POPULATION STRUCTURE AND PATHOGENICITY OF *Colletotrichum gloeosporioides* FROM STRAWBERRY AND NONCULTIVATED HOSTS IN FLORIDA

By

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Colletotrichum crown rot, caused by *Colletotrichum gloeosporioides,* limits strawberry transplant production in Florida summer nurseries and causes moderate plant losses during the winter season. Marker data have shown that *C. gloeosporioides* crown isolates are diverse and recombination contributes to this diversity. *Glomerella cingulata*, the teleomorph of *C. gloeosporioides,* has been observed on petioles, but the role of the meiotic cycle in crown rot disease is unknown. There is little evidence that the primary inoculum for infections comes from imported transplants or from debris from past seasons. *Colletotrichum gloeosporioides* has a broad host range and hosts other than strawberry could contribute inoculum. In the current study, isolates from strawberry crowns, noncultivated hosts, and perithecia were characterized using randomly amplified polymorphic DNA markers, AT-rich DNA banding patterns, pathogenicity assays, and laboratory crosses. Genetic data indicated the population from crowns produced

recombinant ascospores from perithecia and was found on noncultivated hosts close to fields. Pathogenicity to strawberry was variable among perithecia and noncultivated host isolates, but laboratory crosses indicated that sexual recombination occurred between isolates with different pathogenicity phenotypes. The same *C. gloeosporioides* population on strawberry was also found on two native hosts at sites distant from strawberry fields. A low frequency of isolates pathogenic to strawberry at these sites relative to sites close to strawberry fields suggests that there was selection for pathogenicity.

Although strawberry transplants for winter production are rarely propagated in Florida, crown rot resistant cultivars could make this desirable. To identify resistant cultivars and to determine if resistance is isolate specific, plant mortality was evaluated for cultivars following inoculation with an isolate differential. Repeatable differences in resistance were observed among cultivars, but no cultivar × isolate interactions. The lack of a cultivar × isolate interaction suggested limited biotrophic interaction between pathogen and host. Crosses using a cultivar with superior resistance and a susceptible cultivar as parents indicated that major genes contributing to resistance could be useful for breeding. In conclusion, it is unlikely that crown rot introductions into nurseries or fields can be prevented in Florida, but resistant cultivars could help make propagation of transplants between seasons possible.

CHAPTER 1
INTRODUCTION

At least three distinct species of *Colletotrichum* are responsible for anthracnose diseases of strawberry (*Fragaria × ananassa* Duchesne): *Colletotrichum acutatum* J. H. Simmonds (80), *C. gloeosporiodes* (Penz.) Penz. & Sacc. (teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenck) (52) and *C. fragariae* A. N. Brooks (9). All three of these *Colletotrichum* species have been reported to cause fruit rot (49,52,80), although in Florida and strawberry-producing regions around the world, *C. acutatum* appears to be responsible for most fruit rot epidemics (30,92). Colletotrichum crown rot, another economically important anthracnose disease, is caused by *C. gloeosporioides* and *C. fragariae* (10,52). Both species produce a reddish-brown necrosis in crown tissue that eventually causes plants to collapse (49). Infection of roots by *C. acutatum* may also cause plants to collapse, but this pathogen does not appear to colonize the crown tissue (37). *Colletotrichum fragariae* or *C. gloeosporioides* infection can also cause black leaf spot (51), lesions on petioles, and lesions on runners (9,27).

The first report of *C. fragariae* causing crown rot was nearly 50 years ago in 1935 (10). In 1984 isolates described as *G. cingulata*, the teleomorph of *C. gloeosporioides,* were reported to cause crown rot (52). These isolates were referred to by their teleomorphic name based on their ability to form perithecia when grown alone in culture and were distinguished from *C. fragariae* isolates by the color of their conidia in addition to the ability to form perithecia (52). Although *G. cingulata* was reported from strawberry plants in Florida, the plants appeared to have been infected in Arkansas,

Tennessee or North Carolina nurseries (53). *Glomerella cingulata* has also been isolated from strawberry plants in Europe and Japan (30). In 1992, it was noted that, among isolates classified as *C. fragariae,* a subset lacked pointed conidia and failed to produce phialidic setae and these isolates were reclassified as *C. gloeosporioides* (43). Molecular studies have confirmed that these isolates are distinct from those initially described as *C. fragariae* (38). The reason that these populations were not distinguished earlier may have been due to the belief that *C. fragariae* was simply *C. gloeosporioides* from strawberry (51), since they have been considered synonymous (97). Although never explicitly stated in the literature, molecular marker data from self-fertile *G. cingulata* isolates and self-sterile *C. gloeosporioides* isolates indicate that they are from genetically distinct populations (38,43). Currently, the self-sterile population from strawberry appears to be prevalent in Florida, although genotypes of these isolates have not been compared to reference homothallic isolates. Unfortunately, isolates often used in molecular studies to represent the *G. cingulata*/*C. gloeosporioides* population from strawberry were self-fertile and were not representative of the population in Florida, where crown rot caused by *C. gloeosporioides* is a problem (13,30).

A population study examining variation of randomly amplified polymorphic DNA (RAPD) marker data among *C. gloeosporioides* isolates on strawberry in Florida found that the *C. gloeosporioides* population was diverse and recombining, as most markers were in linkage equilibrium (92). *Colletotrichum gloeosporioides* also forms quiescent infections on petioles that can be detected after petiole tissue senesces and the fungus sporulates (66). Some of the isolates derived from these infections are pathogenic to strawberry in crown inoculation tests, whereas others are not. Occasionally perithecia

with morphology consistent with that of *G. cingulata* are found among *C. gloeosporioides* acervuli. Single-ascospore isolates from these perithecia are self-sterile in culture, suggesting that they may be from the same population responsible for crown rot. However, the genetic relationship between ascospore isolates and isolates from diseased crown tissue has not been examined. In addition, the ability of these isolates to cause crown rot has not been confirmed and recombination among progeny from perithecia has not been validated.

In southeastern states such as Florida and Louisiana, strawberries are grown as an annual crop. Plants are established from late September to early November onto raised, methyl-bromide-fumigated beds covered with polyethylene film. Harvest usually begins during the month of December in Florida. In this region, Colletotrichum crown rot is a serious disease and is especially devastating if growers produce their own transplants in summer nurseries (53), because both *C. fragariae* and *C. gloeosporioides* grow and reproduce best under moist conditions at temperatures exceeding 25^{o}C (81). Along with providing chilling to induce floral bud initiation, the high incidence of crown rot in summer nurseries in Florida is one of the primary reasons why transplant production for the winter season has moved to northern states and provinces of Canada (53). Although the movement of summer nurseries out of Florida has dramatically reduced the incidence of crown rot in production fields in this state, a portion of plants still become infected. Analysis of the *Colletotrichum* species infecting crowns revealed that they were primarily *C. gloeosporioides* (92). In contrast, *C. fragariae* accounted for most of the mortality due to crown rot in Louisiana production fields (65).

In Florida, *C. gloeosporioides* does not appear to have the capability of surviving over the summer between seasons on plant debris (93). *Colletotrichum gloeoporioides* cannot be isolated from petioles of transplants shipped from northern latitudes into Florida, although it can be obtained from petiole tissue after plants are set in the field (66). Depending on the nursery source, *C. acutatum* can be isolated from petiole tissue of transplants. Because *C. gloeosporioides* does not appear to be introduced into fields each season on transplants or plant debris from the previous season, alternative host species may play a role in providing primary inoculum for crown rot epidemics. Although there is no genetic or pathogenicity data to confirm this hypothesis, several characteristics of the *C. gleoesporioides* species assemblage indicate that this may be the case. *Colletotrichum gloeosporioides* has a broad host range (69). Cross-inoculations indicate that isolates can produce disease symptoms on hosts other than those from which they were isolated (1). Although use of rapidly evolving genetic markers has identified host-specific subpopulations within *C. gloeosporioides* (47,92), some isolates appear to lack any host specificity (1). *Colletotrichum gloeosporioides* is also a common endophyte on numerous tropical forest plants and there does not appear to be any discernible host specificity among isolates from these plants (60).

The best way to control crown rot in Florida production fields is to eliminate it from nurseries where plants are propagated (53). Movement of transplant production from Florida to temperate regions has effectively done this. If Colletotrichum crown rot is observed during the production season, it is usually at either the beginning or end of the season when temperatures are high and generally no more than a few percent of plants in a field die. Because the conditions for growth and spread of *Colletotrichum*

gloeosporioides are not ideal during the relatively cool weather that coincides with the production season, it is not likely to be the focus of chemical control programs. Weekly fungicide applications are generally designed to control gray mold, anthracnose fruit rot, and powdery mildew. However, both preventive and systemic fungicides used in these programs have activity against *C. gloeosporioides*. Prior to the migration of nurseries to cooler climates, management of soil fertility, fungicide applications, host resistance, and sanitary measures were all employed to control the disease. Measures included reduction of fertilizer to maintenance levels during July and August, two fungicide applications per week and after rain events, careful selection of runners to be used in nurseries, and use of resistant cultivars such as Dover (53). Fungicides with activity against *C. gloeosporioides* include captan, benzimidazoles, and QoI fungicides (J. Mertely, *personal communication*). Resistant cultivars can be successfully propagated in Florida and local production of transplants is one means of lowering costs to growers, but cultivars such as Dover that are relatively resistant to crown rot are not grown on a large scale because they lack desired yield and fruit characteristics. Levels of resistance to crown rot in cultivars currently used for strawberry production in Florida have not been documented and it is conceivable that one or more of these cultivars could be sufficiently resistant to crown rot to justify attempts at local propagation. Identification of cultivars with resistance in a genetic background with desirable fruit and yield properties might also help future breeding efforts. The resistance of cultivars to multiple *C. fragariae* isolates has been investigated (28,48,81). The resistance of cultivars to self-sterile *C. gloeosporioides* isolates that are responsible for most of the crown rot observed in Florida

has not been. This species is more variable than *C. fragariae* and isolate specific resistance could be more prevalent.

This dissertation was designed to address some of the fundamental questions that remain regarding crown rot caused by *C. gloeosporioides* in Florida. Original research is presented in the following four chapters. Chapter 2, the first chapter following this introduction, examines the genetic relationship of single-ascospore isolates from perithecia collected from senescent petioles to isolates known to produce crown rot on strawberry. Both pathogenicity and mating compatibility of isolates are examined to determine if the perithecia observed on strawberry petioles represent the teleomorph of the self-sterile population that causes crown rot. Chapter 3 examines the genetic relationship between isolates from strawberry crown and isolates from noncultivated hosts growing close to strawberry fields. In this chapter, marker frequencies, as opposed to the ability of isolates to mate, are examined along with the occurrence of the pathogenic phenotype to determine if noncultivated hosts can provide inoculum for crown rot epidemics. In chapter 4, *C. gloeosporioides* from two native host species are examined at sites distant from strawberry production and compared to those close to strawberry production. Because the strawberry industry in Florida is highly concentrated (5) and strawberry plants are not native to Florida (26), sampling host populations away from strawberry fields may provide information regarding the origin of the pathogen and whether selection for pathogenicity on strawberry might occur in areas where it is grown in abundance. In chapter 5, resistance of cultivars to a group of *C. gloeosporioides* isolates is examined to determine the mechanism of resistance to *C. gloeoporioides* and to identify cultivars with genes that could be used in future breeding programs. In

chapters 2 and 4, data regarding the inheritance of genes controlling pathogenicity for a cross between *C. gloeosporioides* isolates and resistance to crown rot for a cross between strawberry cultivars are presented.

CHAPTER 2
SEXUAL RECOMBINATION AND PATHOGENIC VARIATION AMONG ISOLATES OF *Colletotrichum gloeosporioides* ON STRAWBERRY

Introduction

Crown rot of strawberry caused by self-fertile *Glomerella cingulata* strains was first reported in Florida in 1984 (52). In addition to the ability of these strains to produce fertile perithecia when grown alone in culture, they were distinguished from *Colletotrichum fragariae* by the production of white rather than salmon-colored conidia. A more recent evaluation of *Colletotrichum* species responsible for strawberry diseases indicated that isolates previously characterized as *C. fragariae* consisted of two morphologically distinct groups of isolates (43). One group of isolates conformed to the initial description of *C. fragariae* and the other group was reclassified as *C. gloeosporioides*. Isolates in the second group produced more oblong conidia that were rounded at both ends and no conidia were formed on the setae. Unlike isolates previously classified as *G. cingulata*, fertile perithecia were not formed when these isolates were grown singly in culture. However, these isolates formed fertile perithecia morphologically similar to *G. cingulata* when paired in culture. No teleomorph has been described for isolates classified as *C. fragariae*. Molecular analysis using AT-rich DNA band patterns, arbitrarily primed polymerase chain reaction (PCR), and sequence data from the internally transcribed spacer 1 region of the rDNA repeat revealed that isolates classified as *C. fragariae* were similar to each other, whereas *G. cingulata*/*C. gloeosporioides* isolates fell into two groups with a high level of similarity among

isolates within each group (38,39,83). These two *G. cingulata*/*C. gloeosporioides* groups were designated Cgl-1 and Cgl-2 (38,39). Isolates found to form the *G. cingulata* state when grown singly fell in the Cgl-1 group whereas those isolates that reproduce only clonally or outcross were in group Cgl-2 (39,43).

In Florida, *C. gloeosporioides* is the *Colletotrichum* species most frequently isolated from the crowns of wilted strawberry plants (92). Self-fertility within this *C. gloeosporioides* population is rare. Analysis of RAPD marker data revealed a high level of diversity among isolates and a low level of linkage disequilibrium among markers (92). Both findings are consistent with genetic recombination within this population, although there is no direct evidence. *Colletotrichum gloeosporioides* also infects strawberry petioles producing asymptomatic quiescent infections in Florida (66). Acervuli are produced after the petiole tissue has senesced. Some isolates from petiole tissue are capable of causing crown rot and plant collapse and others are not. In addition to acervuli of *C. gloeosporioides*, perithecia morphologically similar to *G. cingulata* are occasionally observed on senescent petioles (66). The relationship of isolates produced from ascospores from these perithecia to isolates from crown tissue has not been determined. It is also not certain whether the perithecia on petioles are from a self-fertile strain or the result of recombination between two heterothallic strains nor whether ascospore isolates vary in pathogenicity as do isolates from acervuli.

This study examines the relationship between isolates of *G. cingulata* from perithecia on strawberry petioles to *C. gloeosporioides* isolates known to cause disease on strawberry. AT-rich DNA banding patterns of single-ascospore isolates from perithecia were compared to those from the two genotypically distinct *G. cingulata/C.*

gloeosporioides groups known to cause disease on strawberry. Recombination among progeny was evaluated by comparing RAPD markers of isolates from the same perithecium. The ability of crown rot isolates, pathogenic isolates from single ascospores, and nonpathogenic isolates from single ascospores to recombine was evaluated in laboratory crosses. In addition to studies conducted to define biological species boundaries, segregation analysis of marker data and pathogenicity phenotype was conducted for a cross between a nonpathogenic ascospore isolate and a crown rot isolate.

Materials and Methods

Perithecium Production on Strawberry Petioles

Perithecia were collected from senescent petioles during a study to evaluate the effectiveness of freezing tissue to detect latent infections of *Colletotrichum* spp. on strawberry (66). In the study, healthy petioles with no visible lesions were removed from field-grown plants in plots untreated with any fungicides, cut to lengths of 5 to 7 cm and frozen for 1 to 2 h at –15ºC. After thawing, petioles were surface sterilized for 1 min in 0.5% NaOCl plus 20 µl/L Tween 20, rinsed with sterile water, and placed on moistened filter paper inside petri dishes. Petri dishes were placed in clear plastic boxes and incubated on a laboratory bench at 23-25ºC under continuous fluorescent light. Petioles were monitored for perithecia production for 21 days. When detected, a single perithecium was transferred with a scalpel to a microscope slide and gently crushed in a drop of sterile water between the slide and a cover slip. If ascospores were present in a perithecium and *Colletotrichum* conidia were not observed, as much vegetative tissue as possible was removed and the suspension containing a cluster of asci and ascospores was transferred to 0.75 mL sterile water in a test tube. Ascospore release from asci was

stimulated by repeatedly pipetting the suspension. The suspension was then spread on semi-selective media (16 g potato dextrose broth, 14 g agar, 250 mg ampicillin, 150 mg streptomycin sulfate, 5 mg iprodione, 100 µl tergitol, and deionized water to 1 L) and incubated overnight. Germinating ascospores were subsequently transferred from the semi-selective media with a sterile scalpel and transferred onto potato dextrose agar (PDA).

Imaging and Morphology

Perithecia from field samples were examined using a Zeiss Stemi SV-6 stereo dissecting scope (Carl-Zeiss-Stiftung, Oberkochen, Germany) and asci with a Zeiss Axiolab microscope using brightfield optics at 400× magnification. Microscopic images were captured using a Spot digital camera system (Diagnostic Instruments, Inc., Sterling Heights, MI). Dimensions and morphology of conidia were based on measurements taken on 75 conidia from three isolates.

Fungal Isolates and Analyses

Eight perithecia (P1-P8) were recovered from strawberry cultivars 'Camarosa', 'Strawberry Festival', and 'Rosa Linda' during the 1998-1999 and 2000-2001 growing seasons at the University of Florida Gulf Coast Research and Education Center (GCREC) in Dover, Florida. Seven to ten ascospore isolates were obtained from each perithecium. Pathogenicity to strawberry was determined for all ascospore isolates. The AT-rich DNA banding pattern was determined for one isolate from each perithecium and compared to banding patterns from Cgl-1 and Cgl-2 genotype isolates known to be pathogenic on strawberry. To determine if ascospores from perithecia on petioles were produced by recombination between two or more fungal strains, RAPD DNA bands were amplified

from a subset of four to six isolates from each perithecium. In addition to progeny from perithecia found on petiole tissue, RAPD bands from progeny of laboratory crosses between two pathogenic ascospore isolates, two nonpathogenic ascospore isolates and a cross using pathogenic and nonpathogenic ascospore isolates as parents were analyzed for recombination. Pathogenic ascospore isolates P1-9 and P8-1 and nonpathogenic ascospore isolates P2-6 and P3-3 were used as parents in these crosses. For quick reference regarding the perithecium and pathogenicity phenotype of these isolates, they are also referred to as P1-path, P8-path, P2-nonpath and P3-nonpath respectively. Isolates 97-15A and 99-51, representative of *C. gloeosporioides* genotype Cgl-2 came from crown rot samples submitted to the GCREC diagnostic clinic by local growers in 1997 and 1999. Cgl-1 genotype isolates 311 and 329 were collected by C. M. Howard in Florida. These isolates were characterized previously with respect to morphological traits and AT-rich banding pattern (38,43). Crown isolate 97-15A was also used as a parent in a cross with nonpathogenic ascospore isolate P3-8. A description of all isolates used in this study and specific analysis in which they were employed is given in table 2-1.

Laboratory Crosses

Laboratory crosses were performed in 9 cm diameter plastic petri plates on sucrose-free Czapek-Dox medium (2 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, and 0.01 g/L $FeSO_4 \cdot 7H_2O$) containing 1.5% agar overlaying a piece of Whatman No. 3 filter paper (Whatman International, Maidstone, UK) (21). Isolates were inoculated on opposite sides of a plate approximately 7 cm apart and the plates incubated under fluorescent light at 24ºC. Perithecia formed a line at the point of contact between the two isolates. Few, if any, acervuli were present along the line of intersection between

isolates, greatly reducing the likelihood of conidial contamination. Single-ascospore isolates were obtained from fertile perithecia as described above for isolations from petiole tissue.

DNA Isolation

Mycelia was collected from 2- to 4-day-old cultures grown in 100 mL of Emerson media (4g/L yeast extract, 15 g/L Soluble Starch, 1g/L K_2HPO_4, and 0.5 g/L $MgSO_4$) by vacuum filtration through Whatman No. 3 filter paper. After transfer to 15 mL tubes, mycelia was dried overnight in a centrifugal evaporator. Sixty mg of the dried mycelia were then suspended in 750 µl of DNA extraction buffer consisting of 700 mM NaCl, 50 mM Tris(pH 8.0), 10 mM EDTA(pH 8.0), 1% CTAB, and 1% β-mercaptoethanol and heated to 65ºC for 2 h with periodic shaking. Particulate material was pelleted by centrifugation at 12,000× *g* for 10 min, the supernatant removed, and extracted once with chloroform:isoamyl alcohol (24:1). Two volumes of 100% ethanol were added to the aqueous extract and the mixture incubated at room temperature for 10 min. Nucleic acids were pelleted from the ethanol solution by centrifugation at 12,000× *g* for 10 min. Subsequently the pellet was washed with 100% ethanol and resuspended in 400 µl 1× TE buffer containing 10 µg/mL RNase for 1 h at 37ºC. Ribonuclease was removed from the nucleic acid solution by extraction with 400 µl phenol/chloroform/isoamyl alcohol (25:24:1). To the aqueous extract, 1/10 volume 3 M sodium acetate and 2.5 volumes of ethanol were added to precipitate DNA. This solution was incubated at –20ºC for 1 h and the DNA pelleted at 12,000× *g* for 10 min. The DNA pellet was washed once with 1 mL 80% ethanol, dried, suspended in 100 µl 1× TE buffer, and stored at –20ºC.

Marker Analysis

AT-rich DNA bands were identified by digesting 5 to 10 µg genomic DNA with the restriction enzymes HaeIII or MspI (103). Digested DNA was separated on a 1% agarose gel in 1×TBE buffer for 24 h at 50 V and subsequently stained with ethidium bromide. Successful differentiation of *Colletotrichum* species and subpopulations using this technique has previously been demonstrated (38).

Randomly amplified DNA fragments were obtained using the tetranucleotide repeat primers $(ACTG)_4$ and $(GACA)_4$, the trinucleotide repeat primer $(TCC)_5$, and two short oligonucleotides 5'-GTGAGGCGTC-3' and 5'-GATGACCGCC-3' referred to as OPC-2 and OPC-5 (Operon Technologies, Alameda, CA). Amplifications were carried out under mineral oil in a 20-µl volume containing 1× reaction buffer (50 mM Tris(pH 8.3), 0.25 mg/mL BSA, 2 mM $MgCl_2$, 0.5% Ficoll, and 1 mM Tartrazine), 200 µM dNTP, 1 unit Taq polymerase and 20 ρmol primer/reaction [primers $(ACTG)_4$, $(GACA)_4$, and $(TCC)_5$] or 8 ρmol primer/reaction [primers OPC-2 and OPC-5]. Cycling parameters for the PCR reactions consisted of a 5-min denaturing step at 95°C followed by 30 cycles of 1 min at 95ºC, 1 min at 48ºC, and 2 min at 72ºC for primers $(ACTG)_4$ and $(GACA)_4$; 34 cycles of 1 min at 95ºC, 1 min at 46ºC, and 1.5 min at 72ºC for primer $(TCC)_5$ or 38 cycles of 1 min at 95ºC, 1 min at 35ºC, and 2 min at 72ºC for primers OPC-2 and OPC-5. The amplified products were separated by electrophoresis through a 1.5% high resolution blend agarose (3:1) gel or 2% analytical grade agarose gel in 1× TAE buffer. Gels were photographed on a UV transilluminator after staining with ethidium bromide.

DNA fingerprints were obtained for progeny from the cross between crown rot isolate 97-15A and nonpathogenic perithecium isolate P3-8 using a digoxigenin-labeled

$(CAT)_5$ oligonucleotide fingerprinting probe. For this analysis, 5 to 10 µg of PstI digested DNA were separated on a 1% gel in 1× TAE for 16 to 24 h at 25 to 35 V. Gels were incubated subsequently for 30 min in 1.5 M NaCl/0.5 N NaOH buffer followed by 30-min and 15-min incubations in 1 M Tris (pH 7.4)/1.5 M NaCl. Capillary transfer of DNA to nylon membrane was conducted with 10× SSC buffer overnight. DNA was UV cross-linked to the membrane prior to prehybridization in 5× SSC buffer, 0.1% N-lauryl-sarcosine, 0.02% SDS, and 1% blocking reagent (Roche Applied Science, Mannheim, Germany) at 30ºC for 4 h. Hybridization was carried out in the same buffer with 10 ρmol/mL oligonucleotide probe at 30ºC overnight. Following hybridization, blots were washed twice with 2× SSC/0.1 % SDS for 5 min at room temperature and twice with 0.5× SSC/0.1 % SDS at 30ºC for 5 min. The probe was detected with an anti-digoxigenin Fab fragment conjugated to alkaline phosphatase and NBT/BCIP color substrate according to instructions supplied with a DIG nucleic acid detection kit (Roche Applied Science, Mannheim, Germany).

Resistance to benomyl among progeny of the cross between isolates 97-15A and P3-8 was determined by growing fungi on PDA amended with 5 µg/mL benomyl. Susceptible isolates did not grow at this concentration, whereas growth of resistant isolates was not affected. Benomyl resistance was found to segregate at a 1:1 ratio in crosses with *Glomerella graminicola*, and a point mutation in a β-tubulin gene was shown to confer benomyl resistance in *C. gloeosporioides* f. sp. *aeschynomene* (14,95).

A polymorphism observed among AT-rich DNA bands was identified with the restriction enzyme MspI and used to determine mitochondrial inheritance among progeny of the cross between 97-15A and P3-8. That the polymorphic MspI band was comprised

of mitochondrial DNA was determined by comparison to MspI-digested DNA from purified mitochondria. Purified mitochondrial DNA was isolated from approximately 6 g wet weight mycelia collected onto a filter disk using a modification of the method described for isolation of *Epichloë typhina* mitochondrial DNA (77). Mycelia was ground in 30 mL buffer containing 15% sucrose, 10 mM Tris-HCl(pH 7.5), and 0.2 mM EDTA(pH 7.5) at 4ºC. Nuclei and cellular debris was removed by centrifugation at 1,000× *g* for 10 min and the supernatant was saved. The pellet was re-extracted in 20 mL buffer, ground, and debris removed by centrifugation at 1,000× *g* for 10 min. The supernatant from both centrifugations was pooled and centrifuged at 15,000× *g* for 15 min. The pellet was suspended in 10 mL 20% sucrose, 10 mM Tris-HCl(pH 7.5), and 0.01 mM EDTA(pH 7.5) and centrifuged at 15,000× *g* for 15 min. Following centrifugation, the pellet was resuspended in 5 mL buffer containing 1.75 M sucrose, 10 mM Tris-HCl(pH 7.5), 5 mM EDTA(pH 7.5), 12 mM $MgCl_2$, 100 µg/mL DNase, and 50 µg/mL RNase and incubated for 1.5 h at 4ºC. Mitochondria were pelleted from this solution at 20,000× *g* for 10 min. The pellet was suspended in 5 mL of the same buffer without $MgCl_2$ or nuclease and pelleted a second time at 20,000× g. The pellet was then suspended in 0.5 mL lysis buffer (0.44 M sucrose, 1% SDS) and incubated at 37ºC for 30 min. Lysis buffer was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed by an extraction with chloroform:isoamyl alcohol. DNA was precipitated by addition of 1/10 volume 3 M sodium acetate and 2.5 volumes ethanol. DNA was subsequently washed with 70% ethanol, dried, and resuspended in 1× TE.

Contour-clamped Homogeneous Electric Field Gel Electrophoresis

High molecular weight DNA was prepared from agarose embedded conidia using a modification of the method described for *C. lindemuthianum* (72). Conidia from 5- to 7-day-old cultures were harvested in sterile water and the concentration of conidia determined using a haematocytometer. Conidia were pelleted and suspended in SCE (1 M sorbitol, 50 mM sodium citrate, 50 mM EDTA, pH 5.7, and 20 μM DTT) containing 75 mg/mL glucanex at a concentration of 8×10^8 conidia/mL. An equal volume of 1.2% low melting point agarose in SCE was then added to the conidial suspension at 42ºC and the mixture cast into 10 mm × 5 mm × 0.5 mm wells. Once solidified, plugs (1 to 4 each) were incubated in 0.5 mL SCE buffer at 37ºC overnight. The plugs were then rinsed with 1 mL of EST (0.5 M EDTA, 0.1 M Tris-HCl, and 1% lauryl sarcosyl, pH 9.5) and incubated in 1 mL EST containing 2 mg/mL proteinase K at 50ºC for 48 h with one change of solution. After the proteinase digestion was complete, the buffer was removed and plugs incubated 0.5 h in 1 mL 50 mM EDTA (pH 8.0) at room temperature. This incubation was repeated twice with fresh buffer. Plugs were then stored at 4°C in 50 mM EDTA (pH 8.0) until used. Chromosomes were separated on a CHEF-DR II pulsed field electrophoresis system (Bio-Rad, Hercules, CA) at 200 V in 0.5× TBE buffer using a run time of 24 h and 60 to 120 s ramped switch time. This run condition resolves chromosomes less than 1200 kb in length.

Pathogenicity Tests

To determine if isolates were pathogenic on strawberry, approximately 0.1 mL of 1×10^6 conidia/mL inoculation solution was injected directly into crown tissue of greenhouse grown strawberry plants (cv. Camarosa) as described previously (66).

Conidial suspensions were prepared in sterile water from 7-day-old cultures grown on PDA. Plants injected with sterile water only served as controls. Plants were monitored weekly over 28 days for symptoms of crown rot (wilting and collapse of plants). For single-ascospore isolates from petiole tissue, three plants were inoculated with each isolate on two dates. Isolates that caused the collapse of two or more plants for both inoculation dates were classified as pathogenic and those which failed to cause collapse of at least two plants for both inoculations were classified as nonpathogenic. If an isolate caused collapse of two or more plants after one inoculation date and not the other, the assay was repeated a third time and data from this experiment used to classify the isolate. For progeny from a cross between the crown rot isolate 97-15A and nonpathogenic perithecium isolate P3-8, pathogenicity was determined from inoculation of three plants on a single date.

Statistical Analysis

A hierarchal analysis using theta (θ) was employed to determine if there was population subdivision between perithecia that yielded pathogenic isolates and perithecia that had only nonpathogenic isolates. The perithecium that an isolate was obtained from defined the subpopulation. Values of θ were calculated using the method of Weir and Cockerham (99) with confidence intervals generated by bootstrapping over loci. Theta measures the correlation of alleles from individuals in the same population relative to all populations and is described by the equation $(Q - q)/(1 - q)$, where Q is the probability that two randomly sampled genes within a population are the same allele and q is the probability that genes randomly selected from different populations are the same allele (22). A hierarchal analysis was required due to repeated sampling within perithecia. All

values of θ were calculated using the software program TFPGA (Utah State University, Logan, UT). Chi-square analyses and a Wilcoxon-Mann-Whitney test were done using the SPSS 8.0 statistics package (SPSS Inc., Chicago, IL).

Results

Perithecial Morphology

The proportion of petioles with at least one perithecium ranged from 0% to 10% of those examined, depending on the sample date. Perithecia were typically globose, approximately 100 to 400 μm in diameter, dark, covered with gray mycelia, and found among acervuli of *C. gloeosporioides* (Fig. 2-1A). Each perithecium contained tens of asci holding three to eight ascospores each (Fig 2-1B). Asci were cylindrical to clavate and contained ascospores in both linear and alternately biserrate arrangements. Ostioles were not discernible. Ascospore shape was variable both within and among perithecia. Allantoid, ellipsoid, and oblong ascospores were observed in squashes. No fertile perithecia were observed in cultures of single-ascospore progeny, indicating that isolates were not homothallic when cultured on artificial substrate. The size and shape of conidia from isolates grown in culture were consistent with *C. gloeosporioides* from both strawberry and nonstrawberry hosts. Average conidial length was 16.8 ± 0.32 μm and width 5.07 ± 0.037 μm. Ninety-five percent were oblong with obtuse ends.

AT-rich DNA Analysis

AT-rich DNA banding patterns for the two Cgl-1 genotype isolates from strawberry (isolates 311 and 329) were identical to one another and the two isolates with a Cgl-2 genotype (isolates 97-15A and 99-51) were identical to one another (Fig. 2-2). Dice similarity between the two genotypes was 0.33. Isolates from six of the eight naturally

occurring perithecia (P1, P3, P4, P5, P6, P8) had AT-rich DNA banding patterns identical to the pattern of the Cgl-2 genotype isolates (Similarity = 1.0). The banding patterns of the two unique perithecial isolates (P2 and P7) were more similar to the Cgl-2 genotype than to the Cgl-1 genotype (0.83 vs 0.33 and 0.5) with the gain or loss of a single restriction site able to account for the difference between the pattern for these isolates and those for Cgl-2 isolates from strawberry.

Characterization of Naturally Occurring Isolates

RAPD markers were used to determine if segregation was occurring among isolates from the same perithecium. Using five RAPD primers, eleven polymorphic bands consistently amplified from the template DNA of single-ascospore isolates collected from perithecia on petioles. Bands at 2.15 kb and 1.15 kb for the primer $(ACTG)_4$; 0.9 kb for primer $(GACA)_4$; 2.0 kb and 1.9 kb for primer $(TCC)_5$; 1.9 kb for primer OPC-2; and bands at 2.15 kb, 2.0 kb, 1.8 kb, 1.6 kb, and 1.2 kb for primer OPC-5 segregated among progeny from at least one perithecium (Fig 2-3). For each of the eight perithecia, between one and five of the bands were different among progeny. Four of eight perithecia (P2, P3, P4, P6) contained only nonpathogenic isolates as determined by the greenhouse bioassay and two perithecia contained only pathogenic isolates (P5 and P8) (Table 2-2). Of the two perithecia with progeny that segregated for pathogenicity, only perithecium 1 had multiple representatives of each phenotype. Of the 68 isolates tested, the pathogenicity phenotype was the same in repeated experiments for 62 isolates. Of the six isolates in which there was a conflict in scoring, two were classified as pathogens and four as nonpathogens based on a third assay. Three of the four isolates classified as nonpathogens came from perithecia with progeny that were both pathogenic

and nonpathogenic. Based on RAPD markers, there was no evidence for population subdivision between perithecia with progeny pathogenic on strawberry and those with only nonpathogenic progeny ($\theta = 0.037$, 90% C. I. -0.078 to 0.149), although there was strong support for correlation of RAPD bands among progeny from the same perithecium ($\theta = 0.515$, 90% C. I. 0.393 to 0.634).

Laboratory Crosses

Thirty-six of 80 crosses attempted between single-ascospore isolates or between single-ascospore isolates and isolates from diseased crown tissue yielded perithecia with mature ascospores. Some isolates successfully mated with both parents that were compatible with one another in a third cross, indicating that mating compatibility was not determined by a single mating type locus with two alleles. Viable ascospore progeny were obtained from crosses in which both parents were nonpathogenic on strawberry, both parents were pathogenic on strawberry, as well as with parents that had different pathogenicity phenotypes. Recombination among progeny from one cross for each of these parent combinations was examined using RAPD markers. Of four progeny examined from each cross, at least three were recombinants (Fig 2-4), indicating that recombination had occurred in these crosses.

Forty-six progeny from the cross between isolate 97-15A, from a crown-rot affected plant and isolate P3-8, a nonpathogenic ascospore isolate, were evaluated for pathogenicity using the greenhouse bioassay. Of these 46 isolates, two were nonpathogenic and 44 were pathogenic on strawberry. A 2.5-kb MspI band observed only in mitochondrial DNA of isolate P3-8 was inherited by all progeny (Fig 2-5A, Table 2-3). Putative genomic DNA markers included benomyl sensitivity, three RAPD bands,

and three $(CAT)_5$ repeat bands (Fig 2-5B, 2-5C and 2-5D). Segregation of the 1.6-kb OPC-5 RAPD band, the 0.9-kb $(GACA)_4$ band, and two of the three bands identified by the $(CAT)_5$ repeat probe diverged from the expected 1:1 ratio assumed under the hypothesis that these markers randomly assort and identify a single site within the genome ($P < 0.05$) (Table 2-3). Segregation of the 1.8-kb OPC-5 RAPD band, benomyl resistance, and one of the $(CAT)_5$ repeat bands did not differ significantly from a 1:1 ratio. Of 25 progeny scored for all seven markers, three isolates inherited all of the dominant markers examined. It is unlikely that progeny were derived from contaminating parental material since only one of the progeny had a phenotype that was identical to either parent. The genotypes of the two isolates that were not pathogenic on strawberry were distinct from either parent.

Chromosomes in the size range of 220 to 1100 kb were very different between the parental isolates (Fig 2-6). Isolate 97-15A had four chromosomes ranging from 730 to 940 kb, whereas isolate P3-8 had five chromosomes ranging from 350 kb to 640 kb. The sum of sizes in this range was greater for isolate 97-15A than it was for isolate P3-8 (3310 kb vs. 2240 kb). Chromosome numbers for progeny ranged from three to six. Most progeny had either a different number of chromosomes or different sized chromosomes from either parent. The total length of chromosome DNA for the two isolates that failed to cause crown rot on strawberry was less than it was for ten isolates pathogenic on strawberry (1875 kb vs 3411 kb, $P = 0.03$, Mann-Whitney-Wilcoxon test).

Discussion

Perithecia with morphology consistent with *G. cingulata* were observed on strawberry petioles in a previous study, however the genetic relationship between single-ascospore isolates from these perithecia and isolates known to infect crown tissue was not

investigated (66). Self-sterile *C. gloeosporioides* isolates are most commonly isolated from crown tissue in Florida, although homothallic *G. cingulata* strains have also been isolated from strawberry in this state. It was conceivable that the population that produced the perithecia observed on petiole tissue came from either one of these populations or a population unrelated to those responsible for crown rot. In addition to differences in the ability of isolates to self-fertilize, the *G. cingulata/C. gloeosporioides* populations on strawberry can be distinguished from one another based on AT-rich DNA banding patterns. Self-fertile isolates have a Cgl-1 genotype banding pattern and self-sterile isolates have a Cgl-2 genotype banding pattern. The AT-rich DNA banding patterns observed for perithecial isolates indicate that they are from the same population or are closely related to isolates with the Cgl-2 genotype, which are most frequently isolated from crowns in Florida. Sexual reproduction has been suggested as playing a role in the reproduction of this population, since it is comprised of genetically diverse isolates and linkage disequilibrium among RAPD bands is not observed (92). The occurrence of ascospore isolates with identical or similar AT-rich DNA banding patterns to isolates from crown tissue supports the hypothesis that isolates responsible for crown rot reproduce sexually on strawberry. Further evidence for the occurrence of sexual recombination in this population comes from the analysis of RAPD banding patterns. From each perithecium examined, at least two unique banding patterns were observed among progeny indicating that ascsopores were produced by recombination of parental strains.

Because there is no information on parental genotypes for perithecia collected from field material, it is more difficult to be certain that unique banding patterns do not result

from poor reproducibility of RAPD bands. Evidence that RAPD genotype differences were representative of true genetic differences comes from the ability to reproducibly amplify scored bands from the same isolate and the observation that bands polymorphic among progeny from laboratory crosses were also different between parents. Further evidence that band differences were real and not artifacts of the PCR reaction comes from the correlation of marker data among isolates from the same perithecium. As measured by θ, the correlation of marker data among isolates from the same perithecium was 0.515. Assuming that only one male parent gives rise to ascogenous hyphae, RAPD bands segregate among progeny in a 1:1 ratio, and that there is random mating among individuals within a population, the expected value of θ for a heterothallic fungus would be 0.5. This value is well within the 90% confidence interval calculated for θ. The expected value of θ would be 1 for completely self-fertile isolates, and 0, if ascospores from the same perithecium are unrelated. Assuming that a fungal population is heterothallic, processes such as nonmendelian inheritance of markers would tend to bias measurements of θ towards 1 and multiple male parents toward 0. Although no attempt was made to determine the effect these processes might have on estimates of θ in this study, segregation of markers from crosses conducted in the laboratory indicate that nonmendelian inheritance might bias the estimate of θ upwards, whereas analysis of ascospore isolates collected from the field for other fungi indicate that multiple male parents could bias θ downward (34).

Both pathogenic and nonpathogenic single-ascospore isolates were obtained from perithecia. Most of the perithecia yielded ascospores with only one phenotype or the other. Although three of four perithecia yielding pathogenic ascospore isolates had AT-

rich DNA genotypes identical to three of the perithecia yielding only nonpathogenic ascospore isolates, it is conceivable that isolates with different pathogenicity phenotypes derived from different populations. This would occur if the mutation rate in mitochondrial DNA, which accounts for the bulk of AT-rich bands, was not fast enough to produce detectable polymorphisms to distinghuish the two groups. Several additional lines of evidence, however, support the hypothesis that pathogenic and nonpathogenic isolates derive from the same population. These include the ability of pathogenic and nonpathogenic isolates to produce recombinant offspring when crossed on agar, the occurrence of both phenotypes among isolates from perithecia 1 and 7, and there was no evidence for population subdivision based on RAPD band frequencies, although this analysis probably suffered from reduced statistical power as progeny from the four perithecia in each group only represented eight parental genotypes.

The most frequently occurring AT-rich DNA banding pattern from perithecia isolates matched that for isolates from strawberry crown, indicating that they are both derived from the same population. However, as noted above, mitochondrial DNA may fail to evolve fast enough to identify a recent divergence between the two groups. Additional evidence that crown and perithecia isolates are from the same population comes from the ability of strawberry crown isolates to successfully cross with perithecial isolates on agar. RAPD markers were not amplified from a population of crown isolates in this study. However, in the next chapter band frequencies from a population of strawberry crown isolates are reported. A comparison of these frequencies to those observed among perithecia provided no evidence for population subdivision (data not reported).

The cross between a crown isolate and a nonpathogenic perithecium isolate yielded a highly skewed distribution of progeny pathogenic on strawberry, suggesting that pathogenicity on strawberry is determined by genes at more than one locus or nonmendelian segregation of pathogenicity determinants encoded at a single locus. Plasmids and double-stranded RNA viruses localized within mitochondria have been shown to induce hypovirulence in several species of fungi (29,68). Mitochondrial restriction fragment length polymorphisms (RFLP) also correlate with host preference in *Mycosphaerella graminicola* populations (105). These studies suggest that mitochondrial inheritance of a pathogenicity or hypovirulence factor could account for the skewed distribution of pathogenicity among progeny. However, all progeny examined inherited the 1.6-kb MspI mitochondrial DNA fragment found in nonpathogenic isolate P3-8. Given that inheritance was skewed toward the pathogenic phenotype, it does not appear that factors associated with the mitochondria affect pathogenicity. Inheritance of assumed genomic markers and chromosomes indicate nonmendelian mechanisms govern inheritance in portions of the genome. In most studies examining segregation of markers in fungal crosses, a subset of markers typically deviate from expected ratios (36,76). However, in the cross between 97-15A and P3-8, the number of markers that deviated from a 1:1 segregation ratio was greater than that typically observed. Use of markers that require hybridization to simple sequence repeats might account for the skewed ratios, as a high proportion of bands identified with the $(CAT)_5$ probe fail to segregate at expected ratios in other fungi (32). However, simple sequence repeat loci do not segregate in a nonmendelian fashion at a higher frequency than restriction fragment length polymorphisms in plants (82). The occurrence of dominant markers at more than one

locus, inheritance of more than one homologous chromosome within progeny, or simply preferential inheritance of one or more chromosomes with limited recombination could also account for the skewed ratios. All of the skewed ratios result from a larger proportion of progeny inheriting the dominant marker, suggesting inheritance of more than one copy of the marker or more than one copy of homologous chromosomes. If a significant number of progeny were heterokaryons, one would expect skewed inheritance of dominant markers among progeny, but heterokaryon formation could be excluded for all but three of 25 isolates examined. The meiotic events producing the skewed ratios could also have arisen from genetic incompatibility of the isolates used in the cross as has been observed with geographically isolated strains of *Uromyces appendiculatus* (62) or alternatively they may be a normal occurrence in crosses of *C. gloeosporioides*. Segregation of markers in crosses between perithecial isolates was detected at a high rate by sampling only four progeny, indicating that segregation of markers in these crosses probably did not deviate substantially from mendelian ratios. This suggests that the diseased crown isolate and the ascospore isolate might have lacked genetic compatibility. In crosses of *C. gloeosporioides* from jointvetch and pecan most genomic markers displayed normal mendelian segregation ratios on agar, although use of jointvetch as substrate tended to skew inheritance of markers (21). Ultimately, segregation of co-dominant markers using more than one combination of parents will be required to determine normal mechanisms of segregation among isolates of this species from strawberry.

Distinct electrophoretic karyotypes have been observed for *C. gloeosporioides* isolates from *Stylosanthes* sp., with all of the variation within biotypes due to differences

in the size and number of small chromosomes that range from 0.27 Mb to 1.2 Mb in length and comprise approximately 15% or less of the total genome (63). In this size range, there were also differences in the size and number of chromosomes between isolates 97-15A and P3-8. Most progeny from the cross of these isolates had chromosome banding patterns distinct from each other and both parents. Although recombination appears to have produced some chromosomes that migrate at very different rates from those of the parental isolates, assortment of chromosomes produced most of the differences in banding patterns. This conclusion is based on the observation that most chromosomes inherited by progeny were identical or close to the size of chromosomes from parent isolates. No specific probes were generated for chromosomes, so it is not possible to determine with any certainty which ones might be homologous or if one isolate has coding sequence absent in the other. However, for isolate 97-15A, the sum of chromosome lengths was substantially greater than it was for isolate P3-8, indicating that this isolate may possess genes not found in isolate P3-8. Also, based on the size of progeny chromosomes it appears that chromosomes from parent 97-15A were inherited preferentially. Thus, inheritance of pathogenicity determinants on these chromosomes might account for the high proportion of offspring pathogenic to strawberry. There are a number of examples of genes encoded on chromosomes less than 2.0 Mb in length that are required for virulence on specific hosts, but dispensable for saprophytic growth (24,45). Often the small, dispensable chromosomes that encode these genes display nonmendelian inheritance in crosses (24).

The occurrence of progeny pathogenic to northern jointvetch seedlings has been reported from a cross between a pathogenic isolate from northern jointvetch and a

nonpathogenic isolate from pecan (19). None of the progeny from this cross consistently killed northern jointvetch seedlings. Because there was little evidence from marker data to suggest that nonmendelian mechanisms contributed to the skewed ratio of pathogenic to nonpathogenic progeny, it was suggested that multiple avirulence genes or multiple pathogenicity genes differentiate isolates pathogenic to jointvetch from those that are not and that the genetic requirements for successful infection of jointvetch are complex. In the cross between pathogenic crown isolate 97-15A and nonpathogenic ascospore isolate P3-8 results opposite to those observed for northern jointvetch were obtained, as most of the progeny were pathogenic to strawberry. Discounting the role that nonmendelian inheritance might play in the inheritance of pathogenicity to strawberry, it appears that there may be more plasticity with respect to genetic requirements for causing crown rot on strawberry. However, the nonpathogenic isolate in the current experiment was obtained from a latent infection on strawberry, not a distantly related host, and could already have met most of the physiological requirements for pathogenicity. Also of note, laboratory crosses done in the current study did not support that a single locus with two alleles regulated mating compatibility, a finding consistent with studies examining the mating system of *C. gloeosporioides* and other *Colletotrichum* species (20,94).

The bioassay used to determine pathogenicity phenotype was highly reproducible for most isolates, although several isolates from perithecium 1 were difficult to categorize. This perithecium yielded both pathogenic and nonpathogenic isolates. In field experiments examining plant mortality in response to inoculation with multiple *C. gloeosporioides* isolates presented in chapter 5, the isolates display quantitative differences in aggressiveness. This suggests that multiple genes affect pathogenicity on

strawberry and that the crown injection assay may identify isolates as pathogens only if they possess physiological capabilities that exceed a threshold. Since perithecium 1 yielded both pathogens and nonpathogens, the disease-causing ability of isolates from this source would lie close to the threshold. Also, given that many of the isolates from this perithecium are weak pathogens, one would expect environmental variation between experiments to have a greater effect on the outcome of pathogenicity tests using these isolates. Differences in temperature across experiments is one variable which may account for the conflicting results, as temperature has been shown to alter final mortality of plants inoculated with *G. cingulata* (70).

Colletotrichum gloeosporioides has been reported from a wide range of host species (69). Although reproductively isolated subgroups with narrow host ranges likely exist within the *C. gloeosporioides* species aggregate, there are populations of *C. gloeosporioides* in which isolates from different hosts are not genetically distinct from one another and isolates from different hosts can successfully recombine in culture (19,60). The acquisition of single-ascospore isolates that are able to interbreed and display variation in pathogenic ability on strawberry will benefit future studies examining the specific genetic requirements for pathogenicity and how they are distributed within the population on strawberry and other plant species that the population from strawberry might colonize.

In summary, it appears that *C. gloeosporioides* from strawberry is part of a recombining population that consists of strains both pathogenic and nonpathogenic to strawberry. The mechanism by which the pathogenicity phenotype is inherited in this population remains unclear. It is apparent that a gene or cluster of genes at a single

mendelian-segregating locus does not determine pathogenicity. However, based on the data derived from this study no conclusions could be made regarding wether pathogenicity is determined at a nonmendelian-segregating locus or that it is affected by multiple unlinked genes each having a quantitative effect on pathogenicity.

Table 2-1. *Glomerella cingulata/Colletotrichum gloeosporioides* isolates from Dover, FL used to compare AT-rich DNA banding patterns and for recombination studies

			Analysis[a]		
Isolates	Description	Tissue	AT-rich DNA	Recombination on petiole	Parent – agar crosses
P1-1 to P1-10[b]	Perithecium 1 ascospore	Petiole	P1-9	P1-3 to P1-4, P1-7 to P1-10	P1-9 (P1-Path)[c]
P2-1 to P2-10	Perithecium 2 ascospore	Petiole	P2-6	P2-7 to P2-10	P2-6 (P2-Nonpath)
P3-1 to P3-10	Perithecium 3 ascospore	Petiole	P3-3	P3-7 to P3-10	P3-3 (P3-Nonpath), P3-8
P4-1 to P4-10	Perithecium 4 ascospore	Petiole	P4-7	P4-5 to P4-7, P1-9 to P1-10	
P5-1 to P5-7	Perithecium 5 ascospore	Petiole	P5-3	P5-1 to P5-4	
P6-1 to P6-7	Perithecium 6 ascospore	Petiole	P6-3	P6-1 to P6-4	
P7-1 to P7-7	Perithecium 7 ascospore	Petiole	P7-3	P7-1 to P7-4	
P8-1 to P8-7	Perithecium 8 ascospore	Petiole	P8-1	P8-1 to P8-4	P8-1 (P8-Path)
311	Cgl-1 self-fertile	?	Yes		
329	Cgl-1 self-fertile	?	Yes		
97-15A	Cgl-2 conidial	Crown	Yes		Yes
99-51	Cgl-2 conidial	Crown	Yes		

[a]Subsets of isolates used to compare AT-rich DNA banding patterns, to compare RAPD bands among progeny from perithecia on petioles, and as parents for crosses on agar.

[b]Prefixes P followed by numbers 1 through 8 represent the individual perithecium sampled. Numbers following '-' represent specific ascospore isolates sampled from each perithecium, with only the range of these designations given in column 1. All isolates in column 1 were tested for pathogenicity to strawberry.

[c]Isolate codes in parentheses represent alternate names for isolates to the left. Alternate names identify the perithecium and pathogenicity phenotype on strawberry of the isolate.

Table 2-2. Crown rot pathogenicity phenotype of single-ascospore isolates from eight perithecia from naturally infected strawberry petioles

Perithecium	Phenotype		No. of ascospore isolates evaluated
	Pathogenic	Nonpathogenic	
1	6	4	10
2	0	10	10
3	0	10	10
4	0	10	10
5	7	0	7
6	0	7	7
7	6	1	7
8	7	0	7

Table 2-3. Segregation of mitochondrial DNA, fungicide sensitivity, RAPD bands, and $(CAT)_5$ bands from a cross between pathogenic crown isolate 97-15A and nonpathogenic ascospore isolate P3-8

	97-15A genotype	P3-8 genotype	$P > \lambda^2$
2.5-kb Msp I mitochondrial band	0	39	
Benomyl sensitivity	29	22	0.33
1.8-kb OPC-5 band	11	14	0.55
1.6-kb OPC-5 band	22	3	0.001
0.9-kb $(GACA)_4$ band	7	18	0.04
9.9-kb $(CAT)_5$ band	1	27	0.001
7.7- kb $(CAT)_5$ band	17	11	0.26
3.5-kb $(CAT)_5$ band	20	8	0.02

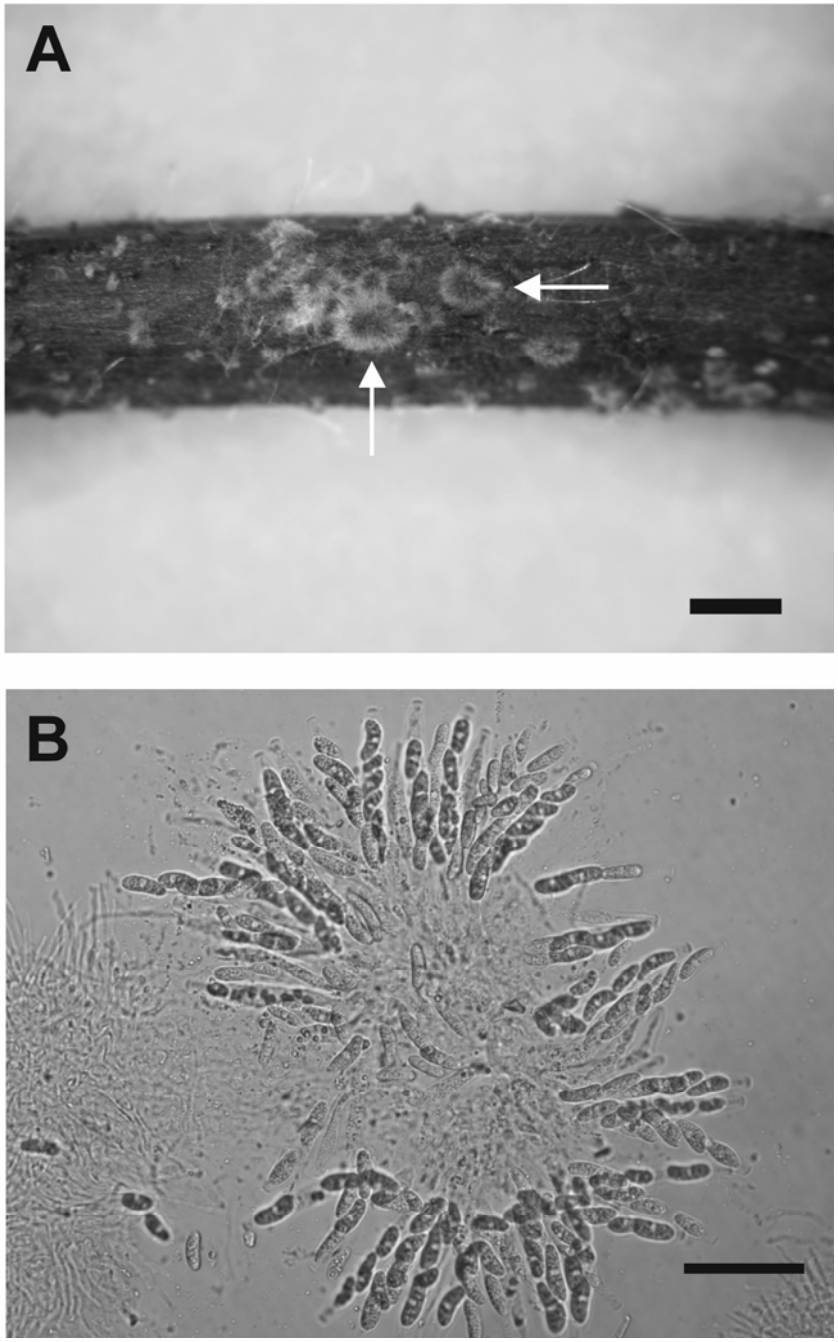

Fig. 2-1. *Glomerella cingulata* from strawberry petiole. (A) Perithecia of *G. cingulata* growing on a field-infected strawberry petiole after the petiole was freeze-killed and incubated at room temperature 2-3 wk. Bar = 1000 μm. (B) Squash showing asci and ascospores of a perithecium collected from a strawberry petiole. Bar = 50 μm.

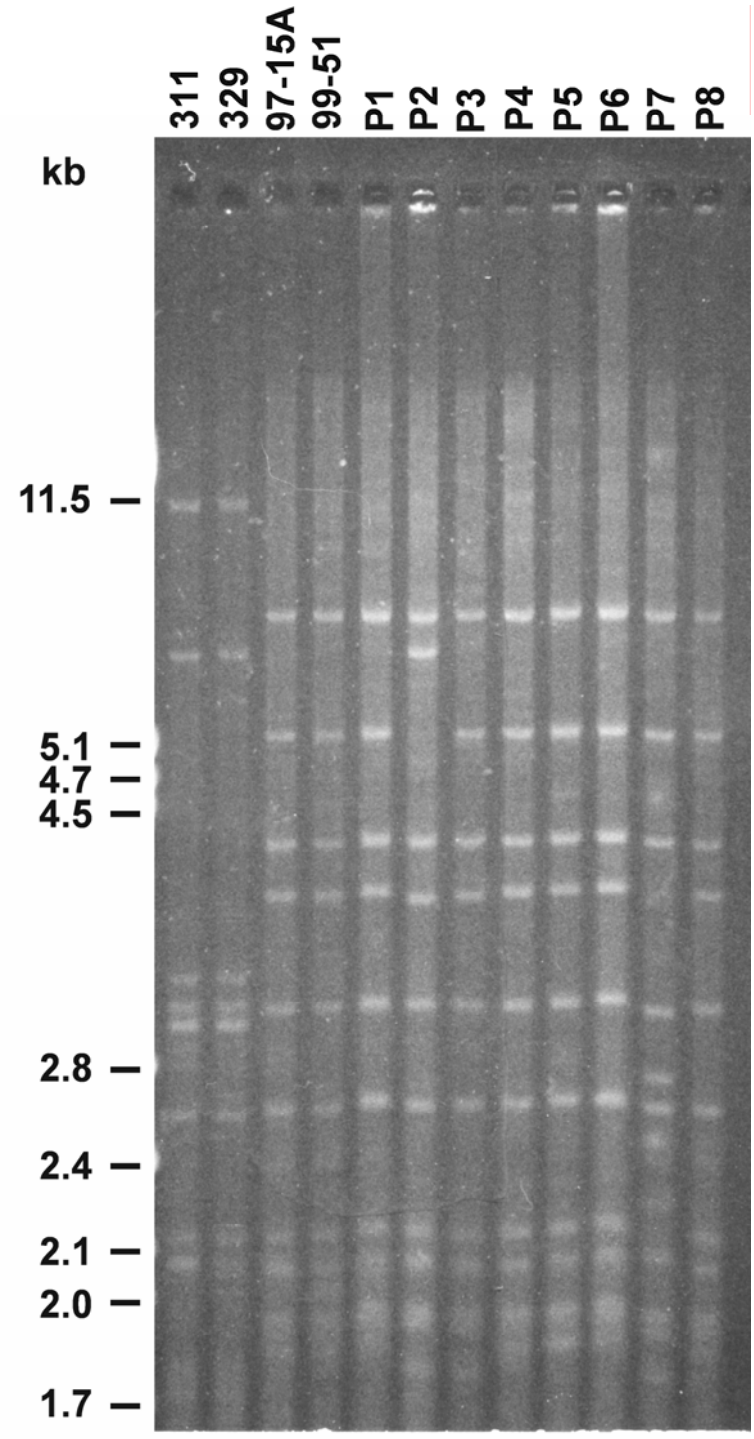

Fig 2-2. AT-rich DNA banding pattern produced by digestion of total DNA with the restriction enzyme HaeIII. Isolates 311 and 329 are Cgl-1 genotype isolates from strawberry. Isolates 97-15A and 99-51 are Cgl-2 genotype isolates from strawberry. Isolates P1 – P8 are perithecium isolates from strawberry petioles.

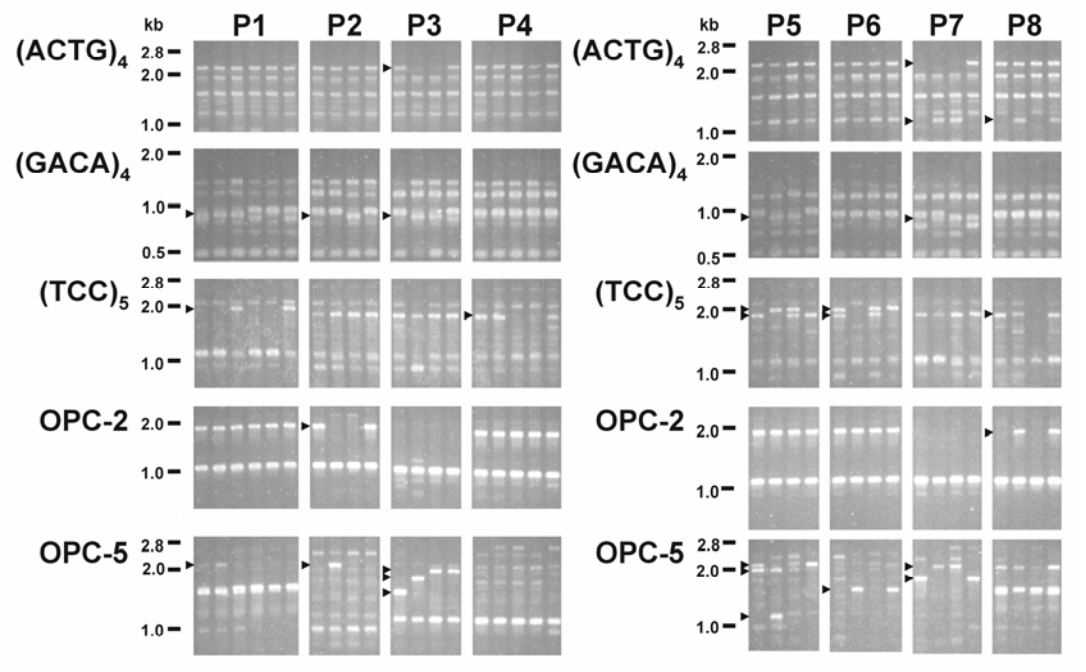

Fig 2-3. Randomly amplified polymorphic DNA banding patterns of single-ascospore isolates from eight *Glomerella cingulata* perithecia removed from strawberry petioles. Frames within columns labeled P1-P8 contain banding patterns of isolates from the same perithecium amplified using primers $(ACTG)_4$, $(GACA)_4$, $(TCC)_5$, OPC-2, or OPC-5. Arrows to the left of the frame show the position of bands that were polymorphic among isolates from the perithecium identified by the label at the top of the column.

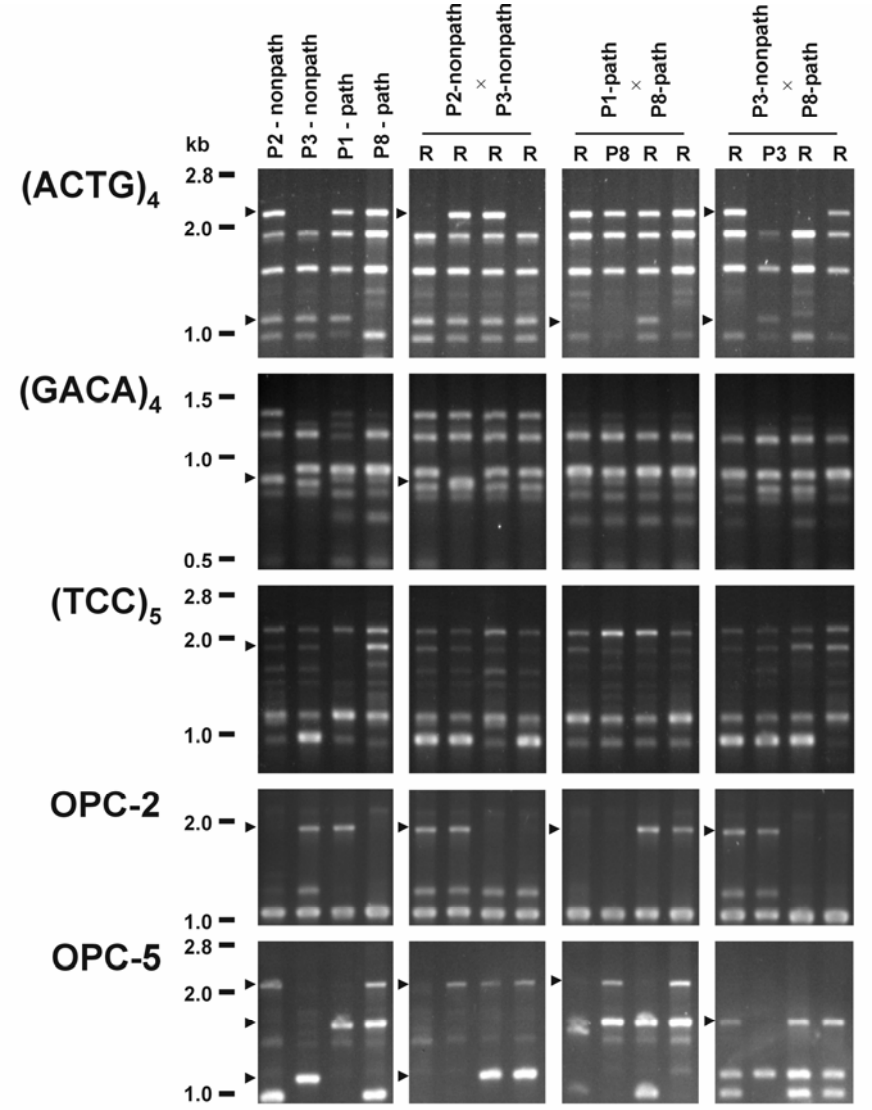

Fig 2-4. Randomly amplified polymorphic DNA banding patterns of two pathogenic and two nonpathogenic isolates to strawberry and RAPD banding patterns of progeny from crosses using these four isolates as parents. The first column of five frames contains bands amplified from the four parents using primers $(ACTG)_4$, $(GACA)_4$, $(TCC)_5$, OPC-2, and OPC-5. The arrows to the left of these frames show the positions of bands polymorphic among parents. The next two columns show bands amplified using the same primers from four progeny of crosses using two nonpathogenic and two pathogenic isolates as parents. The final column shows bands from progeny of a cross using isolates of each phenotype as parents. Arrows to the left of frames show the position of bands polymorphic among progeny. Progeny with banding patterns distinct from both parents are identified by the letter R, those that had banding patterns identical to a parent are identified by the parent perithecium.

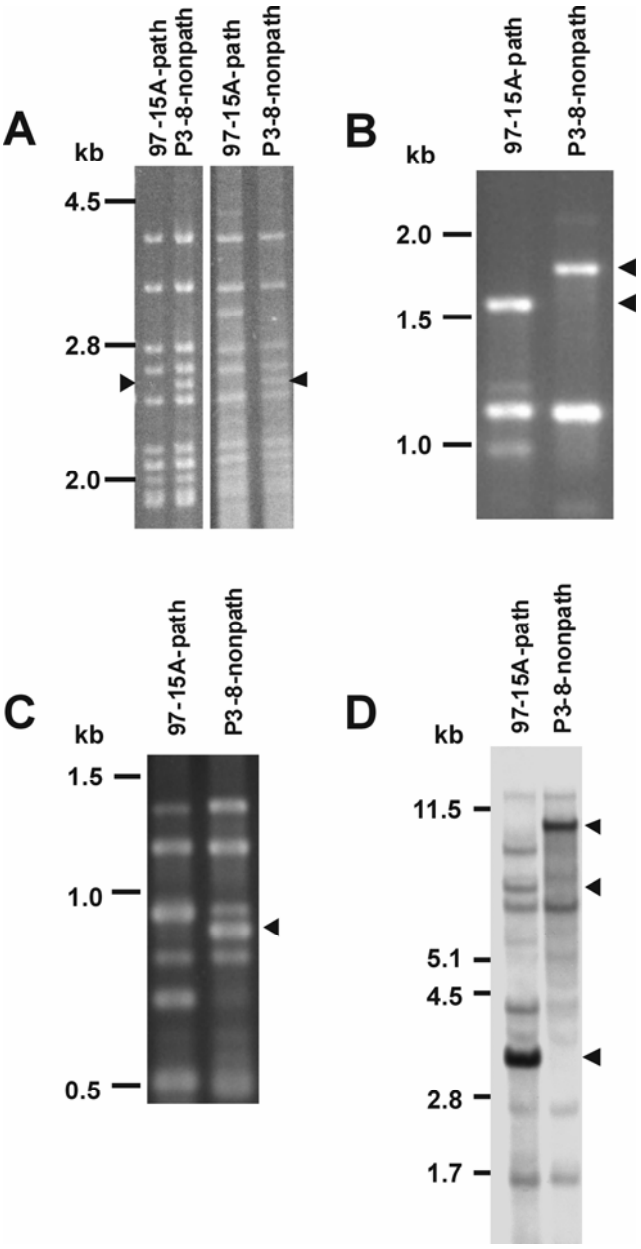

Fig 2-5. Molecular markers for 97-15A, an isolate pathogenic on strawberry**,** and isolate P3-8, an ascospore isolate nonpathogenic to strawberry that were used to examine mitochondrial and genomic inheritance among progeny from a cross between these isolates. (A) The frame on the left is 700-ηg mitochondrial DNA digested with MspI. The frame at the right is 10 μg of total DNA digested with MspI, which was used to identify mitochondrial inheritance among progeny. (B) Bands amplified with the primer OPC-5. (C) Bands amplified with the primer $(GACA)_4$. (D) PstI-digested DNA probed with $(CAT)_5$. Arrows indicate bands used to examine inheritance of mitochondria and recombination among progeny.

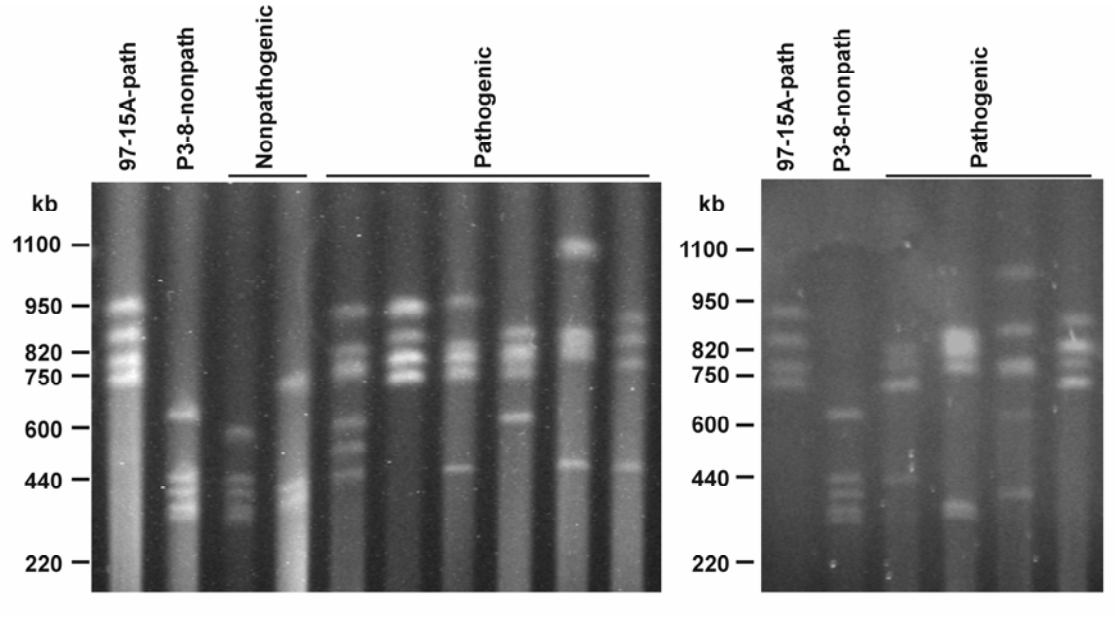

Fig 2-6. Chromosomes ranging in size from 300 kb to 1100 kb for pathogenic crown isolate 97-15A, nonpathogenic ascospore isolate P3-8, and progeny from a cross between these isolates. The only two progeny that were nonpathogenic on strawberry are in lanes 3 and 4 of frame 1. Ten representative isolates of those pathogenic on strawberry are in lanes 5 through 10 of frame 1 and lanes 3 through 6 of frame 2.

CHAPTER 3
GENETIC AND PATHOGENIC ANALYSIS OF *Colletotrichum gloeosporioides* ISOLATES FROM STRAWBERRY AND NONCULTIVATED HOSTS

Introduction

Under west central Florida climatic conditions, *C. gloeosporioides* does not appear to have the capacity to survive on strawberry plant debris in soil during the summer (53,93). In addition, soil is fumigated with a mixture of methyl bromide and chloropicrin before bare-root transplants are set in the fall (3), making transfer of inoculum between plants from different production seasons even less likely. Due to the low probability that infections are transferred directly between plants grown in different seasons, inoculum for the initiation of epidemics in Florida must come from sources outside of production fields. Infected transplants are one potential source and there are some apparent correlations between crown rot epidemics in Florida production fields with the nursery providing transplants (59). Another potential source of inoculum is alternate host species growing in the vicinity of strawberry fields. In Florida, strawberry fields are generally surrounded by noncultivated trees, shrubs, and herbaceous plants and in some fields it

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appears as though strawberry plants with Colletotrichum crown rot symptoms are aggregated near the edge of the field adjacent to these noncultivated plants.

Colletotrichum gloeosporioides has been isolated from numerous host species suggesting that it is a physiologically adaptable fungus (69). This is supported by cross infection experiments using strains of *C. gloeosporioides* from different cultivated plants, the occurrence of crop pathogens on noncultivated plants, and genetic analysis of *C. gloeosporioides* populations from different hosts. *Colletotrichum gloeosporioides* from noncultivated host species have been shown to be pathogenic on black locust (102). Isolates of *C. gloeosporioides* recovered from plant material of seven tropical fruits tended to be more pathogenic on leaves of the plant species from which they are isolated, but they still produced symptoms on alternative host leaves and molecular data did not indicate that isolates from different hosts were derived from genetically distinct populations (1). Among *C. gloeosporioides* living as endophytes on tropical forest trees no host specificity could be detected using molecular fingerprints (60). *Colletotrichum fragariae*, closely related to *C. gloeosporioides*, has been reported on a noncultivated host (50) and was shown to produce disease symptoms on an alternative host (100). In addition to being physiologically adaptive, the *C. gloeosporioides* taxonomic group probably includes more than one biological species as well as clonally reproducing lineages with specific host requirements. Based upon ribosomal DNA sequence analysis, *C. gloeosporioides* isolates cluster into at least two distinct groups (55,78). In addition, RAPD, RFLP, and isozyme markers have been used to identify genetically distinct subgroups on hosts such as citrus and coffee (84,93).

The goal of the study presented in this chapter was to determine whether noncultivated hosts adjacent to strawberry fields serve as potential sources of *C. gloeosporioides* inoculum for infection of strawberry crown. The specific objectives were to collect and identify isolates of *Colletotrichum* spp. from noncultivated plant species adjacent to strawberry fields, evaluate *C. gloeosporioides* isolates from noncultivated hosts for their pathogenicity on strawberry, and determine the genetic relatedness of *C. gloeosporioides* isolates from noncultivated plant species to isolates recovered from diseased strawberry plants.

Materials and Methods

Fungal Isolate Collections

From 1995 to 1998, isolates of *Colletotrichum* spp. with morphology consistent with descriptions for *C. gloeosporioides* (43,81) were collected by staff at the University of Florida, Gulf Coast Research and Education Center in Dover, FL from various noncultivated hosts growing adjacent to strawberry production fields and from diseased strawberry plants in west-central Florida (Table 3-1). Isolations were made from diseased tissues, such as foliar and fruit lesions of noncultivated host plants and diseased crowns of strawberry plants. Diseased plant tissues were surface disinfested for 5 min with 0.525% sodium hypochlorite and plated onto either potato dextrose agar (PDA) amended with neomycin sulfate (20 mg/L), chloramphenicol (6.5 mg/L), tetracycline hydrochloride (25 mg/L), and erythromycin (7.5 mg/L) or a semi-selective medium for *Collectotrichum* (16 g of Difco potato dextrose broth, 14 g of Difco agar, 250 mg of ampicillin, 150 mg of streptomycin sulfate, 5 mg of iprodione, 100 µl of Tergitol, and 1 L deionized water). Isolation plates were incubated under continuous fluorescent light at

room temperature (~22°C) or in an incubator at 24°C for 3 to 7 days. Cultures of *Colletotrichum* spp. were single-spored and stored in 20% glycerol at –85°C.

A total of 53 *Colletotrichum* spp. isolates were obtained between 1996 and 1997 from noncultivated plants growing on land adjacent to strawberry fields at five locations (Table 3-1). Among these sites, there were 16 known and two unknown noncultivated hosts from which *Colletotrichum* spp. were isolated. A *Colletotrichum* species was found growing on both foliar tissue and fruit of one host species, *Smilax rotundifoli*. Isolates coming from strawberry or noncultivated hosts can be identified by the letter "S" for strawberry or "NC" for noncultivated at the beginning of the isolate code (Table 3-1). Approximately half of the isolates included in the noncultivated population were collected from site NC1. This site was sampled once in 1996 and again in 1997. The population of *Colletotrichum* spp. obtained from infected strawberry crowns consisted of 42 isolates collected from 17 sites between 1995 and 1998 (Table 3-1). Site S2 was sampled in both 1995 and 1997. Approximately half of the strawberry crown rot isolates evaluated in the study were from this site. If isolates were collected from a site more than once, the site number is followed by a slash and the year of collection. For isolates from noncultivated hosts, the host species are indicated by the letters "sp" followed by a number referring to the particular host species (Table 3-1 lists species corresponding to the code number). Three *C. gloeosporioides* isolates from mango, four *C. gloeosporioides* isolates from citrus, and one *C. acutatum* isolate from citrus were used as representative outgroup populations for genetic comparisons. These isolates are believed to be from or have previously been shown to be distinct from populations on strawberry (47,92). One *C. acutatum* isolate from strawberry fruit was also included as an outgroup

in the genetic analysis and used to confirm the identity of any *C. acutatum* isolates that may have been isolated from crown tissue.

Extraction of Fungal DNA

Total fungal DNA was extracted from mycelia obtained from cultures grown in 100 mL of Emerson Media (4 g/L yeast extract, 15 g/L soluble starch, 1g/L K_2HPO_4 and 0.5 g/L $MgSO_4$) for 2 to 4 days at room temperature (~22 °C). Mycelium was harvested from the liquid cultures by vacuum filtration through Whatman no. 3 filter paper and transferred into a 15 mL tube. Mycelia were then dried overnight in a centrifugal evaporator and subsequently ground into a fine powder using a sterile glass rod. Sixty milligrams of the dried powder for each isolate was suspended in 750 µl of DNA extraction buffer consisting of 700 mM NaCl, 50 mM Tris(pH 8.0), 10 mM EDTA(pH 8.0), 1% cetyltrimethylammonium bromide, and 1% β-mercaptoethanol for 2 h with periodic shaking at 65°C. Particulate material was pelleted by centrifugation at 12,000× *g* for 10 min, the supernatent removed and extracted once with chloroform:isoamyl alcohol (24:1). Two volumes of 100% ethanol were added to the aqueous extract and the mixture incubated at room temperature for 10 min. Nucleic acids were pelleted from the ethanol solution by centrifugation at 12,000× *g* for 10 min. The pellet was washed with 100% ethanol and suspended in 400 µl 1× TE buffer containing 10 µg/mL RNase for 1 h at 37°C. Ribonuclease was removed from the nucleic acid solution by extraction with 400 µl phenol/chloroform/isoamyl alcohol (25:24:1). One-tenth volume 3 M sodium acetate and 2.5 volumes of ethanol were added to the aqueous extract to precipitate the DNA. This solution was incubated at –20°C for 1 h and the DNA was pelleted at

12,000× *g* for 10 min. The DNA pellet was washed once with 1 mL 80% ethanol, dried, suspended in 50 to 500 µl 1× TE buffer, and stored at –20°C.

Species Identification

A species-specific internal transcribed spacer region 1 (ITS1) primer and the conserved universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') encoded in the 28S ribosomal subunit were used in pairs to identify isolates to species (101). The ITS1 primers used were either the *C. gloeosporioides* specific ITS primer 5'-GACCCTCCCGGCCTCCCGCC-3' or the *C. acutatum* specific ITS primer 5'-GGGGAAGCCTCTCGCGG-3' (83,85). Isolates where assigned to the species group for which a positive amplification with a specific ITS1 primer was obtained. Amplifications were carried out under mineral oil in a 20-µl volume containing 1× reaction buffer (10 mM Tris(pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% Gelatin), 200 µM dNTP, 1 unit Taq polymerase and 10 ρmol of each primer/reaction. The reaction buffer for the *C. acutatum* specific primer also contained 5% glycerol. Temperature cycling parameters for the *C. gloeosporioides* specific/ITS4 pair consisted of a denaturing step for 5 min at 94°C, followed by 26 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. Temperature cycling parameters for the *C. acutatum* specific/ITS4 pair consisted of a denaturing step for 5 min at 94°C followed by 32 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. The amplified products were separated by electrophoresis through a 2% agarose gel containing 1× TAE buffer. Gels were photographed on a UV transilluminator after ethidium bromide staining.

Pathogenicity Tests

Pathogenicity of the 53 *Colletotrichum* isolates recovered from noncultivated plants was evaluated on the susceptible strawberry cv. Camarosa in a greenhouse. Conidial suspensions were made from 7- to 10-day-old PDA cultures grown at 24°C under continuous fluorescent light and adjusted to 1×10^6 conidia per mL in sterile deionized water. Infections were begun by injecting approximately 100 µl of a conidial suspension into the crown of mature transplants with a 25G1 syringe needle. Strawberry plants were evaluated weekly for the development of Colletotrichum crown rot symptoms (i.e., wilting and collapse of plant). An isolate was considered to be pathogenic to strawberry if at least 2 of 3 inoculated plants collapsed within the 4 weeks after inoculation. Each isolate was tested at least twice in separate inoculation experiments. In addition, ten, three, and four isolates of *C. gloeosporioides* recovered from diseased strawberry plants, mango, and citrus, respectively, were also tested for pathogenicity on strawberry in the same manner as described above.

Randomly Amplified Polymorphic DNA Markers

Five primers, including two tetranucleotide repeat primers $(ACTG)_4$ and $(GACA)_4$, the trinucleotide repeat primer $(TCC)_5$, and two short oligonucleotides 5'-GTGAGGCGTC-3' (OPC-2) and 5'-GATGACCGCC-3' (OPC-5) (Operon Technologies, Alameda, CA), were selected for the population studies based on their ability to consistently amplify bands that demonstrated a high level of fluorescence under UV light. DNA amplifications were carried out under mineral oil in a 20 µl volume containing 1× reaction buffer [50 mM Tris (pH 8.3), 0.25 mg/mL BSA, 2 mM $MgCl_2$, 0.5% Ficoll, and 1 mM Tartrazine], 200 µM dNTP, 1 unit Taq polymerase, and 20 ρmol

primer/reaction [primers $(ACTG)_4$, $(GACA)_4$, and $(TCC)_5$] or 8 ρmol primer/reaction (primers OPC-2 and OPC-5). Cycling parameters for the PCR reactions consisted of a 5 min denaturing step at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C for primers $(ACTG)_4$ and $(GACA)_4$; 34 cycles of 1 min at 95°C, 1 min at 46°C, and 1.5 min at 72°C for primer $(TCC)_5$ or 38 cycles of 1 min at 95°C, 1 min at 35°C, and 2 min at 72°C for primers OPC-2 and OPC-5. The amplified products were separated by electrophoresis through a 1.5% high resolution blend agarose (3:1) gel containing 1× TAE buffer. Gels were photographed on a UV transilluminator after ethidium bromide staining.

Statistical Analyses

The SPSS 8.0 statistics package (SPSS Inc., Chicago, IL) was used to perform Fisher's exact tests to determine the association between the pathogenicity of isolates and the site or noncultivated host species from which the isolates were recovered. The genetic relationship of isolates to one another was summarized in a phenogram constructed from dice similarity coefficients using the unweighted pair group method with arithmetic averages (UPGMA) clustering algorithm (NTSYS, PC version 2.0, Exeter software, Setauket, NY). Statistical support for branches was based on 1,000 bootstrapped samples using Winboot (35,71). The probability of obtaining identical genotypes among strains in the sample population assuming a random distribution of alleles was determined using a custom written QBASIC program which shuffles alleles among strains at each locus to mimic recombination and subsequently determines the frequency of the most common genotype observed in the shuffled data set (86,89). Probabilities of obtaining clone frequencies were based on analysis of 10,000 randomized

data sets. Population differentiation was examined using an exact test for population differentiation at each locus and Fisher's combined probability test to obtain a probability estimate over all loci (75). Tests for population differentiation were calculated using the software program TFPGA (Utah State University, Logan).

Results

Identification of Isolates

Of the 53 isolates recovered from noncultivated hosts, 52 produced a characteristic PCR product when the *C. gloeosporioides* specific primer was used and they were therefore identified as *C. gloeosporioides*. One isolate produced an amplification product when the *C. acutatum* species specific primer was used (Table 3-1). The *C. acutatum* isolate obtained from the noncultivated host came from the fruit of *Callicarpa americana*. It was genetically distinct from any of the *C. acutatum* isolates from strawberry (Fig. 3-1) and did not produce either crown rot (Table 3-2) or fruit lesions when inoculated on strawberry (data not shown). Of the 42 isolates recovered from diseased strawberry crowns, 39 were identified as *C. gloeosporioides* and three as *C. acutatum*. The species identities of *Colletotrichum* isolates from mango and citrus (Table 3-1) were also confirmed with *C. gloeosporioides* or *C. acutatum* specific primers.

Pathogenicity Tests

Of the 52 *C. gloeosporioides* isolates from noncultivated hosts, 18 produced typical symptoms of Colletotrichum crown rot (i.e. wilting and collapse of plants) on inoculated plants. Therefore, these isolates were considered to be pathogenic to strawberry crowns. The 18 pathogenic isolates were recovered from nine different noncultivated host species from three separate sites (Tables 3-2 and 3-3). There was a significant association between the pathogenicity of the isolates and the site from which the isolates were

collected (Table 3-2, Fisher's exact test $P = 0.02$). Of the 18 pathogenic isolates, 16 came from a single site (NC1). Half of the pathogenic isolates from this site were collected in 1996 and the other half in 1997. There was no significant association between the pathogenicity of isolates and the noncultivated host species from which the isolates were recovered (Table 3-3, Fisher's exact test $P = 0.23$).

Of ten isolates that were recovered from diseased strawberry plants, nine produced typical crown rot symptoms on inoculated strawberry plants. Two of the three isolates from mango and none of the four citrus isolates were pathogenic to strawberry.

Randomly Amplified Polymorphic DNA Analyses

RAPD amplifications using five primers yielded 60 scorable bands from all isolates examined. Forty-one scorable bands were amplified from *C. gloeosporioides* isolates recovered from strawberry and noncultivated hosts. When isolates from citrus and mango were included, 44 scorable bands were obtained. Thirty scorable bands were amplified from *C. acutatum* isolates. Isolates identified as *C. gloeosporioides* grouped into three clusters with between cluster similarities less than 0.50 (Fig. 3-1). All of the *C. gloeosporioides* isolates from citrus had a level of similarity to one another greater than 0.75 and formed a cluster in 93% of bootstrapped phenograms. A second cluster consisted of two clonal isolates from different noncultivated host species at site NC3 and occurred in 100% of bootstrapped trees. The two isolates in this cluster were homothallic (data not shown). Self-fertility was not observed for any other isolates, although it is possible that low numbers of fertile perithecia may have been overlooked.

A third cluster contained *C. gloeosporioides* from noncultivated hosts, strawberry and mango. This cluster occurred in only 41% of bootstrapped trees, giving weak

support for a monophyletic origin of this cluster. A large amount of the genetic variation within this cluster can be attributed to the mango isolates. The three mango isolates were distinct from most strawberry and noncultivated host isolates based on Dice similarity coefficients, but isolates from this host did not compromise a group of closely related organisms. When mango isolates are excluded from the analysis the cluster containing strawberry and nonhomothallic, noncultivated host isolates occurred in 59% of bootstrapped samples.

Bootstrapping did not provide support for strawberry isolates forming an evolutionary lineage distinct from noncultivated isolates, as strawberry isolates were interspersed among these isolates in the phenogram. However, clustering did occur among subsets of strawberry and noncultivated host isolates. Several of these clusters were supported by relatively high bootstrap values (Fig. 3-1), although the bootstrap values may not be reliable as there are few informative sites separating the clusters from other isolates (46). Seven clusters of nonhomothallic isolates contained two or more clonal individuals based on RAPD profiles. Of these seven clonal lineages, two consisted of isolates from strawberry and five contained isolates from noncultivated hosts. Two of the clonal groups contained three or more isolates. One clonal genotype on strawberry was found at two sites (four isolates at site S2/97; two isolates at S14). Of the five clonal genotypes found on noncultivated hosts, four contained only isolates from the same field and in only one of these fields were all of the isolates from the same host species. Pathogenicity phenotypes were the same among all individuals possessing identical genotypes. Based on probabilities obtained by repeated shuffling of the data set, two or more individuals having the same genotype occurred in 4.39% of the randomized data

sets and three or more individuals with the same genotype did not occur in any of the 10,000 randomized data sets examined.

Allele frequencies were significantly ($P < 0.05$) different between strawberry isolates and nonhomothallic isolates from noncultivated hosts at only four of 36 (11 %) polymorphic loci based on exact tests with α, the level of type I error, equal to 0.05 (Table 3-4). A test for population differentiation combining all polymorphic loci was not significant ($P = 0.29$). The 1.6 kb band amplified with primer OPC-5, one of four loci displaying allele frequency differences between these isolates, also displayed frequency differences between pathogenic and nonpathogenic isolates when only isolates from noncultivated hosts were compared (frequency = 0.50 among pathogens and 0.06 among nonpathogens). There were no significant differences in allele frequencies between pathogenic and nonpathogenic isolates from noncultivated hosts at the three other loci.

Discussion

In this study we found that approximately one-third of the *C. gloeosporioides* isolates recovered from noncultivated hosts grown in the areas adjacent to strawberry fields were pathogenic to strawberry in greenhouse tests. Phylogenetic analysis of RAPD data and tests for genetic differentiation between *C. gloeosporioides* from noncultivated hosts and those from diseased strawberry crowns suggest that they were from a single population. These results indicate that noncultivated hosts growing adjacent to strawberry fields may serve as a source of inoculum for epidemics of strawberry crown rot caused by *C. gloeosporioides.*

Isolates of *C. gloeosporioides* from a wide range of temperate, subtropical, and tropical fruits have shown cross infection potential (1,40). However, pathogenicity tests

in artificial inoculation experiments are not conclusive evidence to support that cross infection occurs under natural conditions. For this reason, in addition to pathogenicity tests, the genetic relationship between isolates from strawberry and different noncultivated hosts was determined in the present study. Based upon bootstrap analysis of a phenogram constructed using RAPD markers, isolates of *C. gloeosporioides* from noncultivated hosts fell into two genetically distinct populations. The largest population consisted of 50 isolates and the smaller population consisted of two genotypically identical isolates. The populations from noncultivated hosts also could be distinguished from one another based upon the presence of homothallism in isolates from the smaller population. Homothallic isolates of *C. gloeosporioides* from strawberry appear to be genetically distinct from interbreeding heterothallic isolates (38,43). However, the homothallic isolates from strawberry do not appear to be related to those isolated from noncultivated hosts (data not shown).

Isolates of *C. gloeosporioides* from strawberry crowns had a high level of diversity and were not genetically distinct from isolates from noncultivated hosts that were not homothallic in culture when all polymorphic loci were included in the analysis. Tests for differences in allele frequencies at single loci revealed a 1.6-kb OPC-5 amplification product that occurred at a higher frequency in the strawberry population relative to the noncultivated host population and also occurred at a higher frequency among pathogenic isolates from the noncultivated host population relative to nonpathogenic isolates. Allele frequencies from this locus do not provide strong evidence for population subdivision, given that the frequency of the vast majority of allelic markers, assumed to be neutral, are not different between populations. The positive correlation between the 1.6-kb OPC-5

product and pathogenicity on strawberry does, however, suggest that it may be linked to a genetic factor conferring pathogenicity on strawberry. In the previous chapter, this marker also displayed highly skewed nonmendelian segregation in a cross between a pathogenic and nonpathogenic isolate.

Although isolates with the same genotype occurred on either strawberry or noncultivated hosts, no identical genotypes were found on both strawberry and noncultivated hosts. Assuming a randomly mating population, the occurrence of even two isolates with identical genotypes would be a relatively rare event given the sample size and polymorphic loci examined in this study. In total, there were seven genotypes detected more than once in isolates from the nonhomothallic strawberry and noncultivated host population. This overrepresentation of specific genotypes is consistent with the important role of clonal reproduction in this species (33). Genetic bottlenecks created by recent colonizing events in spatially subdivided populations can also result in overrepresentation of genotypes at specific sites. However, this is unlikely as the sites from where clonal genotypes were collected contained a substantial amount of genetic diversity. Genetically isolated pathogen subpopulations can also arise relatively rapidly from interbreeding fungal populations due to asexual reproduction and may serve as a mechanism to preserve particularly virulent gene combinations on specific hosts (8). The data provided from this study does not support the hypothesis that this has occurred with *C. gloeosporioides* from either strawberry or noncultivated hosts as clonal isolates tended to occur at specific sites and not on specific hosts. There was, however, one genotype that was isolated from six different strawberry crowns at two separate sites (S2/97 and S14). This was the most common genotype observed and suggests that

selection may be preserving some combinations of genes for pathogencity on strawberry. Alternatively, the occurrence of this genotype may result from selection for a clone at a nursery supplying transplants to farms in the area investigated.

Although a defined group of species harboring pathogenic isolates could not be identified among the nonpathogenic hosts sampled, there was a strong correlation between sampling site and pathogenicity on strawberry. Because not all of the host species were present at each sample site, there may be some bias in the tests for association. This bias might make it difficult to discern whether or not isolates from particular hosts or particular sites differed in pathogenicity. However, eleven of 19 isolates from site NC1 that had hosts identical to those found at sites NC2, NC3, NC4 and NC5 were pathogenic to strawberry, whereas only two of the 22 isolates from the four other sites were pathogenic on strawberry. This result indicates that the analyses were correct in that pathogenicity correlated primarily with the site from which isolates were recovered and not the host species from which they were isolated. Variation in levels of pathogenicity among noncultivated host sampling sites may result from different levels of migration from strawberry fields, where selection for virulence on strawberry would likely occur.

Three *C. gloeosporioides* isolates from mango and four from citrus were included in the phenogram (Fig. 3-1). The mango isolates examined in this study did not appear to form a subgroup genetically distinct from *C. gloeosporioides* isolated from strawberry or noncultivated hosts. These findings were not expected because a study of *C. gloeosporioides* isolates from mangos at locations around the world found the isolates to be relatively homogeneous and genetically distinct from those recovered from other fruit

species (47). However, that study only included one mango isolate from Florida. The Florida isolate had a slightly smaller rDNA size compared to the isolates from other sites. Repeated sampling of *C. gloeosporioides* from mango in Sri Lanka also revealed a greater amount of diversity in rDNA and mtDNA restriction fragment length polymorphisms than was previously thought to exist in that population (2). Also of interest is that the two mango isolates pathogenic on strawberry in greenhouse inoculation tests were more closely related to strawberry isolates than they were to the third mango isolate that was not pathogenic on strawberry. The citrus isolates used in this study were previously demonstrated to be genetically distinct from strawberry isolates (92). Whether or not this genetic divergence is due to geographic isolation or sexual incompatibility was not examined, but test crosses of citrus isolates to apple reference strains have been unsuccessful (23).

A *C. acutatum* isolate was also obtained from a noncultivated host in this study. The isolate was not pathogenic on strawberry and was genetically distinct from strawberry crown isolates, indicating this strain of *C. acutatum* is not responsible for diseases on strawberry.

Table 3-1. Collection sites and host species for *Colletotrichum* spp.

Species, collection site[a]	Host (species code)[b]	Common name	Number of isolates
Colletotrichum gloeosporioides			
NC1/96	*Quercus* spp. (sp12)	Oak	2
	Smilax rotundifolia (sp14)	Smilax	1
	S. rotundifolia, fruit (sp14B)	Smilax berry	3
	Vitis rotundifolia (sp16)	Wild grape	8
NC1/97	*Callicarpa americana,* fruit (sp3)	Beauty berry	1
	Dioscorea bulbifera (sp4)	Air potato	4
	Ipomoea spp. (sp5)	Morning glory	1
	Liquidambar styraciflua (sp6)	Sweet gum	2
	Myrica cerifera L. (sp10)	Wax myrtle	2
	Parthenocissus quinquefolia (sp11)	Virginia creeper	3
	Quercus spp. (sp12)	Oak	1
	Smilax rotundifolia, fruit (sp14B)	Smilax berry	1
	Urena lobata (sp15)	Caesar weed	1
NC2	*Momordica charantia* L. (sp9)	Balsamapple	2
	Richardia brasiliensi (sp13)	Brazilian pusley	1
NC3	*Magnolia virginiana* L. (sp7)	Magnolia, sweet bay	2
	Myrica cerifera L. (sp10)	Wax myrtle	1
	Quercus spp. (sp12)	Oak	1
	S. rotundifolia (sp14)	Smilax	2
	S. rotundifolia, fruit (sp14B)	Smilax berry	1
	V. rotundifolia (sp16)	Wild grape	1
	Unknown species (sp17)	…	2
NC4	*V. rotundifolia* (sp16)	Wild grape	1
NC5	*Asclepias* spp. (sp1)	Milkweed	2

[a]Sites beginning with NC are areas with noncultivated plants adjacent to strawberry fields and sites beginning with S are strawberry fields. Numbers followed by "/" represent year sample was collected at sites sampled more than once.
[b]Species codes given in parentheses are combined with site numbers to identify the sample location and host for isolates displayed in Figure 3-1.

Table 3-1. Continued

Species, collection site[a]	Host (species code)[b]	Common name	Number of isolates
	Bidens bipinnata (sp2)	Bidens	1
	C. americana-fruit (sp3)	Beauty berry	1
	Melia australis Sweet (sp8)	Chinaberry	2
	S. rotundifolia (sp14)	Smilax	2
S1	*Fragariae* × *ananassa*	Strawberry crown	1
S2/95	*Fragariae* × *ananassa*	Strawberry crown	11
S2/97	*Fragariae* × *ananassa*	Strawberry crown	9
S3	*Fragariae* × *ananassa*	Strawberry crown	1
S4	*Fragariae* × *ananassa*	Strawberry crown	1
S5	*Fragariae* × *ananassa*	Strawberry crown	1
S6	*Fragariae* × *ananassa*	Strawberry crown	1
S7	*Fragariae* × *ananassa*	Strawberry crown	1
S8	*Fragariae* × *ananassa*	Strawberry crown	1
S9	*Fragariae* × *ananassa*	Strawberry crown	1
S10	*Fragariae* × *ananassa*	Strawberry crown	2
S11	*Fragariae* × *ananassa*	Strawberry crown	2
S12	*Fragariae* × *ananassa*	Strawberry crown	1
S13	*Fragariae* × *ananassa*	Strawberry crown	1
S14	*Fragariae* × *ananassa*	Strawberry crown	3
S15	*Fragariae* × *ananassa*	Strawberry crown	2
Lake Alfred, FL	*Citrus* spp.	Citrus	4
Homestead, FL	*Mangifera indica*	Mango	3
Colletotrichum acutatum			
NC5	*Callicarpa americana*, fruit (sp3)	Beauty berry	1
S9	*Fragariae* × *ananassa*	Strawberry crown	1
S16	*Fragariae* × *ananassa*	Strawberry crown	1
S17	*Fragariae* × *ananassa*	Strawberry crown	1

Table 3-1. Continued

Species, collection site[a]	Host (species code)[b]	Common name	Number of isolates
Dover, FL	*Fragariae* × *ananassa*	Strawberry Fruit	1
Lake Alfred, FL	*Citrus* spp.	Citrus	1

Table 3-2. Isolates of *Colletotrichum* spp. collected from noncultivated hosts summarized according to site and pathogenicity on strawberry plants

	Number of isolates		
Species, collection site[a]	Pathogenic	Nonpathogenic	Total
Colletotrichum gloeosporioides			
NC1/96	8	6	14
NC1/97	8	8	16
NC2	1	2	3
NC3	1	9	10
NC4	0	1	1
NC5	0	8	8
All Sites	18	34	52
C. acutatum			
NC5	0	1	1

[a] Sites beginning with NC are areas with noncultivated plants in close proximity to strawberry fields in west-central Florida. There was a significant association (P = 0.02) between the pathogenicity of the *C. gloeosporioides* isolates and the site from which the isolates were collected, based on a Fisher's exact test (P = 0.05).

Table 3-3. Isolates of *Colletotrichum* spp. collected from noncultivated hosts summarized according to host species and pathogenicity on strawberry plants

	Number of isolates		
Fungal species, host species[a]	Pathogenic	Nonpathogenic	Total
Colletotrichum gloeosporioides			
Asclepias spp. (sp1)	0	2	2
Bidens bipinnata (sp2)	0	1	1
Callicarpa americana, fruit (sp3)	0	2	2
Dioscorea bulbifera (sp4)	2	2	4
Ipomoea spp. (sp5)	0	1	1
Liquidambar styraciflua (sp6)	0	2	2
Magnolia virginiana L. (sp7)	1	1	2
Melia australis Sweet (sp8)	0	2	2
Momordica charantia L. (sp9)	0	2	2
Myrica cerifera L. (sp10)	2	1	3
Parthenocissus quinquefolia (sp11)	2	1	3
Quercus spp. (sp12)	2	2	4
Richardia brasiliensi (sp13)	1	0	1
Smilax rotundifolia (sp14)	0	5	5
S. rotundifolia, fruit (sp14B)	1	4	5
Urena lobata (sp15)	1	0	1
Vitis rotundifolia (sp16)	6	4	10
Unknown species (sp17)	0	2	2
All host species combined	18	34	52
C. acutatum			
Callicarpa americana, fruit (sp3)	0	1	1

[a]There was no significant association ($P = 0.23$) between the pathogenicity of the *C. gloeosporioides* isolates and the host species from which the isolates were collected, based on a Fisher's exact test ($P = 0.05$). Species codes given in parentheses are combined with site numbers to identify the sample location and host for isolates displayed in Figure 3-1.

Table 3-4. Frequencies of RAPD bands for *Colletotrichum gloeosporioides* isolates from strawberry and noncultivated hosts

	Host		
		Noncultivated	
Primer, length (kb)	Strawberry (*n*=39)[a]	Nonhomothallic (*n*=50)	Homothallic (*n*=2)
$(ACTG)_4$			
2.145	0.49	0.52	0.00
1.9	0.97	1.00	0.00
1.55	0.00	0.00	1.00
1.5	1.00	1.00	0.00
1.12	0.85	0.84	0.00
0.6	0.92	0.84	1.00
0.4	0.33	0.38	1.00
$(GACA)_4$			
1.5	0.05	0.02	0.00
1.35	0.95	0.96	0.00
1.3	0.00	0.00	1.00
1.2	0.97	0.96	0.00
1.15	0.03	0.04	0.00
0.95	0.69	0.74	0.00
0.9	0.46	0.38	1.00
0.8	0.13	0.30	0.00
0.75	0.00	0.02	0.00
0.5	1.00	0.96	0.00
$(TCC)_5$			
2	0.03	0.10	0.00
1.9	0.62	0.62	1.00
1.55	0.23	0.28	0.00
1.15	1.00	0.98	1.00
0.9	0.03	0.02	0.00
0.75	0.13	0.22	0.00

[a]Exact test for population differentiation between strawberry and noncultivated (nonhomothallic) host isolates over all loci was not significant (P=0.29).
[b]Exact test for population differentiation between strawberry and noncultivated (nonhomothallic) host isolates was significant at specified loci ($P < 0.05$).
[c]Exact test for population differentiation between pathogenic and nonpathogenic, noncultivated (nonhomothallic) host isolates was significant at specified loci ($P < 0.05$).

Table 3-4. Continued

Primer, length (kb)	Host		
		Noncultivated	
	Strawberry (*n*=39)	Nonhomothallic (*n*=50)	Homothallic (*n*=2)
OPC-2			
2.2	0.08	0.06	0.00
1.9	0.62	0.64	0.00
1.7	0.03	0.00	0.00
1.2	0.08	0.02	0.00
1.1	1.00	1.00	1.00
0.5	0.03	0.00	0.00
OPC-5			
2.7	0.18	0.34	0.00
2.6	0.00	0.00	1.00
2.5	0.21	0.44[b]	0.00
2.15	0.59	0.82[b]	1.00
2	0.21	0.22	0.00
1.8	0.18	0.16	1.00
1.75	0.08	0.08	0.00
1.65	0.10	0.30[b]	0.00
1.6	0.74	0.22[bc]	1.00
1.55	0.33	0.32	0.00
1.4	0.03	0.06	0.00
1.2	0.38	0.22	0.00

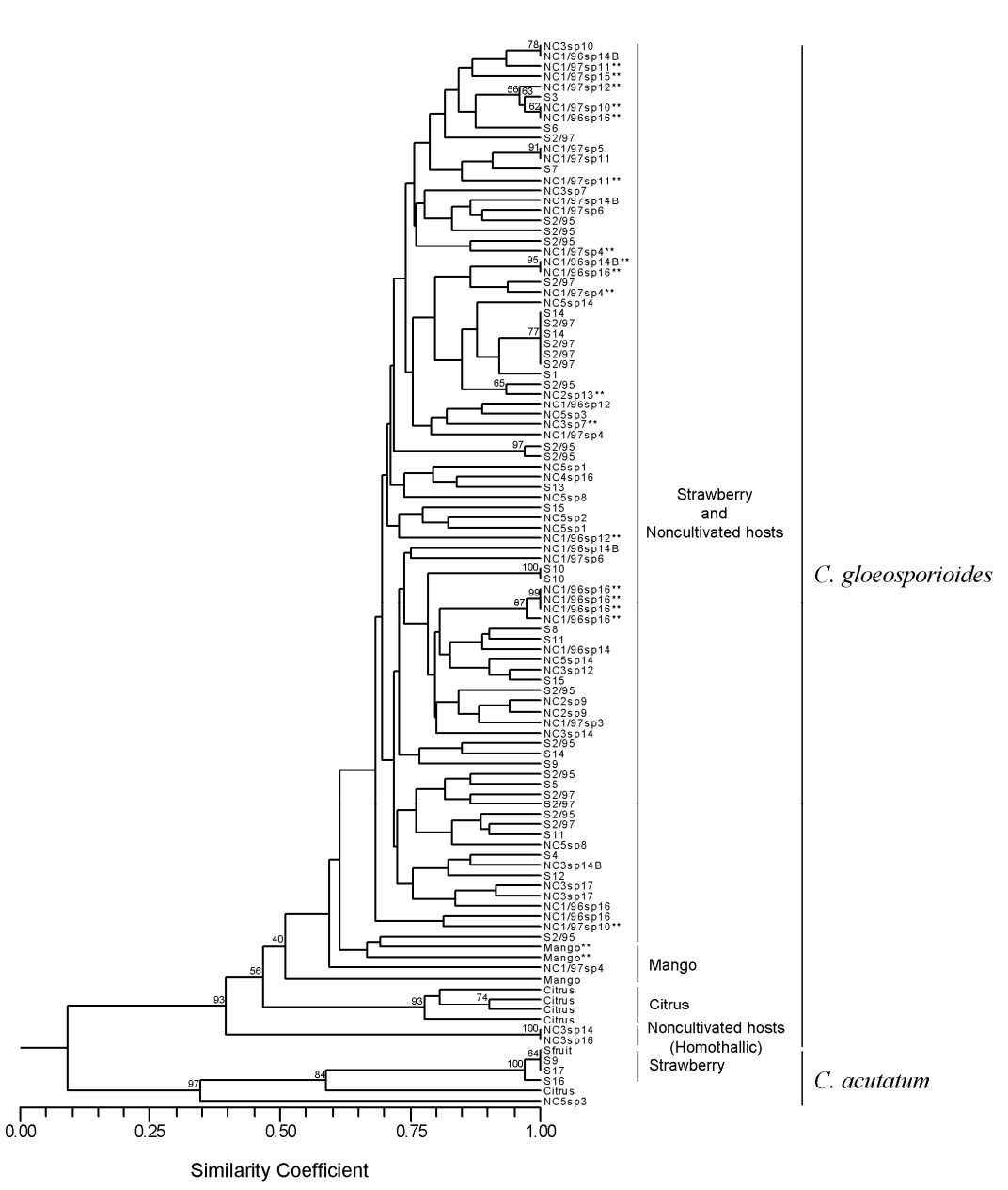

Fig. 3-1. A phenogram using unweighted pair group method with arithmetic averages showing similarity (Dice) between *Colletotrichum gloeosporioides* and *C. acutatum* isolates from noncultivated plants, strawberry crowns, citrus, and mango. Numbers at nodes are the percentage of occurrence of the cluster to the right of the branch in 1,000 bootstrapped samples. All bootstrapped values are reported for clusters that are less than 0.50 similar to other isolates or clusters. For all other clusters, only bootstrapped values greater than 50 are reported. Nonstrawberry *C. gloeosporioides* isolates with asterisks (**) indicate they were pathogenic on strawberry in greenhouse inoculation tests. Isolates are identified in Table 3-1.

CHAPTER 4
SELECTION FOR PATHOGENICITY TO STRAWBERRY IN POPULATIONS OF *Colletotrichum gloeosporioides* FROM NATIVE PLANTS

Introduction

Colletotrichum crown rot of strawberry causes wilting and collapse of strawberry plants in production fields and nurseries in Florida. Currently most of the transplants used for the annual winter production season are propagated in nurseries located in the northern United States or provinces of Canada. This has greatly decreased the incidence of crown rot in production fields, although moderate plant losses still occur. At the present time, the vast majority of *Colletotrichum* species isolated from diseased strawberry crowns in Florida are from a nonhomothallic *C. gloeosporioides* population. This population is genetically diverse and recombination appears to occur at a relatively high frequency (92). As shown in chapter 3, *C. gloeosporioides* can be isolated from foliar lesions of a broad range of introduced and native noncultivated plant species growing adjacent to strawberry fields as well as from strawberry petioles where it forms latent infections (66). Isolates from these sources vary in their ability to cause crown rot with some being aggressive pathogens on strawberry and others lacking the ability to produce crown rot symptoms. Also from results reported in chapter 3, a large proportion of the *C. gloeosporioides* isolate*s* on noncultivated hosts appear to be from the same population as those isolated from diseased strawberry crowns, indicating that noncultivated hosts may provide inoculum for crown rot epidemics. However, populations of *C. gloeosporioides* from noncultivated hosts at sites distant from strawberry production areas have not been studied. Wild strawberry is not known to

occur in the subtropical regions of Florida where commercial strawberries are produced (26). Thus, sampling of *C. gloeosporioides* at sites away from strawberry fields can provide information as to whether isolates on noncultivated hosts are recent migrants from strawberry or if there is an indigenous population of *C. gloeosporioides* on noncultivated hosts and strawberry. In addition, the *C. gloeosporioides*/strawberry pathosystem provides a unique system for examination of local selection for pathogenicity.

In Florida, strawberry production is highly centralized. More than 90% of the area dedicated to strawberry production is located within Hillsborough County, with most plantings concentrated in the Dover/Plant City area (5). If the *C. gloeosporioides* population on strawberry is derived from a widely dispersed population with a wide host range, the cultivation of strawberry in a particular area may influence the frequency of the pathogenic phenotype in the *C. gloeosporioides* population on noncultivated hosts. Studies examining mean host resistance and mean pathogen virulence have shown a positive correlation between these two traits in both natural and agricultural pathosystems (64,88), suggesting directional selection in the pathogen population for increased infectivity. Analogously, genes controlling pathogenicity on strawberry may be selected in *C. gloeosporioides* populations at sites where strawberries are grown extensively.

In this study we genetically characterized *C. gloeosporioides* isolates from two native hosts, oak (*Quercus* spp.) and wild grape (*Vitis* spp.), at four locations. Two locations were immediately adjacent to strawberry fields and two sites were distant from any commercial strawberry production. Sampling at all sites was conducted at least four months after strawberry plants had been removed from fields. The genetic data was used

to identify a population to test the hypothesis that there is local selection for pathogenicity to strawberry in areas where strawberries are grown extensively.

Materials and Methods

Sampling Strategy and Isolate Codes

In Florida, strawberries are grown as an annual crop on raised, plastic mulched beds. Plants are set in late September and harvested from November to late March. At the end of the harvest season, plants are chemically destroyed and the field tilled or a different crop planted on the mulched beds. Sampling was done in late August and early September 2002, 4 to 5 months after plants were removed from fields, to avoid sampling migrants from strawberry fields that may have only transiently established themselves on native hosts. At least 20 isolates identified as *C. gloeosporioides* based on morphology (43) were obtained from necrotic lesions on oak (*Quercus* spp.) or wild grape (*Vitis* spp.) leaves at each of four sites (Fig. 4-1). Samples were taken from leaves of different trees and vines at each site to reduce the possibility of sampling clones. Two sites were adjacent to commercial strawberry fields in Dover, FL. These sites were 3.5 km apart within the strawberry production region in Hillsborough County. The native hosts sampled at these sites were located 9 to 50 m from the edges of fields. Two sites distant from strawberry production fields were sampled: University of Florida, Citrus Research and Education Center in Lake Alfred, FL and a residential area in Sarasota, FL. Both sites contained natural stands of vegetation along edges of roads or citrus groves and in preserved areas. The Lake Alfred and Sarasota sites are located in Polk and Sarasota counties respectively. The combined acreage used for strawberry production in these counties is less than 2% of the acreage used in Hillsborough County. The nearest commercial strawberry farm was approximately 28 km from the Lake Alfred site and 15

km from the Sarasota site. Native host isolates from the two sites in Dover were designated D1-oak, D1-grape, D2-oak, and D2-grape. Those from Lake Alfred were designated LA-grape, LA-oak, and those from Sarasota labeled SS-grape and SS-oak. The site D2 was also sampled in the study presented in chapter 3, where it was designated NS1. Site D1 had not been sampled previously. Twenty *C. gloeosporioides* isolates from diseased strawberry crowns, seven citrus isolates, and one *C. fragariae* isolate from crown tissue were also included in the analysis. Strawberry isolates came from samples submitted by local growers to the diagnostic laboratory at the University of Florida, Gulf Coast Research and Education Center in Dover, Florida from 1995 to 2000 and are coded 'strawberry'. Citrus isolates came from sweet orange or tangelo fruit, twigs, or leaves from plantings near Lake Alfred, Avon Park, Vero Beach, or Frostproof, Florida. These isolates are coded 'citrus'. *C. gloeosporioides* isolates from citrus have been shown to be distinct from those on strawberry (92).

Fungal Isolation

Isolates from strawberry were obtained by placing tissue from necrotic crowns of wilted strawberry plants directly onto CIM media (CIM - 12 g potato dextrose broth, 17 g agar, 100 mg streptomycin, 250 mg ampicillin, and 8 mg iprodione per L plus 0.02 % tergitol). For isolations from native hosts, portions of oak and grape leaves with one or more circular necrotic lesions were surface sterilized with 0.525 % sodium hypochlorite for 1 min, rinsed in sterile water, and placed on CIM media. Plates were incubated under fluorescent light for 3 to 5 days and single-spore isolates made from growing colonies. Cultures were stored at –85 °C in 20% glycerol.

DNA Extraction and PCR Amplifications

Two to 3 days after seeding with an agar plug, mycelium was harvested from 50-mL liquid shake cultures (Emerson media - 4 g yeast extract, 15 g soluble starch, 1 g K_2HPO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ per L) and dried overnight in a centrifugal evaporator. DNA was isolated from 60 mg of the dried mycelia. Mycelia powder was suspended in 750 µl of DNA extraction buffer consisting of 700 mM NaCl, 50 mM Tris(pH 8.0), 10 mM EDTA(pH 8.0), 1% cetyltrimethylammonium bromide, and 1% β-mercaptoethanol for 2 h with periodic shaking at 65°C. Particulate material was pelleted by centrifugation at 12,000× *g* for 10 min, the supernatent removed and extracted once with chloroform:isoamyl alcohol (24:1). Two volumes of 100% ethanol were added to the aqueous extract and the mixture incubated at room temperature for 10 min. Nucleic acids were pelleted from the ethanol solution by centrifugation at 12,000× *g* for 10 min. The pellet was washed with 100% ethanol and suspended in 400 µl 1× TE buffer containing 10 µg/mL RNase for 1 hour at 37°C. Ribonuclease was removed from the nucleic acid solution by extraction with 400 µl phenol/chloroform/isoamyl alcohol (25:24:1). To the aqueous extract, 1/10 volume 3 M sodium acetate and 2.5 volumes of ethanol were added to precipitate the DNA. This solution was incubated at –20°C for 1 h and the DNA was pelleted at 12,000× *g* for 10 min. The DNA pellet was washed once with 1 mL 80% ethanol, dried, suspended in 50 to 500 µl 1× TE buffer, and stored at –20°C.

Isolates were identified to species using a *C. gloeosporioides/C. fragariae* specific ITS1 primer (5'-GACCCTCCCGGCCTCCCGCC-3') and a conserved universal primer encoded within the 28S ribosomal subunit (5'-TCCTCCGCTTATTGATATGC-3'). This primer set amplifies the ITS region from subpopulations within the *C. gloeosporioides*

species complex as well as that of *C. fragariae* (83,92). Amplifications from 5 ng of template DNA were carried out under mineral oil in 20 µl containing 1× reaction buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin), 200 µM dNTP, 1 unit of *Taq* polymerase, and 10 ρmol of each primer/reaction. Cycling parameters consisted of a 5-min denaturing step at 94°C followed by 26 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. RAPD -PCR using four primers $(ACTG)_4$, $(GACA)_4$, $(TCC)_5$, and 5'-GTGAGGCGTC-3' (OPC-2) (Operon Technologies, Alameda, CA) was used to differentiate *C. fragariae* from *C. gloeosporioides* and to identify subpopulations within isolates identified as *C. gloeosporioides*. Reactions were carried out using 5 ng of template DNA under mineral oil in 20 µl containing 1× reaction buffer (50 mM Tris [pH 8.3], 0.25 mg/mL bovine serum albumin, 2 mM $MgCl_2$, 0.5% Ficoll, and 1 mM Tartrazine; Idaho Technology), 200 µM dNTP, 1 unit of *Taq* polymerase, and 20 ρmol primer/reaction (primers $(ACTG)_4$, $(GACA)_4$, and $(TCC)_5$) or 8 ρmol OPC-2/reaction. Cycling parameters consisted of a 5-min denaturing step at 95°C, 30 cycles of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C for primers $(ACTG)_4$ and $(GACA)_4$; 34 cycles of 1 min at 95°C, 1 min at 46°C, and 1.5 min at 72°C for primer $(TCC)_5$ or 38 cycles of 1 min at 95°C, 1 min at 35°C, and 2 min at 72°C for primer OPC-2. Amplified products were separated in 2% agarose gels made with 1×TAE. Samples were randomized before processing and two separate reactions performed for each primer/template combination. Bands were scored manually. Bands that were weak and those that failed to consistently amplify from the same template were excluded from the analysis.

Pathogenicity Tests

Conidia from 7 to 10-day old grape and oak *C. gloeosporioides* cultures grown on PDA were suspended in sterile deionized water, passed through four layers of cheese cloth, and adjusted to 10^6 conidia/mL. Approximately 0.1 mL of this solution was injected directly into the crown of three 'Camarosa' strawberry plants in a greenhouse during the summer of 2003. Under the warm environmental conditions used for the assay, pathogenicity could be determined unambiguously since no isolate caused collapse of an intermediate number of plants. Isolates were categorized as pathogenic on strawberry if they caused collapse of 3 of 3 plants within 4 weeks and nonpathogenic if they failed to affect any of the 3 plants inoculated. The repeatability of the assay was determined from inoculations on subgroups of isolates done during different time periods between the winter of 2002 and the fall of 2004. Based on theses data only one of 89 isolates would have been classified differently (data not shown).

The validity of the assay was confirmed by topically applying 1 mL of 10^6/mL conidia suspension to crowns in ten-plant plots of the cultivar Camarosa under field conditions. Twelve plots were treated with oak or grape isolates determined to be pathogenic in greenhouse tests and twelve plots treated with isolates determined to be nonpathogenic in greenhouse tests. As a positive control, four plots were inoculated with isolates from diseased strawberry crowns and four plots were inoculated with distilled water as a negative control. Transplants were set on October 24, 2003 on plastic mulch covered beds and inoculations conducted on March 12, 2004. Captan 80WP (4.2 kg per ha) was applied to plants weekly, with applications suspended 2 weeks before plants were inoculated and subsequently resumed 2 weeks following inoculations. The proportion of

plants collapsed was recorded for each plot 45 days after inoculation, just before water and nutrient supplies to plants under cultivation were discontinued for the season.

Statistical Analyses

Cluster analysis was performed on Dice similarity coefficients using the unweighted pair group method with arithmetic averages (UPGMA) with NTSYS, PC version 2.0 (Exeter Software, Setauket, NY). All scored bands were used for this analysis. Statistical support for phenogram branches was based on 1,000 replications using the Winboot bootstrap algorithm (71). Values of theta (θ) were estimated using the method of Weir and Cockerham (99), with confidence intervals generated by bootstrapping over loci using TFPGA (Utah State University, Logan). Statistical support for population subdivision was concluded if the lower boundary of the 90% confidence interval generated by 10,000 bootstrap replications exceeded zero. Theta measures the correlation of alleles of different individuals in the same population relative to all populations and is described by the equation $(Q - q)/(1 - q)$, where Q is the probability that two randomly sampled genes within a population are the same allele and q is the probability that genes randomly selected from different populations are the same allele (22). Bands absent or fixed in those populations that were being compared were excluded from this analysis. The effect of proximity to strawberry production, specific sampling site, and native host species on the incidence of pathogenicity was determined using logistic analysis performed with the GENMOD procedure in SAS (SAS Institute Inc., Cary, NC). For this analysis, isolates were categorized as being either close or distant from the strawberry production area. Effects of specific sampling sites were included in the model as nested effects within the variable "proximity to strawberry

production" and were compared to one another using specified contrasts. The incidence of plant collapse in field plots inoculated with isolates determined to be pathogenic or nonpathogenic in greenhouse experiments were compared using one-way analysis of variance or unpaired *t* tests. Proportions were transformed using the arcsine-square root transformation prior to analysis.

Results

Species Identification and Population Structure

Eighteen isolates from oak and grape lesions collected at Dover site 1 and 24 isolates collected at Dover site 2 produced a positive amplification product with the *C. gloeosporioides*/*C. fragariae*-specific ITS1 primer. From the two sites distant from strawberry production, Lake Alfred and Sarasota, 22 isolates and 25 isolates, respectively, were obtained that produced a positive amplification product with the species-specific ITS1 primer. Cluster analysis using data from 38 RAPD bands grouped isolates from grape and oak into 4 distinct clusters with similarity among isolates within the cluster greater than 0.65 and similarity between clusters less than 0.35 (Fig. 4-2). The vast majority (82 of 89) of isolates from these native hosts formed a large cluster along with 20 *C. gloeosporioides* isolates from diseased strawberry crowns. This cluster occurred in 89% of bootstrapped trees. Two homothallic isolates from Sarasota and one from Dover site 1 formed a distinct cluster, an isolate from an oak lesion in Lake Alfred clustered with a *C. fragariae* isolate, and three isolates from Lake Alfred clustered among six citrus isolates obtained from groves at various locations in Florida. Interestingly, one of the seven citrus isolates used as a control clustered among isolates from strawberry crown. The cluster containing only homothallic isolates and the cluster with isolates

related to citrus each occurred in 99% of bootstrapped trees. The cluster that included the *C. fragariae* isolate occurred in 100% of bootstrapped trees.

Of the 38 RAPD bands scored, nine were unique to the cluster defined by isolates of *C. gloeosporioides* from crown tissue, six were unique to the cluster defined by isolates from citrus, four were unique to the homothallic isolate cluster and six were unique to the cluster containing *C. fragariae* (Table 4-1.). Within the cluster that included isolates from crown tissue, there were only two bands that were amplified from native hosts only and these occurred at an overall frequency within these populations of less than 0.04.

When only isolates that clustered with those from crown rot were examined, there was no evidence for population differentiation between isolates from oak and grape hosts at any of the four sites (Table 4-2). In a hierarchal analysis in which native host isolates from all sites were used and sampling site was included in the analysis to delimit subpopulations, there was also no evidence for population subdivision based on host (θ = –0.029, 90% C. I. = –0.054 - 0.000). When sampling sites were examined in pairwise tests for population differentiation, there was evidence for population subdivision between isolates taken from the Sarasota sampling site and samples from Lake Alfred, Dover, and diseased crowns (Table 4-3). There was no evidence for population subdivision for isolates from any of the other samples (Lake Alfred, Dover 1, Dover 2 and the population from diseased crowns). Although there was evidence for population subdivision between the Sarasota sampling site and all others, there were no bands unique to this population nor did it lack any bands present in all of the other populations examined.

Pathogenicity

In greenhouse tests, a higher proportion of isolates from the Dover sites located adjacent to strawberry fields were pathogenic to strawberry than isolates from the sites distant from strawberry fields ($P = 0.002$; Tables 4-4 and 4-5). When the Sarasota population was excluded from the analysis, the proportion of isolates pathogenic on strawberry was still greater at the Dover sites ($P = 0.03$). Isolates from oak did not differ in degree of pathogenicity from grape isolates. There was also no significant difference in the proportion of pathogenic isolates at the two Dover sites located in close proximity to strawberry fields (Contrast $P = 0.59$), nor a difference in the proportion of pathogenic isolates at the Lake Alfred and Sarasota sites distant from strawberry production (Contrast $P = 0.76$). Homothallic *C. gloeosporioides* isolates from native hosts and *C. gloeosporioides* isolates from native hosts that clustered with citrus isolates did not cause crown rot symptoms. The single isolate from Lake Alfred that clustered with a *C. fragariae* isolate also caused collapse of plants when injected into crown tissue, produced tapered conidia, and possessed setae that functioned as phialides. These traits are characteristic of *C. fragariae* (43).

In the field experiment using both nonpathogenic and pathogenic isolates identified by direct injection of inoculum into crown tissue, plots topically inoculated with pathogenic isolates had a higher incidence of crown rot (38.3% vs. 3.3%, $P < 0.001$ unequal variances assumed, $n = 12$ per treatment). The relatively low incidence of crown rot in plots inoculated with isolates determined to be nonpathogenic in greenhouse tests was not statistically significantly different from plots sprayed with water (3.3% vs. 2.5%, $P = 0.783$, $n = 12$ and 4), suggesting that inoculation of plots with nonpathogenic isolates

did not increase disease incidence above that caused by natural sources of inoculum. Incidence of plant collapse was not different in the field for pathogenic isolates from the four different sites ($F = 0.36$, $P = 0.781$, $n = 3$ per site), nor was the percentage of plants collapsed for plots treated with native host isolates different from plots treated with isolates from diseased crown (38.3% vs. 60%, $P = 0.174$, $n = 12$ and 4).

Discussion

Colletotrichum isolates from native grape and oak leaves producing a positive PCR product with *C. gloeosporioides/C. fragariae*-specific ITS1 primers fell into four separate clusters based on RAPD marker data. The majority of the isolates from all sites sampled fell into the same cluster as *C. gloeosporioides* isolates from diseased strawberry crowns. However, one isolate clustered with *C. fragariae*, another pathogen causing strawberry crown rot, and three isolates grouped with *C. gloeosporioides* from citrus. For the oak and grape isolates that grouped with isolates from diseased crowns, band frequencies were not significantly different between the crown rot population and the population distant from strawberry production in Lake Alfred. This supports the hypothesis that the population of *C. gloeosporioides* on strawberry is derived from a population already present on hosts in Florida. Although band frequencies in the Sarasota population were different from the crown rot population, no unique bands were found in the Sarasota population and the phenogram constructed from RAPD data provided no evidence that the population is monophyletic. This suggests that differences in band frequencies are due to restricted gene flow combined with genetic drift rather than a speciation event. Fixation indices may also be estimated using the statistic G_{ST}, which differs from θ as a function of the number of individuals in each sample population and the number of

sample populations used in the calculation (22). This statistic has been used in the past to examine genetic differentiation in pathogen populations. For comparison to other studies, pairwise G_{ST} estimates between the Sarasota population and the other four sampled populations were estimated and ranged from 0.11 to 0.18. These values are in the range of estimates for other fungal populations using RAPD, amplified fragment length polymorphism (AFLP), or RFLP markers (42,67,74). However, in only one study where population structure of the chestnut blight fungus *Cryphonectria parasitica* was examined were distances between populations comparable to those between Sarasota and the other sites (67). No population subdivision was evident between oak and grape hosts at any location. In the study presented in chapter 3, band frequencies were not significantly different between a group of isolates from numerous noncultivated hosts and strawberry crowns. However, due to limited sampling, any host specificity within the noncultivated host population could not be examined. The observation of limited subdivision by host in areas where host species occur in close proximity further supports the hypothesis that the *C. gloeosporioides* population found on both native hosts and strawberry has a broad host range. Similar results were observed in a study examining host specificity of *C. gloeosporioides* isolates classified as endophytes collected from the foliage of trees in a tropical forest (60).

The three homothallic *C. gloeosporioides* isolates obtained from the Dover and Sarasota sites were compared to historical homothallic strawberry isolates (Florida isolates 311-1 and 329-1, C. M. Howard) (38) using RAPD markers and the two groups do not appear to be from the same population (data not shown). In chapter 3, homothallic isolates distinct from historical strawberry isolates were also collected from noncultivated

hosts. Although many of the bands scored for isolates in the study presented here and in chapter 3 were the same, several were not. There also may have been differences in calculated band migration rates. For these reasons, the relatedness of isolates collected in the two separate studies cannot be determined by a simple analysis of marker frequencies reported in tables. However, a side by side comparison of bands amplified from isolates obtained in these two studies using the primer OPC-2 indicates that they are from the same population (data not shown). The single *C. fragariae* isolate was obtained in Lake Alfred, FL approximately 28 km from a commercial strawberry farm. This species was previously shown to colonize *Cassia obtusifolia* growing in and around a strawberry nursery (50), but has never been reported away from a strawberry field. Its occurrence on oak at a considerable distance from any strawberry production suggests that *C. fragariae* may have a wider host range than previously believed and that it may be indigenous to Florida. In Louisiana, *C. fragariae* is responsible for most strawberry crown rot epidemics (79). Contaminated stock appears to be the major source of inoculum for these epidemics, as crown rot is not observed in production fields using disease free transplants (65). However, this does not exclude the possibility that a native host provided the initial inoculum for the population that persists on strawberry. Also, native hosts may still play a role in disease caused by *C. fragariae* as runners taken from plants that were free of disease after one production season develop crown rot symptoms in subsequent years (65).

Isolates of *C. gloeosporioides* from grape and oak that were closely related to citrus isolates were only observed in Lake Alfred. The grape vines and oak trees sampled at this site were next to a citrus grove and immigration of spores from citrus hosts would

likely be high in this area. The fact that only a small proportion of native host isolates from this site clustered with isolates from citrus underscores the specific interaction between the population on citrus and its host. However, the finding of a few citrus type isolates at the Lake Alfred site also demonstrates that, in the presence of a sufficient amount of inoculum from an outside source, alternate hosts can be colonized.

In greenhouse tests, the proportion of isolates from native hosts closely related to those on strawberry that were pathogenic on strawberry was greater at sampling sites close to strawberry fields. This provides support for the hypothesis that local selection for pathogenicity on strawberry occurs where this host is grown in abundance. Experimental studies of pathogen evolution have been conducted in pathosystems where the fungal pathogen displays a high degree of host specificity, such as the *Hordeum vulgare-Rhynchosporium secalis* pathosystem and the wild *Linum marginale-Melampsora lini* pathosystem (56,58,64,88). Within these pathosystems, pathogenic variation is governed by gene-for-gene interactions (15,56). Evidence that the biological relationship between *C. gloeosporioides* and strawberry is different from that found in these other pathosystems comes from research showing that isolates indistinguishable from those on strawberry can be found on numerous hosts. In addition, there is in no differential interaction between *C. gloeosporioides* isolates and strawberry cultivars as demonstrated in the following chapter. No microscopic studies investigating the infection of strawberry crown tissue by *C. gloeosporioides* have been conducted. The best available information on whether the interaction of *C. gloeosporioides* and strawberry is necrotrophic or biotrophic comes from a study examining *C. fragariae* on stolons. In this study, there was only a brief biotrophic phase before the pathogen entered

an extended necrotrophic stage (25). There is also good evidence that *C. gloeosporioides* forms infections in strawberry petioles, citrus twigs, and citrus fruit that remain quiescent until infected tissue senesces (12,66). Taken together, these studies suggest that *C. gloeosporioides* does not form a biologically intimate relationship with its host and gene-for-gene relationships do not play a major role. Because *C. gloeosporioides* has a broad host range and likely uses more than one pathogenic strategy to invade its host, it would be hard to identify an overriding factor shaping the evolution of this species in a natural environment. However, this may not be the case in agricultural areas where the presence of a large, genetically uniform host population at a specific site would select for individuals that can grow on the overrepresented host. This would be consistent with research showing races immune to specific host resistance genes are overrepresented in samples from areas where resistance genes are deployed (98). It is not clear whether the isolates collected in this study were actually pathogens on oak and grape leaves. Isolates from these hosts came from typical anthracnose type lesions, suggesting that they were pathogens. However, it is also possible that isolates were growing as saprophytes on lesions caused by another pathogen, insect damage, or injuries. In greenhouse inoculations of oak leaves, isolates were recovered from inoculated leaves, but produced necrotic symptoms only if the tissue was first wounded (data not shown).

One of the limitations of the crown injection assay may be that resistance to penetration by the pathogen may be circumvented (54). For this reason, topical inoculations of strawberry plots in the field were examined. The field trial indicated that isolates classified as pathogens in greenhouse tests caused plant collapse and those classified as nonpathogens did not. The field experiment also provided quantitative data

to differentiate pathogenic isolates. The aggressiveness of pathogenic isolates from native hosts was not statistically different between sampling sites, nor was aggressiveness of these native host isolates statistically different from crown isolates. However, the comparison of isolates from different sites and hosts suffered from high variability in the number of collapsed plants observed per plot as well as small sample sizes. The mean percentage of plants killed per plot for isolates from diseased crown tissue was 60%, whereas only 38% of plants in plots inoculated with pathogenic isolates from native hosts were killed. These numbers were not significantly different from one another, but it would not be inconsistent with the data on the incidence of pathogenicity that isolates from crown and pathogenic isolates from native hosts would differ from one another using a more quantitative measure for pathogenicity.

In summary, there was no conclusive evidence that *C. gloeosporioides* isolates from diseased strawberry crowns in Florida are genetically distinct from the *C. gloeosporioides* population broadly distributed on oak and wild grape hosts both close to and distant from commercial strawberry fields. Isolates pathogenic to strawberry were also broadly distributed on these native hosts, although they occurred at a higher frequency at sites close to strawberry fields. The results observed in this study are consistent with earlier work indicating that native plants can serve as a source of inoculum for crown rot epidemics. The high incidence of Collectotrichum crown rot that occurs in summer nurseries located in Florida is one of the reasons why transplants used for commercial fruit production in Florida are purchased from nurseries located at higher latitudes. The current study suggests that over the long term using transplants from nurseries not located in Florida will likely reduce the amount of initial inoculum in

growers fields but will not prevent epidemics caused by introduced isolates, since *C. gloeosporioides* isolates pathogenic to strawberry are present away from strawberry fields and the frequency of pathogenic isolates appears to respond to selective pressures.

Table 4-1. Frequencies of randomly amplified polymorphic DNA bands from *Colletotrichum gloeosporioides (C.g.)* and *Colletotrichum fragariae (C.f.)* isolates

	Cluster[a]									
	C.g. Strawberry					*C. g.* Citrus		*C. g.* Homothallic	*C. f.*	
Primer, length (kb)	D1[b] (*n* = 17)	D2 (*n* = 24)	LA (*n* = 23)	SS (*n* = 20)	Strawberry (*n* = 20)	LA (*n* = 3)	Citrus (*n* =6)	SS & D1 (*n* = 3)	LA (*n* = 1)	Strawberry (*n* = 1)
$(ACTG)_4$										
2.145	0.35	0.46	0.89	0.74	0.65	1.00	1.00	0.00	0.00	0.00
1.9	1.00	1.00	1.00	0.91	1.00	0.00	0.00	1.00	0.00	0.00
1.85	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00
1.6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
1.5	1.00	1.00	1.00	1.00	1.00	0.33	1.00	0.00	0.00	0.00
1.45	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.00
1.3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
1.1	0.71	0.83	0.67	0.22	0.80	0.00	0.00	0.00	0.00	0.00
$(GACA)_4$										
1.6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
1.45	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.4	0.12	0.04	0.17	0.70	0.05	0.00	0.00	0.00	0.00	0.00
1.35	1.00	1.00	0.94	1.00	0.95	0.00	0.00	0.00	0.00	0.00
1.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
1.2	0.88	0.96	0.89	0.87	0.90	0.33	0.00	0.00	0.00	0.00
1.1	0.06	0.04	0.11	0.13	0.05	0.67	1.00	0.00	1.00	1.00
0.95	0.65	0.50	0.61	0.87	0.55	0.00	0.00	0.00	0.00	0.00
0.9	0.47	0.63	0.56	0.74	0.60	1.00	1.00	1.00	1.00	1.00
0.8	0.24	0.21	0.11	0.04	0.05	0.00	0.00	0.00	0.00	0.00
0.55	0.00	0.00	0.06	0.09	0.00	1.00	1.00	1.00	0.00	0.00
0.5	1.00	0.96	1.00	0.96	1.00	1.00	1.00	0.00	1.00	1.00

[a]Clusters correspond to the four groups of isolates identified in fig. 4-2.

[b]Isolates from native hosts oak and grape are identified by site code D1, D2, LA, or SS. Isolates from cultivated hosts used as controls are referred to by the name of the host.

Table 4-1. Continued

	Cluster[a]									
	C.g. Strawberry					*C. g.* Citrus		*C. g.* Homothallic	*C. f.*	
Primer, length (kb)	D1[b] (*n* = 17)	D2 (*n* = 24)	LA (*n* = 23)	SS (*n* = 20)	Strawberry (*n* = 20)	LA (*n* = 3)	Citrus (*n* =6)	SS & D1 (*n* = 3)	LA (*n* = 1)	Strawberry (*n* = 1)
$(TCC)_5$										
2.0	0.00	0.08	0.28	0.00	0.05	0.00	0.00	0.00	0.00	0.00
1.95	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	1.00	1.00
1.9	0.59	0.42	0.44	0.61	0.60	0.33	0.83	0.33	0.00	0.00
1.55	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00
1.1	0.59	0.63	0.50	0.87	0.40	0.00	0.00	1.00	0.00	0.00
1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
0.75	0.24	0.17	0.39	0.83	0.25	0.00	0.00	0.33	0.00	0.00
0.7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
OPC-2										
2.4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
2.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
1.9	0.65	0.58	0.67	0.96	0.50	0.00	0.00	0.00	0.00	0.00
1.85	0.00	0.00	0.00	0.00	0.00	0.67	0.50	0.00	0.00	0.00
1.7	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00
1.3	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	1.00	1.00
1.1	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
1.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
0.7	0.00	0.00	0.00	0.00	0.00	1.00	0.33	0.00	0.00	0.00

Table 4-2. Estimates of θ for pairwise comparisons of *Colletotrichum gloeosporioides* isolates from oak and grape hosts at four sites

Site	θ[a]	90% Confidence interval θ[b]
Dover 1	-0.094	-0.114 - -0.069
Dover 2	0.031	-0.032 - 0.091
Lake Alfred	0.056	-0.032 - 0.157
Sarasota	-0.017	-0.057 - 0.040

[a]Thirteen to 15 bands were used to estimate θ.
[b]The 90% confidence interval was determined from 10,000 bootstrap replications.

Table 4-3. Pairwise estimates of θ (above diagonal) for *Colletotrichum gloeosporioides* populations at four sites and the estimated 90% confidence interval for θ (below diagonal)

	Dover 1	Dover 2	Lake Alfred	Sarasota	Strawberry crown
Dover 1	-	-0.021[a]	-0.031	0.218*	-0.005
Dover 2	-0.032 - -0.010[b]	-	0.030	0.275*	-0.007
Lake Alfred	-0.039 - 0.129	-0.017 - 0.094	-	0.168*	-0.004
Sarasota	0.116 - 0.310	0.141 - 0.396	0.087 - 0.239	-	0.281*
Strawberry crown	-0.032 - 0.028	-0.027 - 0.014	-0.025 - 0.023	0.131 - 0.373	-

[a]Fifteen to 17 bands were used to estimate θ. The lower boundary of the 90% confidence interval for estimates followed by an asterisk is greater than zero.

[b]Ninety percent confidence limits were determined from 10,000 bootstrap replications.

Table 4-4. Percentage of isolates pathogenic on strawberry from oak (*Quercus* spp.) and grape (*Vitis* spp.) lesions at four sites

		Number of isolates[a]		Pathogenic
Site	Host	Path	Nonpath	(%)
Dover 1	*Quercus* spp.	4	4	50.0
	Vitis spp.	3	6	33.3
Dover 2	*Quercus* spp.	6	7	46.2
	Vitis spp.	6	5	54.6
Lake Alfred	*Quercus* spp.	2	9	18.2
	Vitis spp.	1	6	14.3
Sarasota	*Quercus* spp.	2	10	16.7
	Vitis spp.	1	10	9.1

[a]Path = pathogenic, Nonpath = nonpathogenic

Table 4-5. Likelihood ratio statistics examining the effect of local strawberry production, specific sampling site and native host species on the proportion of native host isolates pathogenic to strawberry.

Source of variance	df	χ^2	$P > \chi^2$
Proximity to strawberry production[a]	1	9.41	0.002
Site(proximity to strawberry production)	3	0.38	0.828
Host	1	0.17	0.677

[a]Proximity to strawberry production was classified as either near or distant.

Fig. 4-1. Map showing the three locations where *Colletotrichum gloeosporioides* isolates were sampled from lesions on oak and grape leaves. There were two separate sampling sites in Dover. Hillsborough County, the main strawberry-growing county in Florida, is shaded. Map template provided by the Department of Geography, Geology, and Anthropology, Indiana State University, Terre Haute 47809.

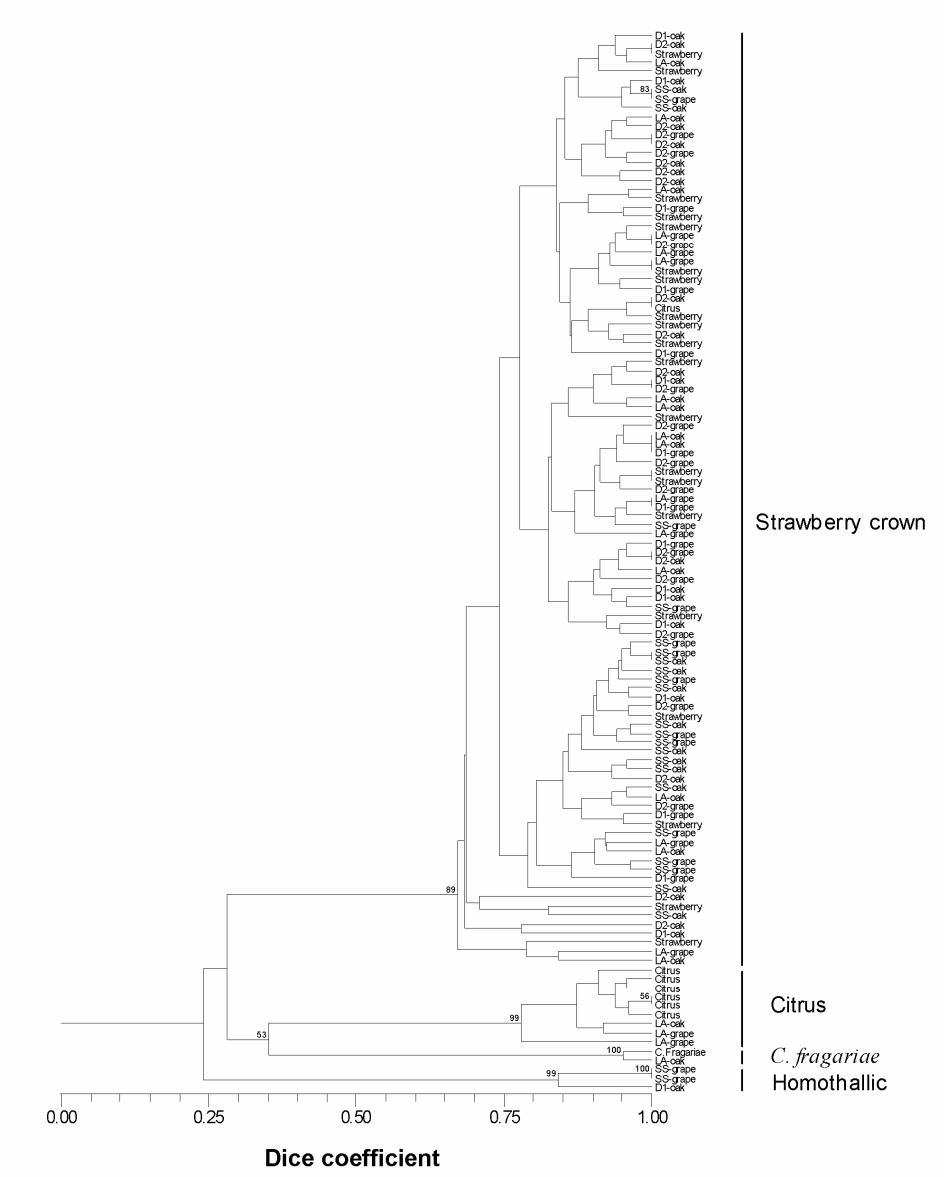

Fig. 4-2. Unweighted pair group method with arithmetic averages phenogram showing genotypic similarity between *Colletotrichum* isolates. *C. gloeosporioides* isolates from oak and grape fell into three separate clusters based on high similarity among isolates within each cluster (similarity ≥ 0.65), low similarity between each cluster and other isolates (similarity ≤ 0.35), and high bootstrap support for clusters (≥ 50%). The three clusters consisted of isolates that were homothallic in culture, isolates similar to strawberry crown isolates, and isolates similar to citrus isolates. An isolate and cluster of *C. fragariae* is identified by name. Isolates coded with the prefix D1 and D2 are from the two sites in Dover. Isolates coded LA are from Lake Alfred and SS from Sarasota. Numbers at branch points indicate the percent occurrence of the cluster to the right of the branch in 1,000 bootstrapped phenograms. Only branches occurring in at least 50% or more of bootstrapped phenograms are labeled.

CHAPTER 5
RESISTANCE OF STRAWBERRY CULTIVARS TO CROWN ROT CAUSED BY *Colletotrichum gloeosporioides*

Introduction

Previous studies examining variation in strawberry resistance to *C. fragariae* found a broad range of susceptibility among cultivars and aggressiveness among isolates (28,48,81). One study found a significant cultivar × isolate interaction (81), implying gene-for-gene interactions between the pathogen and host. Host resistance studies using homothallic *G. cingulata* isolates were not as conclusive, as they included only a few isolates (31,81). One study found significant variation in cultivar susceptibility and isolate aggressiveness, and a significant cultivar × isolate interaction (81). In a later study using a different set of isolates, only cultivar susceptibility varied (31). Variation in cultivar susceptibility to self-sterile *C. gloeosporioides* isolates from Florida has not been investigated. Although closely related to *C. fragariae* (83), genotypes of self-sterile *C. gloeosporioides* isolates are diverse, whereas little diversity is observed among *C. fragariae* isolates (92). In addition, as the name implies, *C. fragariae* is thought to have a much narrower host range (25).

In this chapter, the levels of resistance to crown rot caused by self-sterile *C. gloeosporioides* isolates in cultivars commonly grown in Florida as well as the patterns of virulence and aggressiveness among *C. gloeosporioides* isolates under field conditions are investigated. Resistance data for progeny from crosses using a highly resistant parent and a susceptible parent are also presented. The study was designed to generate

information that can be useful to growers for cultivar selection as well as selection of nursery locations from which to purchase plants. It was also designed to generate information useful to breeders in terms of the mechanism and sources of resistance to crown rot caused by *C. gloeosporioides*.

Materials and Methods

Plant Materials and Cultivation

Cultivars for experiments examining cultivar × isolate interactions were selected from those commercially available as leaf-on, bare-root transplants imported from Canada at the beginning of the 2001-2002, 2002-2003, and 2003-2004 seasons. The cultivars used included the standard cultivars in the industry, several new cultivars, and an advanced selection from the Florida Agricultural Experiment Station breeding program. The cultivars Aromas, Camarosa, Earlibrite, and Sweet Charlie were evaluated during the 2001-2002 season. During the 2002-2003 season, in addition to the above four cultivars, the cultivars Gaviota, Strawberry Festival, and Treasure were also tested. 'Strawberry Festival' and 'Treasure' were evaluated again during the 2003-2004 season, but 'Gaviota' was not available. Additional material used over the 2003-2004 season included the cultivars Camino Real, Carmine, Camarosa, and selection FL 99-164. 'Aromas', 'Camarosa', 'Gaviota', and 'Camino Real' are University of California cultivars; 'Carmine', 'Earlibrite', 'Strawberry Festival', and 'Sweet Charlie' are University of Florida cultivars. The cultivar Treasure is from J & P Research in Naples, FL.

For the experiment examining inheritance of resistance to crown rot, progeny from crosses using 'Treasure' and 'Camarosa' as parents were supplied by the Florida Agricultural Experiment Station breeding program and propagated from runners during

the summer of 2004 in Florida. The parent cultivars were imported from Canada and selected to match crown diameters consistent with those observed among progeny propagated in Florida. The number of progeny for four crosses were: 24 Camarosa × Treasure, 17 Treasure × Camarosa, eleven Treasure × self, and ten Camarosa × self. The number of plants propagated from individual progeny was variable as some produced few runners or transplants died. For the crosses between 'Camarosa' and 'Treasure', the number of plants propagated of each genotype ranged from nine to twelve plants and eight to twelve plants were obtained for progeny from the Camarosa × self. For Treasure × self between nine and twelve plants were propagated for seven genotypes and between two and five plants were propagated for four genotypes. The low numbers of plants propagated for three of these genotypes was due to death of transplants and one genotype only produced three runners.

Plants were grown on raised, plastic mulch covered beds 71 cm wide, 15 cm high at the edge, and 18 cm high in the center. Each bed contained two rows of strawberry plants with 30.5 cm separating rows and plants within the rows separated by 38 cm. The distance between bed centers was 1.22 m. Before planting, the beds were fumigated with methyl bromide/chloropicrin (98:2) at 350 kg/ha. Leaf-on, bare-root transplants were set on Oct 18 for the 2001-2002 season, Oct 16 for the 2002-2003 season, and Oct 29 for the 2003-2004 season for experiments evaluating cultivar × isolate interactions. For the experiment examining inheritance of resistance, plants were set on Oct 21 of the 2004-2005 season. Plants were overhead irrigated for ten to twelve days to facilitate establishment. After establishment, water and fertilizer was provided through drip tape. To prevent secondary spread of crown rot, weekly applications of Captan 80WP (Micro

Flo, Memphis, TN; captan) at 4.2 kg/ha were begun 2 weeks after plants were inoculated. Freeze and frost protection was provided by overhead sprinklers when necessary.

Fungal Isolates and Inoculation Procedure

C. gloeosporioides isolates were selected from those characterized in chapters 2, 3 and 4. Most were from strawberry crown tissue, although during the 2002-2003 season an isolate from a lesion on wild grape (*Vitis rotundifolia*) and during the 2003-2004 season an ascospore isolate from a perithecium emerging from a strawberry petiole were included in the study (Table 5-1). All isolates were determined to be pathogenic on strawberry by injection of inoculum into crown tissue, also reported in chapters 2, 3 and 4. The isolates had distinct RAPD banding patterns and were recovered from seven different strawberry cultivars. Two isolates of *C. fragariae*, one from a strawberry crown and the other from an oak (*Quercus* sp.) leaf lesion in Lake Alfred, FL, were included in the cultivar × isolate interaction study during the 2003-2004 season. Isolate 96-83R was used to inoculate plots in the inheritance of resistance study. This isolate was chosen because it generates numerous spores and has the ability to produce crown rot symptoms on both parents examined.

Inoculum was prepared from 6- to 8-day old cultures grown under continuous fluorescent light at 24°C on potato dextrose agar. Conidial suspensions used for inoculations were prepared in sterile deionized water, filtered through four layers of cheese cloth, and diluted to 5×10^5 conidia/mL. For cultivar × isolate interaction experiments, inoculations were performed by spraying 2 mL of conidial suspension with a hand mister directly into the crown of plants 15 to 17 days after the plants were set in the field. The inoculation date was 2 Nov 2001 for the 2001-2002 season, 31 Oct 2002

for the 2002-2003 season, and 15 Nov 2003 for the 2003-2004 season. For the inheritance of resistance experiment inoculations were delayed until 14 Feb 2005.

Experimental Design and Statistical Analysis

The cultivar × isolate interaction experiment was designed as a two-factor complete block. Blocks consisted of a single 73-m bed for the 2001-2002 season or two parallel 73- to 76-m beds for the 2002-2003 and 2003-2004 seasons. There were four blocks in each experiment. All cultivar-isolate combinations were randomly assigned to ten-plant plots within each block along with one uninoculated plot of each cultivar. After inoculation, the number of collapsed plants within each plot was recorded weekly until the experiment was terminated at the end of the growing season. Termination dates were 15 Mar 2002, 28 Mar 2003, and 26 Mar 2004. The proportion of plants that collapsed in each plot on a specific date was transformed to the arc-sine-square root and used for statistical analysis. The formula for the transformation was *arcsin(sqrt((y + ⅜)/(n + ¾))),* where *y* = the number of collapsed plants per plot and *n* = the total number of plants per plot (4). Data for each season were analyzed separately using PROC MIXED of SAS (SAS institute, Cary, NC). In the analysis using *C. gloeosporioides* and *C. fragariae* data alone, "block" was considered a random effect and "cultivar" and "isolate" were considered fixed effects. In the analysis where *C. gloeosporioides* and *C. fragariae* data were combined, "species", "isolate", and "cultivar" were all considered fixed effects with "isolate" nested within "species". Block was considered a random effect. Uninoculated plots were not included in any reported analysis. The risk of type I error (α) was 0.05 for tests comparing means and was not adjusted for multiple comparisons.

For the inheritance of resistance experiment, progeny and parent clones were randomly assigned to plots on three beds. Each plot contained plants of the same progeny genotype or ten to twelve plants of the cultivars Camarosa or Treasure. There were four plots of each parent genotype and one plot for each progeny genotype. The number of collapsed plants was recorded for plots on Monday and Friday each week after inoculation. The experiment was terminated on 25 May 2005. Proportions of collapsed plants were not statistically analyzed in this experiment due to variation in the number of plants of each genotype and the effect that truncating distributions at 0% and 100% might have on likelihood ratio tests for mixed normal distributions. As an alternative, the sum of average daily temperatures preceding the point where 50% of the plants of each genotype collapsed was estimated from the data by regressing the proportion of plants collapsed over time points immediately before and after this threshold was exceeded. Because temperature affects the rate of collapse (70), an adjustment for this variable was necessary. Assuming that resistance to crown rot is a quantitatively inherited trait determined by genes at multiple loci, progeny of a cross should have resistance clustered around a value close to the midpoint of both parents. If a major gene contributes to resistance in one parent and not the other, then resistance among progeny will be bimodal with equal proportions of progeny in each mode. This assumes that the parent only possesses one copy of the resistance gene. A likelihood ratio test statistic was used to test for bimodality of resistance among progeny from the cross between the susceptible cultivar Camarosa and the resistant cultivar Treasure. Before the statistical analysis, the sum of average daily temperatures between inoculation and the time point at which 50% of plants collapsed was transformed to eliminate any residual skewness in data that could

confound likelihood ratio analysis (61). The formula for the transformation was $(\sum$ *average daily temperature)*$^{1/4}$. Transformed data was described using models fit with the program NOCOM (Ott, J., NOCOM and COMPMIX programs. New York, Rockefeller University. 1992). The test statistic G^2 was calculated from maximum likelihood estimates under the hypothesis of a single normal distribution or a mixture of two normal distributions with equal variance. The formula for G^2 is $2[ln(L_1) - ln(L_0)]$, where L_0 is the maximum likelihood under the hypothesis of a single normal distribution and L_1 is the maximum likelihood under the hypothesis of two normal distributions. When two normal components in a mixture have equal variance, the distribution of G^2 can be approximated by the chi-square distribution with 2 degrees of freedom. However, statistical tests based on this distribution have been shown to be liberal and therefore estimated *P*-values were obtained from simulated data sets (90). In addition to the analysis of progeny from crosses using 'Treasure' and 'Camarosa' as parents, a likelihood ratio analysis on progeny from self pollinated 'Treasure' plants was conducted using the same transformation described above, also under the assumption of equal variances for multiple distributions. Assuming that a dominant gene contributes to resistance in 'Treasure', a bimodal distribution with a 3:1 ratio of progeny in the resistant versus susceptible distribution should be observed.

Results

Disease Progression in Cultivar × Isolate Experiments

The rate at which plants collapsed after inoculation with *C. gloeosporioides* differed during the three seasons (Fig. 5-1A, B and C). During the 2001-2002 season, the majority of plants that developed crown rot symptoms did so in the first 55 days after inoculation. For the 2002-2003 season, rapid plant collapse within this time period only

occurred for ‘Gaviota’ and ‘Camarosa’. During this season, the rate at which plants developed symptoms slowed between day 50 and day 130 after inoculation. However, unlike the 2001-2002 season in which the progression of symptom development also slowed, there was a spike in plant death late in the season. For the 2003-2004 season, symptom development appeared to continue at about the same rate throughout the season for all of the cultivars tested. At each time point, the rankings of plants with respect to susceptibility were approximately the same. However, some cultivars such as Earlibrite in the 2002-2003 season, Strawberry Festival in the 2003-2004 season, and FL 99-164 in the 2003-2004 season initially had relatively low disease incidence that increased at a faster rate relative to other cultivars toward the end of the season. Only ‘Camarosa’ was examined for susceptibility to *C. gloeosporioides* in all three seasons. At the end of each season, the incidence of plant collapse for this cultivar was consistently high ranging from 62% to 84%. Plants were challenged with *C. fragariae* only during the 2003-2004 season (Fig. 5-1D). During this season the progression of symptom development showed a similar pattern to that observed for *C. gloeosporioides*. Secondary spread of pathogens did not appear to affect results in any of the experiments since disease incidence at the end of the season in the control plots ranged from 0% in 2002-2003 to 0.12% in 2003-2004.

Cultivar and Isolate Evaluation

Transformed disease incidence data used for statistical analysis was obtained at different time points after inoculation for each season. Data for analysis were taken on 4 Jan 2002, 28 Mar 2003, and 19 Mar 2004. These dates corresponded to 63, 148, and 125 days after inoculation, respectively. On these dates the absolute value of the difference

between the number of plots with 0% plant mortality and 100% plant mortality was minimized. This reduced compression bias toward one extreme value or the other (0% or 100% mortality). In addition, on these dates the number of plots with 0% or 100% mortality was low and variance among treatment combinations was relatively high. Analysis of data for *C. gloeosporioides* revealed strong isolate and cultivar effects, but no significant cultivar × isolate interaction in each of the three seasons studied (Table 5-2). The variance component "block" was not estimated to be greater than zero in any of the experiments ($P > 0.05$). Graphs displaying disease incidence for each isolate-cultivar combination in all three seasons showed that cultivar rankings were usually consistent across isolates (Fig. 5-2A, B and C). Where rankings did change, the cultivars with different ranks had similar levels of resistance suggesting that random error could account for the changes. There was also a cultivar and isolate effect on disease incidences caused by *C. fragariae* during the 2003-2004 season, but no significant cultivar × isolate interaction (Table 5-3). The graph showing cultivar sensitivity to each *C. fragariae* isolate also shows that cultivar rankings were consistent across isolates (Fig. 5-2D). When the *C. gloeosporioides* and *C. fragariae* data from the 2003-2004 season were combined, in addition to a species, cultivar, and isolate effect on disease incidence, there was also a small but significant species × cultivar interaction (Table 5-3). This interaction resulted from a change in the rankings of 'Strawberry Festival' and 'Camino Real' across fungal species (Fig. 5-3). With the exception of this one rank change, disease reactions to isolates of the different *Colletotrichum* species were very similar.

The cultivars and isolates included in experiments during the different seasons were altered to increase the chance of observing any cultivar × isolate interactions. In

addition, cultivars and isolates were carried over to the next year to assess the repeatability of the assay. During the 2001-2002 season, ‘Camarosa’ was the most susceptible cultivar followed by ‘Aromas’. ‘Earlibrite’ and ‘Sweet Charlie’ were more resistant and had essentially the same level of susceptibility (Table 5-4). The ranking of these cultivars for the 2002-2003 season was the same as for 2001-2002, although statistically ‘Aromas’ could not be separated from ‘Earlibrite’ and ‘Sweet Charlie’. During the 2002-2003 season ‘Treasure’, ‘Strawberry Festival’, and ‘Gaviota’ also were evaluated. ‘Treasure’ was more resistant and ‘Gaviota’ more susceptible than any of the other cultivars examined. ‘Strawberry Festival’ had an intermediate level of resistance, similar to that of ‘Aromas’. During the 2003-2004 season, rankings among cultivars carried over from the previous season remained the same. ‘Treasure’ was once again the most resistant cultivar and ‘Camarosa’ was more susceptible than ‘Strawberry Festival’. Although ‘Camarosa’ and ‘Strawberry Festival’ were not statistically significant from one another for the 2003-2004 season as they were for the 2002-2003 season. During the 2003-2004 season, disease symptoms on ‘Strawberry Festival’ likely had more time to attain the ratings observed on ‘Camarosa’. The three genotypes grown only during the 2003-2004 season, FL 99-164, ‘Carmine’, and ‘Camino Real’ were intermediate between the resistant ‘Treasure’ and the relatively susceptible ‘Strawberry Festival’ and ‘Camarosa’.

During the 2001-2002 season, isolates fell into four groups based on average aggressiveness to the four cultivars tested (Table 5-5). Isolate 97-15A was the most aggressive and isolates 95-63A, 97-45A, and 97-47C had relatively high, comparable levels of aggressiveness. Isolates 98-285 and 97-63 were not as aggressive and 97-63

was even less aggressive than 98-285. Isolates 97-45A, 98-285, and 97-63 were re-evaluated in the 2002-2003 season. These three isolates along with 00-59, 96-83R, and 96-83H also produced four isolate clusters based on aggressiveness. The three isolates from the 2001-2002 season had intermediate levels of aggressiveness similar to the previous year. The rankings of these isolates with respect to one another also remained the same, although in the second season, isolates 98-285 and 97-63 were not significantly different from each other. Isolates 00-59 and 96-83R were highly aggressive and isolate 96-83H, a nonstrawberry isolate, was the least aggressive isolate examined. For the 2003-2004 season only isolate 00-59 was re-evaluated. Once again it was a highly aggressive isolate. Other isolates examined fell into two groups with isolate 02-172 being more aggressive than isolate 96-15A and ascospore isolate 00-117. In separate analyses comparing inoculated plots to uninoculated controls, all *Colletotrichum* inoculated plots had significantly more crown rot than controls (data not shown).

Cultivar rankings for resistance to *C. fragariae* were very similar to those for *C. gloeosporioides* during the season that this species was included in the study (Table 5-4). 'Treasure' was highly resistant to *C. fragariae*; FL 99-164 and 'Carmine' displayed moderate levels of resistance and 'Strawberry Festival' and 'Camarosa' were relatively susceptible. The rankings only differed in that 'Camino Real' appeared to be more resistant and 'Strawberry Festival' more susceptible to *C. gloeosporioides* than to *C. fragariae*, although not dramatically. Only two *C. fragariae* isolates were included in the study. These isolates displayed different levels of aggressiveness: isolate 02-135, a *C. fragariae* isolate from a nonstrawberry host, was more aggressive than C-16, the isolate from strawberry (Table 5-5).

Inheritance of Crown Rot Resistance

Progeny inoculations were not done until February due to differences in the size of transplants. It was also necessary to determine whether any plants were infected with *C. gloeosporioides* during propagation over the summer. During the course of the experiment, average daily temperatures ranged from 9.1ºC to 25.1ºC with a mean daily temperature of 18.8ºC. The average temperature dropped below 15ºC on only eleven days, with all but three of these days occurring before any significant plant death. The experiment extended well beyond the normal growing season until at least 50% of plants had collapsed in all the plots. Once 50% mortality in all plots was achieved, for each progeny genotype the cumulative average temperature was calculated for days up to and including the date when 50% mortality was reached. The mean and standard deviation of transformed cumulative temperature for progeny of crosses Camarosa × Treasure and Treasure × Camarosa was 5.25 ± 0.35 $ºC^{1/4}$. For crosses Camarosa × self and Treasure × self they were 4.82 ± 0.24$ºC^{1/4}$ and 5.24 ± 0.45$ºC^{1/4}$, respectively. The means and standard deviations for parent genotypes were 5.08 ± 0.08$ºC^{1/4}$ for 'Camarosa' and 5.76 ± 0.18$ºC^{1/4}$ for 'Treasure'. In the cross between 'Treasure' and 'Camarosa', female parent did not affect resistance. (Mean ± standard error, Camarosa × Treasure = 5.23 ± 0.08$ºC^{1/4}$ and Treasure × Camarosa = 5.27 ± 0.08$ºC^{1/4}$, $P = 0.67$). Both visual and statistical analysis of the distribution of transformed cumulative temperature for Camarosa × Treasure and Treasure × Camarosa progeny suggests bimodal inheritance (Fig. 5-4, $G^2 = 7.39$, $P = 0.042$). Means for the mixed normal distribution model that maximized likelihood were 4.91 and 5.51 with standard deviation of 0.177. Estimated proportions of progeny belonging to each distribution were 0.45 and 0.55. Analysis of

the distribution of progeny from self pollinated ‘Treasure’ plants suggests that there were eight relatively resistant progeny and three highly susceptible progeny (Fig. 5-4). The log likelihood ratio supports bimodality for this distribution ($G^2 = 8.83$, $P = 0.035$). Means for the mixed normal distribution model that maximized likelihood were 4.60 and 5.483 with standard deviation of 0.170. Estimated proportions of progeny belonging to each distribution were 0.27 and 0.73. Progeny from self pollinated ‘Camarosa’ were mostly susceptible to crown rot with one outlier having a relatively high level of resistance.

Visual analysis of the proportion of collapsed plants for progeny on 28 March 2005 supports conclusions based on the analysis using cumulative average temperature (Fig 5-5). On this date the number of progeny with either 0% or 100% plant collapse was minimal for progeny from crosses between ‘Camarosa’ and ‘Treasure’ (three with 100% mortality and six with 0% mortality of 41 progeny). Plant collapse in most plots of progeny from Camarosa × Treasure and Treasure × Camarosa were close to extreme values of 0% or 100% with few progeny at midpoint values. Approximately half of the progeny had mortality greater than 50% and half had mortality less than 50%. Eight of eleven progeny from self pollinated ‘Treasure’ plants had relatively low or intermediate mortality whereas three progeny had high mortality.

Discussion

Pathogenicity trials conducted over three years using twelve distinct *C. gloeosporioides* isolates and ten strawberry cultivars identified differences in disease resistance among cultivars and differences in aggressiveness among isolates, but failed to identify any cultivar × isolate interactions. A more limited study using two *C. fragariae* isolates inoculated on six strawberry cultivars conducted during the third year identified

differences in disease resistance among cultivars and differences in aggressiveness among the isolates, but failed to identify a cultivar × isolate interaction. When cultivar resistance rankings were compared between the two pathogen species, there was a small but significant cultivar × species interaction.

Horizontal resistance is effective against all isolates of a pathogen, whereas vertical resistance is effective against a subset of isolates (96). These terms are synonymous with race-nonspecific and race-specific resistance, respectively (7). In pathosystems where race-specific resistance occurs, an incompatible interaction between the host and pathogen often requires a dominant host resistance gene and a dominant pathogen avirulence gene. Such interactions are described by the gene-for-gene hypothesis (41). Race-nonspecific resistance is less understood, but is believed to be governed by many host genes that incrementally contribute to the overall resistance of the plant (96). Although both resistance mechanisms can be employed within a pathosystem, race-specific resistance is often associated with biotrophic plant-microbe interactions and race-nonspecific resistance with necrotrophic interactions (41). VanderPlank proposed using analysis of variance to determine the contribution of race-nonspecific and/or race-specific resistance within a host population against a group of pathogen isolates (96). Absence of a cultivar × isolate interaction is evidence for race-nonspecific resistance using this method. A cultivar × isolate interaction suggests that race-specific mechanisms contribute to the resistance observed within the host population, although deviations from additivity could be responsible for the statistical interaction (73). Deviations from additivity result from the scale used to measure resistance, whereas rank changes are consistent with physiological interdependency between the host and isolates.

No cultivar × isolate interactions were detected in this study suggesting that race-nonspecific resistance mechanisms are responsible for the differences in resistance observed between cultivars. Thus, screening with many isolates might not be necessary to examine resistance to *C. gloeosporioides*. Using transformed percentages taken at a time point in which disease incidence was at an intermediate level effectively reduced interactions attributable to deviations from additivity. This finding may be useful in examining resistance to other wilt diseases where use of arbitrary rating scales or measurements of area under the disease progress curve may produce results in which there are no transformations available to eliminate undesirable scale effects. A drawback of measuring resistance using the proportion of collapsed plants is that the rankings of cultivars could change at different time points after inoculation as resistance may occur at different levels of the infection process. Temporally distinct resistance mechanisms have been demonstrated for *Phytophthora palmivora* on cacao (54). The first level of resistance, referred to as penetration resistance, was attributed to morphological factors and the second level of resistance, referred to as post penetration resistance, impeded tissue invasion by the pathogen after colonization. In the current study, rank changes between cultivars over the course of the season were infrequent and occurred only between cultivars displaying similar levels of resistance, suggesting that sequential deployment of resistance mechanisms had little effect on the evaluation of cultivars. However, the relatively high levels of inoculum used could have saturated structural mechanisms that limit ingress into the host. Similarly, individual isolates within the pathogen population might differ in their ability to overcome plant defenses at different times during and after invasion of tissues. However, like the cultivar resistance rankings,

pathogen aggressiveness rankings did not change over the course of the experiment (data not shown).

During the 2001-2002 season, the rate of plant collapse was greater than the 2002-2003 or 2003-2004 seasons. Disease probably progressed more rapidly in the 2001-2002 season because the mean daily temperature in December was 18.3°C, 3.7°C higher than it was in the other seasons. The mean daily temperature of 11.6°C in January of the 2002-2003 season was 2.5°C lower than the mean temperature during January for the other two seasons and may have slowed symptom development.

In a previous study examining resistance to *G. cingulata* and *C. fragariae* in strawberry, cultivar × isolate interactions were observed (81). The population of *G. cingulata* used in that study was homothallic and therefore it was probably distinct from the one we used. Also, in that study, it is conceivable that scale effects produced by the severity rating system used to evaluate disease accounted for the interaction, but the interaction may also have been due to use of isolates or cultivars not included in the current study. We attempted to use isolates from different cultivars and isolates that were genetically distinct. Commercial cultivars shipped from Canada were used to obtain the relatively large number of plants free of crown rot required for the experiment. A disadvantage of using these cultivars was that some of them were closely related, restricting the diversity of the germplasm evaluated. For example, 'Earlibrite' (17) and 'Strawberry Festival' (18) each share 'Rosa Linda' as a parent and 'Strawberry Festival' (18) and 'Treasure' (Plant Patent 12,414) share 'Oso Grande' as a parent. Nevertheless, a great deal of variance in resistance was observed among them.

Race-specific resistance is found most frequently in biotrophic plant-microbe interactions where there is prolonged contact between the pathogen and the living host (41). *Colletotrichum* species use nutritional strategies ranging from necrotrophy to hemibiotrophy (57). Co*lletotrichum* species such as *C. orbiculare*, *C. graminicola*, *C. sublineolum*, *C. destructivum*, *C. truncatum* and *C. linicola* are all considered hemibiotrophs, as there is an asymptomatic biotrophic interaction between these species and host cells before the reaction becomes necrotrophic (57). The occurrence of a number of dominant race-specific resistance genes in bean to *C. lindemuthianum*, a member of the *C. orbiculare* species aggregate, suggest that gene-for-gene interactions play an important role in host resistance to these hemibiotrophic pathogens (104). No microscopic studies of strawberry crown invasion by *C. gloeospioides* have been conducted, although invasion of subtropical fruits (6,11), northern jointvetch (87), and *Stylosanthes scabra* (91) by *C. gloeosporioides* has been investigated. On citrus and avocado fruit, *C. gloeosporioides* is a quiescent epiphyte that resorts to necrotrophy upon ripening of the fruit (6,11). On foliage of *S. scabra* and northern jointvetch, *C. gloeosporioides* has a brief biotrophic phase before entering an extended necrotrophic stage (87,91). The evidence for this biotrophic interaction is not nearly as clear as it is for interactions between *Colletotrichum* spp. commonly referred to as hemibiotrophs and is limited to the occurrence of a spherical vesicle inside an epidermal cell just beneath the appressorium from which infection hyphae emanate. A differential interaction between *S. scabra* cultivars and biotype A *C. gloeosporioides* isolates has also been demonstrated (91), although a great deal of the variation in resistance among cultivars is due to race-nonspecific mechanisms (16). The histopathology of the related pathogen *C. fragariae*

on strawberry stolons showed that a brief biotrophic phase possibly occurred before the pathogen entered an extended necrotrophic phase (25). The biotrophic phase was less than 12 h and it was considered a modification of necrotrophy rather than an example of hemibiotrophy. The lack of race-specific resistance observed in the current study suggests that biotrophic interaction between *C. gloeosporioides* or *C. fragariae* and strawberry is brief and limited. This finding is also consistent with evidence from chapters 3 and 4 that both of these species infect hosts unrelated to strawberry.

An isolate of *C. gloeosporioides* from grape, a *C. gloeosporioides* ascospore isolate from a strawberry petiole, and an isolate of *C. fragariae* from oak were included in this study. Evidence that these isolates came from populations responsible for crown rot on strawberry was presented in chapters 3 and 4. The ascospore isolate was one of the least aggressive isolates during 2003-2004, although it was as aggressive as one of the crown rot isolates. During the 2002-2003 season, the grape isolate was less aggressive than the isolates from strawberry crowns. This may result from more aggressive isolates being selected in the *C. gloeosporioides* population on strawberry. The *C. fragariae* isolate from oak was more aggressive and had essentially the same virulence pattern as the isolate from strawberry. This is further evidence that *C. fragariae* from strawberry is derived from a population with a very broad host range, since the oak isolate was found 28 km from the closest strawberry production area. The only interaction detected was between the species of the isolate and cultivar. This interaction was small and only one rank change occurred between cultivars, suggesting that resistance to *C. gloeosporioides* is correlated with resistance to *C. fragariae* and that large scale screening of plants for resistance to both pathogens may not be necessary.

Only one cultivar, Treasure, was highly resistant to *C. gloeosporioides*. It was also highly resistant to *C. fragariae*. Symptoms eventually developed in response to inoculation with both pathogens and therefore 'Treasure' was not immune. Resistance among progeny from crosses to the susceptible cultivar Camarosa was distributed bimodally with progeny distributed evenly among the two distributions, suggesting that a major gene contributes to resistance. However, the method used to determine resistance had several drawbacks which might cast some doubt on this conclusion. Ideally the experiment would have been conducted at a constant temperature, but due to the large number of mature plants included in the study, temperature could not be controlled. It is well documented that the growth rate of *C. fragriae* and *G. cingulata* is affected by temperature and that low temperatures inhibit progression of crown rot (70,81). For this reason cumulative temperature until 50% of plants collapsed was used to evaluate resistance. The relationship between growth rate and temperature on agar for both *C. fragariae* and *G. cingulata* is approximated by a linear function between 8ºC and 30ºC (81), suggesting that the temperature adjustment is appropriate. However, the growth rate may not be linear in plant tissue or the fungus may fail to grow in crown tissue below a specific temperature. As a result, it is conceivable that the bimodal response was observed when no major gene had an effect on resistance. An example would be if a sudden drop in temperature during the experiment suspended plant death completely, splitting the distribution which otherwise would have been normal. An analysis of temperatures during the time period when few plots reached the 50% threshold suggests that this did not happen, as temperatures were relatively high (data not shown). Data examining proportions of plants collapsed at a time point where plant mortality for most

progeny were at values intermediate between 0% and 100% also supports a bimodal distribution of resistance among progeny, although the distribution was truncated at extreme values for close to 25% of the plots. Progeny from self pollinated 'Treasure' plants supports that a single dominant gene effects resistance in 'Treasure', as statistical analysis indicates a bimodal distribution with the ratio of isolates from the more resistant distribution to the more susceptible distribution very close to 3:1. However, these data must be viewed with caution as several plots had only a few plants and limited progeny were examined. Numerous plants of three of the progeny genotypes collapsed early in the season before inoculation. The cause of death was Colletotrichum crown rot. These three genotypes were also determined to be highly susceptible to crown rot in the experiment. It is conceivable that the plants were already infected with crown rot, but given that they did not develop symptoms during the early part of the season and that plant death occurred relatively synchronously after inoculation it appears that they were infected by applied inoculum. The high susceptibility of these plants also suggests resistance apart from that conferred by a major gene is very low within 'Treasure'. Currently a much larger population of progeny from self pollinated 'Treasure' plants are being propagated for evaluation during the 2005-2006 season.

Sibling families were used in a previous study examining resistance to *C. fragariae* and a bimodal distribution was observed for disease severity among the population (44). That study did not examine bimodality in single crosses and the disease rating scale consisted only of 6 classes. Plants were rated for their ability to resist lesion formation on stems as well as appearance of wilt and collapse. That study also used three *C. fragariae* isolates applied separately to plants and it is conceivable that the bimodality

resulted from differences in aggressiveness of isolates as opposed to resistance. Although the field method used to evaluate progeny in the current study has drawbacks, it provides an objective variable to evaluate genotypes. From a practical perspective, it was able to show that a large proportion of progeny of a cross involving 'Treasure' and a susceptible parent had resistance approaching or surpassing that of 'Treasure'. Three of the 41 progeny were rated as being more resistant to crown rot than 'Treasure'. Given the good fruit quality of this cultivar, crosses to 'Treasure' could produce new genotypes with crown rot resistance and improved horticultural characteristics in just one generation.

Table 5-1. Description of *Colletotrichum* isolates used in inoculation experiments conducted over three seasons in Dover, Florida

Species	Isolate	Host – cultivar	Tissue	Collection site and year	Season used
C. gloeosporioides	95-63A	Strawberry – 'Oso Grande'	Crown	Dover, FL 1995	'01-'02
	96-15A	Strawberry – 'Oso Grande'	Crown	Dover, FL 1996	'03-'04
	96-83H	*Vitis rotundifolia*	Leaf lesion	Dover, FL 1996	'02-'03
	96-83R	Strawberry – 'Selva'	Crown	Dover, FL 1996	'02-'03
	97-15A	Strawberry – 'Sweet Charlie'	Crown	Dover, FL 1997	'01-'02
	97-45A	Strawberry – 'Camarosa'	Crown	Dover, FL 1997	'01-'02, '02-'03
	97-47C	Strawberry – 'Camarosa'	Crown	Dover, FL 1997	'01-'02
	97-63	Strawberry – 'Oso Grande'	Crown	Dover, FL 1997	'01-'02, '02-'03
	98-285	Strawberry – 'Sweet Charlie'	Crown	Dover, FL 1998	'01-'02, '02-'03
	00-59	Strawberry – 'Strawberry Festival'	Crown	Dover, FL 2000	'02-'03, '03-'04
	00-117	Strawberry – 'Rosa Linda'	Ascospore – petiole	Dover, FL 2000	'03-'04
	02-172	Strawberry – 'Gaviota'	Crown	Dover, FL 2002	'03-'04
C. fragariae	C-16	Strawberry – 'Camarosa'	Crown	Dover, FL 2002	'03-'04
	02-135	*Quercus species*	Leaf lesion	Lake Alfred, FL 2002	'03-'04

Table 5-2. Analysis of variance for three experiments evaluating incidence of crown rot in relation to strawberry cultivar and isolate of *Colletotrichum gloeosporioides*

Season	Source of variation	df	*F*	*P* > *F*
2001-2002[a]	Cultivar	3	39.16	<0.001
	Isolate	5	45.04	<0.001
	Cultivar × isolate	15	1.52	0.123
2002-2003[b]	Cultivar	6	35.50	<0.001
	Isolate	5	31.63	<0.001
	Cultivar × isolate	30	1.34	0.134
2003-2004[c]	Cultivar	5	36.66	<0.001
	Isolate	3	21.08	<0.001
	Cultivar × isolate	15	0.65	0.823

[a]*F* values for 2001-2002 season were calculated using a residual variance estimate equal to 0.024 having 69 degrees of freedom.
[b]*F* values for 2002-2003 season were calculated using a residual variance estimate equal to 0.038 having 123 degrees of freedom.
[c]*F* values for 2003-2004 season were calculated using a residual variance estimate equal to 0.035 having 69 degrees of freedom.

Table 5-3. Analysis of variance for 2003-2004 experiment evaluating incidence of crown rot in relation to strawberry cultivar and isolate of *Colletotrichum fragariae* alone or in comparison to *C. gloeosporioides*

Species included	Source of variation	df	*F*	*P* >*F*
C. fragariae[a]	Cultivar	5	24.66	<0.001
	Isolate	1	9.01	0.005
	Cultivar × isolate	5	0.91	0.485
C. fragariae and *C. gloeosporioides*[b]	Species	1	27.10	<0.001
	Cultivar	5	56.91	<0.001
	Isolate(species)	4	19.00	<0.001
	Species × cultivar	5	2.72	0.023

[a]*F* values for the analysis examining *C. fragariae* only were calculated using a residual variance estimate equal to 0.034 having 33 degrees of freedom.
[b]*F* values for the analysis examining both *C. fragariae* and *C. gloeosporioides* were calculated using a residual variance estimate equal to 0.033 having 125 degrees of freedom.

Table 5-4. Mean percent plant collapse of cultivars inoculated with *Colletotrichum gloeosporioides* or *C. fragariae* during three seasons in Dover, Florida

	Disease incidence (%)[a]			
	C. gloeosporioides			*C. fragariae*
Cultivar	2001-2002[b]	2002-2003	2003-2004	2003-2004
Treasure		10.0 A	11.3 A	0.0 A
Sweet Charlie	30.3 A	29.9 B		
Earlibrite	30.7 A	33.9 B		
FL 99-164			33.7 B	12.0 B
Carmine			41.5 BC	17.1 B
Camino Real			50.6 C	56.4 C
Aromas	59.6 B	43.1 BC		
Strawberry Festival		45.9 C	80.6 D	51.1 C
Camarosa	71.8 C	64.7 D	83.8 D	76.3 D
Gaviota		82.2 E		

[a]Statistical tests were conducted on mean transformed disease incidences. Reported values were calculated by back transforming the mean arc-sine-square root disease incidences for all isolates inoculated on a cultivar.

[b]Statistical tests only compare means for plants inoculated with the same species within the same season. Means in each column followed by the same letter are not significantly different, least significant difference ($P = 0.05$).

Table 5-5. Mean percent plant collapse for *Colletotrichum gloeosporioides* or *Colletotrichum fragariae* isolates used to inoculate strawberry cultivars during three seasons in Dover, Florida

		Disease incidence (%)[a]		
Inoculated species	Isolate	2001-2002[b]	2002-2003	2003-2004
C. gloeosporioides	97-15A	86.8 A		
	00-59		69.4 A	71.8 A
	96-83R		66.9 A	
	02-172			59.1 B
	95-63A	53.8 B		
	97-45A	53.8 B	48.6 B	
	97-47C	50.7 B		
	96-15A			36.3 C
	98-285	36.0 C	34.3 C	
	00-117			33.4 C
	97-63	9.5 D	31.4 C	
	96-83H		15.3 D	
C. fragariae	02-135			40.9 A
	C-16			24.7 B

[a]Statistical tests were conducted on mean transformed disease incidences. Reported values were calculated by back transforming the mean arc-sine-square root disease incidences for all cultivars inoculated with an isolate.
[b]Statistical tests only compare means for isolates of the same species within the same season. Means in each column followed by the same letter are not significantly different, least significant difference ($P = 0.05$).

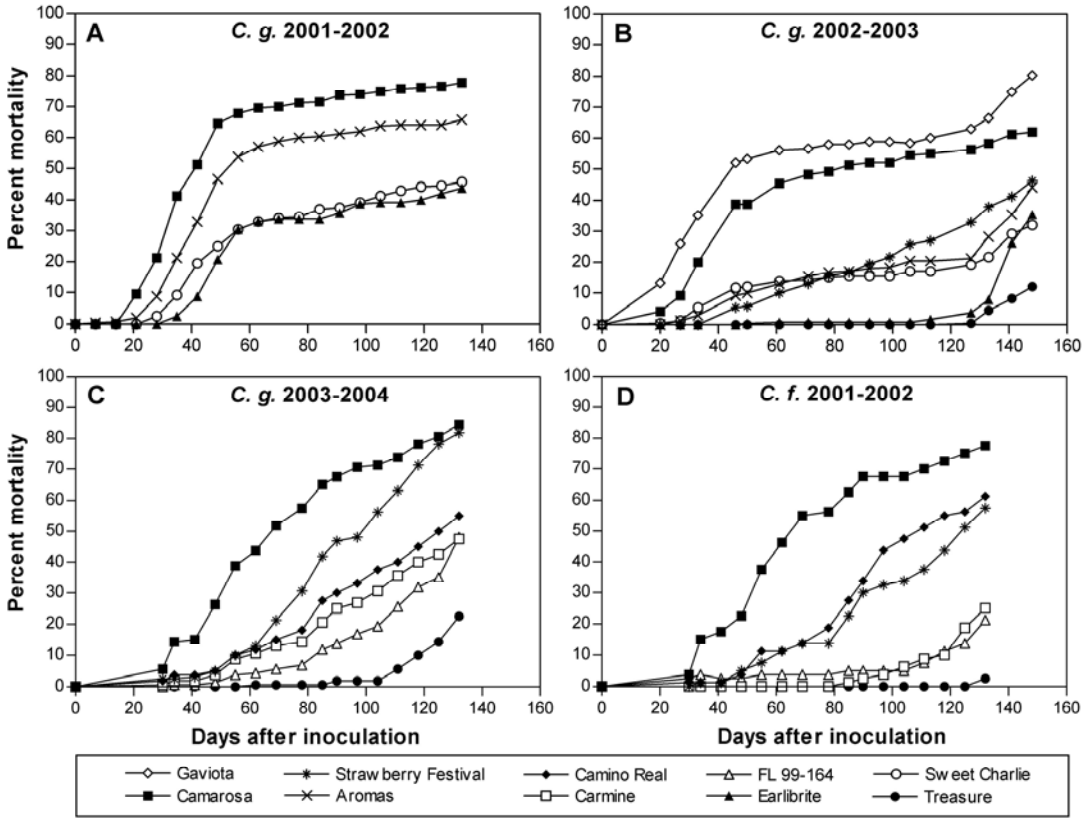

Fig. 5-1. Mean percent mortality for strawberry cultivars calculated at weekly intervals over the course of the growing season using data from all isolates. (A) *Colletotrichum gloeosporioides* inoculated plants during the 2001-2002 season. (B) *C. gloeosporioides* inoculated plants during the 2002-2003 season. (C) *C. gloeosporioides* inoculated plants during the 2003-2004 season. (D) *C. fragariae* inoculated plants during the 2003-2004 season.

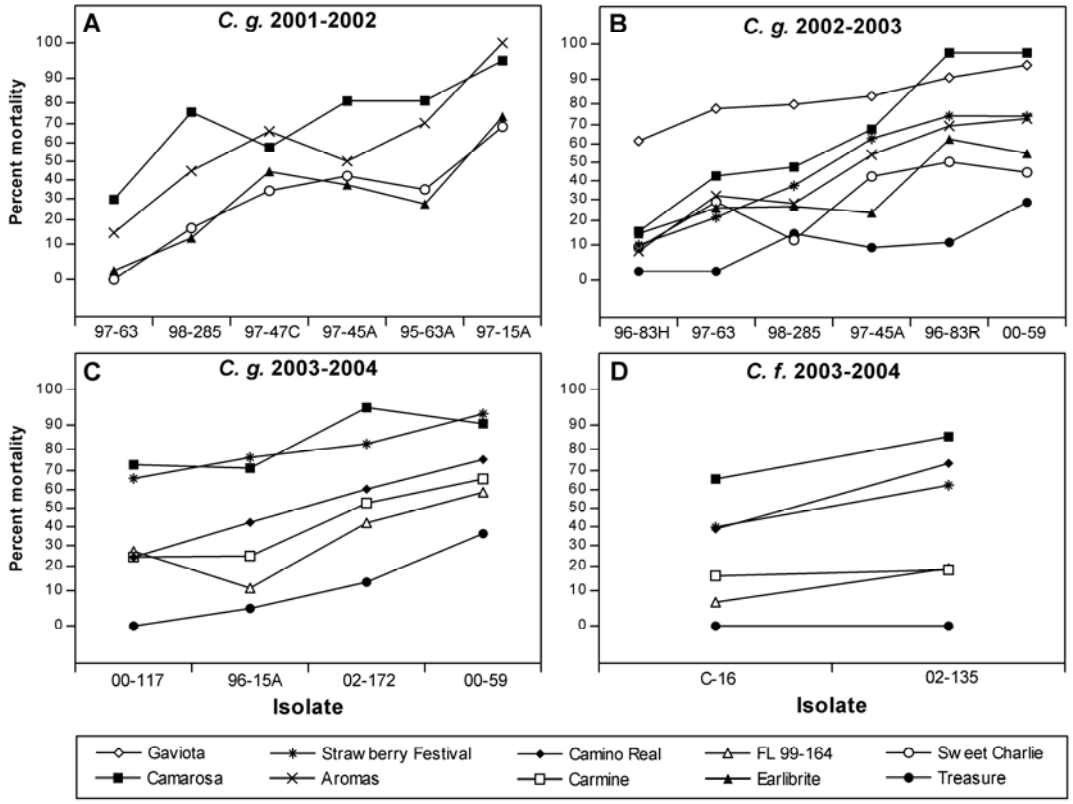

Fig. 5-2. Cultivar × isolate means for arc-sine-square root transformed disease incidences calculated on a specific date during or at the end of the season. Ticks on the y-axis show position of backtransformed incidences ranging from 0% to 100% at intervals of 10%. (A) Disease incidences calculated for plots 63 days after inoculation with *Colletotrichum gloeosporioides* during the 2001-2002 growing season, (B) 148 days after inoculation with *C. gloeosporioides* during the 2002-2003 growing season, (C) 125 days after inoculation with *C. gloeosporioides* during the 2003-2004 growing season, and (D) 125 days after inoculation with *C. fragariae* during the 2003-2004 growing season.

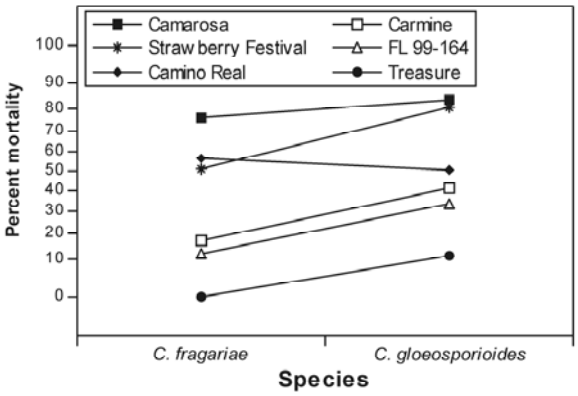

Fig. 5-3. Cultivar × species means for arc-sine-square root transformed disease incidences calculated 125 days after inoculation with *Colletotrichum fragariae* or *C. gloeosporioides* during the 2003-2004 season. The y-axis shows the position of backtransformed incidences ranging from 0% to 100% at intervals of 10%.

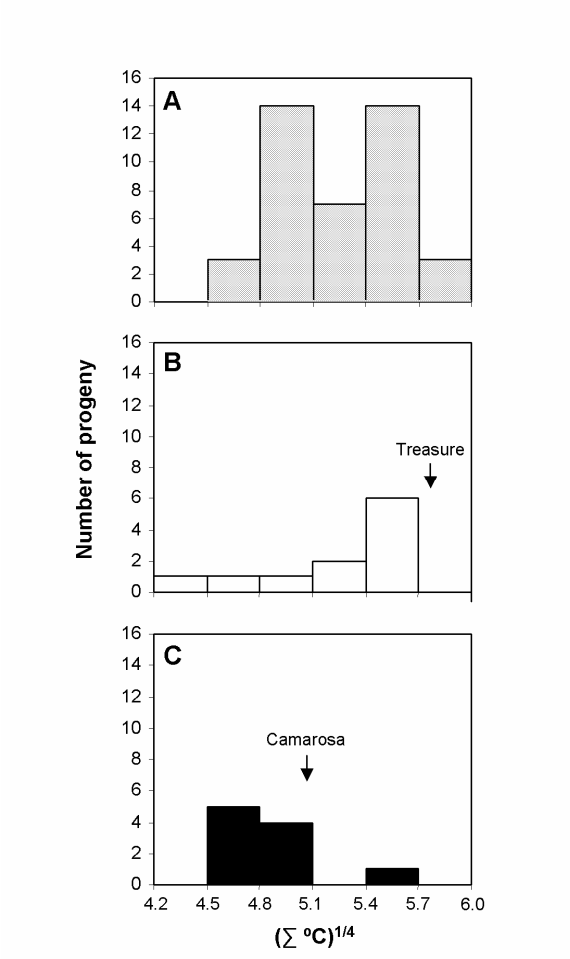

Fig. 5-4. Distribution of transformed cumulative temperature until 50% plant death for progeny inoculated with *Colletotrichum gloeosporioides* from (A) Camarosa × Treasure and Treasure × Camarosa; (B) Treasure × self; and (C) Camarosa × self. Means for the four plots of the parents 'Treaure' and 'Camarosa' are depicted by arrows in graphs B and C, respectively.

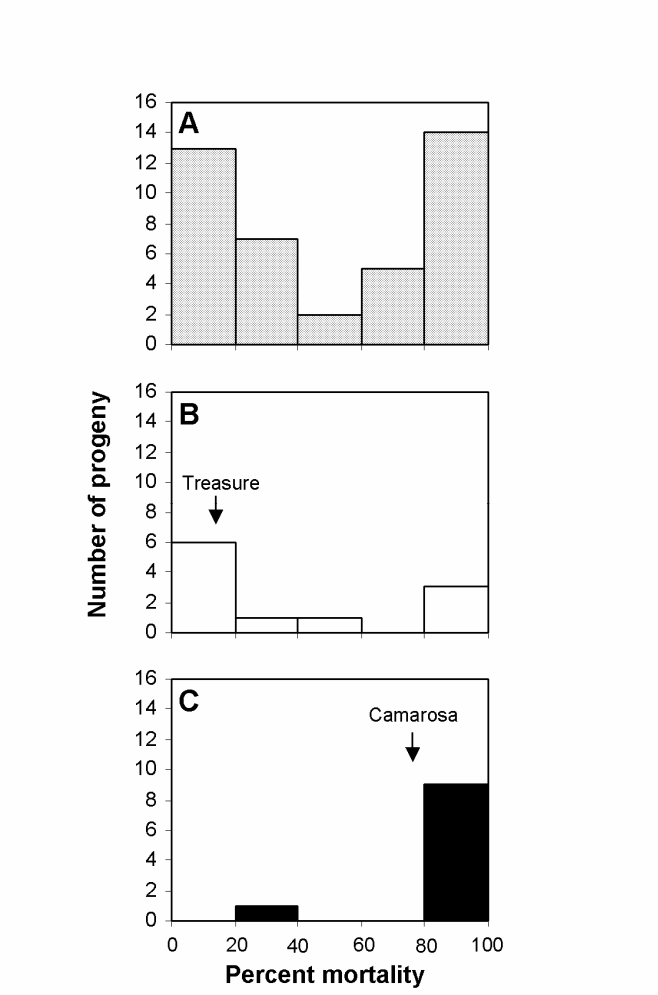

Fig. 5-5. Distribution of percent plant mortality 42 days after inoculation for progeny inoculated with *Colletotrichum gloeosporioides* from (A) Camarosa × Treasure and Treasure × Camarosa; (B) Treasure × self; and (C) Camarosa × self. Means for the four plots of the parents 'Treaure' and 'Camarosa' are depicted by arrows in graphs B and C, respectively.

CHAPTER 6
CONCLUSION

It was clear that *C. gloeosporioides*, not *C. fragariae*, was the species of *Colletotrichum* responsible for most of the crown rot on strawberry observed in Florida (92) prior to the initiation of my research. In addition, genetic data indicated that *C. gloeosporioides* isolates collected from collapsed crowns were genetically diverse and that they were part of a recombining population (92). *C. gloeosporioides* was also known to colonize petiole tissue and some isolates from petioles had been found to be incapable of producing crown rot symptoms (66). On several occasions, perithecia with morphology consistent with that of *G. cingulata* had been found on petioles, but the relationship of isolates from these perithecia to those responsible for crown rot had never been determined (66). Based on the research presented in chapter 2, a clearer picture of the population on strawberry has now emerged. Perithecia found on petioles resulted from recombination and single-ascospore isolates from these perithecia were genetically indistinguishable from self-sterile crown isolates, providing direct evidence that sexual recombination had occured among individuals within the population of *C. gleosporioides* responsible for crown rot. It was also apparent that *C. gloeosporioides* isolates collected from crown tissue were part of the broader population found on strawberry and that some individuals within this population were not capable of causing crown rot. This conclusion was based on the apparent lack of a reproductive barrier between isolates pathogenic and those nonpathogenic to strawberry in laboratory crosses and the

occurrence of progeny with both phenotypes from at least one perithecium on plant material collected from the field.

An important epidemiological question concerning the *C. gloeosporioides*/strawberry pathosystem that had remained unanswered until now was whether primary inoculum could come from sources outside of strawberry fields. Indirect evidence such as disease clusters at the edges of fields, the inability to find infections on transplants (66), and lack of evidence that the fungus could survive on debris between seasons (93) suggested that the primary inoculum was coming from outside of fields. In chapters 3 and 4, the relationship of the *C. gloeosporioides* population from crown tissue to populations of *C. gloeosporioides* from hosts other than strawberry in Florida was examined. Among *C. gloeosporioides* isolates from these hosts, genetically distinct subpopulations were detected. Isolates from the *C. gloeosporioides* population on citrus appeared to be host specific and a homothallic population, distinct from both the self-sterile and self-fertile crown population, was identified from noncultivated hosts. Although these subpopulations existed, the vast majority of isolates from noncultivated hosts were indistinguishable from the self-sterile population on strawberry and, like the population on strawberry petioles, both pathogenic and nonpathogenic isolates were obtained from the noncultivated hosts. This same alternative host population also occurred at sites distant from strawberry production, although the frequency of isolates pathogenic to strawberry was lower. Based on the finding that pathogenic isolates genetically indistinguishable from the population on crowns were present at sites close to and distant from strawberry fields, it appears that primary inoculum for crown rot epidemics can come from alternative hosts. In an

unpublished study conducted in a Florida strawberry field, *C. gloeosporioides* spread from infected to healthy strawberry plants during the season. However, a high proportion of plants had to be infected to observe spread in the field and spread from plants infected early in the season was not detected until the very end of the season. Isolate genotypes from newly infected plants were mostly the same as the genotype of the strain used for the initial inoculation, indicating that disease had spread by conidia. In fields using transplants from Canada, *C. gloeosporioides* isolates from crowns were found to be genetically diverse (92). Since *C. gloeosporioides* has not been recovered from imported transplants and there was a long time lag between infection and spread in field trials, not only is it possible that primary infections come from noncultivated hosts, but it also appears likely that the infections currently observed in production fields come from this source. However, in summer nurseries in Florida, it is probable that spread among strawberry plants perpetuates the disease, since higher temperatures would decrease the length of the disease cycle and rainfall would be more plentiful.

Genetic and pathogenicity data generated in the current study also provided insight into the evolution of *C. gloeosporioides* pathogenicity on strawberry. Whether *C. gloeosporioides* is a pathogen on the native or noncultivated species from which it was isolated is not apparent, since *C. gloeosporioides* was isolated at a high frequency on asymptomatic tissue when epiphytic populations on tropical forest foliage were examined (60). Given that both epiphytic and pathogenic strains of *C. gloeosporioides* were observed on strawberry in the current study and in a previous study (66), both saprophytic and pathogenic nutritional strategies are probably employed. The genetic requirements for pathogenicity on strawberry occurred at a detectable frequency within

the *C. gloeosporioides* population present on native hosts, even though strawberry is not native to Florida. An increase in the pathogenic phenotype was also observed where strawberries were abundant. These observations were consistent with the idea that *C. gloeosporioides* occurs as a broadly distributed epiphytic population on numerous hosts with a portion of individuals possessing pathogenicity genes for some but not all hosts. In the presence of a host that is suitable for colonization, selection for pathogenicity genes might then occur. Experiments examining the inheritance of pathogenicity did not give clear results. The ratio of pathogenic to nonpathogenic progeny in a cross between parents with different pathogenicity phenotypes deviated from the expected 1:1 ratio under the assumption that a single segregating locus determines pathogenicity. This suggests that multiple genes contribute to pathogenicity on strawberry. However, nonmendelian segregation of whole chromosomes and genetic markers was also observed indicating that nonmendelian inheritance of one or a cluster of pathogenicity genes could also explain the skewed inheritance. It is conceivable that these genes lie on a dispensable chromosome as has been observed for other fungal species (24,45).

No cultivar × isolate interactions were observed in field trials reported in chapter 5. This finding is consistent with the hypothesis that *C. gloeosporioides* employs a necrotrophic nutritional strategy, with little biological interaction between the pathogen and its host. The occurrence of *C. gloeosporioides* on numerous hosts other than strawberry is also consistent with this strategy. Resistance to *C. gloeosporioides* and *C. fragariae* among cultivars was positively correlated, indicating that resistance mechanisms to these fungi overlap. In previous studies examining resistance to crown rot, *C. fragariae* was used to screen for resistance (28,44,48,81). The positive correlation

between resistance to *C. fragariae* and *C. gloeosporioides* among cultivars suggested that cultivars identified in previous studies as being resistant to *C. fragariae* would also be useful in breeding programs for *C. gloeosporioides* resistance. Although a cultivar immune to crown rot was not identified, resistance in the cultivar 'Treasure' appeared superior to others examined. When crossed with a susceptible cultivar, segregation of resistance among progeny indicated that a major gene contributes to resistance in 'Treasure'. Given that resistance in 'Treasure' is conferred by a major gene product, breeding and selection of crown rot resistant offspring from 'Treasure' is possible in a short time.

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BIOGRAPHICAL SKETCH

Steven was born in Mount Clemens, Michigan, and grew up in Grand Haven, Michigan, on the shore of Lake Michigan. He received a Bachelor of Science degree in biology from the University of Michigan, Ann Arbor in 1988. From 1989 to 2000, he was employed at the Detroit Medical Center, where he was a research assistant in the Department of Endocrinology. He also received a Master of Business Administration degree from Wayne State University in 1997, while living in Detroit. His mother, Marjory MacKenzie, was a psychiatric nurse before retirement and his father, Donald MacKenzie, was a general surgeon until his death in 2000. He has one brother and three sisters. He married Dr. Enas Sallam in 2000. Their daughter, Leila, was born in 2004.