Summary

Anthracnose fruit rot (AFR) caused by *Colletotrichum acutatum* and crown rot caused by *C. gloeosporioides*, are major limiting factors in strawberry production in North America. Quiescent infections on foliage often serve as an inoculum source for fruiting field plants and the ripening berries and may cause severe crown rot or fruit infection under favorable weather conditions. Assessment of the incidence and severity of quiescent infections on foliage may provide growers some directions whether to adopt a preventive measure. We developed and optimized a real time PCR system to detect and quantify *C. acutatum* and *C. gloeosporioides* and discriminate these two species based on melting temperature through melt curve analysis of the amplicons. Quiescent infection of leaves at young middle and older stages from inoculation with same number of spores indicated middle aged leaves were the best for assessing quiescent infections. The leaf was proven to be a significantly bigger reservoir of inoculum of quiescent infections compared to petioles, making these findings essential considerations for sampling. *C. acutatum*. Quantification with the newly developed protocol in an inoculated strawberry fungicide screening trial and growers’ field showed higher precision and accuracy compared to a traditional paraquat protocol. Assessment of levels of *C. acutatum* quiescent infections at the pre-fruiting stage from three strawberry fields showed significant correlation with anthracnose fruit rot outbreak under favorable weather conditions. Quantification of *Colletotrichum* spp quiescent infections in strawberry foliage can be effectively used for plant health as well as risk assessment and prediction of disease outbreaks with an assumption of favorable weather conditions for anthracnose.
INTRODUCTION

The devastating losses caused by anthracnose fruit rot (AFR) and crown rot, in combination with cultivation practices, the regional climate, and the susceptibility of the current favored cultivars, make AFR one of the most economically important diseases of strawberry in most of the strawberry growing regions of North America (Howard et al., 1992). The dominant strawberry production system, annual hill plasticulture, typically utilizes plug-plants rooted from tips or bare root plants grown in a nursery. Plugs or bare root plants are planted in the fall as twin rows on raised beds, typically pre-fumigated, for fruit production in the following spring. In the case of AFR, 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 (Legard 2000) if inoculum is present on foliage as quiescent infections and favorable weather conditions follow. The disease is aggravated by wind driven rain splash and long wetness hours. Crown rot caused by C. gloeosporioides also causes severe losses in the Southeast as observed in recent years (MacKenzie et al., 2007). Although inoculum sources for fruiting fields may be diverse, non-symptomatic infected planting stocks is the most important source of inoculum (Freeman et al., 2001; Leandro et al., 2001). Contaminated planting stock is not symptomatic at lower levels of infestation (Parikka and Lemmetty 2004; Nam et al., 2004) and this can lead to the inadvertent introduction of the pathogen(s) to the fruiting field, a major challenge for the fruit growers. Currently, supply of apparently disease-free plants relies on inspecting nursery fields for symptomatic plants followed by isolation and identification of the causal organism by studying the colony/spore morphology, resulting in risk of disease transfer to fruiting fields. Low levels of asymptomatic plants is particularly devastating in plug production facilities due to the constant misting and other favorable conditions that can lead to an epidemic in the plug production facility. Detection of the anthracnose pathogens on quiescently infected foliage with a highly sensitive DNA-based diagnostic tool can therefore be used as an essential decision making tool for the growers to determine if nursery plants pose risk and to adopt preventive control measures. Assessing the level of quiescent infection and defining the parameters that lead to inoculum multiplication in the field can forseeably be used in risk assessment tools for anthracnose outbreaks and provide growers crucial information to make management decisions. The objectives of this research were to: 1) optimize a recently developed real time PCR protocol for discriminating two major anthracnose causing species of Colletotrichum, 2) determine the optimum foliar tissue or leaf stage for the best result for detecting quiescent infections on strawberry, and 3) relate the quiescent inoculum levels with anthracnose fruit rot outbreaks in the field.

MATERIALS AND METHODS

Optimization of Real Time PCR Protocol

A previously developed real-time PCR protocol was optimized for maximum detection and quantification of quiescent infections on strawberry foliage by testing effect of inhibitors, leaf age, petiole or leaf blade. Optimized spatial analysis was also done by sampling from different distances from the source of inoculum.

Species Discrimination

Different forward primers were tested in combination with a conserved reverse primer to produce amplicons of different sizes. Forward primers located upstream of the single nucleotide polymorphic (SNP) sites were selected for this study that provided a net plus of more than 33.8 F (1 C) in post PCR melting temperatures for amplicons produced from C. acutatum template DNA compared to amplicons from C. gloeosporioides. Following a final extension at 161.6 F (72 C) for 3 min, melt curves were generated using the default settings in high resolution melt (HRM) in an ABI 7900 HT machine (ABI-Foster City, CA).
**Effect of Host Metabolite on PCR Efficiency**

Conidia of *C. gloeosporioides* were produced on PDA in petri dishes and dislodged by a rubber polishman in dd H₂O. The conidial suspension was quantified using a hemocytometer to have 100,000 conidia in two replicates. The conidial suspension was centrifuged at a speed of 12,000 g to form a pellet at the bottom of the tube. An aliquot was poured off and conidial pellets were frozen by liquid N₂ as pure suspensions or by mixing .0018 oz (50 mg) of leaf tissue. The DNA was extracted with QiaGen kits (Valencia, CA 91355). Initial template concentration was used to create a 10⁻²-10⁻¹ standard curve by a 1:10 dilution series for both extracts. This experiment was conducted twice. Standard curves were developed using regression analysis and PCR efficiency was calculated and compared.

**Identification of Leaf Stage that Supports Highest Detection and Quantification**

Strawberry plants from North Carolina State University registered stock were grown in the greenhouse controlled environment to make sure the plants remained free from any kind of quiescent infection. For a preplanned inoculation and sampling, leaves from four replicate plants were selected to represent three different growth stages such as young (fully opened), middle age (~30 days after full opening) and old (~60 days or older after full opening). Leaves were inoculated with five different concentrations (10, 20, 50, 500, and 1000 conidia/leaf disk) by placing small droplets on pre-selected areas. Immediately after inoculation, plants were covered with plastic bags for 72 hr after which time leaves were sampled by cutting leaf disks with a scissors from the inoculation sites and DNA extracted immediately. A real time PCR Taqman cycle was run in ABI 7000/7900 machine following default cycle parameters.

**Assessment of Petiole and Leaf Blade for Potential Reservoir of Quiescent Infections**

This experiment was conducted under both field and Phytotron conditions.

**Field experiment.** Petioles and leaf blades were sampled from a field experiment conducted at the Horticultural Crops Research Station, in Castle Hayne, NC, which was set to evaluate the efficacy of fungicides for controlling anthracnose fruit rot. Rooted plug plants (20 in each plot) were planted on October 22, 2008, on four 6 in high, 27 in wide, plastic mulched beds on 60 in centers. Plots were 10 ft long and contained 20 plants on a 12 in spacing staggered in two rows and individual plots were separated by 3.5 ft within beds and were arranged in randomized complete block design (RCBD). Highly AFR susceptible Chandler plug plants were inoculated with 5 x 10⁵ conidia.ml⁻¹ (equally mixed using three *C. acutatum* single-conidia strains, JVL7-01, JCP7-02 and PDIC7-745) by spraying on the foliage to run off followed by incubation in a humid chamber for 48 hr at ambient room temperature (70±35.6 F). Two of these inoculated plants were then planted in October at both ends of each field plot to allow “natural” spread of inoculum throughout the growing season. Immediately after transplant, the plants were overhead irrigated with sprinklers for three days (5, 3 and 2 hr, respectively) to aid plant establishment. Commercially recommended pre-plant fertilization and other cultural practices were followed. All other irrigation and fertilization was done through the drip tape that was pre set at the middle during bed formation. No fungicides were applied during the growing season. Leaf and petiole samples from four selected plants 12 in interior to the plots from the inoculum source from 40 replicate plots were collected at 60 days after planting. From each trifoliate leaf sample, one arbitrarily selected leaflet and half the petiole was used for DNA extraction and the rest of the tissue was used for the paraquat assay (induction of senescence to surface sterilized leaves/petioles by dipping in paraquat followed by a short incubation on metal screens inside a crisper layered with moist paper towel to enhance acervular growth).
Phytotron experiment. Ninety-six ‘Chandler’ strawberry plug plants were obtained from the North Carolina State University Micropropagation Unit, and transferred to the NCSU Phytotron growth chambers. Twenty-four plants were placed in each of two 9.84 ft² walk-in growth chambers set at 78.8/71.6 F day/night temperatures under 14 hr of fluorescent light to suppress flowering and encourage vegetative growth. The remaining 48 plants were transferred to a Phytotron greenhouse under the same temperature conditions with natural light plus 3 hr of supplemental lighting. All plants were watered once daily with a standard nutrient solution (Thomas et al., 2006). The plants were then spray-inoculated with a C. acutatum conidial suspension as described for the field experiment and covered with clear plastic for 48 hr to maintain high humidity. One set of 96 leaves and petioles (two from each plant) were collected 60 days after inoculation and processed as described for evaluating the relative incidence of quiescent infections. The chambers were sterilized, and a duplicate experiment was performed with the remaining forty-eight plants from the greenhouse. The whole experiment was repeated in the Phytotron one more time.

Tracing Quiescent Inoculum from Field Leaf Samples and Determining the Relationship with Anthracnose Fruit Rot Outbreaks

Leaf samples were collected from three different fields in North Carolina, twice in the fall (November 15 and December 1, 2008) and three times in the spring (February 10, 20 and March 15, 2009). Out of three fields, one was an anthracnose fruit rot spray trial experiment in the Horticultural Crops Research Station, in Castle Hayne, NC, with the plots arranged and inoculum introduced as described above. Samples were collected from non-treated control plots only from this location. The other two fields were certified organic strawberry productions in Franklin County and Wake County, NC, where no fungicides were applied during the growing season.

Statistical Analysis

Cycle threshold (Ct) values obtained from three different leaf stages at different conidial concentrations were subjected to two way analysis of variance (ANOVA) using the general linear models procedure (PROC GLM) of the statistical analysis system (SAS 9.1.3, SAS Institute Inc., Cary, NC). Fisher’s protected least significant difference (LSD) test ($P = 0.05$) was used to compare individual Ct means at each spore concentration and leaf stage and estimate standard errors of means.
RESULTS

Species Discrimination

Amplicon sequences when aligned with CLUSTALW showed several polymorphic differences (Figure 1) that could be exploited to use melting temperature to provide the capability to discriminate the two major anthracnose causing species of *Colletotrichum*, thus alleviating the need for multiplexing. Post amplification high resolution melt curve analysis revealed the melting temperature for *C. gloeosporioides* was 183.4 °F (84.3 °C) compared to 185.7 °F (85.4 °C) for *C. acutatum* (Figure 2).

* glo- *C. gloeosporioides*, polymorphic sites are shown in italics.
**acu- *C. acutatum*

![Figure 1](image1.png)

**Figure 1.** Target amplicon sequence alignment in CLUSTALW showing polymorphic sites in *C. gloeosporioides* and *C. acutatum* that can be utilized for discrimination of these two species based on difference in melting temperatures of amplicons.

![Figure 2](image2.png)

**Figure 2.** Characterization of amplicons produced in real-time polymerase chain reaction (PCR) by high resolution melt curve analysis with the following templates: i) *C. gloeosporioides*; ii) *C. acutatum*; iii) nuclease-free water (no template control).
Effect of Host Metabolites on PCR Efficiency

Despite using the same number of *C. gloeosporioides* conidia, amplification efficiency markedly decreased when leaf tissues were mixed with spores. This result indicated that quantification of quiescent infections on strawberry leaves should factor in the amplification efficiency to have a correct assessment of initial inoculum. Our results indicated that the primers and probe set developed in this study provided high amplification efficiency (99.86%) if pure *Colletotrichum* templates were used for standard development. However, extracts obtained from the same number of spores when mixed with strawberry tissue had 86.39% efficiency (Figure 3).

![Graph](image)

**Figure 3.** Real-time PCR amplification efficiency for standard DNA extracted from a known number of spores vs. amplification efficiency for standard DNA from the same number of spores co-extracted with strawberry leaf tissue.
Identification of Leaf Stage that Supports Highest Detection and Quantification

Leaf age significantly (Pd<0.003) influenced detection of C. acutatum. After inoculation, no leaf stage showed any disease symptoms (black spot or necrosis) after 72 hr of incubation under high humidity in the greenhouse controlled environment. Direct extraction of DNA from the middle aged leaf tissue followed by real-time PCR showed significant correlation with initial conidia number and Ct values obtained. Cycle threshold values from the young leaf stage showed higher variability among replicates and less correlation with the number of conidia placed on preselected leaf areas. The lower inoculum levels placed on older leaves could not be detected with any of the real time assays. Relatively high numbers of conidia (50 and above) on old leaves were detected with the TaqMan protocol at higher Ct values without any correlation with increasing number of conidia. Lack of strong correlation of Ct values with number of conidia or inability of detecting lower conidia number indicated that very young and old stages of leaf maturation might be less suitable for quantification of inoculum load on foliage. The TaqMan assay showed superior detection ability from middle aged leaves at each inoculum level with significantly lower Ct values and less variability among replications and experiments (Figure 4).

Figure 4. Effect of leaf stage on detection and quantification of Colletotrichum propagules by real time PCR. Cycle threshold value as determined by TaqMan real time PCR for different leaf ages at different levels of initial spore inoculations.
Assessment of Petiole and Leaf Blade as a Potential Reservoir of Quiescent Infection

The traditional paraquat bioassay and real time PCR both clearly showed that leaf blades had significantly higher inoculum compared to petioles in either directly inoculated plants in the Phytotron greenhouse or natural spread of inoculum in the field from an initial inoculum source. In the Phytotron/greenhouse, 61% of the petioles showed positive detection for C. acutatum compared to 89% in the leaf blades. Only 26.7% field sample petioles were positive compared to 52.8% of the leaf blades. Besides being quantitative, real time PCR showed higher detection percentage in both petioles and leaf blades in the greenhouse and field conditions compared to the paraquat bioassay. Detection in petioles by real time PCR from the Phytotron and field were 34% and 9.6% higher, respectively, compared to the bioassay. Leaf blades had 26% and 21% higher detection by real time PCR compared to the paraquat assay from the Phytotron and field samples, respectively (Table 1).

Table 1. Comparison of C. acutatum quiescent infection incidence (%) using the paraquat bioassay and real-time PCR for detection from artificially inoculated strawberry leaves and petioles in the Phytotron/greenhouse and naturally infected tissue under field conditions

<table>
<thead>
<tr>
<th>Phytotron / Greenhouse</th>
<th>Petiole: n=120 (values are expressed as percentage)</th>
<th>Leaf: n=120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay Method</strong></td>
<td><strong>Bioassay +</strong></td>
<td><strong>Bioassay -</strong></td>
</tr>
<tr>
<td>Real-time PCR +</td>
<td>35.8</td>
<td>25.0</td>
</tr>
<tr>
<td>Real-time PCR -</td>
<td>0.7</td>
<td>48.5</td>
</tr>
<tr>
<td><strong>SUM</strong></td>
<td>36.5</td>
<td>63.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Field</th>
<th>Petiole: n=80</th>
<th>Leaf: n=80</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay method</strong></td>
<td><strong>Bioassay +</strong></td>
<td><strong>Bioassay -</strong></td>
</tr>
<tr>
<td>Real-time PCR +</td>
<td>11.5</td>
<td>15.2</td>
</tr>
<tr>
<td>Real-time PCR -</td>
<td>5.6</td>
<td>67.7</td>
</tr>
<tr>
<td><strong>SUM</strong></td>
<td>17.1</td>
<td>82.9</td>
</tr>
</tbody>
</table>

1 Leaf and petiole samples were collected from the same plant and cut separated in the laboratory before processing. Data are means from three different experiments pooled together.
2 Samples were collected from inside the plots that had inoculated plants at both ends and inoculum spread inside the plot occurred naturally. Data are means from two different samplings.
Tracing Quiescent Inoculum from Field Leaf Samples and Determining Relationship with Anthracnose Fruit Rot Outbreaks

Inoculum load was high in the spray trial at the research station. There was a large increase in the multiplication of inoculum at the time of flowering and final AFR incidence was 37% from all the harvests over nine weeks (Figure 5). The last sampling in the spring followed by real time PCR quantification showed 60% of the middle aged leaf samples had quiescent infections. No inoculum was traced at the Franklin County site, and no incidence of AFR was observed. However, at the Wake County site, a low inoculum load was detected but did not translate to AFR incidence (Figure 5). This specific organic field had a very low level of fertility as well. As weather and fertility levels were different at three different locations, these two factors might have played a role in Wake County site, together with a low inoculum load.

Figure 5. Leaf quiescent infections and AFR incidence in three strawberry fields in NC. Twenty five leaf samples were collected at each sampling time to evaluate with real time PCR.
DISCUSSION

This study provides evidence in favor of the usefulness of a highly sensitive molecular diagnostic tool such as a real
time PCR assay to assess risk and to implement preventive disease management practices. This is specifically important
for the pathogens that remain quiescent in host tissues for an extended period of time after infection. The primers and
probe set previously developed by us was further optimized for detecting and quantifying an extremely low number of
propagules of major strawberry anthracnose causing fungal species of Colletotrichum. Strawberry anthracnose is primarily
caused by two major species and their infection mechanism, dispersal and management are very similar. As such, this
tool will be equally effective against C. acutatum and C. gloeosporioides. In most cases, quiescent infections in the fruiting
field does not cause any concern until the sudden appearance of crown rot, expressed by plant collapse, or fruit rot,
putting the growers off guard. We have determined the foliar tissue type that provides highest detection and quantification
to reduce the risk of a false negative. This tool will also help nursery growers in California and other nursery stock
producing areas to check for quiescent infections and certify for plant health or to let their customers know levels of risk
with the planting stock. However, an effective sampling protocol for the nursery still needs to be validated before the
recommendation can be made to the growers for sampling size and best sampling design.

The reason quiescent infections occur in strawberry is yet to be proven empirically, however, it is presumably due to
the presence of preformed antifungal compounds (phytoanticipins) and inhibitors of cell wall degrading enzymes such as
polygalacturonase inhibitor proteins (PGIPs) in host cells. The primers and probe set used for this study can be used for
any of the two major species of Colletotrichum, and is very effective with leaf tissue extracts. Different amplification
efficiency of DNA extract from a known number of Colletotrichum conidia in the presence and absence of strawberry leaf
tissues followed by real time PCR indicated partial PCR inhibition may occur by strawberry metabolites (Figure 3) especially
if the optimum leaf stage is not correctly chosen during sampling. The real-time PCR assay yielded consistently higher
detection capability than the bioassay for artificially inoculated plants as well as plants from field experiments. In the
Phytotron experiments, only 36.5% of the petioles showed positive detection compared to 60.8% by real time PCR in the
paired sampling design. Positive detection on leaves using the bioassay was 65.3% compared to 89.2% with real time
PCR. These numbers indicate that even in artificially inoculated plants under optimum conditions, petioles harbored
significantly lower amounts of quiescent infections compared to the leaf blades. The probability of a false negative is very
high using the bioassay if inoculum level is too low to be visible even after multiplication of the pathogen on senescent
leaves after the paraquat assay.

Similar trends were observed in the field samples that received inoculum due to natural spread from inoculated
plants set at the end of the plots. However, average levels of positive detection in field samples were lower than directly
inoculated plants in the Phytotron. Variability of positive detection within samplings was also higher in the field. This clearly
indicates the importance of optimized sampling techniques to be employed for the assessment of quiescent infections in
field grown strawberry plants either in a nursery or fruiting field.

In our leaf stage experiment, we did not get any false negatives, even at the lowest level of conidia placed on leaf
disk, indicating the PCR assay is sensitive enough for detecting very low levels of quiescent infections when tissues
harbor inoculum. None of these extracts from 10 to 1,000 conidia containing tissues showed any amplification in a regular
PCR using species specific primers (data not shown).
This large difference in sensitivity makes this tool a clear choice over regular PCR for tracking quiescent inoculum in leaf tissue to ascertain risk assessment in the case when weather patterns favor a disease outbreak. This result is consistent with the findings of Cullen et al. (2002) that 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 from potato. Our results indicate that leaf stage can greatly influence the detection and quantification of quiescent infections. Thus consistency of selecting the optimum leaf stage during sampling will play a key role in obtaining meaningful results. The plausible explanation for differences in detection from different leaf stages receiving the same initial inoculum in our study could be that young stage leaves may not be providing enough nutrients for the growth and development of the fungus and old stage leaves may contain higher concentrations of inhibitory metabolites that interfere with PCR amplification.

While real-time PCR provides the advantage of being 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 and exploring how quiescent infections can lead to a disease outbreak when the environment becomes favorable or inoculum load crosses a threshold. In our experiment for tracing quiescent infections, the Wake County site had a very low level (5%) of detection and a very low level of severity as indicated by Ct values. The lowest level of foliar quiescent infection and severity that causes significant fruit infection needs to be determined empirically and of course is impacted by variety, crop fertility, weather patterns and other external factors. The real time primers and probe set designed by us will provide scientists enough flexibility to use a highly sensitive tool required in disease forecasting experiments that are based on the presence or absence of inoculum in strawberry foliar tissues that can subsequently serve as an inoculum source for ripening berries. We were able to monitor low levels of leaf infections in our experimental plots, saw a peak at bloom, and this translated into a severe epidemic, a preventable problem given current fungicide programs we have developed for our clientele. Adoption of a sampling technique capable of reducing the likelihood of false negatives for the presence of quiescent infections will provide assurance to growers to avoid expensive fungicide sprays in the absence of inoculum in the field.
REFERENCES


