum concentration experiment. A) Selection of immature strawberry fruit. Fruit located on the far left were selected for inoculation. B) Arrangement of inoculated detached fruit for each replication and cultivar. Photos courtesy of Bruna B. Forcelini.

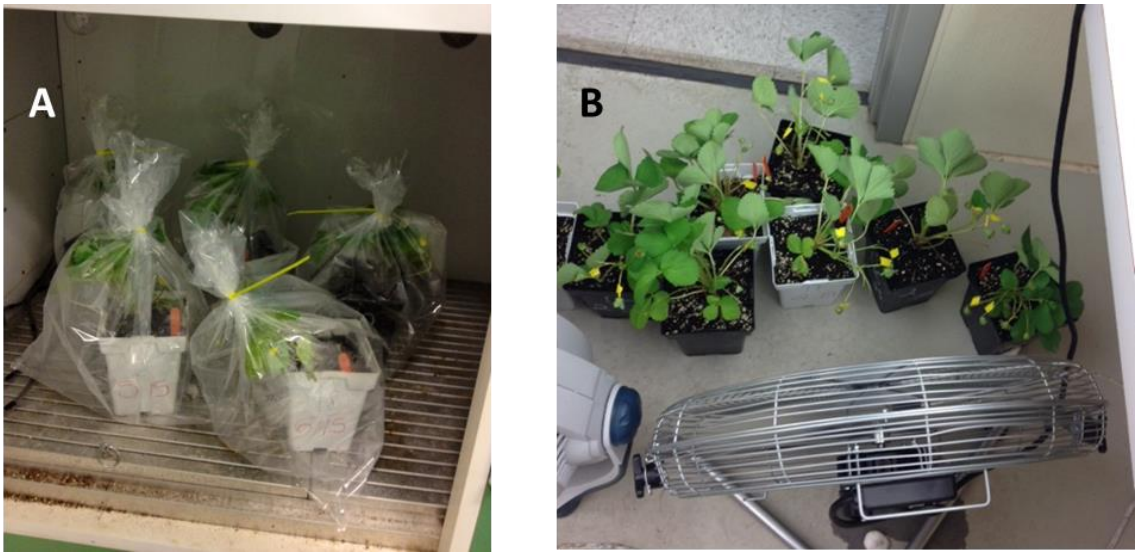

Figure 2-3. Controlled wetness duration and temperature experiment. A) Inoculated and bagged plants inside the growth chamber set at the designated treatment temperature. B) Use of fans to dry strawberry plants after different wetness periods. Photos courtesy of Bruna B. Forcelini.

CHAPTER 3
RESULTS

For all of the experiments, the statistical analysis using disease incidence or the arcsine transformed data showed similar results.

Effect of Inoculum Concentration on Anthracnose Fruit Rot Development

Field Trial

Disease incidence increased with increasing inoculum concentration from 0 to 10^6 conidia/ml for the three cultivars in the 2011-2012 and for 'Camarosa' in the 2012-2013 strawberry seasons. The effects of strawberry cultivar, inoculum concentration and plant organ were significant ($P < 0.05$), while all interactions (cultivar x concentration, cultivar x plant organ and concentration x plant organ) were not (Table 3-1). For 'Camarosa' and 'Treasure', a low disease incidence was observed on flowers and immature fruit on non-inoculated plants (Figure 3-1). 'Camarosa' and 'Treasure' had higher disease incidence than 'Strawberry Festival' and did not differ from each other (Table 3-2).

Inoculum concentration was highly significant ($P < 0.0001$) for the three cultivars and the highest disease incidences were observed for all cultivars when flowers and fruit were inoculated with conidia at 10^6/ml. A second order polynomial regression indicated that disease incidence on immature fruit and flowers for the three cultivars increased with an increase in inoculum concentration from 0 to 10^6 conidia/ml. However, the lowest inoculum concentration required for disease development differed among cultivars and plant organs (Figure 3-2). First AFR symptom appearance for

‘Strawberry Festival’ was observed on flowers inoculated with 10^3 conidia/ml, whereas for immature fruit, a higher inoculum concentration (10^4 conidia/ml) was needed.

Flowers were more susceptible to AFR than immature fruit (*P*=0.0147). Disease incidence for flowers reached 83% on the more susceptible cultivars (Camarosa and Treasure), whereas for immature fruit the maximum disease incidence was about 40%. For ‘Strawberry Festival’, incidence on flowers and immature fruit were not significantly different (*P* = 0.2037).

AFR incubation period (days from inoculation to symptom appearance) differed between trial one and two. Since trial one produced inconsistent results and had a low R^2, only the results of the second trial are shown. The AFR incubation period was shorter for flowers than for immature fruit for all three cultivars. However, when incubation period was compared within cultivars, ‘Strawberry Festival’ had a significantly longer incubation period than ‘Treasure’ or ‘Camarosa’ on both plant organs (Figure 3-3). The incubation period for ‘Treasure’ on immature fruit and flowers were 5.75 and 5.25 days, respectively, whereas for ‘Strawberry Festival’, the incubation period was 8 days for immature fruit and 7.25 days for flowers. Data from pink and mature fruit were not used in the statistical analysis because fruit was over ripened before the end of the evaluation period.

Detached Fruit Trial

In the detached fruit experiment, disease incidence also increased with increasing inoculum concentration and ‘Camarosa’ had a significantly higher disease incidence (80%) than ‘Strawberry Festival’ (55%) at 10^6 conidia/ml (Table 3-3). The highest disease incidence was observed at the highest inoculum concentrations (10^5 and 10^6 conidia/ml) for both cultivars (Figure 3-4). In addition, the minimum inoculum

concentration required for disease development was lower for ‘Camarosa’ than for ‘Strawberry Festival’. A concentration of 10^4 conidia/ml was sufficient for symptom development on ‘Camarosa’, whereas for ‘Strawberry Festival’, symptoms were only observed at 10^5 conidia/ml. The data shown for ‘Strawberry Festival’ are from the first two trials (fruit selected according to size and color), since these trials did not differ. Data from the additional trials (fruit selected according to age) are not shown because trials were different among each other and from the first two experiments.

Correlation between Detached Fruit and Field Trial

For each cultivar, a polynomial regression of second order was used to fit the curves for the detached fruit and field experiments. Disease incidence was lower for ‘Strawberry Festival’ in both trials than for ‘Camarosa’, but the curves for detached fruit and field experiments were similar to each other and between cultivars. A value equivalent or less than 1.96 is required to determine if the curves are similar and the analysis of covariance indicated a value of 0.6 (Tables 3-4 and 3-5). Therefore, the trials were correlated and similar results can be achieved in laboratory and in field trials. Even though trials are correlated, inoculated detached fruit had a shorter incubation period than those inoculated in the field for both cultivars. For ‘Camarosa’ and ‘Strawberry Festival’ field experiments had an average incubation period of 8 days, whereas with detached fruit, the period was less than 7 days.

Effect of Temperature on Mycelial Growth

Temperature affected mycelial growth of the three *C. acutatum* isolates (2-163, 2-179 and 3-32) similarly. At 5 and 35^0C, no mycelial growth was observed for any of the three isolates (Figure 3-5). Growth of *C. acutatum* increased with increasing temperature from 10 to 25^0C and then rapidly decreased at 30^0C. Highest growth was

observed at 25^{0}C and colony diameter ranged from 49.2 to 53.4 mm and averaged 7.32 mm/day. Seven days after mycelial plug was transferred to the culture media, colony diameter averaged 4.8, 22.3, 43.8 and 34 mm at 10, 15, 20 and 30^{0}C, respectively.

Effect of Temperature on Anthracnose Fruit Rot Development on Detached Fruit

The effect of temperature was highly significant ($P < 0.0001$), whereas cultivar and the interaction between temperature and cultivar were not. Cultivars were analyzed separately for homogeneity within the four experiments and there was no statistical difference between them ($P > 0.9366$ for 'Camarosa' and $P > 0.9262$ 'Strawberry Festival'). Therefore, experiments were combined and disease incidence was averaged for each treatment.

At 5, 10 and 35^{0}C, no AFR symptoms were observed on detached immature fruit of 'Strawberry Festival' and 'Camarosa' even with a prolonged incubation period of 19 days (Figure 3-6). A second order polynomial regression showed an increase and subsequent decline on disease incidence when temperatures ranged from 15 to 30^{0}C. However, at 15^{0}C, disease incidence was low (4.15 %) compared to 20, 25 and 30^{0}C. Disease incidence increased rapidly when fruit were exposed to 20^{0}C, but at 25^{0}C cultivars were different. Disease incidence was the same at 20 and 25^{0}C for 'Camarosa' (91.67%), whereas for 'Strawberry Festival' it was 84.5 and 47%, respectively. Out of the four experiments, two had high disease incidence (83.3 and 71.4%) and two had low incidence (16.6 and 16.6% at 25^{0}C). Therefore, when averaged, the mean incidence for the 25^{0}C treatment of 'Strawberry Festival' was lower than for 'Camarosa'. At 30^{0}C, cultivars had similar mean disease incidence, 73.2% ('Strawberry Festival') and 80.3% ('Camarosa'), and the difference was not significant between 20 and 25^{0}C

for ‘Camarosa’. At the highest temperature (35^0C), immature fruit dried out rapidly and no fruit were infected. The maximum disease incidence was at 20 and 25^0C (91.7%) for ‘Camarosa’, whereas the highest incidence for ‘Strawberry Festival’ (84.5%) occurred with immature fruit at 20^0C. Even though cultivars were not significantly different from each other, ‘Camarosa’ always had a disease incidence equal to or greater than the mean incidence for ‘Strawberry Festival’. Disease severity values were not analyzed and are not shown because infected fruit of ‘Strawberry Festival’ were only observed with inoculum concentrations of 10^5 and 10^6 conidia/ml and therefore the only severity data available were at the two highest concentrations.

Effect of Wetness Duration and Temperature on Anthracnose Fruit Rot Development

The ANOVA showed a significant effect of temperature, wetness duration (WD) and their interaction on the development of anthracnose fruit rot (Table 3-6). In addition to the ANOVA, an analysis of covariance confirmed that cultivars did not differ within temperatures and at all WDs. The data of all three experiments of each cultivar were combined. Disease incidence increased non-linearly for ‘Camarosa’ and ‘Strawberry Festival’ from 0 to 48 hours of wetness for most temperatures (Table 3-7 and Figure 3-7). A second order polynomial regression best fit the data and resulted in high R^2 values for both cultivars at all temperatures, except for ‘Camarosa’ at 15^0C (Figure 3-8). The R^2 values for the combined data of ‘Strawberry Festival’ were 0.8997, 0.9819 and 0.9435, and for ‘Camarosa’ were 0.6069, 0.9493 and 0.9195 at 15, 20 and 25^0C, respectively (Table 3-8).

No diseased fruit were observed in the control treatments (inoculum + 0 hour WD, and no inoculum + 48 hours of WD) at any temperature. With 3 hours of WD, mean

disease incidence (28.3%) for ‘Camarosa’ was higher at 15^{0}C than at the other temperatures, whereas for ‘Strawberry Festival’, the highest mean disease incidence (16.6%) was at 25^{0}C. Mean disease incidence of ‘Strawberry Festival’ with a WD of 6 hours was the same at 20 and 25^{0}C (11.1%) and for ‘Camarosa’ it was higher at 20^{0}C (30%) than at 25^{0}C (23.3%). At 12 hours of WD, mean incidence was 58.8% at 25^{0}C for ‘Strawberry Festival’ and 77.5% for ‘Camarosa’. The mean disease incidence with 24 h of WD ranged from 46.83% to 76.6% from 15 to 25^{0}C for ‘Strawberry Festival’ and from 31.6 to 80% for ‘Camarosa’. The highest mean disease incidences were observed with 48 h of WD at 20^{0}C for ‘Camarosa’ (100%) and ‘Strawberry Festival’ (79.4%) (Figure 3-7). The 24 and 48 h WD treatments did not differ, but were statistically different from the shorter wetness periods. For ‘Strawberry Festival’, for the treatments with 6, 12 and 24 hours of WD, disease incidence increased with the increase in temperature, whereas in the 3 h treatment, incidence decreased at 20^{0}C, followed by an increase at 25^{0}C. At 48 h for both cultivars and at 6 and 24 hours for ‘Camarosa’, disease incidence increased rapidly from 15 to 20^{0}C, but declined slightly at 25^{0}C. Unlike the other treatments, disease incidence for treatments with 3 and 12 hours of WD for ‘Camarosa’ decreased from 15 to 20^{0}C and then increased at 25^{0}C (Figure 3-8). During the 2011-2012 season, a 12h light/12h dark photoperiod was used in the growth chambers. Later, it was found that the temperature inside the bags with inoculated plants were on average 5^{0}C above the selected temperature treatment when the lights were on. Therefore, data from this experiment was not used in the statistical analysis.

Discussion

Development of AFR of strawberry is greatly affected by the concentration of inoculum on the plant, temperature and wetness duration. The knowledge of the minimum inoculum concentration, optimum temperature and wetness duration required for *C. acutatum* infection on cultivars with different levels of susceptibility such as Strawberry Festival, Camarosa and Treasure are necessary to adapt disease forecasting systems for Florida's production and to develop more efficient disease management programs. A forecasting system to predict AFR incidence based on temperature and wetness duration has been developed to advise Florida strawberry growers on timing fungicide applications when weather conditions are favorable, instead of following a preventive program of calendar (weekly) applications (MacKenzie and Peres, 2012a). However, information about the effect of temperature and wetness duration for the strawberry cultivars commonly grown in Florida was not available; hence, the importance of this research project.

Anthracnose fruit rot incidence increased with the increasing inoculum concentration for all strawberry cultivars and plant organs. Diéguez-Uribeondo et al. (2011), Trapero-Casas and Kaiser (1992) and Chungu et al. (2001) reported similar results with anthracnose of almond, Ascochyta blight on chickpea, and *Septoria tritici* blotch on wheat, respectively, where disease severity increased non-linearly with increasing inoculum concentration. Diéguez-Uribeondo et al. (2011) observed that when inoculating *C. acutatum* on almond cultivars with different levels of susceptibility, 'NePlus Ultra (more susceptible) and 'Nonpareil' (less susceptible), both required a minimum inoculum concentration of 10^4 conidia/ml for disease development. In our study, the highly susceptible cultivars Camarosa and Treasure required a lower

inoculum concentration for initial disease development and had higher percentage of infected immature fruit and flowers when compared to ‘Strawberry Festival’, which is considered less susceptible to AFR. ‘Strawberry Festival’ is one of the most planted strawberry cultivars worldwide, due to its high berry quality and low susceptibility to common strawberry diseases like AFR and Botrytis fruit rot. Thus, our results are different than those of Diéguez-Uribeondo et al. (2011) who reported that the less and more susceptible almond cultivars required the same inoculum concentration for anthracnose development.

The difference in cultivar susceptibility and the minimum inoculum concentration required for disease development is extremely important to understand, since strawberry transplants may arrive from nurseries already infected with *C. acutatum.* If transplants arrive at production fields infected with as little as 10^3 conidia/mg, it would be enough for the more susceptible cultivars like Camarosa and Treasure to start developing symptoms after planting. However, symptoms would not develop on ‘Strawberry Festival’ until inoculum concentration builds up, by secondary conidiation, to approximately 10^4 conidia/mg. Polymerase Chain Reaction (PCR) or quantitative Polymerase Chain Reaction (qPCR) could be used to detect *C. acutatum* on strawberry transplants. The use of qPCR would be more efficient since it not only detects the presence or absence of the fungus but also quantifies the pathogen when compared to a DNA standard (Postollec et al. 2011). Unfortunately, qPCR is an expensive method to use on a regular basis when testing a large number of transplants. Recently, researchers have been working on developing an inexpensive, highly specific, efficient assay, called the loop-mediated isothermal amplification (LAMP), which is an alternative

to qPCR (Zhang et al., 2013; Notomi et al., 2000). Preliminary results have shown that the LAMP method is able to detect *C. acutatum* on symptomless plants within one hour by using two sets of primers to amplify the internal transcribed spacer (ITS) such as G1 and the tubulin 2 genes (tub2) (Zhang et al., 2013). With the use of these molecular tools, researchers and extension agents will be able to detect the amount of inoculum concentration present in samples of transplants and recommend initial spray applications according to that and to the level of susceptibility of the strawberry cultivar planted.

In addition to cultivar susceptibility, plant organ is an important factor for AFR development. Strawberry flowers were more susceptible than immature fruit of 'Camarosa' and 'Treasure'. This is in agreement with Maas (1998), who also reported the high susceptibility of strawberry flowers to *C. acutatum.* Furthermore, a study conducted from 2002 to 2004 to evaluate the susceptibility of flowers and fruit of 'Camarosa' and 'Strawberry Festival' to *C. acutatum* according to their age (open flower, 4, 8, 12, 16, and 20-day-old fruit) showed that disease incidence for flowers reached almost 100% for 'Camarosa' and 90% for 'Strawberry Festival' and then sharply declined when fruit were 8 days old (J. Mertely, *unpublished*). In our study, open flowers and 8-day-old fruit with no petals attached were also used. Wilson et al. (1990) as well as Mertely (*unpublished*) observed that disease incidence increased with fruit maturation. Therefore, immature fruit are less susceptible to AFR than flowers and mature fruit. However, even though our results showed that AFR symptoms on flowers of all three cultivars were observed with a lower inoculum concentration than the required for infection of immature fruit, for 'Strawberry Festival' there was no significant

difference between the susceptibility levels of plant organs. Information about the higher susceptibility of flower versus immature fruit can help growers to schedule fungicide applications. Since flowers are more susceptible than immature fruit for ‘Treasure’ and ‘Camarosa’, growers should follow a strict spraying schedule when flowers are predominant compared to immature fruit, and use fungicides with protective and curative effects, such as the QoI fungicides azoxystrobin and pyraclostrobrin.

Strawberry cultivar and plant organ highly affect AFR incubation period. Flowers of ‘Strawberry Festival’, ‘Camarosa’ and ‘Treasure’ had shorter incubation periods than immature fruit. This may be correlated with the higher susceptibility of flowers compared to immature fruit but not for ‘Strawberry Festival’. These findings are also in agreement with Mertely’s results, that 8-day-old fruit had a longer incubation period than open flowers for ‘Camarosa’ (J. Mertely, *unpublished*). In our study, incubation period was shorter for ‘Camarosa’ and ‘Treasure’ than for ‘Strawberry Festival’, independent of plant organ. These results may be associated with the lower susceptibility of ‘Strawberry Festival’ to *C. acutatum* and also agree with Mertely’s unpublished data, where flowers of ‘Strawberry Festival’ had a longer incubation period than those of ‘Camarosa’. Thus, plant organs and cultivars with shorter incubation periods may harbor more disease cycles and build up inoculum quicker for subsequent tissue infection.

The same trend observed in the detached fruit trials was observed in the field trials. Disease incidence in detached fruit was highly affected by inoculum concentration and increased with increasing concentration for both cultivars. A non-linear increase in disease incidence occurred from 10^4 and 10^5 conidia/ml to 10^6 conidia/ml for ‘Camarosa’ and ‘Strawberry Festival’, respectively. ‘Camarosa’ had higher disease incidence than

‘Strawberry Festival’ at all inoculum concentrations. However, the minimum inoculum concentration required for AFR symptom development was higher in detached fruit than field trials for both cultivars. This may be explained by the difference in fruit surface exposed to the inoculum. In the field trials, fruit were inoculated with a higher quantity of inoculum suspension and over the whole fruit surface. The results from detached fruit and field trials were correlated and show that similar results can be generated in laboratory conditions using detached fruit. Some of the advantages of the detached fruit assay are: the control of environmental conditions in the laboratory, since potted plants in growth rooms and the greenhouse do not flower and fruit well; and there can be space limitations in greenhouses and growth room/chambers for potted plants. Therefore, the use of detached fruit in the laboratory occupies less space and biotic and abiotic factors such as pests, wind, and temperature fluctuation are better controlled. Detached fruit senesced and matured faster than attached fruit. Symptoms developed on average 7 days after fruit inoculation on detached fruit trials and 8 days in the field. This may be because fruit were under ideal conditions (23 ± 1^0C and high humidity) in the laboratory, whereas fruit inoculated in the field were exposed to fluctuating temperatures.

Temperature highly affected *C. acutatum* mycelial growth and infection of strawberry fruit. Mycelial growth of the three *C. acutatum* isolates used in our study was only observed when they were exposed to temperatures ranging from 10 to 30^0C. The optimum temperature for growth was at 25^0C, which agrees with Wilson et al. (1990). Even though Wilson et al. (1990) used a different isolate of *C. acutatum*, they reported

no mycelial growth at 5 and 35^{0}C and observed an increase in growth from 10 to 25^{0}C and a sudden decline at 30^{0}C.

In our trials with detached fruit, disease incidence generally increased with the increase in temperature from 15 to 30^{0}C for both cultivars. This is in partial agreement with Wilson et al. (1990), who reported that AFR incidence increased with increasing temperatures from 6 to 25^{0}C. The difference of temperature ranges at which symptoms were seen can be related to the strawberry cultivar used. Wilson et al. (1990) used the cultivar Midway (not commonly grown in the Florida annual production system) and evaluated disease incidence under different wetness periods, whereas our laboratory study used continuous wetness for the nine day period.

Although disease incidence increased rapidly from 15 to 20^{0}C for both cultivars, optimum temperature was different between cultivars. The highest disease incidence for 'Camarosa' was for fruit exposed to 20 and 25^{0}C, and was 20^{0}C for 'Strawberry Festival'. Our results from 'Camarosa' agree with findings from King et al. (1997), who observed maximum *C. acutatum* sporulation on strawberry plants at 25^{0}C up to 4 days after production of conidia and that the increase in temperature decreased the incubation period. Incubation period was not evaluated in these trials; however, we did observe that symptoms appeared first on the treatments 20, 25 and 30^{0}C (data not shown).

Wetness duration (WD) has been reported in many studies to influence *C. acutatum* infection on strawberry and other hosts (Wilson et al., 1990; Leandro et al., 2003a; Diéguez-Uribeondo et al., 2011). Our results confirmed that it directly affects AFR development on 'Camarosa' and 'Strawberry Festival'. However, the effect of WD

also depended on temperature, and the interaction of wetness duration-temperature (Wilson et al., 1990). Results of our study and those of Diéguez-Uribeondo et al. (2011) with almond showed that infected fruit were only observed when plants were exposed to a wetness period after inoculation. Disease incidence on immature fruit generally increased non-linearly from 15 to 25^{0}C and from 0 to 48 hours of wetness for both cultivars. Similar results were observed by Wilson et al. (1990) with *C. acutatum* infection on immature and mature strawberry fruit of the cultivar Midway. They reported that the increase of disease incidence was positively correlated with an increase in temperature from 6 to 25^{0}C and wetness duration from 0.5 to 51 hours.

At 15^{0}C, disease symptoms had developed with only 3 hours of WD for 'Camarosa' and 'Strawberry Festival'. In addition, disease incidence for 'Camarosa' was higher than 'Strawberry Festival' for most of the wetness periods at 20 and 25^{0}C. At 15^{0}C, 'Strawberry Festival' disease incidence increased non-linearly with the increase of WD, however for 'Camarosa' disease incidence was higher at 3 hours than at 6 hours of WD, likely an artifact of the experiment, and this caused a low coefficient of determination (R^2). Moreover, even though for most of the treatments, 'Camarosa' had higher disease incidence, no difference in the covariance analysis was observed between the two cultivars. This was also seen in the study testing the effects of temperature on detached immature fruit of 'Camarosa' and 'Strawberry Festival'.

A second order polynomial regression was used to describe the effect of temperature and WD for both cultivars, except for 'Camarosa' at 15^{0}C. For 'Camarosa' at 20 and 25^{0}C and for 'Strawberry Festival' at all three temperatures, the resulting equations described well the effect of temperature at all wetness durations for both

cultivars. Wilson et al. (1990) developed an equation to predict AFR incidence on immature and mature strawberry fruit based on temperature and wetness duration for the cultivar Midway. MacKenzie and Peres (2012a) monitored temperature and wetness duration in Florida, and evaluated the equation by Wilson et al. (1990) to predict AFR. Different thresholds for the predicted proportion of infected fruit (INF) were evaluated for timing fungicide applications with captan and pyraclostrobin on two strawberry cultivars, Camarosa and Strawberry Festival. Since 'Strawberry Festival' is considered less susceptible to AFR than 'Camarosa' (Chandler et al., 2006), two *INF* thresholds, a lower (*INF* ≥ 0.15) and a higher one (*INF* ≥ 0.5), were evaluated to determine whether 'Strawberry Festival' could tolerate a higher *INF* and require fewer fungicide applications. High disease incidence was observed when strawberry fruit were sprayed when the *INF* exceeded 0.5 for both cultivars and thus a low threshold was selected for both 'Camarosa' and 'Strawberry Festival'. These results were used to build the web-based SAS that collects data from weather stations at different locations in Florida and automatically calculates the risk for AFR development based on the *INF* (≥0.15 or ≥0.5) (MacKenzie and Peres, 2012a). Our study aimed to determine whether the infection threshold used in the model built in the SAS should be the same for a highly susceptible cultivar such as Camarosa and a less susceptible cultivar such as Strawberry Festival. Our findings suggest that the disease threshold used in the model built in the SAS (MacKenzie and Peres, 2012a) should be the same for cultivars with different levels of susceptibility since weather conditions (wetness duration and temperature) required for initial disease development was similar for 'Camarosa' and 'Strawberry Festival'.

However, disease incidence curves were higher for ‘Camarosa’ than ‘Strawberry Festival’ for most WD and temperature combinations.

This research indicates that inoculum concentration influences disease outcome depending on the cultivar susceptibility. In practical terms, it means that even low levels of latent infection coming with the strawberry transplants can induce symptom development on highly susceptible cultivars. Even though final disease incidence was higher for ‘Camarosa’, disease development curves of different cultivars did not differ with different wetness durations and temperatures. Therefore, the same proportion of diseased fruit threshold can be used for the highly and moderately susceptible ‘Camarosa’ and ‘Strawberry Festival’, respectively. In addition to cultivar susceptibility, plant organ susceptibility and incubation period should be taken into account when using disease forecasting systems to control AFR. Since flowers are more susceptible than immature fruit of most cultivars, growers should follow the fungicide spray recommendations strictly when flowers are predominant. Finally, AFR incidence values for detached fruit inoculations agreed with field trials which allows better control of the environmental conditions and is more practical to perform than field experiments. Even though the SAS can use the same thresholds for cultivars with different susceptibility levels, growers could take more risks when planting less susceptible cultivars since disease incidence is unlike to reach an epidemic when using the system. However, for highly susceptible cultivars, a preventative spraying schedule may be more efficient when controlling AFR.

Table 3-1. Analysis of variance of the effects of cultivar, inoculum concentration, plant organ and all the interactions on the incidence of Anthracnose Fruit Rot of strawberry in field trials.

Effect	Num DF	F Value	Pr > F
Cultivar	2	5.97	0.0259
Inoculum Concentration	5	19.08	0.0003
Plant organ	1	9.59	0.0147
Cultivar*Concentration	8	1.08	0.4591
Cultivar*Plant organ	2	1.90	0.2118
Concentration*Plant organ	5	1.68	0.2444

Table 3-2. Mean disease incidence of strawberry cultivars when inoculated with concentrations of conidia from 10^3 to 10^6 conidia/ml in field trials.

Cultivar	Disease Incidence (%)
Camarosa	54.35 A[a]
Treasure	51.64 A[b]
Strawberry Festival	39.53 B

[a] Mean separation within rows followed by the same letter are not significantly different according to *F* test (LSD) ($P \leq 0.05$).

[b] Percentage data were transformed by arcsine prior to analysis, but non-transformed data are presented.

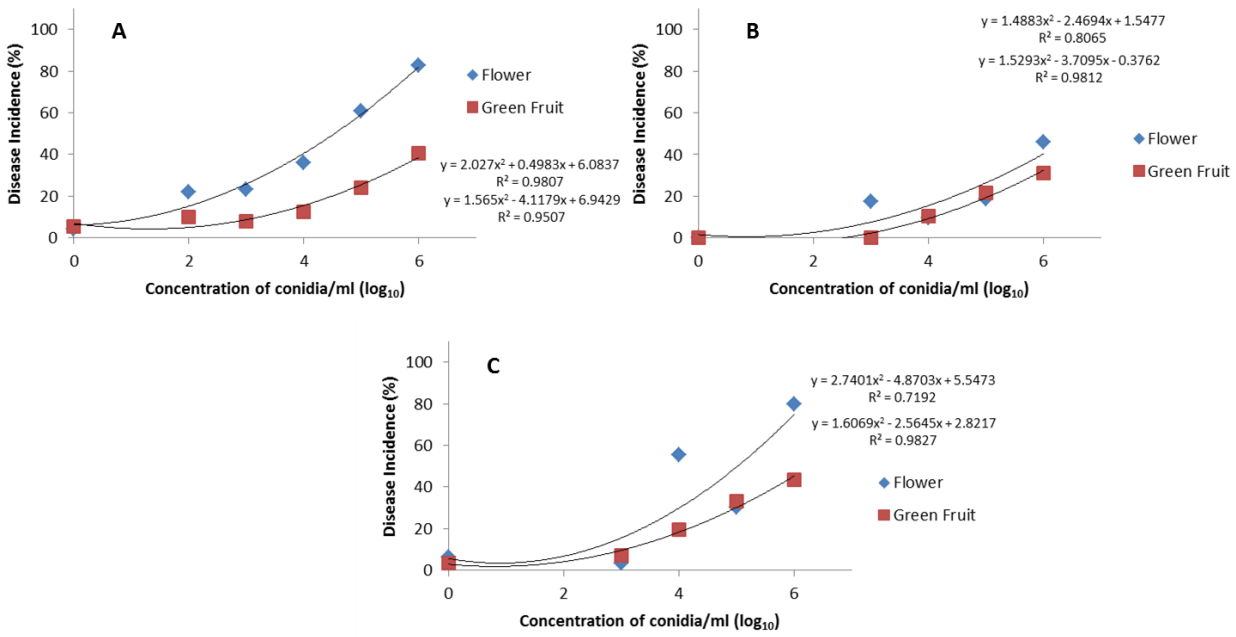

Figure 3-1. Regression of *Colletotrichum acutatum* inoculum concentration on Anthracnose Fruit Rot incidence of flowers and immature fruit on different strawberry cultivars. A) Cultivar Camarosa. B) Cultivar Strawberry Festival. C) Cultivar Treasure. Results represent the mean incidence of three experiments for ‘Camarosa’ and two experiments for ‘Strawberry Festival’ and ‘Treasure’.

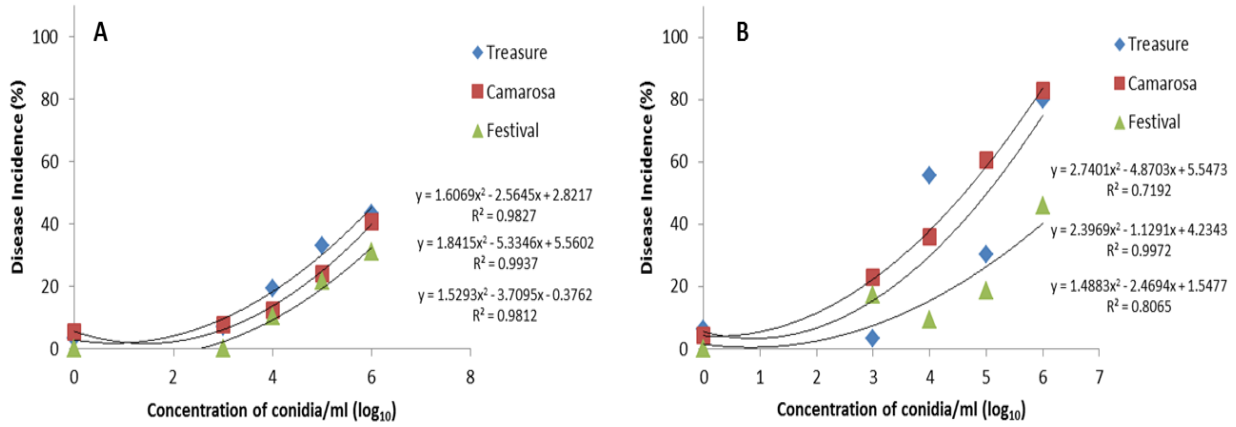

Figure 3-2. Regression of inoculum concentration of *Colletotrichum acutatum* on Anthracnose Fruit Rot development of strawberry cultivars on different plant organs. A) Inoculated immature fruit. B) Inoculated flowers. Data are the means of two field trials for 'Strawberry Festival' and 'Treasure' and three for 'Camarosa'.

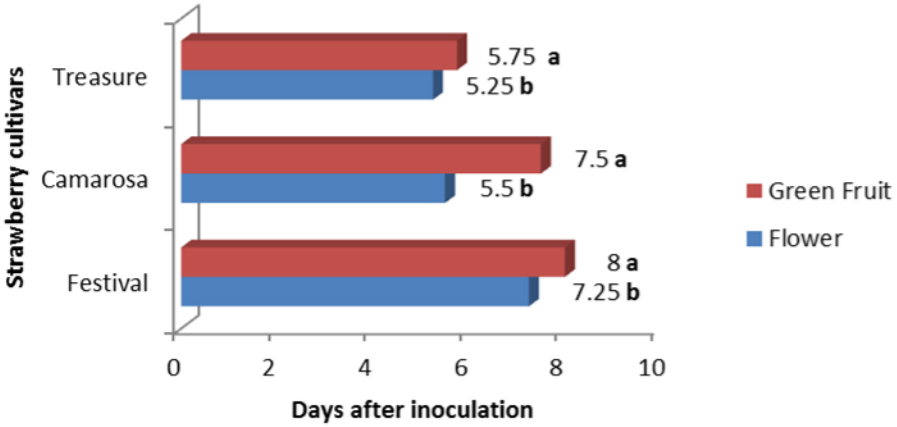

Figure 3-3. Incubation period (days from inoculation to symptom development) for immature fruit and flowers of different strawberry cultivars inoculated with 10^6 conidia/ml of *Colletotrichum acutatum.* Data is the mean of one field trial.

Table 3-3. Effect of strawberry cultivar on mean disease incidence of detached strawberry fruit inoculated with different inoculum concentrations.

Cultivar	Disease Incidence (%)
Camarosa	23.96 A[a]
Festival	13.54 B[b]

[a] Mean separation with rows followed by the same letter are not significantly different according to *t* test (LSD) ($P \leq 0.05$).

[b] Percentage data were transformed by arcsine prior to analysis, but non-transformed data are presented.

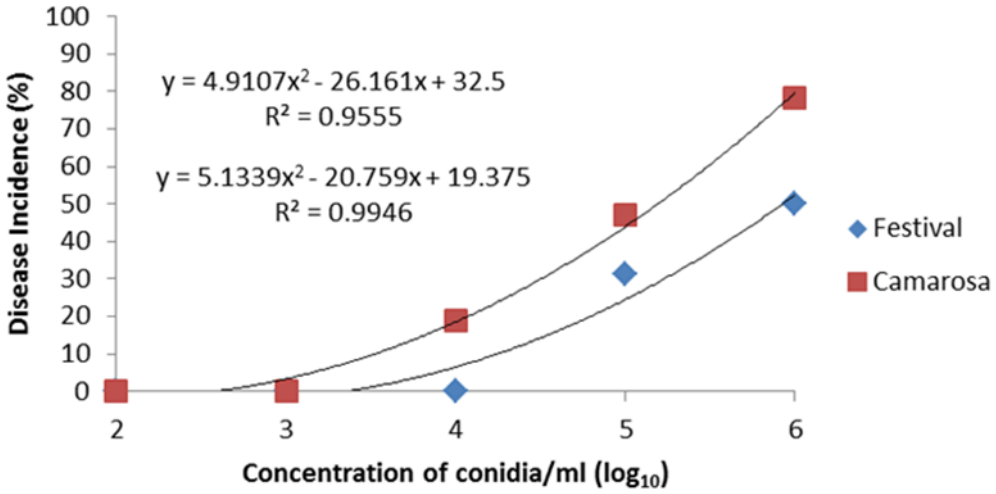

Figure 3-4. Regression of inoculum concentration of *Colletotrichum acutatum* and Anthracnose Fruit Rot development on detached immature strawberry fruit. Plants were inoculated with concentrations from 0 to 10^6 conidia/ml and maintained at continuous wetness and room temperature for nine days. Data are the means of four trials.

Table 3-4. Analysis of covariance of the parameters for field inoculation of *Colletotrichum acutatum* on immature strawberry fruit.

Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept	0.06449	0.1122	2	0.57	0.6234[a]
Treatment	0.07335	0.01120	96	5.76	<0.0001

[a] Percentage data were transformed by arcsine prior to analysis, but non-transformed data are presented.

Table 3-5. Analysis of covariance of the parameters for detached fruit inoculation of *Colletotrichum acutatum* on immature strawberry fruit.

Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept	0.08366	0.07658	1	1.09	0.4719 [a]
Treatment	0.1107	0.01402	93	7.90	<0.0001

[a] Percentage data were transformed by arcsine prior to analysis, but non-transformed data are presented.

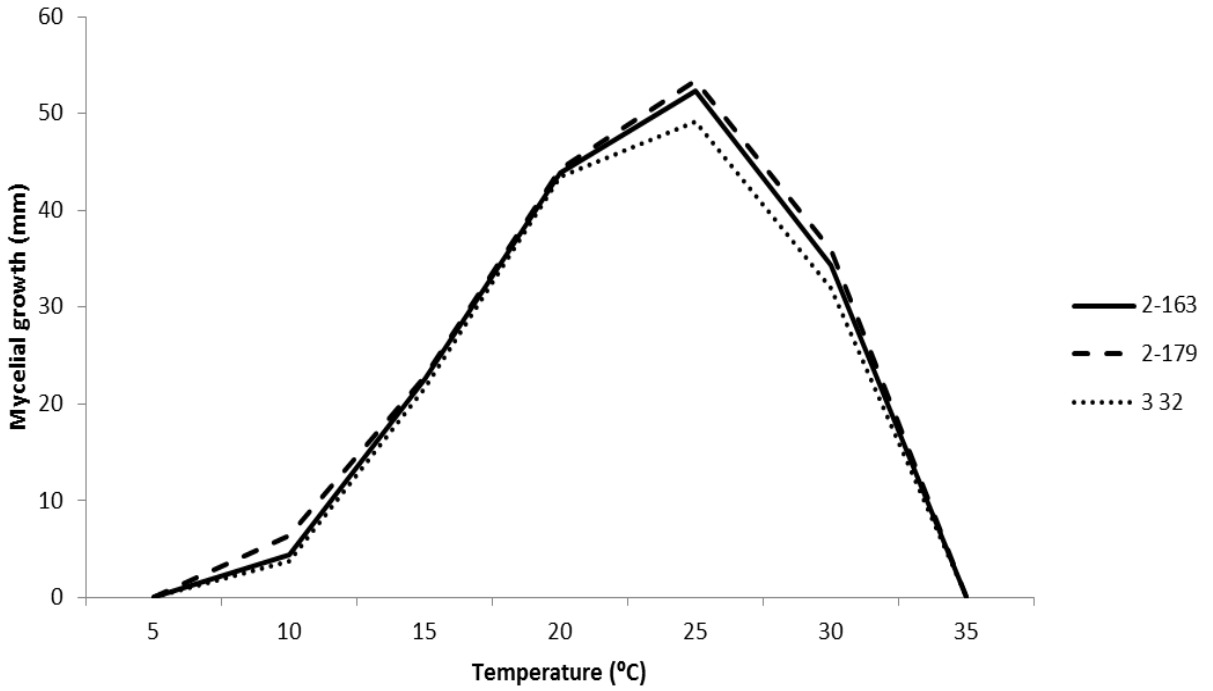

Figure 3-5. Effect of growth chamber temperature on *Colletotrichum acutatum* mycelial growth at seven days after incubation. Results represent the mean incidence of three experiments for each fungal isolate.

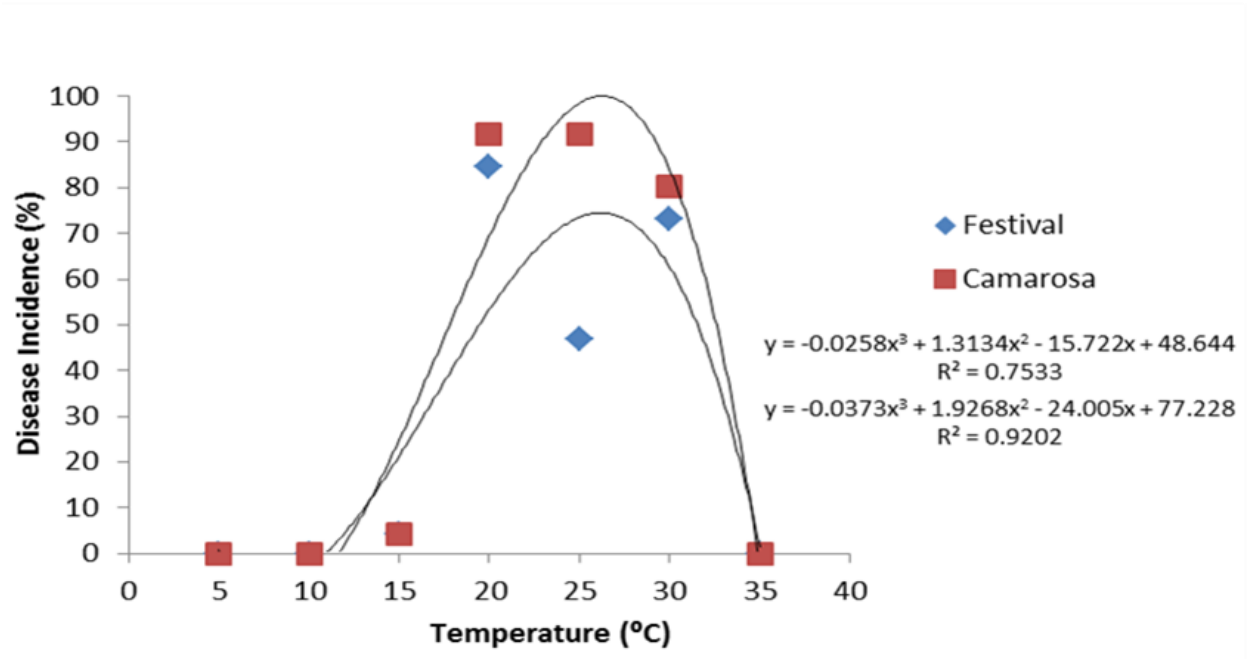

Figure 3-6. Regression of growth chamber temperatures from 5 to 35^{0}C on Anthracnose Fruit Rot development on detached immature strawberry fruit for 'Camarosa' and 'Strawberry Festival'. Curves were produced using a second order polynomial regression. Results represent the mean incidence of four experiments.

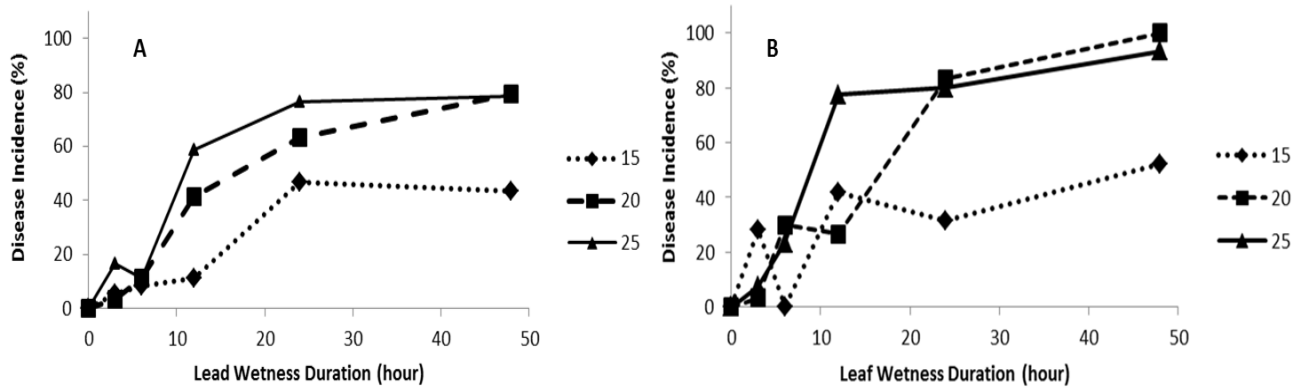

Figure 3-7. Infection of immature fruit of strawberry cultivars with different levels of susceptibility to Anthracnose Fruit Rot by *Colletotrichum acutatum* for wetness durations between 0 and 48 hours at 15, 20 and 25^{0}C. A) Cultivar Strawberry Festival. B) Cultivar Camarosa. Results represent the mean incidence of three experiments.

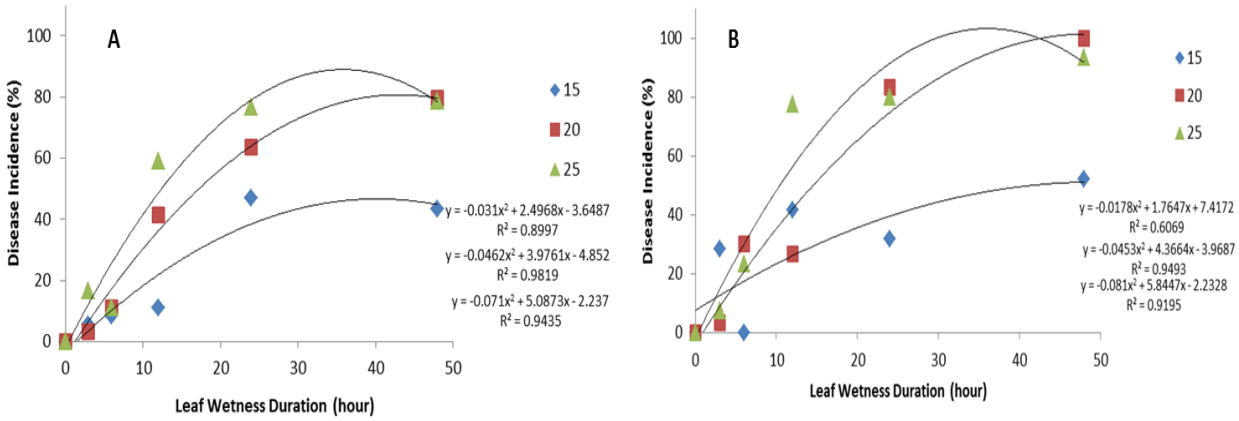

Figure 3-8. Regression of wetness duration on Anthracnose Fruit Rot development on immature fruit of strawberry cultivars with different levels of susceptibility after inoculation with *Colletotrichum acutatum* and incubation at temperatures between 15 and 25^{0}C and wetness periods from 0 to 48 hours. A) Cultivar Strawberry Festival. B) Cultivar Camarosa. Results represent the mean disease incidence of three experiments.

Table 3-6. Analysis of Variance for the effects of cultivar, wetness duration and temperature on the incidence of Anthracnose Fruit Rot of immature strawberry fruit.

Effect	DF	F Value	Pr > F
Cultivar	1	3.37	0.0706
Wetness Duration	4	32.98	<0.0001
Temperature	2	11.1	<0.0001
Cultivar x Wetness Duration	4	0.25	0.9105
Cultivar x Temperature	2	0.01	0.9937
Wetness Duration x Temperature	8	2.56	0.0167

Table 3-7. Analysis of Covariance for the effect of cultivar on the development of Anthracnose Fruit Rot of immature strawberry fruit at 15, 20 and 25^{0}C and at all wetness duration periods

Temperature (^{0}C)	Estimate	Standard Error	DF	T Value	Pr>t
15	0.09135	0.1097	32	0.38	0.4114[a]
20	0.09712	0.1472	32	0.66	0.5142
25	0.07407	0.1517	32	0.49	0.6287

[a] Percentage data were transformed by arcsine prior to analysis, but non-transformed data are presented.

Table 3-8. Regression equations for Anthracnose Fruit Rot incidence on strawberry cultivars after inoculation of immature fruit with 10^6 conidia/ml of *Colletotrichum acutatum* and incubation at different temperatures and wetness duration periods.

Cultivar	Temperature (^{0}C)	Regression equation	R^2
Festival	15	$y= -0.031x^2 + 2.4968x – 3.6487$	0.8997
	20	$y= -0.0462x^2 + 3.9761x – 4.852$	0.9819
	25	$y= -0.071x^2 + 5.0873x – 2.237$	0.9435
Camarosa	15	$y= -0.0178x^2 + 1.7647x + 7.4172$	0.6069
	20	$y= -0.0453x^2 + 4.3664x – 3.9687$	0.9493
	25	$y= -0.081x^2 + 5.8447x – 2.2328$	0.9195

CHAPTER 4
CONCLUSION

Anthracnose fruit rot has affected strawberry production in commercial fields in Florida and other areas where strawberry is grown for many decades. Weather conditions, such as moderate temperatures (≈ 20 and 25ºC) and humidity are present in Florida and are extremely conducive for AFR development. Growers usually rely on fungicides and cultural practices to control AFR. However, the excessive use of fungicides can be detrimental to the environment and lead to fungicide-resistance. Therefore, a disease forecasting system that predicts AFR outbreaks and provides growers with spray recommendations has been developed. However, more information about the weather effects on AFR development on strawberry cultivars and plant organs with different susceptibility levels was necessary to better adapt models to Florida's production systems.

In this project, we evaluated the effect of temperature, wetness duration and inoculum concentration on AFR development on flowers and fruit of highly and moderately susceptible strawberry cultivars. Increases in temperature, wetness duration and inoculum concentration generally increased disease incidence non-linearly for both cultivar and plant organ. The highly susceptible cultivars, Treasure and Camarosa, had higher disease incidence than 'Strawberry Festival' (moderately susceptible) and required a lower inoculum concentration for initial AFR symptom development. Flowers were more susceptible to *C. acutatum* than immature fruit for 'Treasure' and 'Camarosa', but were not different in susceptibility for 'Strawberry Festival'. The minimum inoculum concentration necessary for symptom development was lower for flowers than for immature fruit for 'Strawberry Festival' and incubation period was longer

for immature fruit than for flowers for all cultivars. Since infected transplants are the main source of inoculum in strawberry commercial fields, the quantification of *C. acutatum* on strawberry transplants with the aid of molecular tools will be helpful to determine initial spray applications. Furthermore, detached fruit trials generated similar results to those conducted in the field and allow the control of environmental conditions and require less space.

Mycelial growth of *C. acutatum* and AFR development on detached fruit of 'Camarosa and 'Strawberry Festival' were directly affected by increasing temperatures from 10 to 30ºC. Wetness duration and temperature were confirmed to be important microclimatic factors for AFR development on immature strawberry fruit. AFR symptoms for 'Camarosa' and 'Strawberry Festival' were observed when fruit were exposed to wetness durations after inoculation at all temperatures. A non-linear increase in disease incidence was observed for both cultivars as wetness duration periods and temperatures increased. Regression curves for AFR development for the different cultivars were not different and minimum wetness duration for symptom development was 3 hours at all temperatures. Even though there was no difference among the slopes of the curves for the two cultivars, disease incidence was always higher for 'Camarosa' than 'Strawberry Festival'. Based on our results, the disease infection threshold used in the model adapted for the "Strawberry Advisory System" should be the same for cultivars with different levels of susceptibility, such 'Camarosa' and 'Strawberry Festival'.

AFR is a major threat to Florida strawberry production and the excessive number of fungicide sprays and possibility of fungicide resistance concerns growers and

researchers. The use of a disease forecasting system adapted to Florida climatic conditions helps to limit fungicide applications to only when conditions are conducive to AFR development. The results of this project will be used to adapt the “Strawberry Advisory System” and advice growers to establish spray schedules according to plant organ susceptibility.

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BIOGRAPHICAL SKETCH

Bruna Balen Forcelini was born in Passo Fundo, Brazil. She lived in Gainesville, FL from 1994 to 1997 where her father got his Doctor of Philosophy degree in plant pathology at the University of Florida. In 2006, Bruna enrolled in the University of Passo Fundo where she would get her bachelor's degree in agronomy. In the fall of 2010, she decided to return to Gainesville for her final undergraduate internship at UF, where she started working with Dr. Natalia Peres. Then, in May, 2011 she began to pursue her graduate degree in plant pathology under the supervision of Dr. Peres. Her project consisted of the evaluation of the effects of temperature, wetness duration and inoculum concentration on anthracnose fruit rot development on flowers and fruit of strawberry cultivars with different levels of susceptibility.