Title: Epidemiological significance of *Colletotrichum gloeosporioides* infestation of nursery plants on crown rot of strawberry and management in the Southeast

Final Report

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Objectives:
The objectives of this research were to:- i) Determine dispersal gradient of *C. gloeosporioides* in strawberry nursery from inoculated mother to daughter plants and assess crown rot severity in fruiting fields from known amounts of nursery infestation; ii) Assess potential risk involved with plug production from infested tips; iii) Implementation of PCR-based technology to detect and quantify *C. gloeosporioides* in strawberry production systems especially from asymptomatic infection; iv) Develop tools to manage anthracnose.

Objective 1.

Methodology: A strawberry nursery was established at the Horticultural Crops Research Station Clinton, NC on May 14, 07 by using mother plants from the NCSU certification program for 2007-2008 growing season. This experiment was repeated in 2008-2009 growing season following the same experimental protocol with a nursery planting date on April 23, 08. Four treatments were inoculation levels of 5%, 10%, 25% of the mother plants in RCBD in 4 replicates. Leaf samples were collected at 30 and 60 days after inoculation (DAI) from mother and daughter plants and evaluated for the presence of quiescent infections (QI). For dispersal study, in 2007, leaf samples were collected from all 3 levels of inoculation, but in 2008 sampling was done only from 5% inoculation towards a control plot that allowed us to collect sample form up to 5 meters. Plug plants were produced from tips of the 25% inoculation treatment. Bare root plants from each treatment were collected separately from within a 1' radius of the point of inoculation or outside of the 1' area, which constituted 2 different treatments from each inoculation percentage in the fruiting field as shown in **Fig 1**. Plants from 10% I were subjected to fungicide dip prior to planting in fruiting field.
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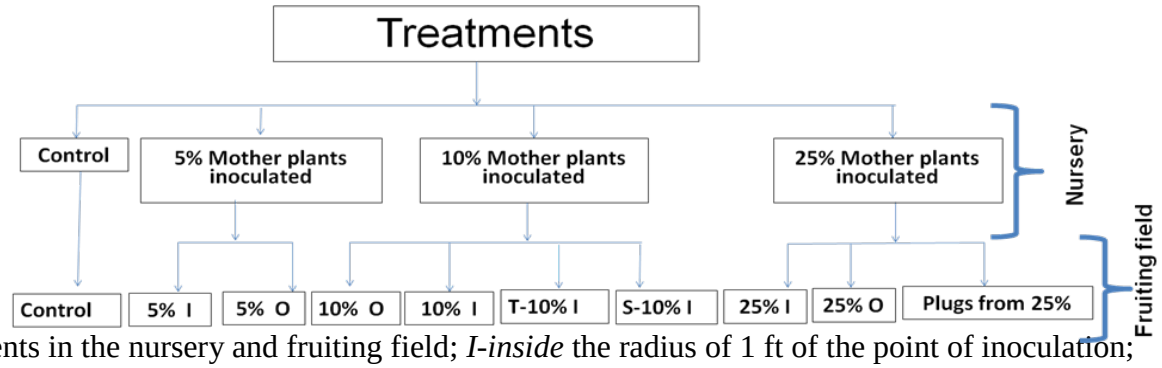

Fig.1. Treatments in the nursery and fruiting field; *I-inside* the radius of 1 ft of the point of inoculation; *O-outside* 1 ft radius, T-Topsin-M, S-Switch

Results: i) In 2007, after 30 days of inoculation, QI incidence in the nursery had a very strong significant correlation with level of inoculation of plants. In 2008, due to high rainfall, inoculum dispersal was faster and reached a high level within 30 days of *C. gloeosporioides* inoculation but correlation of incidence with level of inoculation was lower compared to 2007 (**Fig. 2**). Quiescent infections on leaves of daughter plants in both years reached at the same level or higher compared to mother plants (**Fig. 3**) but the severity on daughter plant leaves were significantly (P≤0.003) lower (**Fig. 4**).

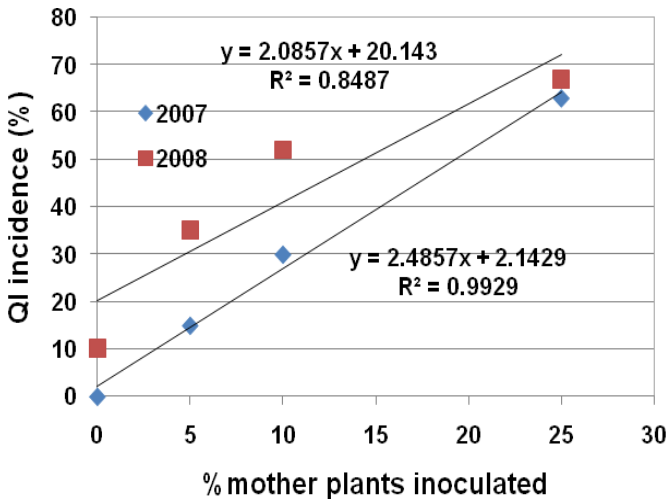

Fig.2. Correlation of *C. gloeosporioides* incidence (QI) in leaves at 30 DAI with % mother plants inoculated in nursery.

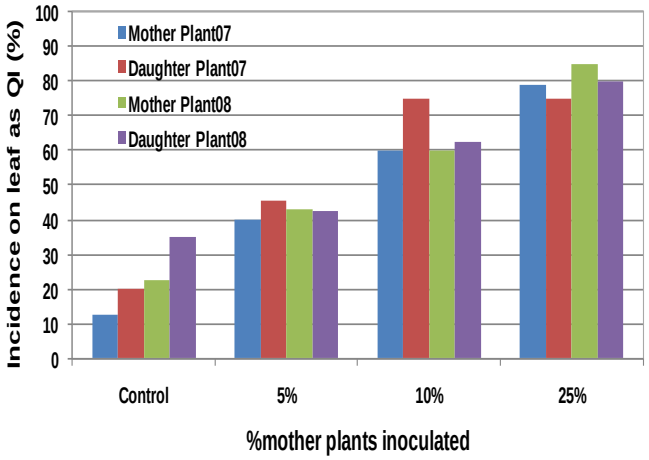

Fig.3 . Incidence of QI in leaves of mother and daughter plants at 60 DAI before taking bare root plants to the fruiting field

In 2007, which was a dry and hot year, leaf samples at 60 DAI indicated that dispersal of inoculum declined sharply from the point of inoculation to a 2' distance (**Fig. 5B**). However, in 2008 due to high rain splash early in the nursery, inoculum dispersal was faster compared to 2007 level. (**Fig. 5A**). Based on the inoculum dispersal data obtained in consecutive 2 years, distance for roguing of infested plants should consider weather variables but plants collected from 3 meters or farther from infection foci should be considered relatively safe.

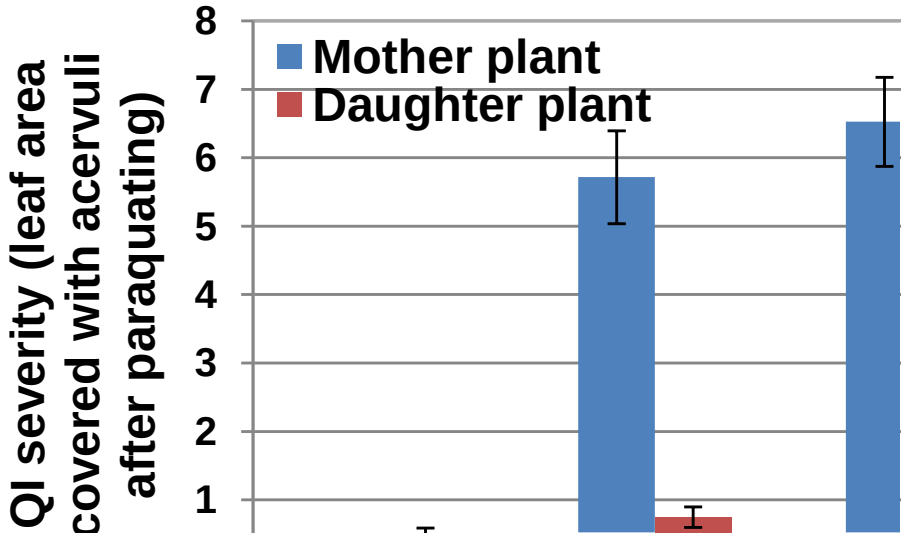

Fig. 4. Severity of quiescent infection on leaves of mother and daughter plants collected from nursery that was inoculated with *C. gloeosporioides* conidial suspension at different levels on mother plants (Data pooled from 2007 & 2008 after checking homogeneity of variance)

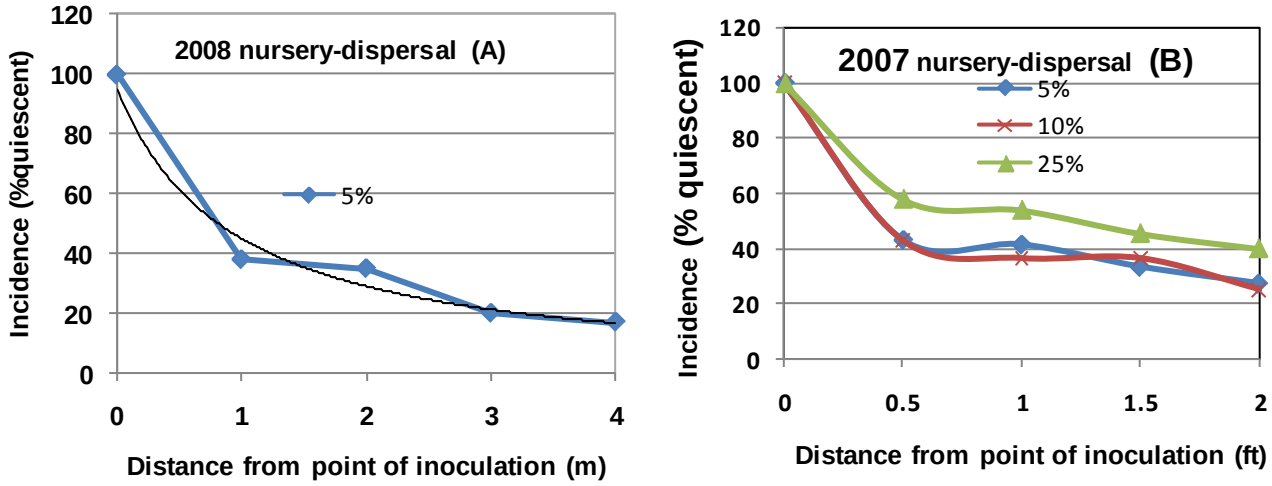

Fig. 5. Dispersal of *C. gloeosporioides* in strawberry nursery from the source of inoculation at 60 DAI in 2008 and 2007

In the fruiting field in 2007-2008, bare root plants that was collected from inside the 1' radius had significantly higher plant mortality compared to the outer side of the 1' radius. Although 10% and 25% nursery inoculation at 60 DAI had similar incidence on daughter leaves, in the fruiting field only 25% I treatment caused plant mortality in an alarming rate throughout the season (**Fig. 6**). Fungicide dipped plants from 10%-I treatment showed significant ($P<0.0001$) reduction in area under disease progress curve compared to non dipped treatments **(Fig. 7).** Level of infestation in the nursery significantly affected fruit yield in the field. Although at the end of the nursery season all levels of

inoculation had incidence of *C. gloeosporioides*, only higher levels of severity caused plant mortality and reduced yield.

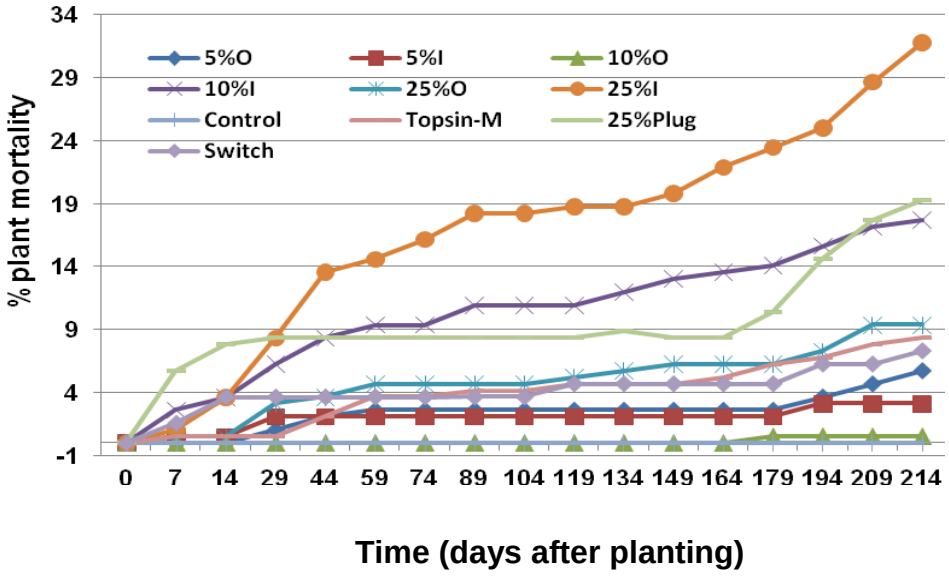

Fig. 6. Plant mortality in the fruiting field as affected by level of infestation in the nursery and fungicide dip before planting

In 2008-2009, overall plant mortality was lower than 2007-2008 most likely due to a cooler fall and winter but plant mortality showed similar trend (data not shown).

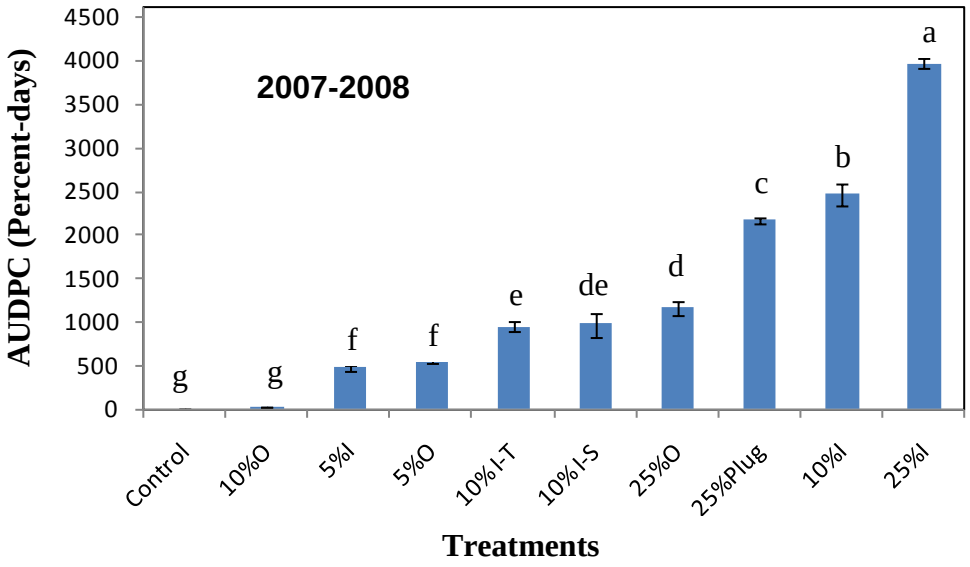

Fig. 7. Area under disease progress curve for the plant mortality in different treatments recorded from planting through the last harvest

Overall yield was higher in 2008-2009 due to lower plant mortality, but plant growth and yield was significantly affected by level of quiescent infections (Table 1)

Table 1. Marketable Fruit yield and plant biomass production in different treatments originating from the nursery inoculation at different levels followed by daughter plants collected from different distances as shown in Fig. 1.

Treatment	Yield (lb/A)		Plant dry wt (g/5 plants)	
	2007-2008	2008-2009	2007-2008	2008-2009
5% Outer	22848 abc	24570 c	242.96 a	179.4 ab
5% Inner	20008 abcd	20722 d	179.96 b	158.9 ab
10% Outer	21510 abc	24539 d	174.87 b	146.4 ab
10% Inner	14405 d	22331 e	159.48 b	173.1 ab
25% Outer	18415 cd	17295 g	179.71 b	134.5 b
25% Inner	17765 cd	17698 g	173.74 b	131.5 b
Control	25559 a	25338 b	265.41 a	180.2 ab
10% I-Topsin M	20379 abc	21083 ef	163.65 b	155.0 ab
25% plug	23813 ab	27833 a	280.37 a	234.4 a
10% I-Switch	19943 abcd	19759 fg	273.42 a	209.2 ab
LSD (α = 0.05)	***5744***	***706***	***48.28***	*93.24*

ii) Plant under mist in the plug trays showed wilting symptom in the first week and continued throughout the plug production. After 4 weeks plant mortality reached 30% (Fig. 5) in 2007-2008 but the mortality was significantly lower in 2008-2009. In spite of using only apparently healthy plants from the tray for field planting, another 20% of these plug plants died in the field until at the end of harvest in 2007-2008 and 11.25% in 2008-2009.

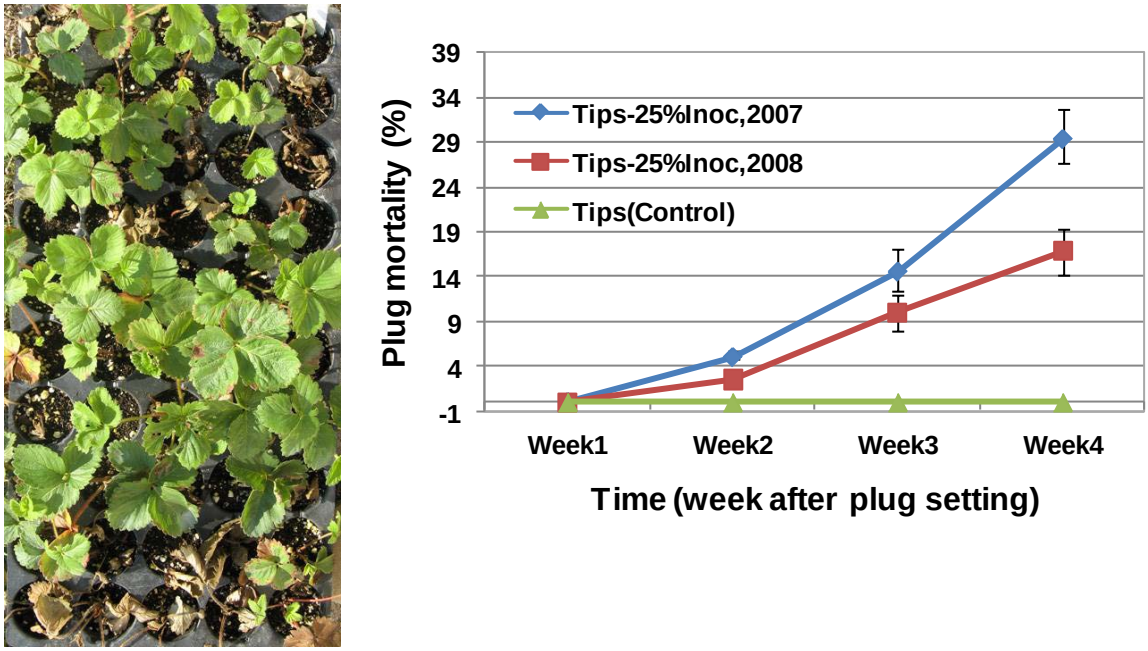

Fig.8. Plant mortality in the plug tray from tips during the 4-week propagation phase in 2007 and 2008; planting was done by using only the apparently healthy plants.

Objective iii, Methodology

Purification of *C. gloeosporioides* template from leaf tissue by magnetic capture hybridization (MCH). Strawberry leaves were inoculated with four different levels of spores (200, 1000, 2000 and 5000) on pre-selected areas (marked by drawing circles with a sharpie) in 4 replicates. Leaves were covered with Ziploc bags for 72 h after which time 50 mg tissues were cut out from inside the circle. Tissue samples from each of 4 spore concentrations were subjected to DNA extraction with Quiagen DNeasy plant mini kit (Valencia, CA 91355). Two replicate sample extracts were directly used in PCR and the rest two replicates were further purified with magnetic capture hybridization (MCH) probe as described below.

MCH protocol. A 40 base hybridization probe was designed and synthesized 150 bases upstream of the real time PCR target in the 5.8S-ITS2 region of *Colletotrichum gloeosporioides* ribosomal gene. Genomic DNA extract from infected strawberry leaf tissue (50µl) was first sheared by sonication to have smaller fragments of the genome followed by MCH following manufacturer's instruction.

Results: Two replicate samples from all three spore concentrations subjected to MCH were positively detected with TaqMan real time PCR protocol whereas non purified samples did not show any amplification regardless of the initial spore concentrations used for leaf inoculation. MCH technology provided marked improvement in template detection and quantification from quiescently infected foliage of strawberry.

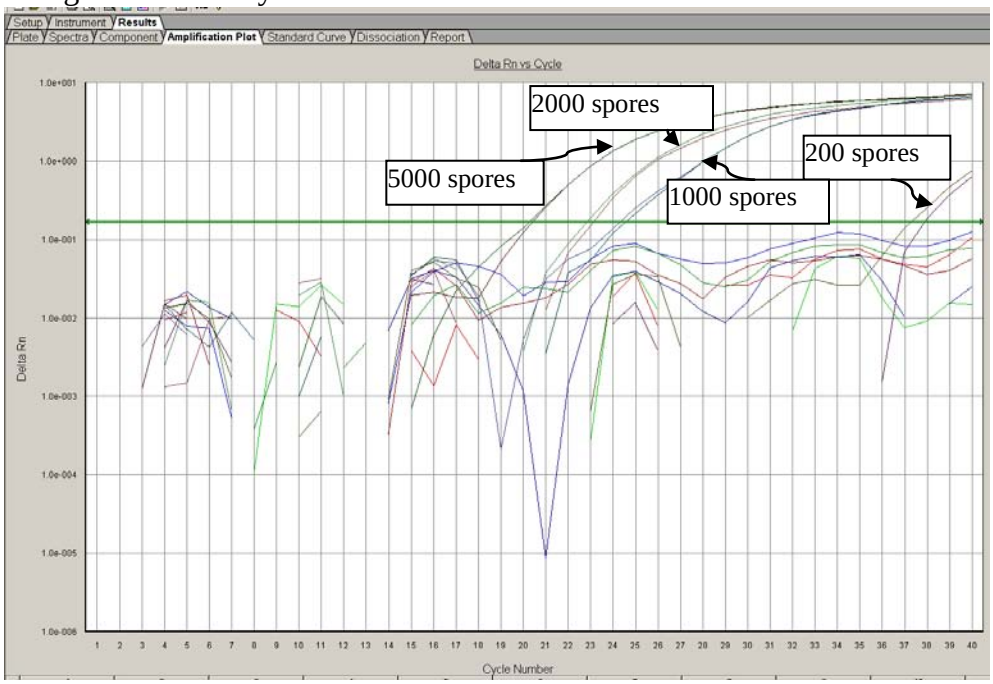

Fig. 9. TaqMan Real time PCR amplification plot showing amplification of MCH purified samples with 200-5000 spores but no amplification of extracts were observed for non-purified samples.

I**dentification of leaf stage that supports highest detection and quantification**. Strawberry plants from NCSU registered stock were grown in the greenhouse control environment to make sure the plants remain free from any kind of quiescent infection. For a preplanned inoculation and sampling, leaves from 4 replicate plants were selected to represent 3 different growth stages such as young (fully opened), middle age (~30 days after full opening) and old (~60days or older after full opening). Leaves were inoculated with 5 different concentrations of *C. gloeosporioides* (10, 20, 50, 500, and 1000) spores/leaf disk by placing small droplets on pre-selected areas. Immediately after inoculation, plants were covered

with plastic bags for 72 h after which time leaves were sampled by cutting leaf disks with a scissors from the inoculation sites and were subjected to paraquat protocol (induction of senescence of 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). After a week of incubation leaf disks from inoculated areas were cut out and DNA extracted immediately followed by MCH purification. A real time PCR Taqman cycle was run in ABI 7000/7900 machine following default cycle parameter.

Results: Paraquat protocol could effectively increase inoculum on leaf tissues that in turn reduced the detection limit for all leaf stage. However, middle aged leaf showed the best amplification in all spore concentrations (Table 2).

Table 2. qPCR Ct values obtained from leaf samples of different growth stages

Leaf stage	*No of spores placed on preselected areas on leaf surface*				
	10	*20*	*50*	*500*	*1000*
young	27.7*	27.5*	28.1*	28.49*	20.6*
Middle age	20.5	19.2	17.7	15.95	13.15
Old	28.15	28.5	28.13	25.36	19.05

*Ct value as determined by TaqMan real time PCR for different leaf age at different levels of initial spore inoculations.

Objective iv-Methodology

Invitro efficacy of fungicide active ingredients either currently recommended for crown rot or pending registration were determined from the hyphal growth suppression compared to non-amended control on PDA.

Results EC_{50} value of these fungicide active ingredient premixes such as Quadris Top (premix of Azoxystrobin and Difenoconazole), Inspire Super (premix of Cyprodinil and Difenoconazole) including with Sportak (a.i. Prochloraz) showed superior efficacy against crown rot causing fungus *C. gloesporioides*. EC50 value of currently recommended fungicide active ingredients Captan, Pyraclostrobin, Captan + Pyraclostrobin, or Captan + Thiophanate-methyl were significantly higher indicating newer fungicides may be more effective in managing this disease (**Fig. 10**).

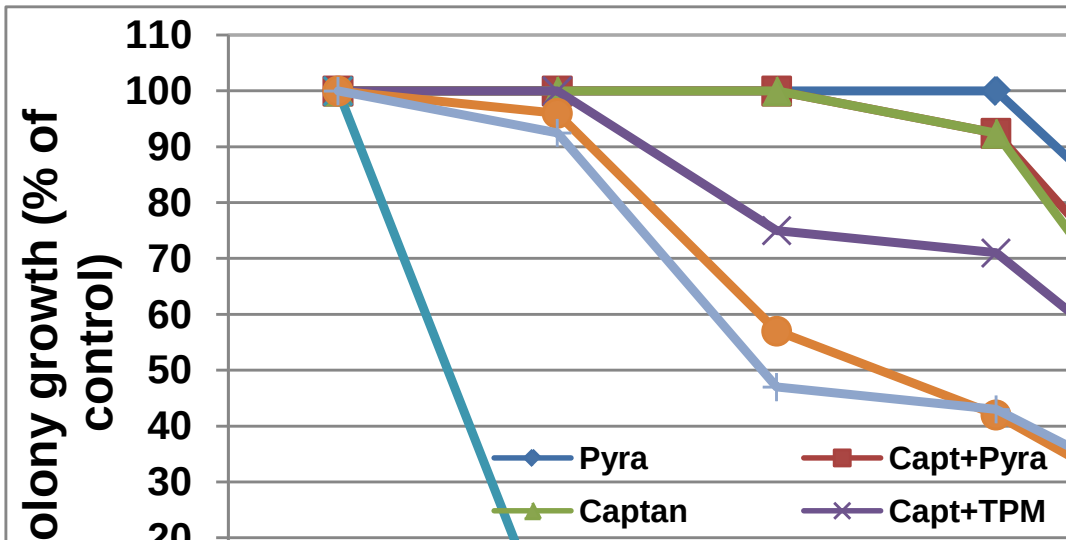

Fig. 10. Invitro efficacy of fungicides against *C. gloeosporioides*

Disease control with fungicide dip of infested plant: in 2008, 10 and 12% disease reduction was achieved by drip treatment with Topsin-M and Switch, respectively. However, fungicides that showed more efficacy invitro compared to the currently recommended ones need to be tested in the field. **Conclusions:** The biology, ecology and management of *Colletotrichum gloeosporioides* were studied in the nursery and associated risk in fruiting fields. Our research discovered new information about this serious pathogen in the nursery and fruiting fields suggesting the pathogen maybe much more manageable with the chemicals expected to be registered in the future. Steep dispersal gradients suggest rouging could play an important role in the nursery; however, weather factors play an important role and needs to be considered for determining rouging distance. Whole plant dips are helpful just prior to transplanting if plants are infested and a low infestation rate is of minor risk in the fruiting field. PCR based approaches to detect this serious pathogen will help in selecting true healthy plants.

Impact statement: Results from this study contributed to the early and sensitive diagnostics of anthracnose crown rot. We made recommendations to extension agents and growers the best possible way to keep this disease under control. Most importantly this study could assure growers that this disease is manageable if the diagnosis is done on time and preventative actions are taken.

Publications from this project:

1) **Rahman, M.** and Louws, F. J. 2008. Epidemiological significance of *C. gloeosporioides* infestation of nursery plants on crown rot of strawberry. Phytopathology 98:S129 (Abstr.).

2) Mahfuzur Rahman and Frank Louws. 2008. Pre-plant disease alert for 2008-2009 strawberry season. The strawberry Grower (North Carolina Strawberry Growers Association Newsletter) 14 (9): 2-3. http://www.smallfruits.org/Newsletter/Vol8-Issue4.pdf

3) Mahfuzur Rahman and Frank Louws. 2009. Important aspects in strawberry disease management: From nursery to berry harvest. Pp 13-19, Proceedings-2009 Southeast Strawberry Expo