

ABSTRACT

SCHWEGEL, ROSEMARY. Strategies for the Management of Strawberry Anthracnose. (Under the direction of Dr. Frank J. Louws.)

Strawberry anthracnose, which can be caused by *Colletotrichum acutatum*, *C. dematium*, *C. fragariae*, or *C. gloeosporioides*, is an economically important disease in North Carolina. As a response to the Fall 2003 anthracnose epidemic in NC resulting from the use of latently infected Canadian planting stock, three related studies were conducted to present management options for this disease, which is difficult to control chemically and is often introduced through planting stock.

First, to secure a source of uninfected plants, three strawberry transplant sources (NC Registered, NC Certified, and noncertified Canadian) were evaluated in the field for the presence of anthracnose and horticultural characteristics. None of the plants showed symptoms of anthracnose. The Canadian plants showed significantly greater leaf dry weights, root dry weights, and leaf areas before planting, but this difference decreased over time. Crown dry weights of the Canadian plants remained greater on average than those of the NC plants over the course of the growing season. Root health ratings for the Canadian plants were significantly greater than those of the NC plants at the peak bloom and harvest samplings. The Canadian plants produced a significantly greater total berry number (69% greater) and weight (53% greater) but a slightly lower average berry size than the North Carolina-produced plants. This difference in yield may be due to the difference in transplant size, the difference in root health, or the possibility that NC-grown plants require more frequent overhead watering after planting for good establishment.

In a second study designed to evaluate diagnosis and control measures for the disease, petioles were sampled at two sites in NC, each containing 30,000 plug plants, 105 petioles from the first site and 100 from the second. The level of infection was evaluated visually in the field and by a bioassay of the collected petioles in which the petioles were treated with herbicide to induce senescence of the plant tissue and encourage fungal sporulation. Spore suspensions of isolates collected from the sampled petioles were plated on media containing the strobilurin fungicide pyraclostrobin, and the percent germination was compared to the incidence of germination for isolates which had never been exposed to strobilurin fungicides. The collected isolates were not found to be resistant to pyraclostrobin, indicating that this fungicide may prove to be a viable management option for strawberry anthracnose in fruiting fields.

In the third study, a real-time PCR protocol for detection of *Colletotrichum* spp. in strawberry petiole tissue was developed and compared to the previously described bioassay method. Petioles were sampled from strawberry plants grown in a controlled environment and artificially inoculated with *C. acutatum*, and from field-grown strawberry plants naturally infected with *C. gloeosporioides*; the sampled petioles were screened using both assays. The real-time PCR protocol was more rapid and showed significantly greater sensitivity than the bioassay when evaluating the artificially inoculated petioles. A lack of sensitivity in the real-time PCR assay was observed when evaluating the naturally infected petioles; this problem was likely due to poor tissue disruption of the tougher field-grown petioles during the DNA extraction protocol. Once issues of contamination and poor tissue disruption are addressed, real-time PCR may

become an important tool for sensitive and specific detection of *Colletotrichum* spp. in strawberry tissue.

STRATEGIES FOR THE MANAGEMENT OF STRAWBERRY ANTHRACNOSE

by
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DEDICATION

To Nahks Tr'Ehnl, for figuring out where to find the wheelbarrow and holocaust cloak.

BIOGRAPHY

Rosemary Schwegel, born on June 10th 1981, is originally from Philadelphia, PA. Her interests in horticulture go back to a project involving the restoration of the greenhouse at her high school, Mount St. Joseph Academy. The summer after graduation, she assisted her older sister at Merck Pharmaceuticals, getting hands-on experience in a molecular biology lab. Rosemary began her undergraduate work at the Pennsylvania State University in Fall 1999. At Penn State, Rosemary obtained a variety of experience, including work with Dr. Richard Arteca in phytoremediation of soil contaminated by the gasoline additive MTBE, with Dr. Paul Backman in evaluating the inhibitory effect of various herbal extracts on the growth of *Alternaria solani* *in vitro* and *in vivo*, and with Dr. Richard Craig in studying the floral longevity of Regal Pelargonium cultivars and the relationship of floral longevity to ethylene evolution and sensitivity. She graduated in May 2003 with a degree in horticulture and a minor in plant pathology. In June she married Nahks Tr'Ehnl, also a graduate of Penn State. Rosemary began her masters program at the North Carolina State University in Fall 2003 under the guidance of Dr. Frank Louws. Her research centered on developing and evaluating control measures for the management of strawberry anthracnose, including a real-time PCR protocol for the detection of the causal organism in strawberry tissue.

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1. CHAPTER I:

General Introduction

1.1. LITERATURE REVIEW

North Carolina is the fourth leading producer of strawberries in the United States, after California, Florida, and Oregon. NC strawberry production in 2002 was 1800 acres at 12,500 pounds per acre, for a total crop of 22.5 million pounds (17). The value of North Carolina strawberry production in 2002 was estimated at 19.1 million USD (14).

Strawberry production in North Carolina consists mainly of small plasticulture operations with a high proportion of pick-your-own, roadside and farmer's market sales (16). In the plasticulture method, strawberries are grown in an annual system where the soil is fumigated and formed into black plastic-covered raised beds with drip irrigation tubing (16). Bare-root or plug plants are transplanted in late September or early October: bare-root plants are less expensive, but plug plants establish themselves more quickly, require less post-planting watering and are less perishable than bare-root plants, among other advantages (5, 10).

The close plant spacing (12) and increased water splash (13, 15) in the plasticulture system create ideal conditions for the spread of anthracnose fruit rot and crown rot. These diseases, collectively referred to as strawberry anthracnose, are a leading cause of losses for strawberry growers in the southeastern United States (6, 11).

Strawberry anthracnose was first described based on *C. fragariae* isolates collected from Florida strawberries by Brooks in 1931 (3). Symptoms of strawberry anthracnose include a black or irregular leaf spot, necrotic petiole and runner lesions,

flower blight, fruit rot, and bud and crown rot (6, 11). Of primary economic importance is the fruit rot phase, which can be caused by any of the four *Colletotrichum* species associated with strawberry, but is most commonly caused by *C. acutatum* J.H. Simmonds (11). The brown or black sunken lesions on the berries render them unmarketable, and can cause over 50% yield loss even under the most stringent management programs (6). *C. dematium* (Pers.) Grove can occasionally cause fruit rot, but this species is not as virulent as *C. acutatum* (1). The crown rot phase of the disease, associated with *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz. & Sacc, can also cause economically important losses (8). *C. gloeosporioides* has been recently reported in North Carolina (9), but *C. fragariae* has not been reported as a common pathogen in North Carolina since 1986 (R. Milholland, North Carolina State University, personal communication).

The most popular strawberry cultivars in NC ('Chandler' and 'Camarosa') are highly susceptible to this disease, though cultivars showing ripe fruit rot resistance such as 'Bish' and 'Sweet Charlie' are also grown (2, 4). Chemical control of anthracnose fruit and crown rot sometimes produces unsatisfactory results; as a consequence, sanitation is the most important management strategy for this disease (11). However, the presence of latent infections complicates the implementation of the most important sanitation strategy: the use of disease-free planting stock (7, 11). The devastating losses caused by *Colletotrichum*, in combination with cultivation practices, the regional climate, and the susceptibility of the currently popular cultivars, makes anthracnose one of the most economically important diseases of strawberry in the southeastern United States (6, 8, 11).

1.2. RESEARCH OBJECTIVES

This thesis was designed to investigate alternative measures for the management of strawberry anthracnose. Chapter II compares certified anthracnose-free planting stock from the North Carolina Strawberry Certification Program to stock from a noncertified Canadian source. Chapter III examines the resistance of selected *Colletotrichum* isolates from a local epidemic to the recently introduced fungicide pyraclostrobin. Chapter IV presents a novel real-time PCR protocol for the diagnosis of strawberry anthracnose and evaluates the protocol for sensitivity and specificity in detecting *Colletotrichum* from artificially and naturally infected petioles.

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2. CHAPTER II:

Assessment of Three Strawberry Transplant Sources for Performance and Presence of Anthracnose

2.1. ABSTRACT

The North Carolina Strawberry Certification program began producing anthracnose-free strawberry planting stock in 2001. As a response to the Fall 2003 strawberry anthracnose epidemic produced by infected Canadian planting stock, three strawberry transplant sources (NC Registered, NC Certified, and noncertified Canadian) were evaluated in the field for the presence of anthracnose and horticultural characteristics. The three sources were distributed in a randomized complete block design with five replications and grown under standard conditions during the 2003-2004 growing season. One plant was sampled from each plot before transplanting, at peak bloom, and at harvest, and horticultural data such as leaf area were collected. None of the plants showed symptoms of anthracnose. The Canadian plants showed significantly greater leaf areas, leaf dry weights, and root dry weights before planting, but this difference decreased over time. Crown dry weights of the Canadian plants remained greater on average than those of the NC plants over the course of the growing season. Root health ratings for the Canadian plants were significantly greater than those of the NC plants at the peak bloom and harvest samplings. Berries were collected twice weekly, and the number of berries and the total berry weight was recorded. The Canadian plants produced a significantly greater total berry number (69% greater) and weight (53% greater) but a slightly lower average berry size than the North Carolina-produced plants. This difference in yield may be due to the difference in transplant size,

the difference in root health, or the possibility that NC-grown plants require more frequent overhead watering after planting for good establishment.

2.2. INTRODUCTION

Strawberry fruit rot and crown rot caused by *Colletotrichum* spp., collectively referred to as strawberry anthracnose, are a leading cause of losses in strawberry production fields in the southeastern United States (3, 6). The most popular strawberry cultivars in NC ('Chandler' and 'Camarosa') are highly susceptible to anthracnose, though cultivars showing fruit rot resistance such as 'Bish' and 'Sweet Charlie' are also grown (1, 2). Chemical control of anthracnose fruit and crown rot sometimes produces unsatisfactory results; as a consequence, sanitation is the most important management strategy for this disease (6). However, the presence of latent infections complicates the implementation of the most important sanitation strategy: the use of disease-free planting stock (4, 6).

The statewide anthracnose epidemic in 1990 devastated the NC strawberry nursery industry, forcing fruit production growers to buy planting stock from non-local nurseries such as those in Canada (9). The North Carolina Strawberry Certification program began producing certified anthracnose-free transplants in 2001 in response to this situation. The program's foundation plants are produced by the North Carolina State University Micropropagation Unit. These foundation plants are used to produce registered planting stock, which is in turn used to produce certified planting stock under the guidelines provided by the North Carolina Crop Improvement Association (7). The number of certified transplants produced yearly has increased dramatically since 2001; 1.3 million certified transplants were produced for the 2005-06 growing season (Z. Pesic-Van Esbroeck, North Carolina State University Micropropagation Unit, personal communication).

In Fall 2003, the southeastern US experienced an epidemic of strawberry anthracnose on plug plants grown from Canada-produced runner tips, causing many growers to discard their infected plug plants and order bare-root planting stock (5, 8). In order to secure a source of reliably uninfected planting stock, this study compares the performance of bare-root transplants from the North Carolina Strawberry Certification program with that of bare-root Canadian transplants.

2.3. MATERIALS AND METHODS.

2.3.1. Experimental design. In Fall 2003, 200 strawberry bare-root transplants (cv. 'Chandler') were obtained from each of three sources: a North Carolina certified grower, a North Carolina registered grower, and a noncertified Canadian grower (Prince Edward Island). The transplants were installed in a field at the North Carolina State University experiment station in Clayton, NC on October 6 and grown in black plastic-covered raised beds under drip irrigation at 30 cm spacing with two rows per bed. The soil was fumigated with methyl bromide:chloropicrin 67:33 prior to planting. The field was laid out in three rows and subdivided into 5 blocks. The transplant sources were distributed in a randomized complete block design with 40 plants in each plot and grown according to standard cultural, fertility and disease and pest management practices.

2.3.2. Whole plant sampling. One plant was arbitrarily sampled from each plot before transplanting (October 6), at peak bloom (March 17), and at the final harvest (June 7). Each plant collected on a given sampling date was taken from the same location within each plot. The horticultural characteristics recorded from the sampled plants were leaf area, leaf dry weight, flower and bud number, flower and bud dry weight, crown number, crown dry weight, root dry weight, root health rating, and root rot rating. The plants were divided into leaf, flower, crown, and root sections and placed in brown paper bags. Leaf area was measured using a LI-3100C Leaf Area Meter (LI-COR, Lincoln, NE). Root health ratings were performed by comparing the ratio of fibrous branch roots to large adventitious roots: a high percentage of fibrous roots corresponded to a high root health rating, and was assigned a score of 1-12 by a modified Horsfall-Barrett scale. Root rot ratings were performed by estimating the percentage of decayed roots. Root

health and root rot ratings were not performed at the preplant sampling time due to the dryness of the root systems. After the leaf area, root health and root rot ratings were completed, the bagged plant material was placed in a drying oven for one week, and dry weights were recorded. Flower number and dry weight were recorded only at peak bloom due to the almost complete absence of flowers at the other sampling times.

2.3.3. Yield assessment. Berries were collected twice weekly from the central 24 plants in each plot during the harvest season (April 26-June 7) and the number and weight of marketable and unmarketable berries were recorded. A berry was considered unmarketable if it weighed less than 10 g or showed blemishes.

2.3.4. Data analysis. Results from whole plant sampling were transformed to the \log_{10} of the data to minimize the increase of variance over time and analyzed by strip-plot analysis of variance using Student's t-tests to compare means at each time. The exception to this was the root health ratings, which were not a measure of growth and so did not display an increase in variance over time and were analyzed without transformation. Total berry yields at each collection date were analyzed using the general linear model (GLM) for analysis of variance and Tukey's Studentized Range test to compare means. Both analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC).

2.4. RESULTS

2.4.1. Whole plant sampling. The significant date x source interaction made it necessary to analyze the sources at each time of sampling. The conversion of the data to \log_{10} minimized this situation but did not eliminate it. The leaf areas (Figure 2.1) were significantly different ($p < 0.004$) in all three cultivars at the preplant sampling, with the Canadian source being the highest, then NC Registered and NC Certified. At peak bloom, the Canadian source was significantly greater ($p = 0.004$) than the NC Certified but not greater than Registered ($p = 0.09$), and at harvest the differences were not significant (Figure 2.1). The leaf dry weights showed a similar pattern (Figure 2.2). At the preplant sampling, the Canadian source was significantly higher ($p < 0.004$) than the two NC sources. At the peak bloom sampling, the Canadian source was significantly greater ($p = 0.005$) than the NC Certified, but not the NC Registered, and at harvest the differences were not significant.

The flower numbers and dry weights (*data not shown*) were not significantly different at peak bloom, the only time at which they were measured.

Crown numbers were not significantly different at any sampling time. Crown dry weights (Figure 2.3), however, did show differences: at preplant, Canadian and Certified sources were significantly greater ($p < 0.0002$) than Registered, at bloom the Canadian source was significantly greater ($p = 0.005$) than the Certified source but not the Registered source, and at harvest the Canadian was greater ($p < 0.01$) than both NC sources.

Canadian root dry weights (Figure 2.4) were significantly greater ($p < 0.01$) than the two NC sources at preplant, but no significant differences were observed at the other

sampling times. Root health and root rot ratings were not taken at preplant. The Canadian root health ratings (Figure 2.5) were significantly greater ($p < 0.04$) than that of the two NC sources at bloom and at harvest. The root rot ratings (*data not shown*) were not significantly different at any sampling time.

2.4.2. Yield assessment. Canadian plants showed significantly greater ($p < 0.05$) marketable berry number (Figure 2.6), marketable berry weight (Figure 2.7), unmarketable berry number, and unmarketable berry weight than the two NC sources, which were not significantly different from each other. The average berry weight for Canadian plants was less than for the NC plants (17.4 g vs. 19.4 g), but the difference was not significant at 0.05 ($p = 0.11$). The total berry weight at each collection date is shown in Figure 2.8.

2.5. DISCUSSION

The leaf area and leaf weight of the Canadian plants were significantly greater at the first two samplings, but the difference became less distinct as the growing season progressed. This could be due either to variation increasing with plant growth or to a true decrease in differences once the sources were placed under the same conditions. If the lack of significance were due to increasing variation, the early advantage may have enabled the Canadian plants to store more carbohydrates over the winter and so contributed to the significantly higher yield observed.

The flower numbers and dry weights of the three sources were not significantly different at the time of sampling (March 17). As shown in Figure 2.8, the yields from the three sources were similar at the beginning and end of the harvest period, but the Canadian source had greater yields in the peak harvest season (early to mid-May). A sampling around May 10 would be more likely to show a difference in the flower number and weight.

The crown numbers were not significantly different, but Canadian crown weights were greater at all sampling times. This result is consistent with the larger leaf areas, leaf dry weights and yield observed for the Canadian plants.

The Canadian root dry weight was greater at the preplant sampling, but was not significantly different at the other samplings. As with the leaf area and leaf weight results, this effect could be due either to variation increasing with plant growth or to a true decrease in differences once the sources were placed under the same conditions. The root health of the Canadian plants was significantly greater than that of the two NC sources at bloom and at harvest, indicating improved water uptake in the Canadian plants

which may have contributed to the increased yield of the Canadian plants. The root rot ratings were not significantly different at any time.

The Canadian plants produced 69% more berries on average than the NC sources, and the total weight of the Canadian berries was 53% greater. The p-values for the berry number and berry weight were very low, indicating a distinct difference between the Canadian and the NC sources. The average Canadian berry weight was slightly less, but this was not found to be significant.

Overall, the Canadian plants showed more desirable horticultural characteristics and a much greater yield than the NC-grown plants. This observed difference may be due to the fact that NC-grown plants, unlike Canadian plants, are not dormant at the time of planting. The higher metabolic rate of NC plants may require an increased amount of overhead watering after planting in order for the plants to establish well. The advantage of this characteristic is that, if the initial watering is sufficient for good establishment, the plants start growing immediately without the post-planting lag characteristic of Canadian stock. However, the post-planting watering schedule used for this study had been designed for Canadian-grown plants and may have placed the NC-grown plants at a disadvantage. In order to verify this hypothesis, a similar experiment should be performed using an increased initial watering schedule.

Other questions which might be addressed are whether transplant size and root health are consistently different between Canadian and NC sources, and whether these characteristics can be correlated with higher berry yield, as discussed by Stapleton *et al.* (10).

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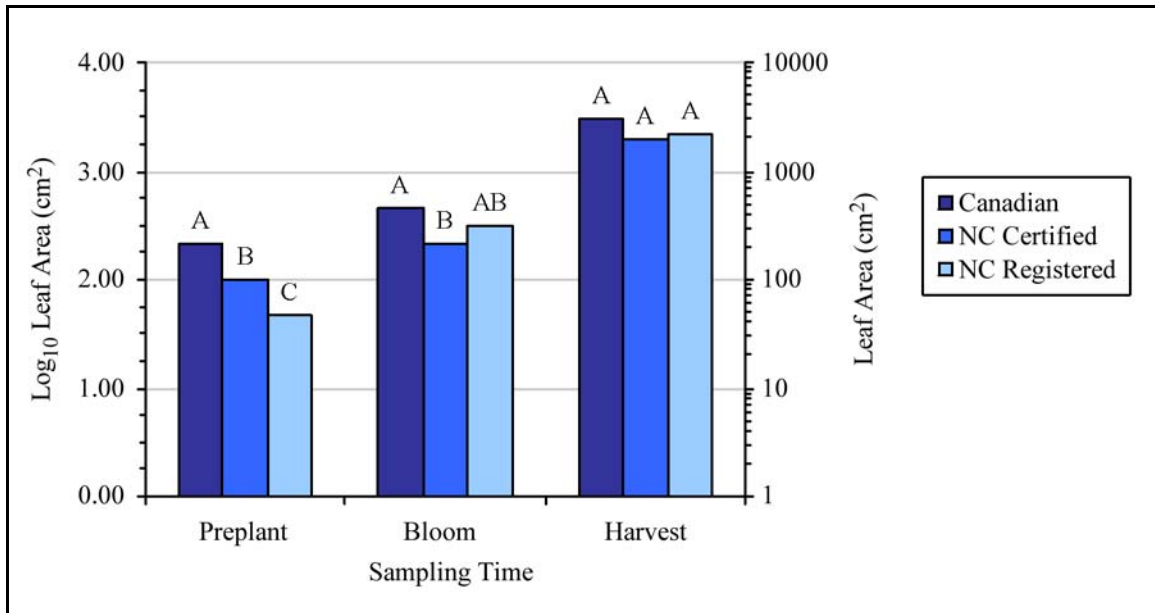


FIGURE 2.1. Average leaf area for sampled whole plants by transplant source (Canadian, NC Certified, NC Registered) at three sampling times (preplant, bloom, harvest) during a 2003-2004 field trial^{y, z}

^y Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. One plant was sampled from each of the 5 replicate plots before planting, at peak bloom, and at harvest.

^z Significance of differences ($p < 0.05$) was evaluated with a strip-plot ANOVA using the \log_{10} of the leaf area; transformed data is shown above.

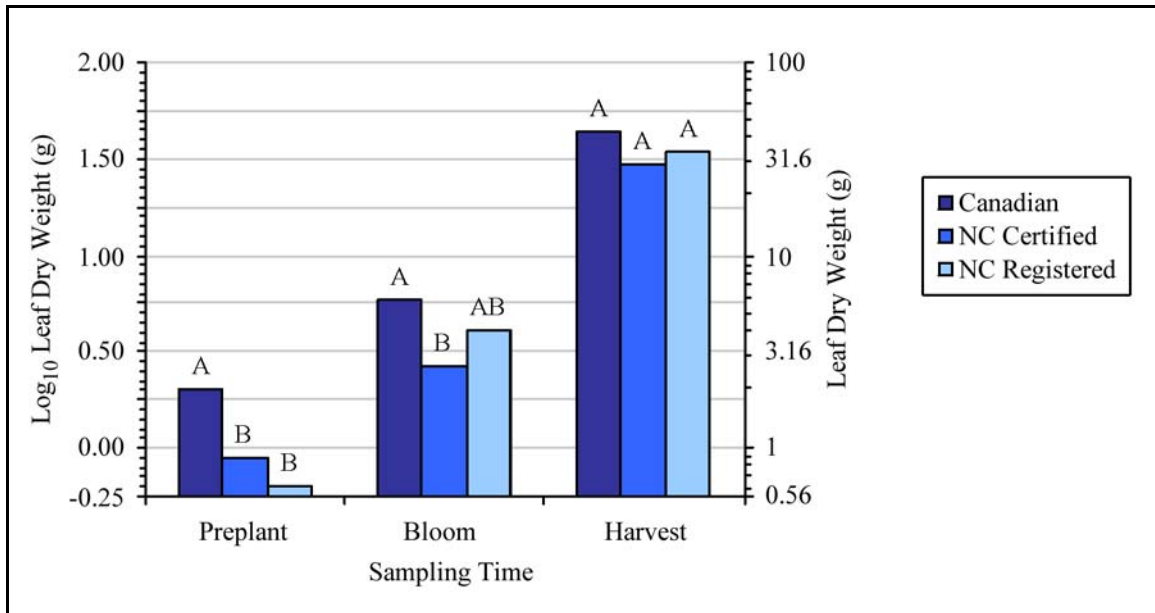


FIGURE 2.2. Average leaf dry weight for sampled whole plants by transplant source (Canadian, NC Certified, NC Registered) at three sampling times (preplant, bloom, harvest) during a 2003-2004 field trial^{y, z}

^y Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. One plant was sampled from each of the 5 replicate plots before planting, at peak bloom, and at harvest.

^z Significance of differences ($p < 0.05$) was evaluated with a strip-plot ANOVA using the log_{10} of the leaf dry weight; transformed data is shown above.

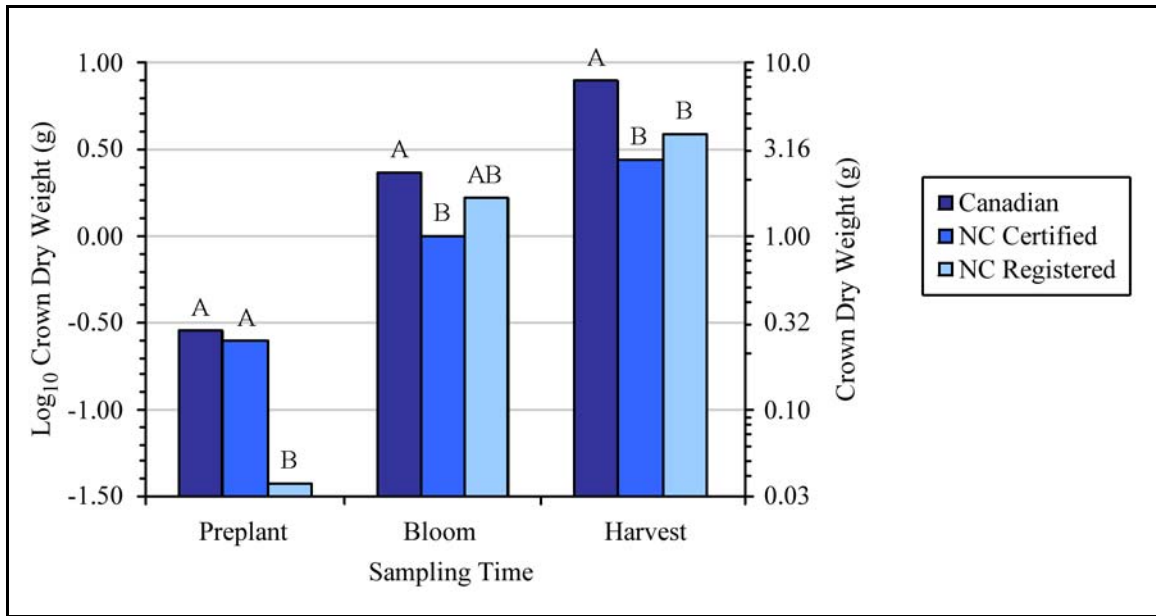


FIGURE 2.3. Average crown dry weight for sampled whole plants by transplant source (Canadian, NC Certified, NC Registered) at three sampling times (preplant, bloom, harvest) during a 2003-2004 field trial^{y, z}

^y Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. One plant was sampled from each of the 5 replicate plots before planting, at peak bloom, and at harvest.

^z Significance of differences ($p < 0.05$) was evaluated with a strip-plot ANOVA using the \log_{10} of the crown dry weight; transformed data is shown above.

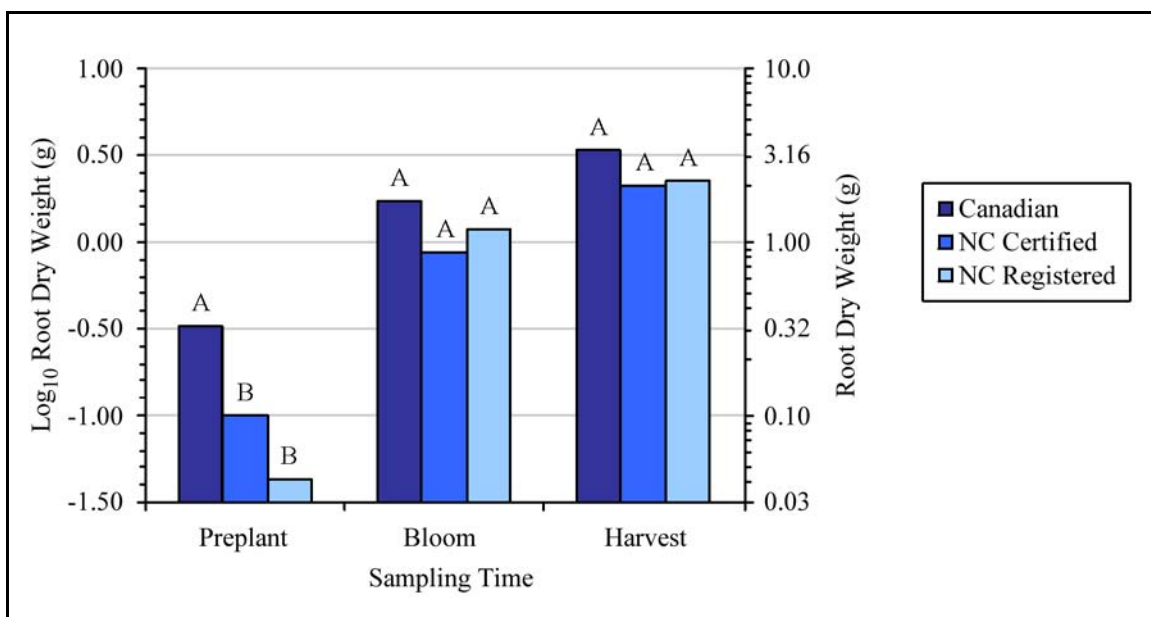


FIGURE 2.4. Average root dry weight for sampled whole plants by transplant source (Canadian, NC Certified, NC Registered) at three sampling times (preplant, bloom, harvest) during a 2003-2004 field trial^{y, z}

^y Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. One plant was sampled from each of the 5 replicate plots before planting, at peak bloom, and at harvest.

^z Significance of differences ($p < 0.05$) was evaluated with a strip-plot ANOVA using the \log_{10} of the root dry weight; transformed data is shown above.

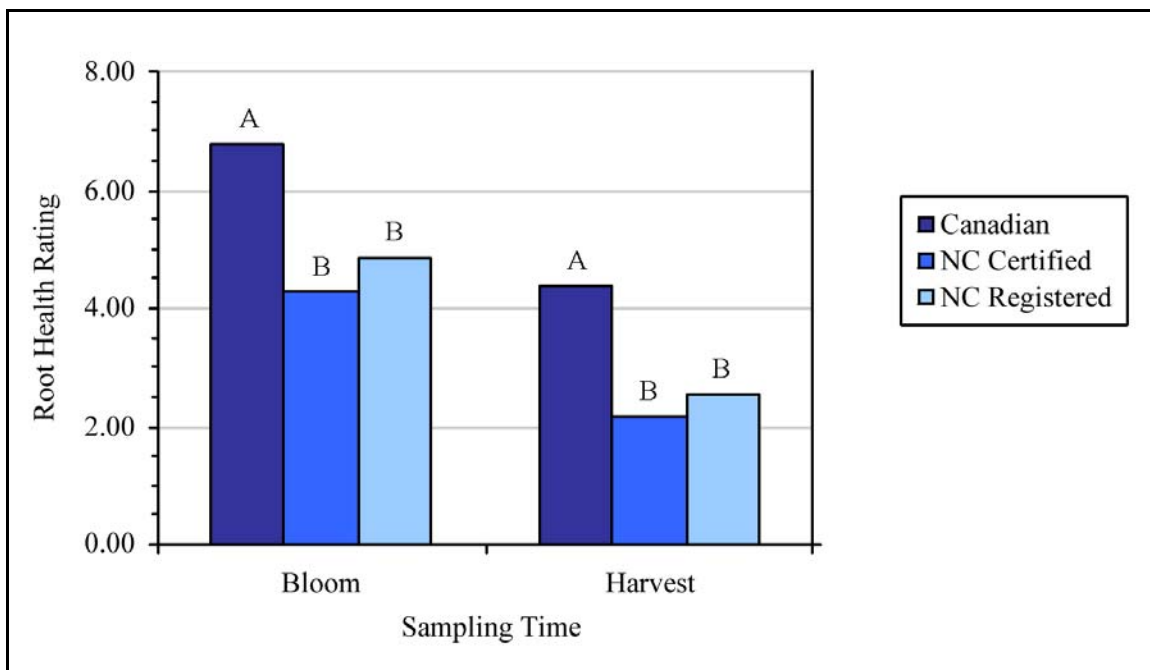


FIGURE 2.5. Average root health rating for sampled whole plants by transplant source (Canadian, NC Certified, NC Registered) at two sampling times (bloom, harvest) during a 2003-2004 field trial^{x, y, z}

^x A modified Horsfall-Barrett scale was used for estimation of root health where the score was based on the percentage of fibrous roots: 1=low, 2=1-3%, 3=4-6%, 4=7-12%, 5=13-25%, 6=25-50%, 7=51-75%, 8=76-89%, 9=88-94%, 10=95-97%, 11=98-99%, 12=100%.

^y Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. One plant was sampled from each of the 5 replicate plots at peak bloom and at harvest; root health ratings could not be taken before planting due to the dryness of the roots.

^z Significance of differences ($p < 0.05$) was evaluated with a strip-plot ANOVA based on the Horsfall-Barrett root health ratings.

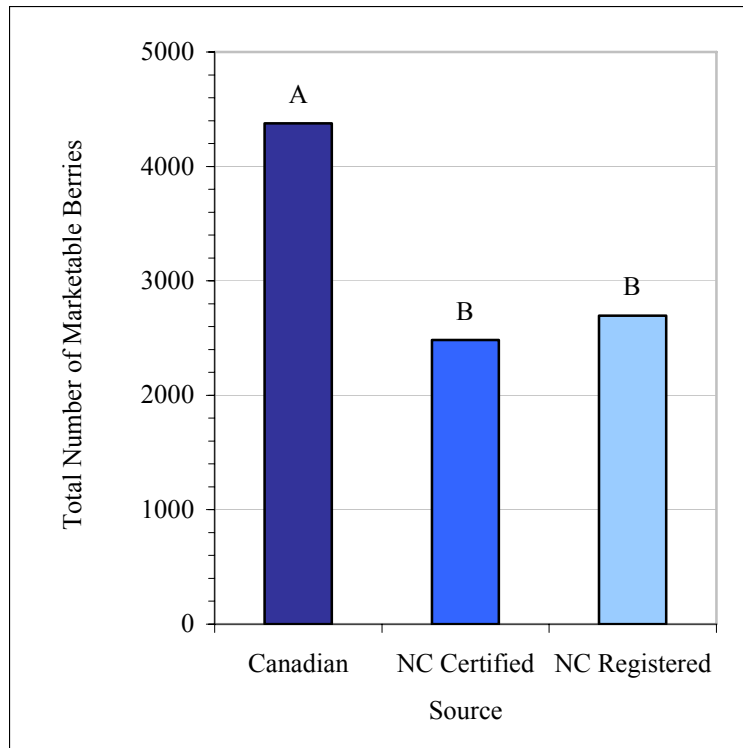


FIGURE 2.6. Total number of marketable berries produced by plants from three transplant sources (Canadian, NC Certified, NC Registered) during the 2003-2004 growing season^z

^z Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. Berries were collected twice weekly from the central 24 plants in each plot during the harvest season (April 26-June 7) and the number and weight of marketable and unmarketable berries were recorded.

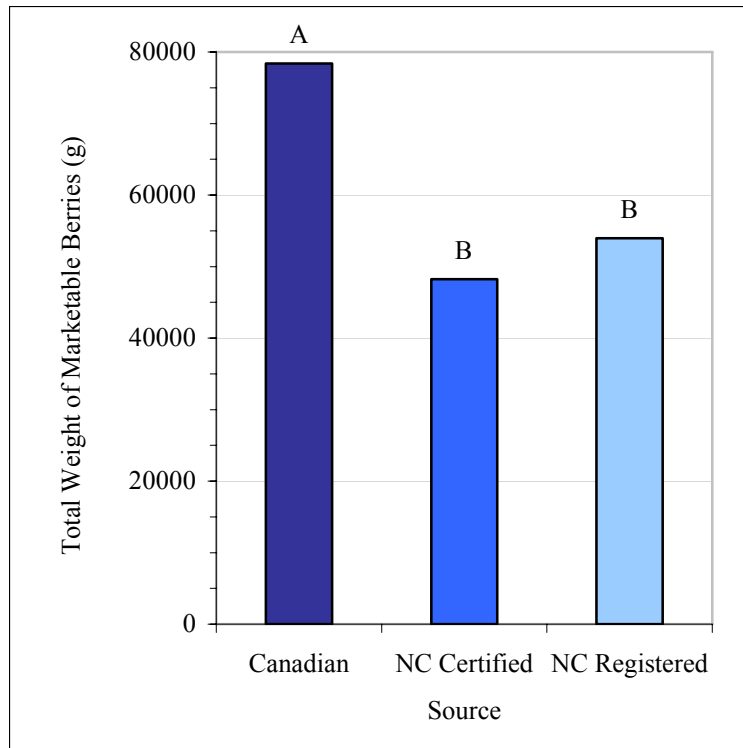


FIGURE 2.7. Total weight of marketable berries produced by plants from three transplant sources (Canadian, NC Certified, NC Registered) during the 2003-2004 growing season^z

^z Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. Berries were collected twice weekly from the central 24 plants in each plot during the harvest season (April 26-June 7) and the number and weight of marketable and unmarketable berries were recorded.

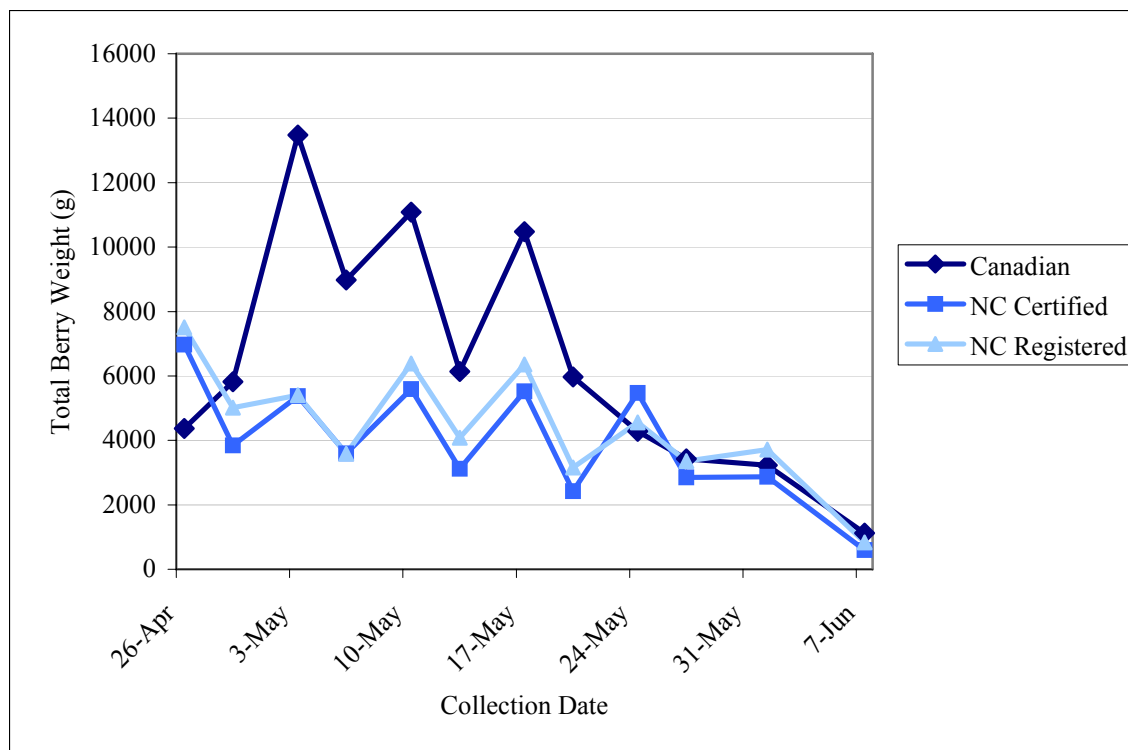


FIGURE 2.8. Total berry weight produced by plants from three transplant sources (Canadian, NC Certified, NC Registered) on seven collection dates during the 2003-2004 growing season^z

^z Three transplant sources (Canadian, NC Certified, and NC Registered) were distributed in the field in a randomized complete block design with 5 replications and grown under standard conditions during the 2003-2004 growing season. Berries were collected twice weekly from the central 24 plants in each plot during the harvest season (April 26-June 7) and the number and weight of marketable and unmarketable berries were recorded.

3. CHAPTER III:

Assessment of strawberry anthracnose levels in plug plant production operations and evaluation of collected *Colletotrichum acutatum* isolates for sensitivity to strobilurin fungicides

3.1. ABSTRACT

In North Carolina and surrounding states in the southeastern US, Fall 2003 was marked by an epidemic of strawberry anthracnose fruit rot (*Colletotrichum acutatum*) in plug plants. In an effort to evaluate diagnosis and control measures for the disease, petioles were sampled at two sites in NC, each containing 30,000 plug plants, 105 petioles from the first site and 100 from the second. The level of infection was evaluated visually in the field and by a bioassay of the collected petioles in which the petioles were treated with herbicide to induce senescence of the plant tissue and encourage fungal sporulation. Spore suspensions of isolates collected from the sampled petioles were plated on media containing the strobilurin fungicide pyraclostrobin, and the percent germination was compared to the incidence of germination for isolates which had never been exposed to strobilurin fungicides. The collected isolates were not found to be resistant to pyraclostrobin, indicating that this fungicide may prove to be a viable management option for strawberry anthracnose in fruiting fields.

3.2. INTRODUCTION

Inoculum of *Colletotrichum acutatum*, the causal organism of strawberry anthracnose, has been shown to be most commonly introduced to a field in or on planting material (7, 8). As a result, the most effective technique for management of this disease is the use of disease-free planting stock (15). This means of control is difficult to implement, however, due to the presence of latent infections in planting stock and the higher cost and limited availability of certified disease-free stock. When the disease is present in the field, the fungicides azoxystrobin (Abound, Quadris) and captan are commonly recommended for management (11). Pyraclostrobin (Cabrio), a strobilurin fungicide like azoxystrobin, was chosen for investigation because of its potential for management of strawberry anthracnose (6).

Strobilurins, also known as quinine outside inhibitors (QoIs), are a recent addition to the arsenal of fungicides used to manage strawberry diseases (6). QoI-type fungicides inhibit respiration by binding to the Qo site in cytochrome *b*, which is a part of complex III in the mitochondria (2, 3, 13). Because QoIs have a single target site, qualitative resistance may develop by way of single or multiple amino acid substitutions (1, 9). Because the target site is the same, resistance to one strobilurin indicates a strong possibility of resistance to other strobilurins (5). The risk of resistance development is minimized by applying strobilurins preventatively, limiting the number of applications per growing season, and applying in conjunction with other fungicides (17). As a further preventative measure, strobilurin fungicides are labeled for use in fruit production fields but not in nursery fields due to the potential for the spread of a resistant isolate.

Since strobilurin fungicides inhibit respiration, the most easily observable effect is the suppression of fungal growth. In this study, conidial germ tube elongation was used as a measure of growth. This approach results in good statistical significance and has the advantage of requiring only an overnight incubation.

Salicylhydroxamic acid (SHAM) is added to the media when evaluating isolates for strobilurin sensitivity to prevent respiration by way of the alternative oxidase pathway, which enables the electron transport chain to bypass cytochrome *bc₁* at the cost of a much lower metabolic efficiency (16). Because the throughput of the alternative oxidase pathway is indistinguishable from the throughput of the standard electron transport chain, disabling the alternative oxidase pathway enables a more accurate assessment of the sensitivity of the organism to the strobilurin.

The alternative oxidase pathway is not generally considered a type of strobilurin resistance because strobilurins are used primarily to prevent fungal germination, and the alternative oxidase pathway generally does not play a significant role during germination (16). Plant secondary metabolites may play a role in the inability of the alternative oxidase pathway to compensate for the inhibition of standard respiration: flavonoids of various plant species have been shown to indirectly inhibit the induction of the alternative oxidase pathway (17).

In Fall 2003, multiple locations in North Carolina demonstrated unusually high incidences of *C. acutatum* on strawberry plug plants grown from Canadian tips (12), indicating a high probability that the inoculum was introduced from the planting stock. Plug plants are a type of transplant produced locally from unrooted strawberry runner tips with 3-4 leaves obtained from field nurseries. The tips are rooted in potting mix within

50-cell transplant trays under frequent misting and are ready to be transplanted into the field within 4-5 weeks.

Growers who had not yet planted the infected plugs generally chose to discard them and use fresh-dug bare-root plants as replacement planting stock, but many growers were not familiar with the methods required for planting fresh-dug material. Extension's response to this need is detailed by Poling *et al.* (12). However, growers who had planted the infected plugs before noticing disease required a publication detailing recommended strategies for managing the disease in the field. For this reason, the results of the study described below were released in a preliminary form as part of North Carolina Cooperative Extension Service Pest Alert 43 (10).

3.3. MATERIALS AND METHODS

3.3.1. Visual assessment of disease levels. A grid was drawn illustrating the first transplant production site evaluated in which each cell represented one flat of plug plants (Figure 3.1). A mark was placed in each cell corresponding to a flat of plants showing signs of infection: large areas of visible infection were marked with circles, and individual plants or small areas of visible infection were marked with a cross. The overall level of infection was measured by comparing the number of infected flats to the total number of flats. This graphical assessment was not performed at the second site due to the lower level of disease.

3.3.2. Petiole assay of disease levels. Two commercial strawberry production sites containing an estimated 29,000-30,000 plug plants each were divided into a sampling grid with 21 sectors for the first site and 20 for the second. Five petioles were collected from each grid sector using clippers which had been disinfested with 6.15% NaOCl between each sampling. The five petioles from each sampling area were placed in a 50mL plastic tube. The petioles were then screened for strawberry anthracnose according to a modification of the bioassay protocol used by Freeman *et al.* (4). The petioles were placed in a -20°C freezer for 3-4 hours and defrosted at room temperature. The petioles were then disinfested in a solution of 8.5 mL of 6.15% NaOCl and 72 µL Tween 20 in 1 L of sterile water by adding 25 mL of solution to each tube and inverting for 2 minutes. The five petioles from each tube were placed parallel to one another in a Petri plate containing a piece of filter paper moistened with 0.75 mL of sterile water. The Petri plates were then sealed with Parafilm and placed in clear plastic boxes which were placed under fluorescent light at room temperature for 5 days. After the incubation

period, the petioles were removed and examined under a dissecting microscope for the presence of *Colletotrichum* acervuli. The identity of the acervuli was confirmed by examining conidia from the acervuli under a compound microscope.

3.3.3. Screening of isolates for resistance to pyraclostrobin. Baseline germination rates for 23 *C. acutatum* isolates which had not been exposed to strobilurin fungicides were obtained from a previous study performed by Hendrik Ypema and Yuejian Lu (14). In that study, 0.1 mL aliquots of a 1×10^4 conidia/mL spore suspension were placed on each of three Petri plates containing 2% water agar, 100 ppm SHAM, and one of the 8 concentrations of pyraclostrobin (0, 0.001, 0.004, 0.016, 0.063, 0.25, 1, and 10 ppm). Plates were incubated for 24 hours at 25° C and the first 100 conidia viewed in each plate were scored for the number of germinated and ungerminated spores. Germination was defined as the production of a germ tube whose length was equal to or greater than the conidial length.

In the study responding to the 2003 epidemic, two isolates from the assayed commercial sites and five isolates from samples submitted to the NCSU Plant Disease and Insect Clinic during the epidemic were evaluated using the same method with 3 concentrations of pyraclostrobin (0 ppm, 0.063 ppm, and 1 ppm). Three plates of each concentration were used for each strain, and the first 100 conidia viewed in a plate were scored for the number of germinated and ungerminated spores.

3.4. RESULTS

3.4.1. Visual assessment and bioassay for *Colletotrichum*. Visual inspection resulted in an estimate of 30.6% infected trays in the first commercial site (Figure 3.1). Visual symptoms were found in less than 5% of the trays at the second site. Similarly, the petiole assay indicated 15% infected petioles at the first site and 7% at the second. Microscopic examination of the spores confirmed the preliminary identification of the pathogen as *C. acutatum*.

3.4.2. Screening of isolates for resistance to pyraclostrobin. Average ED₅₀ and ED₉₅ values using the 23 baseline isolates were 0.011 ppm and 0.072 ppm, respectively (Table 3.1). The isolates collected from the 2003 epidemic yielded similar results. The germination rate for all seven strains from the 2003 epidemic was 98-100% at 0 ppm pyraclostrobin and 0-1.3% at 1 ppm; germination rates at 0.063 ppm ranged from 0-55.7% (Table 3.2). The conidial germination rates at 0.063 and 1 ppm were significantly lower than the rates at 0 ppm. The two data sets were compared graphically in Figure 3.2.

3.5. DISCUSSION

The bioassay method demonstrated the presence of latent infections in non-symptomatic tissue, but the two infected fields investigated showed only 7-15% positive results. Since the frequency of positive results was low even when disease levels were high, this result indicates the importance of sampling many petioles in order to decrease the likelihood of a false negative. However, increasing the number of petioles sampled increases the time and labor required for diagnosis. This requirement creates a potential difficulty for diagnosticians and highlights the need for alternative detection systems.

The high germination rates at 0 ppm pyraclostrobin demonstrate the normal rate of conidial germination under favorable conditions. The germination rates at 0.063 and 1 ppm pyraclostrobin were significantly lower than for the 0 ppm control and fell within the range predicted by the ED_{50} and ED_{95} values from the baseline isolates, indicating that these strains were not resistant to pyraclostrobin. These results are consistent with current research, which has found strobilurin resistance in other species of *Colletotrichum* (1), but not in *C. acutatum*. This lack of resistance supports the recommendation of pyraclostrobin as an effective measure for the prevention and control of strawberry anthracnose in fruiting fields.

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TABLE 3.1. ED₅₀ and ED₉₅ values for baseline *Colletotrichum* isolates on media containing pyraclostrobin^x

Isolate	Year	ED ₅₀ (ppm)	ED ₉₅ (ppm)
SM-4 ^y	2002	0.011677	0.072426
SM-5 ^y	2002	0.010005	0.060763
SM-6 ^y	2002	0.008159	0.061427
CHC-2 ^y	2002	0.014104	0.091742
CHC-3 ^y	2002	0.016712	0.164953
CHC-4 ^y	2002	0.019275	0.161118
VC-4 ^y	2002	0.009549	0.04648
VC-5 ^y	2002	0.009128	0.061282
VC-6 ^y	2002	0.007498	0.040897
VO-6 ^y	2002	0.010277	0.055415
VO-7 ^y	2002	0.0098	0.053749
VO-8 ^y	2002	0.009363	0.04815
CHU-5 ^y	2002	0.019664	0.14291
CHU-6 ^y	2002	0.010771	0.152254
CHU-7 ^y	2002	0.005694	0.034148
00-966 ^z	2000	0.012632	0.10584
01-1524 ^z	2001	0.0315	0.089114
01-1601 ^z	2001	0.004392	0.012745
99-783-A1 ^z	1999	0.008429	0.043738
99-884-A ^z	1999	0.006404	0.035452
99-1085-D2 ^z	1999	0.009307	0.050339
99-1708 ^z	1999	0.007312	0.040847
99-1974 ^z	1999	0.007126	0.04036

^x ED₅₀ and ED₉₅ values were calculated based on percent germination of conidia plated on water agar containing 8 concentrations of pyraclostrobin and incubated for 24 hours at 25°C.

^y Isolates were collected from North Carolina strawberry fields.

^z Isolates were collected from samples submitted to the North Carolina State University Plant Disease and Insect Clinic.

TABLE 3.2. Percent spore germination of *Colletotrichum acutatum* isolates from the 2003 southeastern US strawberry anthracnose epidemic on media containing pyraclostrobin^{y,z}

Isolate	Pyraclostrobin concentration (ppm)		
	0	0.063	1
03-2401A	99.0	3.3	1.3
03-1974-1D	100.0	0.0	0.0
03-2362-1B	100.0	6.0	0.0
03-2384-1A	99.0	55.7	0.0
03-2349A	99.3	1.0	0.0
03-2399B	100.0	28.0	0.0
L5	99.3	5.0	0.0

^yIsolates were collected from commercial fruit production fields in North Carolina.

^z Percent germination was observed for conidia plated on water agar containing 3 concentrations of pyraclostrobin and incubated for 24 hours at 25°C.

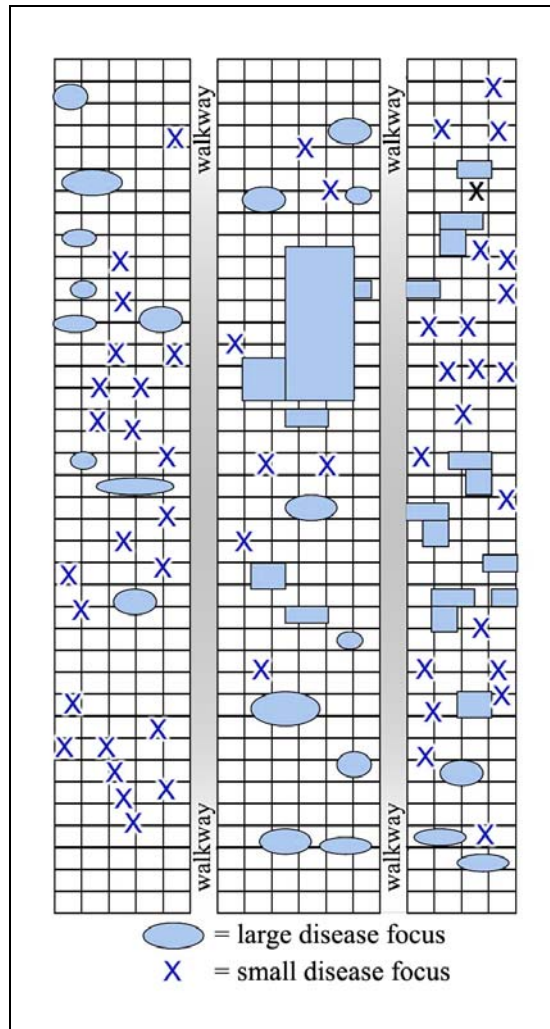


FIGURE 3.1. Visual representation of disease levels observed at a commercial plug plant production operation, during the 2003 southeastern US strawberry anthracnose epidemic^z

^z Each rectangle represents a tray containing 50 rooted strawberry tips. Tips were rooted 12 days prior to the creation of this figure.

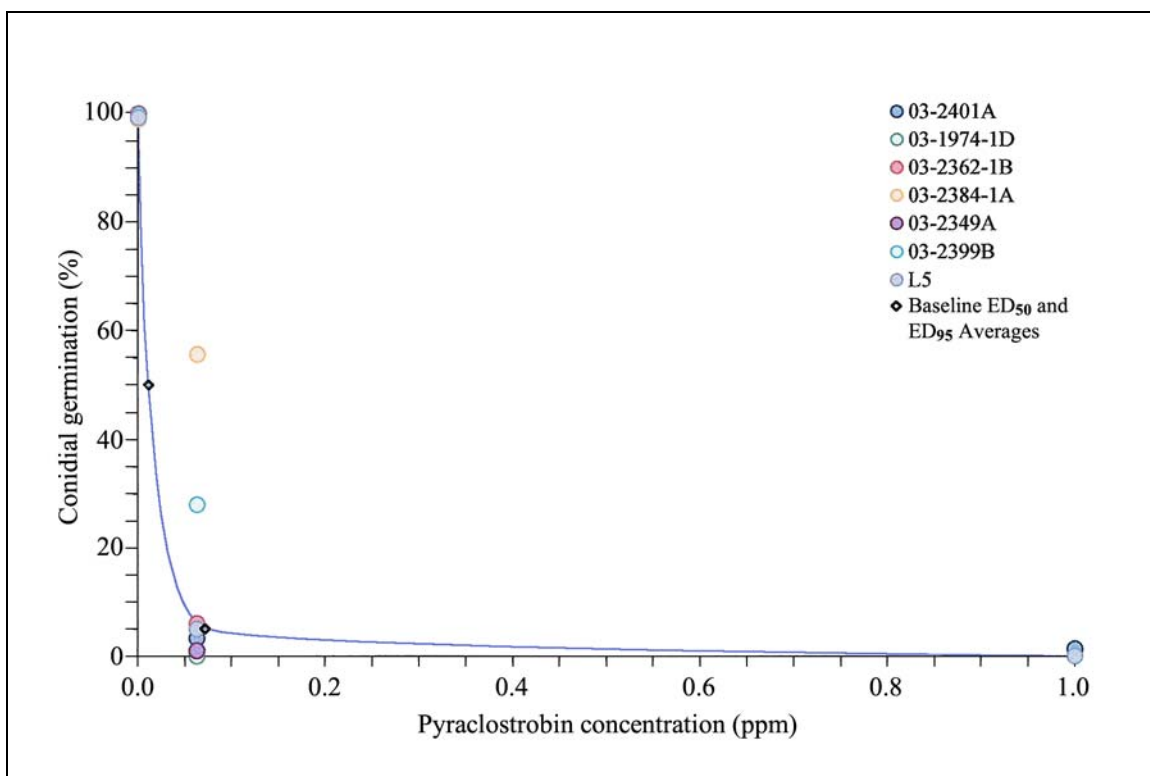


FIGURE 3.2. Percent conidial germination of *Colletotrichum acutatum* isolates collected from the 2003 southeastern US strawberry anthracnose epidemic compared to ED₅₀ and ED₉₅ values for baseline isolates^{y,z}

^y Percent germination was observed for conidia plated on water agar containing 3 concentrations of pyraclostrobin and incubated for 24 hours at 25°C.

^z The curve describes the average percent conidial germination at each concentration of pyraclostrobin for baseline isolates.

4. CHAPTER IV:

Development and Assessment of a Realtime-PCR Protocol for Detection of *Colletotrichum* spp. on Strawberry Petioles

4.1. ABSTRACT

Anthracnose is an economically important disease of strawberry most commonly caused by the fungal species *Colletotrichum acutatum*, *C. fragariae*, and *C. gloeosporioides*. Planting stock carrying *Colletotrichum* is the primary source of inoculum in fruiting fields, making detection of latent infections an important management strategy for this disease. A real-time PCR protocol for detection of *Colletotrichum* in strawberry petiole tissue was developed and compared to a previously developed bioassay method in which the plant tissue is treated with herbicide to induce sporulation of the pathogen. Petioles were sampled from strawberry plants grown in a controlled environment and artificially inoculated with *C. acutatum*, and from field-grown strawberry plants which were naturally infected with *C. gloeosporioides*; the sampled petioles were screened using both assays. The real-time PCR protocol was more rapid and showed significantly greater sensitivity than the bioassay when evaluating the artificially inoculated petioles, demonstrating 89% detection vs. 11% from the bioassay in the first experiment, and 56% vs. 7% in the second. A lack of sensitivity in the real-time PCR assay was observed when evaluating the naturally infected petioles; this problem was likely due to poor tissue disruption of tougher, field-grown petioles during the DNA extraction protocol. Once issues of contamination and poor disruption are addressed, real-time PCR may become an invaluable tool for sensitive and specific detection of *Colletotrichum* in strawberry tissue.

4.2. INTRODUCTION

The devastating losses caused by strawberry anthracnose, in combination with cultivation practices, the regional climate, and the susceptibility of the current favored cultivars, make it one of the most economically important diseases of strawberry in the southeastern United States (24, 33, 36).

Symptoms of strawberry anthracnose include a black or irregular leaf spot, necrotic petiole and runner lesions, flower blight, fruit rot, and bud and crown rot (24, 36). Of primary economic importance is the fruit rot phase, which can be caused by any of the four *Colletotrichum* species associated with strawberry, but is most commonly caused by *C. acutatum* (36). The brown or black sunken lesions on the berries render them unmarketable, and can cause over 50% yield loss even under the most stringent management programs (24).

The most important source of *Colletotrichum* inoculum in fumigated strawberry fruiting fields is contaminated planting stock (12, 13, 14, 48). Often, contaminated planting stock is not symptomatic; the infection is latent until conditions are conducive to disease development (14, 31, 32, 41, 44). The detection of the pathogen on latently infected planting stock is therefore an essential control measure for this disease.

Current methods for detecting latent infections rely primarily on bioassay techniques: strawberry tissue is killed to encourage the pathogen to sporulate, and the tissue is then evaluated for the presence of the pathogen (25, 26, 41, 42, 44). The primary disadvantage of these protocols is that they rely on the sporulation of the pathogen. The tissue samples are generally incubated for 5 to 10 days to allow for sufficient sporulation (26, 41), which results in a time-consuming protocol. Also, sporulation may be inhibited

by previously applied fungicides or by the surface disinfestation common to these assays. Secondary conidiation on the leaf surface is an important means of survival and reproduction in latent infections (31, 32), and so inhibition of sporulation creates a potential for false negatives. However, if the disinfestation step were to be removed, the growth of contaminating organisms such as *Botrytis* would obscure the results.

Reliance on sporulation can be avoided by the use of molecular techniques which are sensitive enough to detect the pathogen without the need for a bio-amplification step. Sequence comparison by PCR (10, 11, 13, 23, 28, 37, 39, 43, 46, 50, 52, 54, 56), arbitrarily-primed PCR (15, 16, 17, 18, 20-22), labeled oligonucleotide probes (40), isozyme comparisons (3, 5), and DNA fingerprinting by RAPD (6, 8, 9, 11, 29, 30, 38, 43, 49, 53, 57) and RFLP analysis (5, 9, 15, 16, 17, 20, 22, 34, 53) have all been used for detection and differentiation of *Colletotrichum* species. Most of these techniques were primarily designed for taxonomic studies, but Sreenivasaprasad *et al.* (52) and Parikka and Lemmetty (46) designed PCR assays for detection of *C. acutatum* on strawberry, and Mills *et al.* (43) designed an assay for *C. gloeosporioides* on tomato. These assays have proven to be sensitive, specific, and more rapid than the bio-amplification-type assays (46, 52).

Real-time PCR offers an even greater potential for sensitivity and specificity than conventional PCR. The type of real-time PCR used in this experiment, the 5' nuclease-based assay, determines DNA amplification by measuring the amount of fluorescence produced during the thermal cycling, which is directly proportional to the amount of amplification, enabling accurate quantification (2, 45). An additional way in which the 5' nuclease-based assay improves quantification is that it measures amplification during the

early cycles, thus minimizing the degree to which cycling efficiency affects the results (1, 45). The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence of the reporter dye exceeds the baseline fluorescence (1). The 5' nuclease-based assay also increases specificity because the fluorescent probe, as well as the primers, is specific for the target sequence: fluorescence will not be produced in response to nontarget products (2). An additional advantage is that the lack of an electrophoresis step results in a less time-consuming procedure.

Cullen *et al.* (10) designed a real-time PCR assay for detection of *C. coccodes* on potato tubers and compared it to conventional nested PCR. The results demonstrated that real-time PCR was more rapid than conventional nested PCR and offered the advantage of accurate and reproducible quantification and detection of *C. coccodes* (10).

Real-time PCR detection is desirable for use on *C. acutatum*, *C. fragariae* and *C. gloeosporioides* for the same reasons as it is for *C. coccodes*. Potatoes and strawberries are both vegetatively propagated, and planting stock often harbors the pathogen, so sanitation is an essential management strategy (27). The techniques currently used to screen planting stock for *Colletotrichum* are time-consuming, and so the development of a rapid, sensitive diagnostic assay is essential.

4.3. MATERIALS AND METHODS

4.3.1. Design of a *Colletotrichum*-specific primer/probe set. A consensus sequence was developed from 45 *Colletotrichum acutatum*, *C. fragariae*, and *C. gloeosporioides* DNA sequences of the ITS1-5.8S rRNA-ITS2 region obtained from the EMBL Nucleotide Sequence Database (Table 4.1). Beacon Designer software (Premier Biosoft, Palo Alto, CA) was used to design a dual-labeled TaqMan primer/probe set from the consensus sequence. The primer/probe set developed (Table 4.2) was located in the ITS2 region and produced an amplicon of 95 bp in length.

4.3.2. Petiole sampling from artificially inoculated strawberry plants. In May 2005, ninety-six strawberry plug plants were obtained from the North Carolina State University Micropropagation Unit and transferred to the NCSU Phytotron growth chambers. After one week, these were transplanted to six-inch pots in a 50% peat-lite (4), 50% sand potting medium. Twenty-four plants were placed in each of two 3 m² walk-in growth chambers set at 26/22°C day/night temperatures under 14 hours of fluorescent light to suppress flowering and encourage vegetative growth. The remaining forty-eight plants were transferred to a Phytotron greenhouse under the same temperature conditions with natural light plus three hours of supplemental lighting. All plants were watered once daily with a standard nutrient solution (55).

Three cultures of *Colletotrichum acutatum* (01-1218, 01-1601, and 01-1608) were obtained from the NCSU Plant Disease and Insect Clinic. Conidia were collected with a variation of the protocol used by Smith and Black (51). The cultures were grown for 7 days under continuous fluorescent lighting on Petri plates containing potato dextrose agar (39 g/L PDA and 25 mg/L ampicillin). Conidia were collected by adding 10 ml of

0.002% Tween 20 solution to each plate and scraping gently with a bent glass rod. The conidial suspensions were combined, quantified using a hemacytometer, and diluted to 10^6 spores/mL. The plants were then spray-inoculated to runoff with the conidial suspension and covered with clear plastic after inoculation for 48 hours to maintain humidity.

One 15 cm petiole sample was collected from each plant at four sampling times. The first petiole sampling was performed prior to inoculation, and additional samplings were conducted 5, 12, and 19 days after inoculation. Petioles were collected using scissors and forceps sterilized with 70% ethanol, and were stored individually in 10 mL tubes for same-day processing. Equal numbers of symptomatic and asymptomatic petioles were preferentially collected. After the final sampling, the first set of forty-eight plants was removed from the growth chambers. The chambers were sterilized, and a duplicate experiment was performed with the remaining forty-eight plants from the greenhouse.

4.3.3. Processing of artificially infected petioles. The petioles were notched twice to designate a central 4 cm segment of interest and sliced longitudinally with a flame-sterilized scalpel. One petiole half was assigned to the bioassay, leaving the parts exterior to the notching for ease of handling. The other half was shortened to the central 4 cm to optimize the tissue weight for DNA extraction and assigned to the real-time PCR assay. Bioassay petioles were processed immediately, and real-time PCR petioles were stored for later DNA extraction in 2 mL plastic tubes at -80°C .

4.3.4. Petiole sampling from naturally infected strawberry plants. In August 2005, petioles were collected from strawberry plants in a North Carolina nursery where

strawberry anthracnose had been reported. The North Carolina State University Plant Disease and Insect Clinic identified the causal agent as *C. gloeosporioides*. Sixty 15 cm petiole samples of each cultivar were collected: 12 petioles from each of the five rows of ‘Bish’ plants, and 10 petioles from each of the six rows of ‘Chandler’ and ‘Sweet Charlie’ plants. Symptomatic petioles were preferentially collected, and the ‘Chandler’ petioles were not diagnosed by the bioassay due to the 100% rate of symptom development. Each set of 12 or 10 petioles was placed in a separate plastic bag for processing.

4.3.5. Processing of naturally infected petioles. A 2 cm segment was removed from the end of each petiole for use in the real-time assay, and the remainder of the petiole was replaced in the plastic bag for use in the bioassay. Bioassay petioles were processed immediately, and real-time PCR petioles were stored for later DNA extraction in 2 mL plastic tubes at -80°C .

4.3.6. Visual assessment and bioassay. In the visual assessment, the petioles were evaluated for the presence of the dark-brown, sunken lesions characteristic of strawberry anthracnose. The bioassay was performed according to a modification of the bioassay protocol used by Freeman *et al.* (19). Each petiole was numbered and placed in a single layer in a wire mesh cage. The petioles were disinfested for 30 seconds in a solution of 8.5 mL of 6.15% NaOCl and 20 μL Tween 20 in 1L of sterile DI water, rinsed with sterile DI water, and immersed in herbicide (paraquat, 0.3% a.i.) for 1 minute. The petioles were then dried under a fume hood, sprayed to runoff with a *Botrytis*-specific fungicide solution (iprodione, 2.5 μg a.i. L^{-1}), and placed in clear plastic boxes incubated under fluorescent light at room temperature for 7 days. After the incubation period, the

petioles were removed and examined under a dissecting microscope for the presence of *Colletotrichum* acervuli and conidia. Only the central 4 cm section of interest was scored. The identity of the conidia was confirmed by examination under a compound microscope.

4.3.7. DNA extraction. Multiple methods for DNA extraction were evaluated in preliminary tests, and a modified protocol for the Qiagen DNeasy Plant Mini kit developed by Parikka and Lemmetty (46) was selected. Tissue disruption was accomplished by adding two 5 mm glass beads to each 2 mL tube, freezing the tubes in liquid nitrogen, shaking them in a Mini-BeadBeater-1 (Biospec Products, Carl Roth, Germany) for 30 seconds, and repeating the freezing and shaking steps. DNA from the disrupted tissue was then extracted using the protocol developed by Parikka and Lemmetty.

4.3.8. Standard development. Standard DNA for quantification was created by cycling the real-time PCR primers in a conventional PCR reaction mix to produce a high concentration of amplicon. This amplicon was quantified on an agarose gel by comparison to a commercially available plasmid standard and diluted to 0.33 pM so that 5 μ L of standard would contain 10^7 amplicons. This 10^7 standard was used to create a 10^6 - 10^1 standard curve by a 1:10 dilution series. Potential inhibition by strawberry-produced metabolites was evaluated by comparing the average amplification efficiency for four standard curves created using standard DNA diluted in sterile DI water against the amplification efficiency of a standard curve created using standard DNA diluted in strawberry DNA extracted using the protocol developed by Parikka and Lemmetty (46).

Amplification efficiency (E_x) was calculated by the formula $E_x = 10^{(-1/m)} - 1$, where m is the slope of the standard curve (1).

4.3.9. Real-time PCR amplification. All supplies (primers, probe, TaqMan Universal PCR Master Mix, Exogenous Internal Positive Control reagents and MicroAmp optical 8-tube strips) were obtained from Applied Biosystems (Foster City, CA). The probe was labeled with the reporter dye FAM and the quencher dye TAMRA. The reactions were performed at a volume of 50 μ L (5 μ L of DNA in 45 μ L master mix). Primer concentration was optimized at 300 nM and probe concentration at 200 nM. The reactions were cycled with the ABI Prism 7000 Sequence Detection System using the default thermal cycling protocol: 50° C for 2 minutes for AmpErase uracil-*N*-glycosylase (UNG) digestion, 95° C for 10 minutes for denaturation of UNG and activation of AmpliTaq Gold DNA polymerase, and 40 cycles of 95° C for 15 seconds and 60° C for 1 minute for DNA replication. The Ct values and initial concentrations of template DNA were calculated according to the standard curve by the ABI Prism Sequence Detection System software. Two thresholds for a positive result were defined: one with a calculated initial concentration of greater than 10 and one with greater than 100 copies of template DNA per reaction.

4.3.10. Primer/probe specificity testing. Primer/probe specificity was evaluated using isolates of *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* obtained from the NCSU Plant Disease and Insect Clinic and from Dr. Stanley Freeman of the Volcani Center in Israel. Specificity was also evaluated for other fungal species isolated from strawberry by the NCSU PDIC, including *Fusarium oxysporum*, *F. solani*, *Phytophthora cactorum*, *Pythium irregulare*, *P. ultimum*, *P. dissotocum*, and *Rhizoctonia*

fragariae. A BLAST search was also performed to search for matches to the primer and probe sequences.

4.4. RESULTS

4.4.1. Standard development. The goodness of fit (R^2 value) for all standard curves was 0.9886 or better, indicating a high reproducibility and accurate quantification (Figure 4.1). The amplification efficiency was not decreased by the addition of strawberry DNA to the standards: the efficiency for the standards in plant DNA was actually slightly higher than the average efficiency for standards diluted in water (Figure 4.1).

4.4.2. Primer/probe specificity testing. The real-time PCR primer/probe set was found to amplify all *Colletotrichum* isolates tested and no other fungal species (Table 4.3). The BLAST search found matches for the primer/probe set in isolates of *C. dematium*, *C. caudatum* and *C. capsici*, and identified isolates of *C. acutatum* and *C. gloeosporioides* from olive and soursop which did not match the full sequence.

4.4.3. Visual assessment and bioassay of artificially inoculated petioles. Disease levels were higher during the first experiment than during the second (Figure 4.2), and so the data from the two experiments are shown separately rather than being pooled. Visual assessment showed that 26 of the 144 petioles sampled post-inoculation during the first experiment were symptomatic, compared to only 5 of the petioles sampled during the second (Tables 4.4, 4.5). The level of symptomatic petioles decreased over time in both experiments. Neither the visual assessment nor the bioassay found infected petioles at either baseline sampling. The bioassay showed a consistently low level of detection (8-15%) at all post-inoculation samplings except for the final sampling, which showed no positive results. Microscopic examination of the conidia confirmed that the recovered organism was *C. acutatum*.

4.4.4. Real-time PCR of artificially inoculated petioles. The real-time PCR assay showed 6-8% detection at the baseline samplings using the >10 copies-per-reaction threshold and 0-2% detection using the >100 threshold (Figure 4.2). At the >10 threshold, the detection for the first experiment after inoculation was uniformly high (81-94%), but the detection in the second experiment decreased over time from 96-29% (Figure 4.2). At the >100 threshold, the detection incidence after inoculation for the first experiment decreased from 71% to 40%, and the incidence decreased from 48% to 4% in the second experiment (Figure 4.2). This trend can also be observed in the initial concentrations as shown in Figure 4.3.

4.4.5. Bioassay of naturally infected petioles. The 60 petioles collected from ‘Chandler’ plants, which are highly susceptible to anthracnose (47), all showed characteristic lesions and were not scored by the bioassay. The level of symptoms on the other two cultivars, which both show field resistance to anthracnose fruit rot (7, 35), was much lower; the bioassay showed 12% infection of the ‘Bish’ petioles and 33% infection of ‘Sweet Charlie’ (Figure 4.4). Microscopic examination of the conidial morphology supported the previous diagnosis of the pathogen as *C. gloeosporioides*.

4.4.6. Real-time PCR of naturally infected petioles. The real-time PCR assay showed a lower percent of positive results than the bioassay for the naturally infected plants (Figure 4.4). For the ‘Chandler’ petioles, 73% positives were found at the >10 threshold, and 40% at the >100 threshold. In the ‘Sweet Charlie’ petioles, 13% positives were found at the >10 threshold, and none at the >100 threshold. No positive results were detected in the ‘Bish’ petioles. Poor tissue disruption was observed in these

samples, and so the number of freezing/disruption cycles was increased from two to three, but incomplete disruption was still observed.

4.5. DISCUSSION

The high amplification efficiencies observed demonstrate the absence of PCR inhibition by strawberry or fungal metabolites (Figure 4.1). The high R^2 values indicate high reproducibility between replicate samples.

Real-time PCR primer/probe specificity testing resulted in amplification of the three target *Colletotrichum* species which cause strawberry anthracnose and none of the non-*Colletotrichum* isolates. A BLAST search added *C. dematium*, which causes strawberry anthracnose but is not an economically important pathogen, as a probable candidate for amplification. However, the BLAST search also indicated that some isolates of *C. capsici* (pepper anthracnose), *C. caudatum* (turfgrass anthracnose) and *C. graminicola* (anthracnose of turfgrass and corn) may be amplified by this primer/probe set. Additionally, some isolates of *C. acutatum* and *C. gloeosporioides* from olive (Portugal) and soursop (Colombia) might not be amplified according to the BLAST search. Despite these known limitations, the real-time PCR testing and the BLAST search indicate that the primer/probe set should amplify all *C. acutatum*, *C. gloeosporioides*, *C. fragariae* and *C. dematium* isolates from strawberry. A specific primer/probe set for each species could be developed by targeting a more variable region of the genome.

The lower disease level indicated by all diagnostic methods during the second experiment may be due to variation in the success rate of infection (Figure 4.2). The real-time assay showed low levels of detection at the pre-inoculation samplings, indicating the presence of background contamination. However, none of the negative PCR controls showed amplification at the >10 threshold, indicating that the contamination may have

taken place during petiole sampling or DNA extraction. Pre-PCR contamination could be minimized by decontamination of all instruments and work surfaces with bleach solution and the use of aliquots of the DNA extraction reagents.

Separate pre- and post-PCR handling areas are not necessary for real-time PCR due to the use of AmpErase UNG and the fact that the tubes are not normally opened after thermal cycling. The most important sources of real-time PCR contamination would be in air currents, particularly as aerosols produced by the DNA standards. Dedicated pipettes, barrier tips, aliquotted reagents, and DNA removal solution were used to decrease the risk of contamination, but the risk could be further decreased or eliminated by the use of air filters, DNA-free hoods, and loading the DNA standards after the DNA samples.

The real-time PCR assay yielded consistently higher detection capability than the bioassay for artificially inoculated plants. The PCR detection rate at both thresholds was higher than that of the bioassay at every sampling except for Day 12 of Experiment #2, where the >100 PCR threshold and the bioassay produced the same incidence of detection. Only 9 (>10) and 4 (>100) petioles out of the 384 total petiole samples showed a positive bioassay result but a negative real-time PCR result. Conversely, 194 petioles (>10) and 86 petioles (>100) were positive according to the real-time assay but not according to the bioassay. Even taking into account the low rate of background contamination, these results indicate that the real-time PCR assay is more sensitive than the bioassay.

Of the petioles sampled after inoculation in the first experiment, 11.1% (>10, Table 4.4) and 50.0% (>100, Table 4.5) did not produce a positive result by the real-time

PCR assay, while 43.8% (>10, Table 4.4) and 79.2% (>100, Table 4.5) did not during the second experiment. These figures indicate that even in infected plants rates of non-detection may be fairly high, and a large number of petioles must be sampled in order to decrease the probability of a false negative.

Incidence of real-time PCR detection was lower than that for bioassay detection for all three cultivars of naturally infected plants. This problem was likely due to the observed poor disruption of the field-grown petioles, which were noticeably thicker and tougher than petioles from the growth chamber. The poor disruption could be improved by using steel or tungsten-carbide beads for disruption rather than glass beads. Also, liquid disruption in DNeasy Buffer AP1 could prove to be an effective alternative to liquid nitrogen disruption with the addition of an anti-foaming agent. Use of an internal positive control designed to amplify a region of the strawberry genome would differentiate between poor DNA extraction and poor amplification. Another factor which may have contributed to the difference in detection rate was the higher amount of petiole tissue assigned to the bioassay. The overall decrease in disease level over time may have been due in part to the removal of inoculum during petiole sampling.

Real-time PCR could be used to inspect and diagnose plant material from nurseries, planting stock, fruit production fields, and imported shipments. Scientific applications of real-time PCR also include tracking the spread of disease through a field or following the progress of infection within a single plant. Once problems related to contamination and tissue disruption are further addressed, this real-time PCR protocol has the potential to become an important tool in the detection of *Colletotrichum* spp. in strawberry tissue.

4.6. LITERATURE CITED

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TABLE 4.1. DNA sequences used for the design of a TaqMan primer/probe set for the detection of *Colletotrichum* spp. by real-time PCR^z

EMBL accession number	Organism	Reference	Host	Origin
AF272780	<i>G. cingulata</i>	Freeman <i>et al.</i> 2001	Strawberry	United States
AF272784	<i>G. acutata</i>	Freeman <i>et al.</i> 2001	Strawberry	United States
AF272785	<i>G. acutata</i>	Freeman <i>et al.</i> 2001	Strawberry	France
AF272789	<i>G. acutata</i>	Freeman <i>et al.</i> 2001	Strawberry	Spain
AF489556	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Raspberry	United States
AF489557	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	Switzerland
AF489558	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	United States
AF489559	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Grape	United States
AF489560	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Tomato	United States
AF489561	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	France
AF489562	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	France
AF489563	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Blackberry	Switzerland
AF489564	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	France
AF489565	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Tomato	United States
AF489566	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	France
AF489567	<i>G. acutata</i>	Denoyes-Rothan <i>et al.</i> 2003	Bilberry	France
AF489568	<i>G. cingulata</i>	Denoyes-Rothan <i>et al.</i> 2003	Strawberry	Spain
AJ536199	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	France
AJ536200	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States
AJ536201	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Switzerland
AJ536202	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States
AJ536203	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Costa Rica
AJ536204	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United Kingdom
AJ536205	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Japan
AJ536206	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Australia
AJ536207	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Australia
AJ536208	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Australia
AJ536209	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Spain
AJ536210	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Netherlands
AJ536211	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United Kingdom
AJ536212	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	New Zealand
AJ536213	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	New Zealand
AJ536214	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States

TABLE 4.1. (continued).

EMBL accession number	Organism	Reference	Host	Origin
AJ536215	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	New Zealand
AJ536216	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	New Zealand
AJ536217	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	France
AJ536218	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	France
AJ536219	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	New Zealand
AJ536220	<i>G. acutata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	France
AJ536221	<i>C. fragariae</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States
AJ536222	<i>C. fragariae</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States
AJ536223	<i>C. fragariae</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States
AJ536224	<i>G. cingulata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	Canada
AJ536225	<i>G. cingulata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States
AJ536226	<i>G. cingulata</i>	Martinez-Culebras <i>et al.</i> 2003	Strawberry	United States

^zSequence data was obtained from the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk/embl/>)

TABLE 4.2. TaqMan primer/probe set designed for real-time PCR detection of *Colletotrichum* spp.^z

Oligonucleotide	Sequence (5' to 3')	Length (bp)
Sense (ColTqF1)	GGCCCTTAAAGGTAGTGGCG	20
Anti-sense (ColTqR1)	GGTTTACGGCAAGAGTCCCT	21
Probe (ColTqP1)	CCCTCCCGGAGCCTCCTTTGCGTA	24

^z Sequence data from Table 4.1 was used to develop a consensus sequence using MegAlign software. The primer/probe set was designed from the consensus sequence using Beacon Designer software.

TABLE 4.3. Fungal species used to test the specificity of the TaqMan primer/probe set for real-time PCR detection of *Colletotrichum* spp.^{y,z}

Species	Isolate	Source	Real-time PCR Amplification
<i>Colletotrichum acutatum</i>	Ca29	S. Freeman ^y	+
<i>Colletotrichum acutatum</i>	01-1218	NCSU PDIC ^z	+
<i>Colletotrichum acutatum</i>	01-1601	NCSU PDIC ^z	+
<i>Colletotrichum acutatum</i>	01-1608	NCSU PDIC ^z	+
<i>Colletotrichum fragariae</i>	Cf163b	S. Freeman ^y	+
<i>Colletotrichum gloeosporioides</i>	Cg272a	S. Freeman ^y	+
<i>Fusarium oxysporum</i>	B104	NCSU PDIC ^z	-
<i>Fusarium solani</i>	V2AB	NCSU PDIC ^z	-
<i>Phytophthora cactorum</i>	03-3192	NCSU PDIC ^z	-
<i>Pythium dissotocum</i>	C-11-D2	NCSU PDIC ^z	-
<i>Pythium irregulare</i>	C-11-4P	NCSU PDIC ^z	-
<i>Rhizoctonia fragariae</i>	AGA-NS2	NCSU PDIC ^z	-

^y Dr. Stanley Freeman, The Volcani Center, Bet Dagan, Israel

^z North Carolina State University Plant Disease and Insect Clinic, Raleigh, NC, USA

TABLE 4.4. Comparison of post-inoculation detection incidence (%) for bioassay and real-time PCR at the >10 copies-per-reaction threshold for detection of *Colletotrichum acutatum* from artificially infected strawberry petioles^{y, z}

	Experiment #1				
	Symptomatic at collection: n=26		Asymptomatic at collection: n=118		SUM
	Bioassay +	Bioassay -	Bioassay +	Bioassay -	
Real-time PCR +	5.6	9.7	3.5	70.1	88.9
Real-time PCR -	2.1	0.7	0.0	8.3	11.1
SUM	7.6	10.4	3.5	78.5	100.0

	Experiment #2				
	Symptomatic at collection: n=5		Asymptomatic at collection: n=139		SUM
	Bioassay +	Bioassay -	Bioassay +	Bioassay -	
Real-time PCR +	2.1	0.7	4.2	49.3	56.3
Real-time PCR -	0.0	0.7	0.7	42.4	43.8
SUM	2.1	1.4	4.9	91.7	100.0

^y In both experiments, 24 strawberry plants were placed in each of two controlled-environment chambers. A baseline sampling of one petiole per plant was conducted, after which the plants were spray-inoculated with a 10⁶ spores/mL suspension of *Colletotrichum acutatum* in water. Additional petiole collections were conducted at 5, 12, and 19 days after inoculation.

^z Visual assessment was conducted by examining each petiole for the presence of the dark sunken lesions characteristic of *C. acutatum* infection. The collected petiole was then sliced longitudinally. One petiole half was assigned to the bioassay, where the petiole was disinfested and treated with herbicide, then incubated at room temperature for 7 days and examined for the formation of acervuli. The protocol developed by Parikka and Lemmetty (46) was used to extract the DNA from the other petiole half, and the DNA was amplified in an ABI Prism 7000 thermal cycler with the primer/probe set described in Table 4.2. Initial concentrations of 10 and 100 copies per reaction were established as thresholds for a positive result.

TABLE 4.5. Comparison of post-inoculation detection incidence (%) for bioassay and real-time PCR at the >100 copies-per-reaction threshold for detection of *Colletotrichum acutatum* from artificially infected strawberry petioles^{y, z}

	Experiment #1				
	Symptomatic at collection: n=26		Asymptomatic at collection: n=118		SUM
	Bioassay +	Bioassay -	Bioassay +	Bioassay -	
Real-time PCR +	5.6	9.7	2.8	31.9	50.0
Real-time PCR -	2.1	0.7	0.7	46.5	50.0
SUM	7.6	10.4	3.5	78.5	100.0

	Experiment #2				
	Symptomatic at collection: n=5		Asymptomatic at collection: n=139		SUM
	Bioassay +	Bioassay -	Bioassay +	Bioassay -	
Real-time PCR +	2.1	0.0	1.4	17.4	20.8
Real-time PCR -	0.0	1.4	3.5	74.3	79.2
SUM	2.1	1.4	4.9	91.7	100.0

^y In both experiments, 24 strawberry plants were placed in each of two controlled-environment chambers. A baseline sampling of one petiole per plant was conducted, after which the plants were spray-inoculated with a 10⁶ spores/mL suspension of *Colletotrichum acutatum* in water. Additional petiole collections were conducted at 5, 12, and 19 days after inoculation.

^z Visual assessment was conducted by examining each petiole for the presence of the dark sunken lesions characteristic of *C. acutatum* infection. The collected petiole was then sliced longitudinally. One petiole half was assigned to the bioassay, where the petiole was disinfested and treated with herbicide, then incubated at room temperature for 7 days and examined for the formation of acervuli. The protocol developed by Parikka and Lemmetty (46) was used to extract the DNA from the other petiole half, and the DNA was amplified in an ABI Prism 7000 thermal cycler with the primer/probe set described in Table 4.2. Initial concentrations of 10 and 100 copies per reaction were established as thresholds for a positive result.

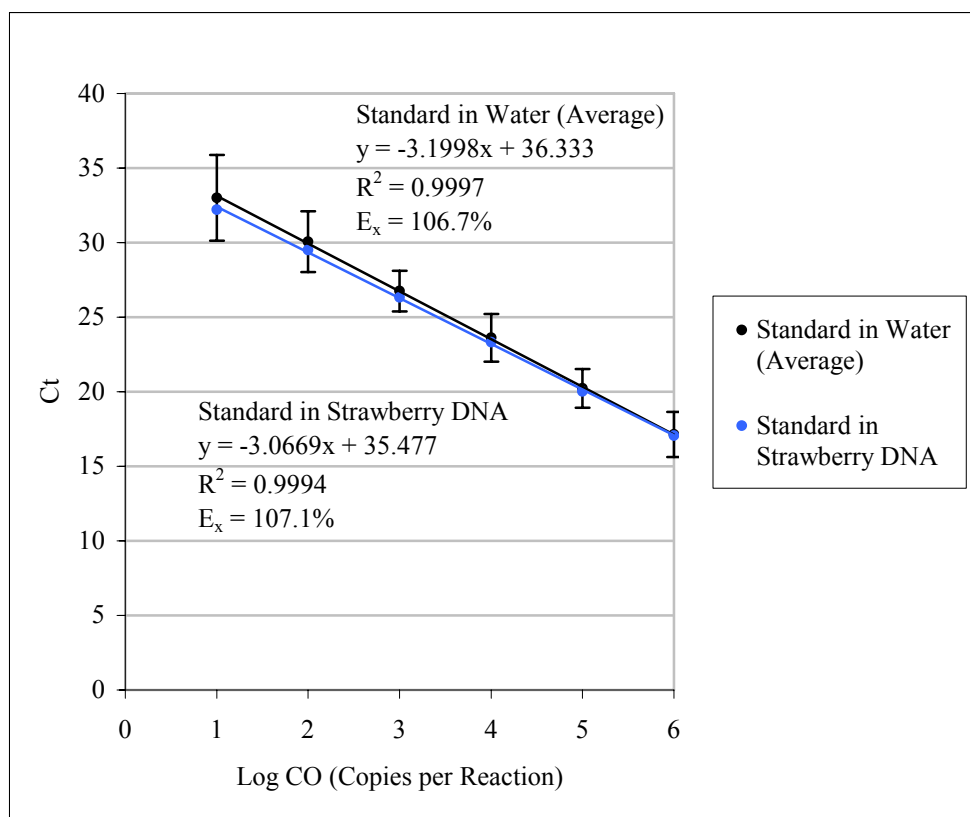


FIGURE 4.1. Average real-time PCR amplification efficiency for standard DNA diluted in sterile DI water vs. amplification efficiency for standard DNA diluted in strawberry DNA^z

^z Standard DNA for quantification was created by cycling the real-time PCR primers in a conventional PCR reaction mix to produce a high concentration of amplicon. The average values at each concentration for four standard curves of DNA diluted in water are shown compared to a standard curve of DNA diluted in strawberry DNA. Error bars represent the 95% confidence interval for the Ct values of the DNA diluted in water at each concentration. Amplification efficiency (E_x) was calculated with the equation $E_x = 10^{(-1/\text{slope})} - 1$ using the slope of the regression lines for the standard curves, above.

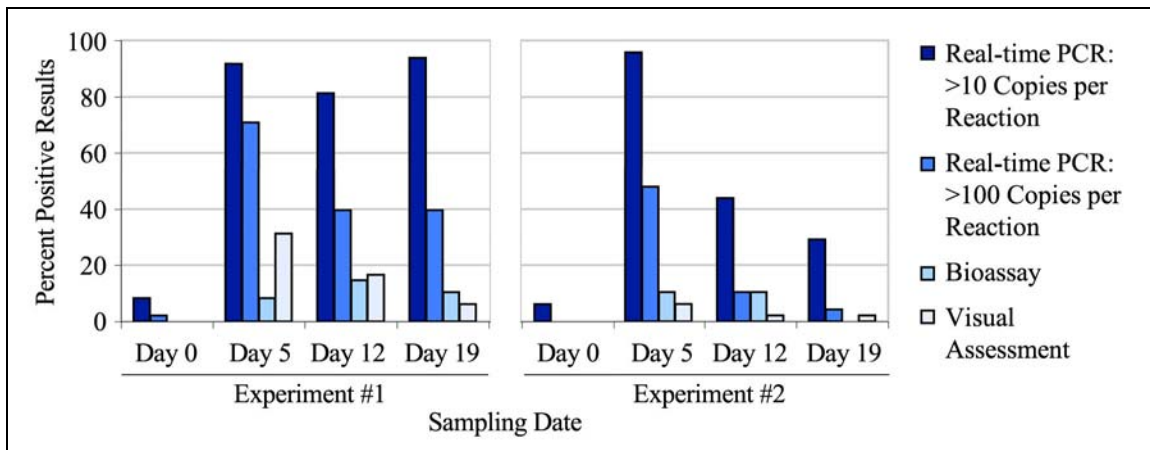


FIGURE 4.2. Percentage of positive results for the detection of *Colletotrichum acutatum* in artificially infected petioles by visual assessment, bioassay, and real-time PCR at two thresholds^{x,y,z}

^x In both experiments, 24 strawberry plants were placed in each of two controlled-environment chambers. A baseline sampling of one petiole per plant was conducted, after which the plants were spray-inoculated with a 10^6 spores/mL suspension of *Colletotrichum acutatum* in water. Additional petiole collections were conducted at 5, 12, and 19 days after inoculation.

^y Visual assessment was conducted by examining each petiole for the presence of the dark sunken lesions characteristic of *C. acutatum* infection.

^z Each collected petiole was sliced longitudinally. One petiole half was assigned to the bioassay, where the petiole was disinfested and treated with herbicide, then incubated at room temperature for 7 days and examined for the formation of acervuli. The protocol developed by Parikka and Lemmetty (46) was used to extract the DNA from the other petiole half, and the DNA was amplified in an ABI Prism 7000 thermal cycler with the primer/probe set described in Table 4.2. Initial concentrations of 10 and 100 copies per reaction were established as thresholds for a positive result.

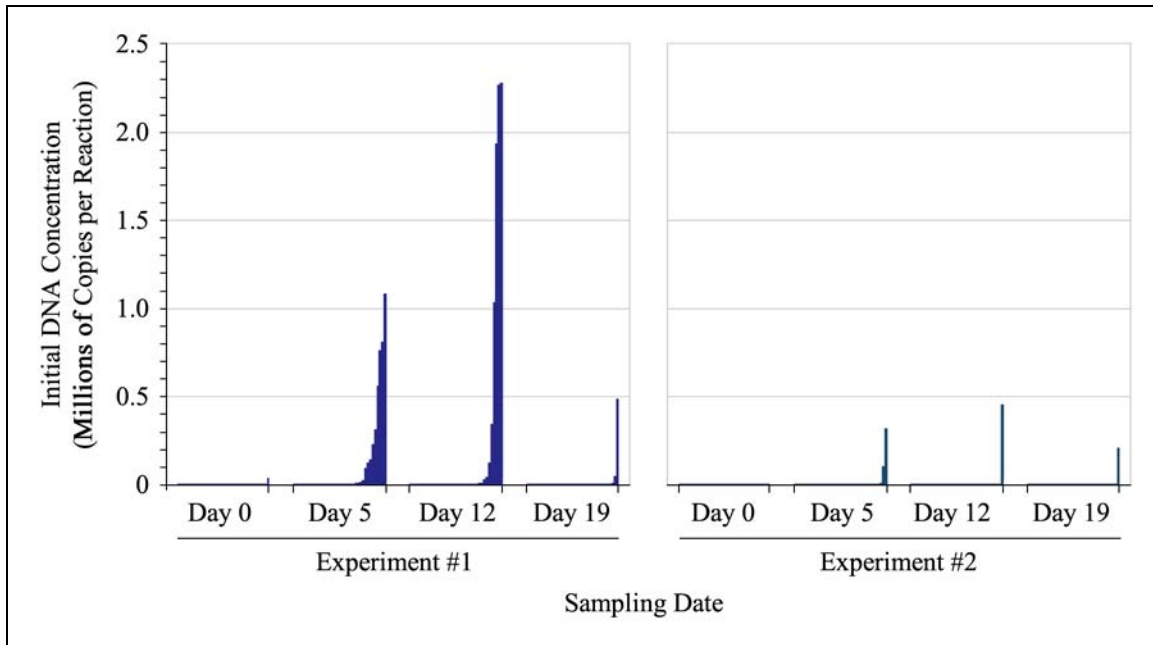


FIGURE 4.3. Initial concentrations of *Colletotrichum acutatum* DNA extracted from artificially infected strawberry petioles determined by real-time PCR^{y,z}

^y In both experiments, 24 strawberry plants were placed in each of two controlled-environment chambers. A baseline sampling of one petiole per plant was conducted, after which the plants were spray-inoculated with a 10^6 spores/mL suspension of *Colletotrichum acutatum* in water. Additional petiole collections were conducted at 5, 12, and 19 days after inoculation.

^z Each collected petiole was sliced longitudinally. The protocol developed by Parikka and Lemmetty (46) was used to extract the DNA from one petiole half, and the DNA was amplified in an ABI Prism 7000 thermal cycler with the primer/probe set described in Table 4.2. The initial DNA concentration was calculated according to the standard curve, and the concentrations for 48 samples from each sampling date were sorted in increasing order, as shown above.

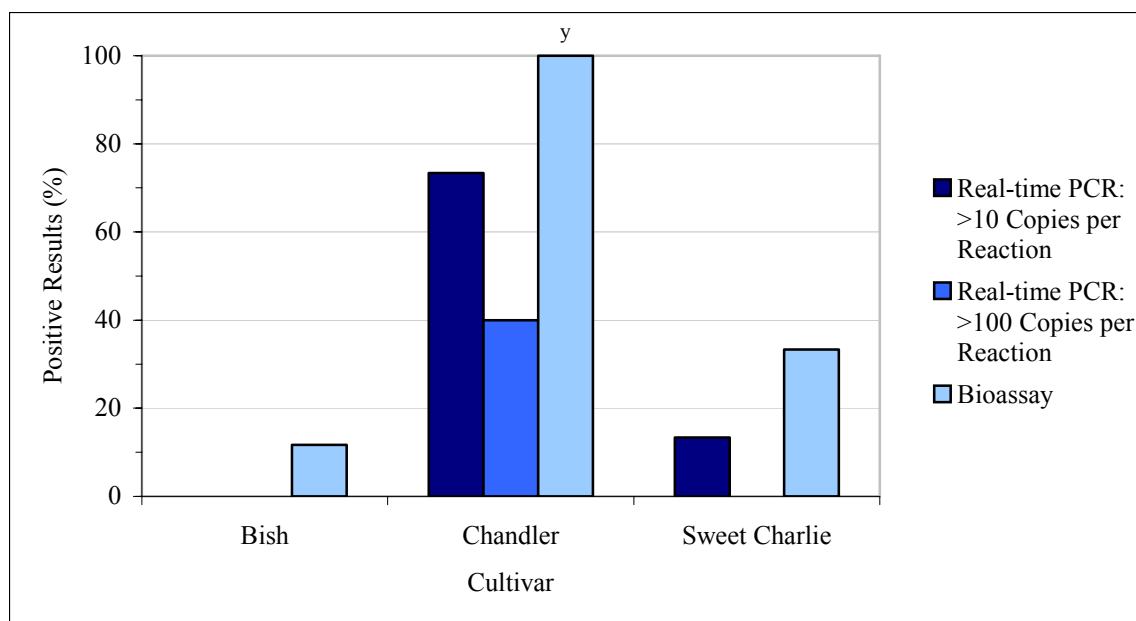


FIGURE 4.4. Percentage of positive results for detection of *Colletotrichum* spp. in naturally infected petioles by bioassay and real-time PCR for three strawberry cultivars^z

^y Chandler petioles were not screened by the bioassay because all petioles sampled were symptomatic.

^z Sixty petioles of each cultivar ('Bish,' 'Chandler,' and 'Sweet Charlie') were collected from strawberry plants in a North Carolina nursery where *C. gloeosporioides* had been reported. A 2 cm segment was removed from the end of each petiole for use in the real-time assay, and the remainder of the petiole used in the bioassay. The protocol developed by Parikka and Lemmetty (46) was used to extract the DNA from the 2 cm segment, and the DNA was amplified in an ABI Prism 7000 thermal cycler with the primer/probe set described in Table 4.2. Initial concentrations of 10 and 100 copies per reaction were established as thresholds for a positive result. The remainder of the petiole was disinfested and treated with herbicide, then incubated at room temperature for 7 days and examined for the formation of acervuli according to the bioassay protocol.