Title: Screening Strawberries for resistance to Anthracnose crown (Colletotrichum gloesporiodes) fruit rot (C. acutatum)

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Public Abstract:
Anthracnose diseases of the strawberry are rotting diseases caused by fungal pathogens of the Colletotrichum genus. These diseases have complex lifecycle with a hidden (hemibiotrophic) stage in the leaves and a visible (necrotrophic) stage in the fruit (Anthracnose fruit rot AFR) and other parts of the plant (Anthracnose crown rot ACR). Each year strawberry growers experience significant losses to both stages of these diseases in the Southeastern US. The resistance to the visible phase is well known, but there is little information for resistance to the hidden infection and no genetic markers been identified. We screened 280 plants that were known to have variable resistance to these diseases in the field and in growth chambers. We identified 12 plants with high resistance to ACR, 8 to AFR, and 5 and 11 to one or the other form of hidden form of the disease. We are also using statistics and genetic analysis to look for markers that we

can use to screen plants for in the future. Figure 1 illustrates the hidden and visible stages of this disease.

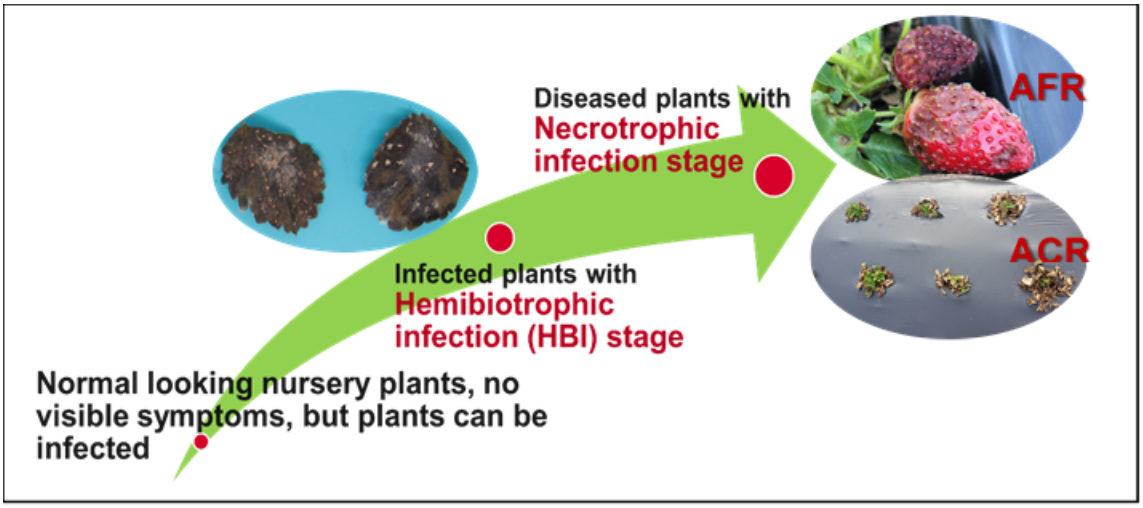

Figure 1. Depiction of strawberry anthracnose diseases, the visible stages, anthracnose fruit rot (AFR) and crown rot (ACR) are on the right. The hidden (HBI) stage found in leaves is in the middle image. This image was used in extension talks at NCSU field day and grower meetings to explain the difference between the stages of this disease.

Introduction:

For the past 25 years, two cultivars, 'Chandler' and 'Camarosa' have been the backbone of the southeastern US strawberry acreage. However, both cultivars are highly susceptible to two forms of anthracnose. *Colletotrichum acutatum* (*Ca*) and *C. gloeosporioides* (*Cg*) cause anthracnose fruit rot (AFR) and crown rot (ACR) of strawberry. Anthracnose diseases are highly destructive to the strawberry plant nursery and the fruit production industry and have been identified as among the most important pathosystems in North Carolina and the SEUS (Howard *et al*. 1992; Peres *et al*. 2005; Poling 2008, Louws pers comm.).

The NCSU strawberry breeding program has 68 selections that have been screened in the greenhouse for *Cg*. Based on previous research, one selection, NCS 10-147 exhibited *Colletotrichum* hemibiotrophic infection (HBI) resistance to both *Ca* and *Cg* and transmits it to its progeny (Jacobs 2015). A hemibiotrophic organism (e. g., *Ca* and *Cg*) will infect and live in the host strawberry but does not kill the host. We think that resistance to these two *Colletotrichum* species is controlled by the expression of common genes in strawberry leaf tissue. Therefore, we hypothesize that selecting for increased resistance to HBI for one *Colletotrichum* species should produce gains in resistance to the other. However, this theory was not tested on a *mapping population* in the field or phytotron. Therefore, in this study we evaluated resistance for HBI in a large breeding population.

Materials and Methods:

A breeding population of 286 seedlings from NCS 10-080 (*Cg* susceptible) × NCS 10-147 (*Cg* and *Ca* resistant) was established in tissue culture in 2016-17. Copies of these plants have been generated and used in a number of studies.

Inoculum preparation. *Colletotrichum* isolates were obtained from the laboratory of Dr. Frank Louws (Plant Pathology and Entomology Dept., NCSU). Multiple isolates were obtained from strawberry plants that had been proven to be pathogenic in growers fields. The isolates were stored at 4 °C in sealed plastic dishes with Potato Dextrose Agar (PDA) media. Each isolate was prepared by adding 10 mL of deionized sterile water with 0.05% Tween 20 per dish, scratched and filtered with a double layer of sterile cheesecloth to remove the mycelial debris. The inoculum concentration of each isolated adjusted to 1.0×10^5 conidia mL^{-1}.

ACR field experiment. Three replicates of 280 clonal plants produced *in vitro* from the biparental population were acclimatized in plastic trays of 50 cells. The plants were transferred to the Horticultural Research Station at Castle Hayne, NC, and planted the 30th of April 2018 on white plastic covered rows fumigated with Pic-Clor 60 EC. The experimental design was a randomized complete block design with two repetitions of five plants each. At both ends of the rows a plot of five 'Albion' plants were planted, but not inoculated, as negative controls. Plants were fertilized according to standard commercial practices in North Carolina (NCDA&CS 2011) and water was applied via drip irrigation. The plants were inoculated the first week of June and weekly evaluation started at 7 days after inoculation (DAI) and continued for six weeks. Prior to inoculation the plants were overhead irrigated at 3:00 pm, then inoculated at 7:00 p.m. directly to the crown of each plant. The disease was scored for each plant inside the plots using a disease index from 0 to 5, where 0 = no disease, 1 = basal leaves wilted, 2 = 25% of leaves wilted, 3 = 50% of leaves wilted, 4 = 75% of leaves wilted, 5 = completely or almost collapsed plant. The scores were entered directly into the app Field Book. At the end of the study, one or two plants per plot were uprooted and the crown was cut longitudinally, then pictures of each were taken with the same Tablet and the Field Book app at the end of the six week period.

AFR field experiment. One plant per genotype was maintained *in vivo* and transferred to a 6" pot, fertilized with approx. 2.5 g of slow release 19-6-12 NPK. Tips were harvested from the plants and were planted in 50 cell trays. The rooted plants were maintained in the greenhouse with overhead irrigation for 6 weeks. These plants were set in the ground at Castle Hayne Research station during the 3th week of October 2018 on black plastic covered rows fumigated with Pic-Clor 60 EC. The trial was a randomized complete block design with two replications of four plants. Each row had 4 control plots of four 'Albion' plants, one positive control at each end and one positive randomly placed inside and a negative plot placed randomly in the row. Plants were inoculation was the last week of February 2019. The number total fruits and number of anthracnose affected fruits per plant were recorded in the Field Book app weekly after 28 days after inoculation for six weeks. The percentage of AFR affected fruits per plot was calculated per week using Microsoft Excel.

HBI phytotron essay. Per standards required by the NCSU Phytotron, plants from *in vitro* culture were transferred in the NCSU Phytotron to 32 wells trays with sterile media. The plants were maintained the chamber at 24 +0.25 °C, photoperiod of 16:8 hours light – darkness. Nine

weeks after planting, the leaves of one plant of each genotype was sprayed to run off with *C. gloeosporioides* or *C. acutatum*, maintained in opposite sides of the chamber on plastic carts and covered with clear plastic domes and the relative humidity was increased to 100% with a humidifier for 72 hours. From each plant 3 leaflets from mature composite leaves were collected in plastic bags at 7, 14 and 21 DAI. The leaves were treated with the herbicide Diquat dibromide using the established protocols for paraquat (Cerkauskas and Sinclair, 1982: Rahman et al*.,* 2013). The leaflets were placed adaxial side up upon wire mesh frames inside clear boxes with a double layer of paper towels in the bottom and 100 mL of sterile deionized water, then exposed to natural sunlight near a window for one day. The leaves were evaluated for sporulation at 7, 14 and 21 after diquat treatment and photographed with a Canon DSLR camera. The photographs were analyzed with ImageJ software. The percentage of sporulation leaf area (PSLA) was calculated using Microsoft Excel.

Secondary metabolites measurement. Leaf content of flavonoid and anthocyanins was measured in 3 young mature leaves of on two plants of each genotype planted in the field at the end of May 2019 using the Dualex Scientific+™ Chlorophyll and Polyphenol-Meter (Force-A, France).

Results and Discussion:

These studies were conducted in field conditions where humidity and temperatures are among the highest recorded in Southeastern coast of North Carolina. The conditions in the highly controlled Phytotron enabled us to evaluate with confidence HBI infection.

The necrotrophic phase of the infection of ACR were evaluated in the field using a scale. The symptoms started with the wilting of lower leaves and progressed to total collapse of the plant with fast dried foliage, but frequently the youngest leaves were still alive. Damage to the crown was observed as a reddish – brown discoloration, this symptom was present in all plants that were longitudinally sliced for evaluation. The compiled AUDPC, flavonoid and anthocyanin content levels are seen in Figure 2. Correlation analysis of the traits revealed no correlation between resistance to ACR, AFR, HBI for both pathogens or the leaf concentration index of flavonoids or anthocyanins. In addition, we found no significative correlation of flavonoid and anthocyanin basal content to resistance.

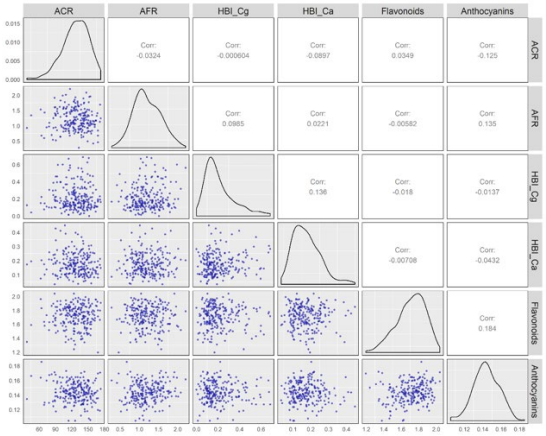

Figure 2. Correlation matrix for traits in the biparental strawberry population: AUDPC for AFR, ACR, HBI *C. acutatum* and HBI *C. gloeosporioides*, and the index values for flavonoids and anthocyanins.

We were able to detect segregation in the resistance of strawberry to multiple pathogens in a single population (Figure 3), indicating that individuals with a full range of resistance through susceptibility. Futher, we identified 12 genotypes with high resistance to ACR, 8 to AFR, and 5 and 11 to one or the other form of HBI. These latter results are useful for the breeding program as these genotypes that can be used as parental material in the breeding program.

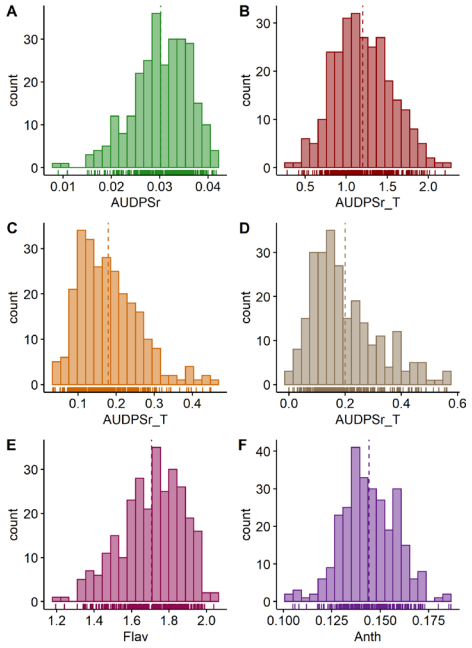

Figure 3. Distribution of traits in the biparental strawberry population *AUDPS* for: A) AFR, B) ACR, C) HBI *C. acutatum* and D) HBI *C. gloeosporioides*, and the index values for E) flavonoids and F) anthocyanins. Note: dashed line marks average.

These results form a basis that aim to uncover the genetic basis of the HBI traits in the octoploid strawberry. Ultimately, the results from these studies will allow for the development of molecular markers that will assist strawberry plant breeders to reduce the cost and time for multiple disease resistance breeding.

NOTE: This research and the findings reported above are a part of a graduate student, Jose Guillermo (Memo) Chacon-Jimenz's PhD dissertation. He is scheduled to defend his dissertation on Dec 12, 2019 and plans to remain in our labs to continue this work for 2 more years as a Post-Doctoral Scholar.