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CONSORTIUM

Title: Epidemiological Applications to Manage Anthracnose Crown Rot of Strawberry in the Southeast

Progress Report.
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Research Proposal
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Objective:
The objectives of this research proposal were to:

1) Determine the temporal and spatial dynamics of *Colletotrichum gloeosporioides* in strawberry nurseries
2) Validate a novel adaptive cluster sampling strategy and real-time PCR assay to quantify distribution of *C. gloeosporioides* in strawberry nursery, and
3) Assess potential risk associated with plug production from infected tips collected from nursery or mother plants and test plugs with novel fungicides to manage crown rot severity.

Justification: Anthracnose fruit rot and crown rot, incited by *Colletotrichum acutatum* and *C. gloeosporioides*, respectively, are major fungal diseases of strawberry production (Maas, 1998). In the nursery, lesions are formed on stolons that eventually girdle runners, and wilt and die (Freeman, 2008). Mother plants affected by anthracnose may collapse entirely due to anthracnose crown rot (Freeman, 2008). The disease epidemic is mainly favored by warm and moist conditions and splash dispersal of conidia is the primary source of dissemination of inoculum from plant to plant in the fields (Madden and Hughes, 1995). Based on the literature and previous work we have done, we hypothesize that the emergence of anthracnose crown rot (ACR) in the fruiting field is connected with quiescent infections on foliage in the nursery. On the other hand, attempts to detect these pathogens in nurseries (and early in fruiting fields) is difficult due to the lack of sensitive detection methods. Thus, more supporting data is needed to determine whether anthracnose epidemics can develop from quiescent infections or infected plugs, to determine the level of risk when hot spots occur in the nursery, and there is a need for more rapid and sensitive tools to detect the pathogens in quiescent infections.

The use of “disease-free transplants” is the most effective strategy for managing both fruit rot and crown rot in production fields. Unfortunately, many nursery industries faced serious challenge with crown rot incited by *C. gloeosporioides* possibly due to the

quiescent infection of plants that escaped visual inspection and widespread problems led to the collapse of the NCSU certification program. This affects strawberry growers throughout the region since many growers as far south as Florida now procure their plants from North Carolina as well. We have conducted research work on *C. gloeosporioides* in small-plot work evaluating source strength of inoculum and spread (Rahman et al. 2015). The knowledge gained from such epidemiological studies is essential to understand the movement of the pathogen from transplants or 'plugs' to neighboring plants and its impact on the crop.

Characterization of the spatial and temporal dynamics of crown rot is a necessary step for quantitative epidemiological analysis. Information obtained from spatial and temporal analyses, is not only useful for the quantification of fungal biomass, and adoption of a comprehensive inspection protocol but also able to aid management decisions by nursery producers and minimize the risk to fruit producers. Several statistical methods are available to quantify spatial patterns, which can provide important insights into plant disease epidemiology. More recently, an adaptive cluster sampling has gained considerable popularity compared to a simple random sampling (Ojiambo and Scherm, 2010). An adaptive cluster sampling occurs preferentially in the neighborhood of random sampling quadrats in which the species of interest is detected during the sampling bout. Furthermore, this method is most appropriate when the characteristic of interest is highly aggregated or clustered, consistent with anthracnose in strawberry. A core question for us is "How do we (or inspectors) reliably sample nurseries to reduce risk of the pathogen and/or institute mitigation measures?

Traditionally, diagnosis of *Colletotrichum* spp. was primarily based on using classical mycological methods. Several molecular tools have been developed and utilized for the characterization and differentiation of between *Colletotrichum* species (Bonde et al., 1991). Although these techniques have a several advantages over classical methods, they are time-consuming and are not specific for routine diagnosis use. Recently, a sensitive and specific method called "real-time PCR" has been developed for the detection and quantification of *C. gloeosporioides* from infected plants by our group and others (Garrido et al., 2009). This molecular tool would allow us to rapidly assess and quantify *C. gloeosporioides* over time and space. One of the important strategies to manage crown rot is the use of QoI fungicides (e. g., Pristine, Cabrio, Abound). Our goal is to better link the presence of the pathogen to well-timed and efficacious control practices, including the use of fungicides. In general, strawberry growers do not spray fungicides in plug production. Enhancing our knowledge about the dynamics and the biology of the pathogen, combined with the development of control practices, we hope to provide better recommendations for growers, particularly in the Southeast. This proposal builds on previous work we have done.

METHODOLOGIES:

Objectives: Determine the temporal and spatial dynamics of *Colletotrichum gloeosporioides* in strawberry nurseries AND to validate a novel adaptive cluster sampling strategy and real-time PCR assay to quantify distribution of C. *gloeosporioides* in strawberry nursery:

A commercial strawberry nursery in NC was selected and sampled in 2012 and 2013. Commercial cultivar Chandler susceptible to *C. gloeosporioides* was planted in the nursery. As an example of the experimental methodology and in 2012, there were 29 rows and each row was ~ 260' long. Disease samplings were performed four times (July 24, August 12, August 23, and September 11, 2012) approximately at 2 week intervals. Crown rot symptoms of selected plants were confirmed by laboratory tests and microscopic examination. Disease incidence (number of diseased plants divided by total number of plants in the row), was recorded as presence or absence of crown rot symptoms in each plant by visual inspection. Adaptive clustering was used for 10 hotspots within the field. A similar methodology was used in 2013 except the row lengths and number of rows were slightly different. To detect the fungus in asymptomatic leaves or quiescent infections in the nursery, we used two methods: ***Paraquat method***: To detect *C. gloeosporioides* in quiescent infection, six leaves per plant and five plants in each row were collected randomly at each sampling time. Leaf samples were placed in plastic zip-lock bags in ice and brought to the laboratory. Prior to paraquat assay, tissues were taken from four sites (top, bottom, left and right) in each leaf using cork-borer for DNA isolation. Leaf tissues were placed in 2-ml tubes and stored at -80° C. Leaf samples were processed for quiescent infection using a paraquat assay as described previously (Mertely and Legard, 2004). Fungal spore induction on paraquat-treated leaves were performed at room temperature and recorded 7 to 10 days after incubation using modified Horsfall and Barratt disease rating scale. To confirm identify, the spore of *C. gloeosporioides* developed on paraquat-treated leaves were isolated and grown in acidic PDA. These spores were further examined under a microscope and genomic DNA was extracted from fungal culture and confirmed by regular PCR using the primers specific to *C. gloeosporioides*. ***Real-time PCR method*:** We employed different DNA extraction methods such as CTAB and SDS-based phenol-chloroform extraction procedures and several other extraction kits prior to our objective to detect and estimate DNA of *C. gloeosporioides* in quiescent infections. After optimizing the DNA extraction and PCR protocols, and testing new specific primer set, the same leaf samples used for paraquat assay were used. The DNeasy plant mini kit (Qiagen, Inc.; Valencia, CA) appeared to be effective for extracting DNA from strawberry. Freeze-dried and ground leaf tissues were used for DNA extraction. Primer specific *C. gloeosporioides* was selected and analyzed using real-time PCR based on SYBR Green assay (Garrido et al., 2009). Duplicate or triplicate of each sample was analyzed and threshold cycle (C_T) values were recorded. Data were analyzed and quantity of DNA in each sample was expressed as pg fungal DNA in 100 mg of leaf tissue used.

RESULTS

Disease incidence: In the first year of the work, plants with ACR initially appeared in an apparent random fashion in the field. Plants with symptoms were scored each 14 days from

July to September and several large hotspots occurred in the field (Fig. 1). Up to 10% of the mother plants wilted by the end of the season (last tip harvest). Harvested tips were collected from these hotspots and rooted to evaluate management practices (Obj 3 above). However, no symptoms ensued in the plug production phase. Ironically, the entire experiment was repeated the second year and no symptoms were observed in the field, although the paraquat assays and qPCR assays yielded positive hits (data not shown). Tips in this second year were deployed in commercial settings and a high incidence of ACR occurred in those plug production facilities. Data requires further temporal and spatial analysis by cooperators with advanced statistical expertise.

Paraquat method: Frequency of spore production and detection of *C. gloeosporioides* varied with sampling dates. The highest fungal spore production on paraquat-treated leaves was detected in July 24 and August 12 samples compared to August 23 and September 11of 2012 (Fig. 1). In addition, *C. gloeosporioides* was detected in most rows sampled in early sampling times. Only a few leaves (5 out of 180 leaves treated) developed spores in Sept. 11 sampling time. The spores of *C. gloeosporioides* from paraquat-treated leaves were isolated on acidic PDA. DNA was extracted and confirmed by regular PCR method. The distance of spread and utility of the adaptive sampling method are still under analysis.

Real-time PCR method: The detection of the fungus in asymptomatic leaves also varied with sampling dates (Fig. 2). The C_T values >40 in each sample was regarded as no fungal growth on the leaves sampled. Although visible symptoms were absent on sampled leaves, fungal DNA was detected and quantified by the real-time PCR method, indicating the presence of *C. gloeosporioides*. In general, the highest fungal DNA was detected in July 24 samples and the lowest was quantified in Sept. 11.

Conclusions:
Asymptomatic planting material could be an important inoculum for *C. gloeosporioides*. Once the fungus is introduced to fields, quiescent infection of strawberry leaves can rapidly build up and can potentially serve as sources of inoculum for further infection of leaves and fruits. The paraquat assay and real-time PCR method could be valuable tools for detecting and quantifying the fungus from symptomless plants. The findings of this study provide insight into epidemiology and dynamics of the anthracnose pathogen in the field and will be useful to develop management strategies to reduce both the initial inoculum and the latent infection of strawberry production. However, our results proved challenging to interpret. In the first year, a high incidence of crown rot occurred over time and assays for quiescent infections did not seem to prove more informative than visual observations of hotspots (and avoidance of these areas), although the assays frequently detected the pathogen in asymptomatic tissue. Tips collected from these hotspots did not result in ACR of plug plants during the plug production phase of our work. In contrast, during the second year no ACR was visually observed but the assays for quiescent infections were highly positive from July to Sept and tips rooted in commercial operations had a high incidence of ACR. The link between inspections and risk was not clear.

Impact Statement: Management of ACR is critical. We designed assays to assess for the presence of the pathogen in large fields. Quiescent infections are clearly a major source

of risk in the nursery and these infections can occur without showing symptoms. Securing data on incidence and linking this to risk is essential toward developing rational sampling methods and mitigation efforts. More work is needed in this pathosystem to understand this link and reduce losses due to ACR in the nursery and subsequently in fruiting fields.

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Fig. 1. Incidence of anthracnose crown rot in the nursery. Symptoms appeared as individual plants then spread as hotspots (insert). Total incidence approached 10% of all mother plants.

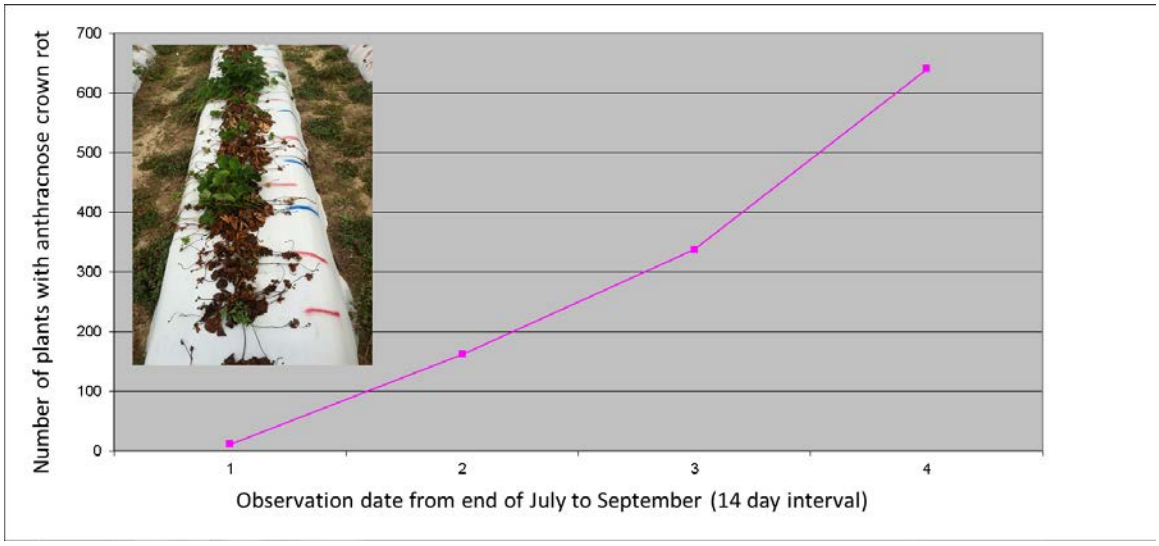

Fig. 2. Detection of *C. gloeosporioides* in asymptomatic leaves of strawberry cv. Chandler from a nursery, NC in 2012 using the paraquat assay.

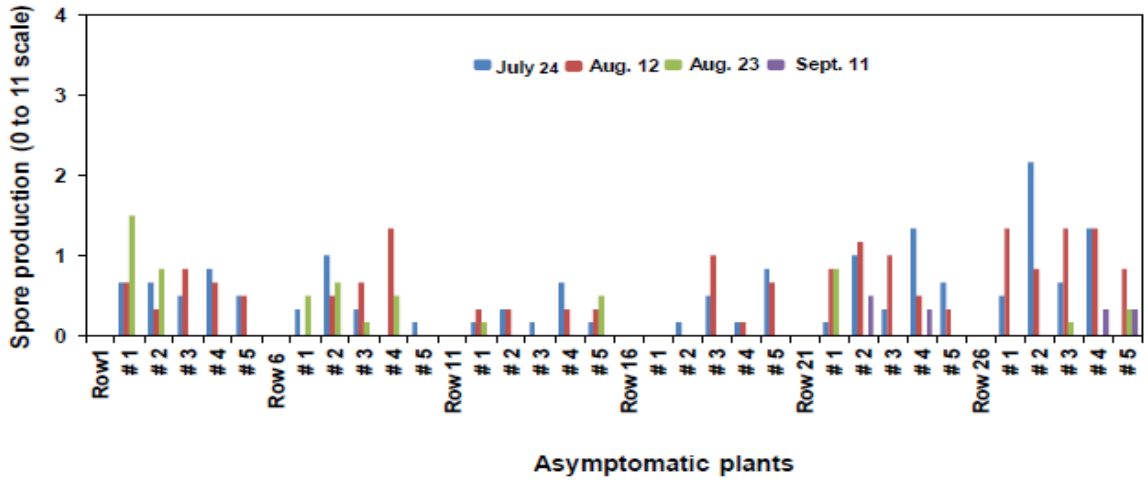

Fig. 3. Detection and quantification of *C. gloeosporioides* asymptomatic leaves of strawberry cv. Chandler from a nursery, NC in 2012 using the real-time PCR method. Quantity of DNA in each sample was expressed as picogram (pg) of leaf tissue analyzed.