Preservation of *Monilinia fructicola* Genotype Diversity Within Fungal Cankers

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Abstract

Monilinia fructicola is a destructive pathogen causing brown rot on stone fruits worldwide. Though it is best known as a fruit rot pathogen, *M. fructicola* also causes blossom blight and, subsequently, twig cankers in the spring. Orchard management strategies often overlook cankers as an inoculum source, though they are an inoculum source of both blossom and fruit infections. In this study, we analyzed the role of cankers as storage structures for diverse genotypes of *M. fructicola*, examining whether multiple genotypes can be transmitted from blossom to canker. Fungal spores from blossoms, and 2 months later from their corresponding cankers, were collected from a conventional and an unsprayed orchard in 2015 and 2016. Simple sequence repeat markers were used to genotype 10 to 20 single spores from each of four blossom/canker pairs per orchard. Individual blossoms and cankers were detected containing up to

four and five genotypes, respectively. The average number of genotypes in blossoms and corresponding cankers were not significantly different ($P = 0.690$) across both years and farms, showing that a bottleneck for genetic diversity was not generated during the transition from blossom to canker. The average number of genotypes unique to blossom or canker was not significantly different ($P = 0.569$) and no significant effect of farm ($P = 0.961$) or year ($P = 0.520$) was observed, although blossoms had a numerically greater number of unique genotypes in both cases. In conclusion, a single blossom may be infected by one or more genotypes of *M. fructicola*, and this diversity is being preserved in the corresponding canker. This information implicates *M. fructicola* cankers as diversity storehouses, and may also apply to other *Monilinia* spp. and fungal diseases that initiate in reproductive tissue.

Monilinia fructicola, one of the causal agents of brown rot, is an important flower and fruit-infecting pathogen on stone fruits worldwide (Byrde and Willetts 1977; Martini and Mari 2014; Willetts and Harada 1984). It is particularly important to the \$137 million peach industry in the southeastern United States, where high humidity, frequent rain events, and warm temperatures produce an ideal environment for brown rot infections (Holb 2008; Martini and Mari 2014; USDA 2017).

The lifecycle of *M. fructicola* begins in the spring when either ascospores or conidia infect newly formed flower tissue. Sexual spores are produced on apothecia growing from the previous year's stromatized peach mummies on the orchard floor and are commonly considered the primary inoculum resulting in blossom blight. However, some studies have shown that ascospore production is limited in fields that are well managed with limited vegetation below trees (Holtz et al. 1998; Landgraf and Zehr 1982). Therefore, the amount of *M. fructicola* sexual recombination may vary by farm and often depends on the presence of mummies under wild plums nearby (Landgraf and Zehr 1982). Sources of flower-infecting asexual spores include mummies, cankers, and infected peduncles overwintering in the tree from previous years (Byrde and Willetts 1977). After flower infection, the fungus often travels through the peduncle into the twig to form a canker. Both blighted blossoms and cankers act as a bridge for fruit infections later in the season (Byrde and Willetts 1977; Landgraf and Zehr 1982). Cankers often survive in the tree and are capable of producing inoculum for multiple years (Byrde and Willetts 1977). In one study, up to 31% of the twig cankers collected from previous seasons sporulated under conducive conditions of temperature and humidity (Watson et al. 2002).

M. fructicola often has high diversity in stone fruit producing regions, even in locations with limited ascospore production. This high

diversity is important because it maintains genotypes in the population that may be fungicide-resistant or highly virulent (Chen et al. 2013a, b). Studies of *M. fructicola* in Switzerland, China, and California showed high levels of diversity, even in areas where the pathogen was comparatively recently introduced (Fan et al. 2010; Jänsch et al. 2012). This diversity was attributed to sexual reproduction in *M. fructicola* known to occur in these areas. However, in other locations, including Greece as well as Georgia and South Carolina in the United States, sexual reproduction appeared to be limited based on index of association (I_A) values (Dowling et al. 2016; Everhart and Scherm 2015; Papavasileiou et al. 2015).

Though many studies have investigated genetic diversity of *M. fructicola* and its relationship to sexual recombination, none have examined cankers as potential storage reservoirs for genetic diversity. Multiple genotypes may infect a single blossom (Dowling et al. 2016), but it is unknown how many of these genotypes are transferred to the twig during canker formation. If cankers can maintain diverse genotypes, they could then serve as a continuous source of diverse inoculum for fruit later in the season, and possibly for future years as well. Though this does not explain where diverse genotypes originate, it does give a mechanism for maintenance of diverse genotypes in years where sexual reproduction is limited. In this study, we genetically analyzed spores infecting blossoms and later producing twig cankers to determine if cankers could harbor and reproduce diverse fungal genotypes over a season.

We developed three hypotheses for how fungal genotypes might be affected while moving from flower to twig tissue (Fig. 1). It is possible that (I) a single genotype dominates the blossom and is recovered from the canker. This hypothesis is reasonable if one flower-infecting genotype is most fit and outcompetes other genotypes, preventing them from becoming established on blossom or woody tissue. Likely, other genotypes would be present infecting both blossom and canker, but would not be detected in our sample due to their limited presence. Another hypothesis is that (II) multiple genotypes infect the flower, and an equal number of genotypes are recovered from the canker. This hypothesis is reasonable if several genotypes can coexist without outcompeting each other in both flower and canker tissue. Lastly, it is possible that when (III) multiple genotypes infect the blossom, a bottleneck occurs and either reduces the number of genotypes, or a single genotype is recovered from the canker. This hypothesis is most likely if competition for resources and change in environment results in some genotypes

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being more prevalent in the blossom while others are more prevalent in the canker (Fig. 1).

Our goals in this study were to determine if (1) multiple genotypes of *M. fructicola* infecting a flower can survive to produce secondary inoculum on a canker, (2) orchard management strategies during bloom affect the number of genotypes present in the blossom and canker, and (3) how genotype diversity is affected as the canker forms.

Materials and Methods

Setup of field plots. All samples were collected from an experimental orchard at the Musser Fruit Research Farm in Clemson, SC, and at a commercial farm approximately 7 miles away in Sandy Springs, SC. At both farms, blocks consisted of approximately 20 trees with 10 trees in a row. At the commercial farm, peach cultivars “Big Red” and “Southern Pearl” were used in 2015 and 2016, respectively. Two different cultivars were used due to limited blossom blight in the “Big Red” block in 2016. Prior to 2013, fungicides and herbicides were applied at both farms according to standard commercial practice (Blaauw et al. 2018). However, in 2013 through 2016, no fungicides were applied to the experimental farm block to avoid fungicide selection pressure imposed on the pathogen population. At the commercial farm, applications included 2% spray oil and chlorpyrifos (Lorsban; Dow Agrosciences, Indianapolis, IN) insecticide at late dormant; a mixture of chlorotholani (Bravo Weather Stik; Syngenta, Greensboro, NC) and phosmet (Imidan, Gowan Co., Yuma, AZ) at petal fall, shuck split, and shuck off; Captan 80WDG and phosmet (Imidan) for cover sprays; and propiconazole (Tilt, Syngenta) for preharvest protection. Four trees were randomly picked from each block at each location, and in 2016, the trees were rerandomized. In April 2015 and 2016, blighted flower frequencies were determined for 10 twigs approximately 0.5 m long in each experimental tree and farm. The calculated average blight frequency of the 10 twigs from each tree was considered the blossom blight incidence.

Sample collection and isolation. To track genotypes moving from blossom to canker, blossoms were tagged at the beginning of the season, and cankers originating from those blossoms were later collected (Fig. 2). For each farm, blighted blossoms were detected in the canopy of four trees, and a total of 15 blossoms were used for all farms and years in this study, since one blossom and canker pair was lost from the experimental farm in 2016. The twig containing each blighted blossom was marked using a permanent paint marker as well as label tags on either side of the blossom. All other blossoms nearby on the branch were stripped off to prevent

confusion during collection. *M. fructicola* spores were collected after petal fall from conidial tufts on blighted blossoms in April 2015 and 2016 as described previously (Dowling et al. 2016). In mid-May, twig segments approximately 12 cm long containing cankers were harvested from the tagged locations where blossom samples had been collected. Cankers were surface sterilized and placed in moist chambers, and then spores were obtained as described previously (Dowling et al. 2017). If sporulation was minimal, spores were washed into a centrifuge tube using sterile distilled water, and the resulting solution was spread on water agar in preparation for single spore isolation. Otherwise, spores were collected with cotton swabs. Spores on swabs were dislodged onto individual water agar plates by tapping. After 24 h in the dark at 22°C, 10 to 20 germinating conidia were transferred from the water agar to individual potato dextrose agar (PDA) plates and permanently stored. To prevent bias, equivalent numbers of spores were obtained from each blossom and corresponding canker. A total of 160 and 186 isolates were obtained in 2015 and 2016, respectively.

DNA extraction and SSR analysis. DNA was extracted from all isolates according to the method described by Chi et al. (2009). A previously described modification to the cleaning steps in this method was used (Dowling et al. 2016). An average of 11.6 isolates were chosen from each tree for simple sequence repeat (SSR) analysis using the forward and reverse primers for the loci SED, SEF, SEL, SEN, SEP, and SEQ described by Everhart et al. (2012). Polymerase chain reaction (PCR) to amplify fragments began with an initial denaturation step of 95°C for 2 min and 30 s, followed by a second cycle repeated 30 times which included denaturation at 95°C for 20 s, annealing at 60°C for 20 s, with a temperature decrease of 0.5°C at each annealing step, then extension at 72°C for 30 s. The third cycle was repeated 15 times and began with denaturation at 95°C for 20 s, annealing at 50°C for 20 s, and 72°C for 30 s. A final extension step ended the reaction at 72°C for 5 min. Analysis of fragments was performed using the software Genemapper 4.0 (Life Technologies, Carlsbad, CA) according to the methods described by previously (Dowling et al. 2016).

Data analysis. A statistical model was developed to relate the mean number of genotypes, number of shared genotypes, and number of unique genotypes to the factors in the experiment, namely: farm, tree within farm, year, and tissue type. ANOVA was used to determine if the factors and/or their interactions had a significant effect on mean number of genotypes, number of shared genotypes, and number of unique genotypes. If a factor or an interaction was significant, then *t* tests were used to further study the nature of the significant effect.

Fig. 1. Schematic of possible genotype interactions when single or multiple genotypes infect a flower and subsequently produce a canker. Spores of the same color represent a single genotype, while spores of different colors represent different genotypes. In I, a single genotype infects the blossom and is recovered from the canker. In II, multiple genotypes infect the blossom and all genotypes are recovered from the canker. In III, multiple genotypes infect the blossom and a bottleneck effect either reduces the number of genotypes or a single genotype is recovered.

Additionally, linear regression models were developed to determine if a relationship existed between the number of genotypes in blossoms and disease incidence; and if a relationship existed between the number of genotypes in cankers and disease incidence. These models were evaluated in each year and farm. Since results from the first model suggested no difference in disease incidence between blossom and canker, final regression models were estimated for the number of genotypes and disease incidence using combined blossom and canker data. The significance level for each test was $\alpha = 0.05$, and all tests were performed using the software JMP Pro 12 (SAS Institute, Inc., Cary, NC). The genotype accumulation curve and index of association values were calculated using the software R. The diversity measures of alleles per locus, effective alleles per locus, private alleles, and haploid diversity were calculated for each farm and year and for the pooled population using the software Genalex 6.5 (Peakall and Smouse 2006).

Fig. 2. Schematic showing the experimental design used to investigate interactions of genotypes collected from blossoms and cankers.

Results

Comparison of *M. fructicola* multilocus genotype numbers across blossom and canker tissues. Over both years, a total of 346 isolates were collected from blossoms (173) and cankers (173). An average of 11.6 spores were recovered from each blossom and canker. Each spore was genotyped at six microsatellite loci, and a genotype accumulation curve was produced to ensure that isolates were sufficiently differentiated. The curve showed a plateau beginning at five loci, indicating that enough loci had been used to resolve isolates from each other (data not shown).

Eight of the blossoms and six of the cankers harbored more than one genotype. A maximum of four and five genotypes were detected in blossoms and cankers, respectively, though the average number of genotypes was approximately two for both tissues. The average number of genotypes did not significantly change between each blossom and corresponding canker ($P = 0.690$) across both years and farms (Table 1). Most clonal genotypes were found within single blossoms and their corresponding cankers. However, one clonal genotype was isolated from both blossom and canker tissue in 2015 and blossom tissue in 2016 at the experimental farm.

For both years and locations and in all blossoms and cankers, a dominant genotype was present. The average frequency of the dominant genotype was 85.8% in the blossom and 87.7% in the canker. The number of unique genotypes, or genotypes found only in the blossom or the canker was calculated, as well as the number of shared genotypes, which gave the number of isolates recovered from both blossom and canker in the same tree (Table 1). In 2015, only one canker contained a genotype that was not detected in the blossom. However, in 2016, only one blossom and canker pair shared a genotype. In all other infection sites screened from 2016, cankers contained different genotypes from those found in blossoms (Table 1).

Genetic diversity of *M. fructicola* isolates collected from blossoms and cankers. A total of 52 genotypes were found when data for all populations were pooled, and seven of these were unique genotypes that only appeared once in the pooled population (Table 2). Six of the unique genotypes occurred in blossom tissue, and only one unique genotype in canker tissue. However, canker isolates had significantly larger number of alleles ($P = 0.015$), number of effective alleles ($P = 0.044$), and unbiased haploid genetic diversity values ($P = 0.050$) than blossom isolates. Most (92.3%) private alleles were also present in isolates collected from canker tissue. All loci were polymorphic in the pooled populations, and were also polymorphic for most of the individual populations broken down by farm and year. However, not all loci were polymorphic for the population of isolates collected from blossoms at the experimental farm in 2016, blossoms at the commercial farm in 2016, and cankers collected from the experimental farm in 2016 (Table 2). These three populations also exhibited numerically lower allele numbers and unbiased haploid genetic diversities than the other populations. Index of association (I_A) values were calculated for each farm to analyze linkage of loci as a measurement of the amount of sexual recombination occurring. I_A is a measure of linkage disequilibrium occurring in a group of individuals. Values significantly different from zero indicate a lack of sexual recombination and high linkage disequilibrium. I_A values for blossoms (0.066, $P = 0.309$) and cankers (0.277, $P = 0.097$) at

Table 1. Average number of genotypes found in blossom and canker tissue from the two farms. Data are not split out by farm, since no significant effect of farm was detected for any category in either year.

Year ^y	Number of isolates	Number of total genotypes recovered		Number of unique genotypes recovered		Shared genotypes
		Blossom	Canker	Blossom	Canker	
2015	160	1.88 a ^z	1.50 a	0.5 a	0.13 a	1.38 a
2016	186	2.00 a	2.00 a	2.00 b	1.86 b	0.14 b
Average	173	1.94 a	1.75 a	1.25 a	1.00 a	0.76 a

^y Total, unique, and shared genotype designations refer to all recovered genotypes, tissue-specific genotypes, or genotypes found in both tissues, respectively.

^z Letters indicating significant difference are valid within rows and columns of each genotype category, but not between categories.

the commercial farm were not significantly different from zero, indicating that sexual recombination was occurring in this location. However, at the experimental farm, I_A values for blossoms (0.42, $P = 0.045$) and cankers (0.616, $P = 0.002$) were significantly different from zero, indicating a lack of sexual reproduction.

Correlation between blossom blight incidence and genotype numbers. To determine if there was a correlation between the disease incidence, type of farm, and number of genotypes found infecting blossoms and cankers, we determined blossom blight incidence values in 2015 and 2016 for all four trees at each farm (Table 3). In 2015, disease incidence was higher in the commercial farm than the experimental farm ($P = 0.001$). However, in 2016, blossom blight incidences were more characteristic, and the commercial farm had significantly less blossom blight than the no-spray experimental location ($P = 0.001$). Linear regression analysis revealed a significant positive correlation between the number of genotypes and disease incidence at the commercial farm in 2015 ($P = 0.002$). There was also a positive correlation in 2016; however, it was not statistically significant. In the experimental farm in 2015, disease incidence and number of genotypes had a significant negative correlation ($P = 0.026$). There was also a negative correlation in 2016, but it was not statistically significant.

Discussion

The primary goal of this study was to determine if individual cankers could function as storehouses of genetically diverse inoculum over a single season. In this study, 40% of the cankers analyzed contained more than one genotype, one containing five genotypes, showing that cankers can store genotypically different fungal isolates within a season. Past studies have indicated that peach blossoms can be infected by multiple genotypes of *M. fructicola* (Dowling et al. 2016), but how these genotypes were affected as the fungus traveled through the peduncle into the twig tissue was unknown. In the current study, we observed no significant genetic bottleneck effect occurring during the transition of the fungus from blossom to canker tissue, and genetic diversity of canker isolates was even higher than in blossom isolates. Though this higher genetic diversity in cankers was likely an artifact of the optimal conditions for canker sporulation in the lab versus blossom sporulation in the field, it still indicates that cankers can produce highly diverse populations of spores under ideal conditions. This agrees with the initial hypothesis (Fig. 2, II) that no major reduction of genotypes occurs after multiple genotypes infect a single blossom, though we found that the genotypes present in the blossom are not necessarily the same as those in the canker.

The apparent lack of a bottleneck was surprising, since bottlenecks generally occur when pathogen isolates compete, encounter host defenses, physical barriers, nutritional limitations, or new environments, all of which likely occur as the fungus moves from blossom to canker tissue (Abel et al. 2015). Though there was no significant bottleneck effect in our data, we did observe a numerical decrease in

number of genotypes between blossom and canker in all locations and years except the experimental farm in 2016. It is possible that the amount of diversity present in both blossom and canker is so high that this study only detected the most common genotypes, and that any minor bottleneck between tissues was not detected. Our observations in 2016 of different genotypes occupying canker and blossom tissue agree with the idea that some genotypes present in the blossom were not detected, but became dominant when they entered canker tissue. A preliminary in vitro experiment testing this hypothesis indicated that fitness in canker tissue alone did not fully explain this result. Perhaps weather and other environmental variables were suitable to produce this phenomenon in 2016 but not in 2015 (data not shown).

Comparison of commercial and experimental farms was performed to determine if a difference in management strategies resulted in differing numbers of genotypes present in the canker and the blossom. However, in 2015, heavy frost severely affected the commercial farm, but not the experimental farm, likely increasing secondary infection by *M. fructicola*, and leading to higher disease incidences in

Table 3. Disease incidence and number of genotypes found in each blossom and canker by farm, year, and tree

Farm	Year	Tree	Number of genotypes		Blossom blight incidence ^y (%)
			Blossom	Canker	
Experimental	2015	1	1	1	21
		2	2	1	21
		3	2	2	9
		4	1	1	18
		Average	1.5	1.3	17.3
	2016	1 ^z	...	...	...
		2	3	1	48
		3	1	2	43
		4	1	5	27
		Average	1.7	2.7	39.3
Commercial	2015	1	2	1	28
		2	2	1	41
		3	4	4	65
		4	1	1	30
		Average	2.3	1.8	41.0
	2016	1	4	2	24
		2	1	1	9
		3	3	1	17
		4	1	2	28
		Average	2.3	1.5	19.5

^y The average number of diseased blossoms out of the total number of blossoms found on branches measuring approximately 0.5 m long.

^z No spores were recovered from any of the cankers collected from tree 1 in the experimental farm in 2016.

Table 2. Genetic variation in 346 *Monilinia fructicola* isolates collected from blossom and canker tissue in 2015 and 2016 at the experimental and commercial farms

Farm	Year	Tissue	Number of isolates ^y	Polymorphic loci (%) ^z	Alleles per locus ^z	Effective alleles per locus ^z	Private alleles ^y	Haploid gene diversity ^z	Genotypes
Experimental	2015	Blossom	40	100	2.67	2.22	0	0.62	6
Experimental	2015	Canker	40	100	2.83	2.22	4	0.63	5
Experimental	2016	Blossom	39	66.7	1.67	1.54	0	0.37	5
Experimental	2016	Canker	39	100	3.33	2.44	1	0.54	8
Commercial	2015	Blossom	40	100	3.00	2.59	1	0.64	7
Commercial	2015	Canker	40	100	4.00	3.64	6	0.83	6
Commercial	2016	Blossom	54	83.3	2.17	1.90	0	0.47	9
Commercial	2016	Canker	54	83.3	2.67	2.40	1	0.59	6
Pooled		Blossom	173	100	2.38	2.06	1	0.52	27
Pooled		Canker	173	100	3.21	2.67	12	0.65	25

^y Data not clone-corrected.

^z Clone-corrected data.

the conventional orchard than in the no-spray location. In 2016, however, disease incidence results were more characteristic, with the commercial location having significantly less blossom blight incidence than the experimental farm. The unusual conditions in 2015 led to our inability to determine if the number of genotypes retained in canker is limited by conventional management practices. However, in 2016, the number of genotypes in each farm was not significantly different ($P = 0.84$).

Though production methods could not be effectively correlated with genotypes present in the canker for this study, correlation of disease incidence and number of genotypes present in the canker was performed for both locations. At the experimental farm, the number of genotypes was most often negatively correlated with disease incidence, though not consistently across years and tissues. This lack of consistency at the no-spray location may be a result of primarily clonal reproduction occurring as evidenced by the high I_A values detected in this location. If this were the case, disease incidence would increase, but genotype number would decrease as the most viable clones spread throughout the orchard. It is also possible that this lack of correlation is an artifact of the small number of blossoms (four) sampled within each location.

Because cankers have the capacity to harbor diverse genotypes across a season, they may form a more important part in the epidemiology of *M. fructicola* than was previously believed, particularly if this diverse inoculum is preserved and released for multiple consecutive years. Though this study did not investigate the capability of cankers to retain diverse genotypes across years, it is reasonable to assume that they can do so, since past studies have shown that cankers often produce viable inoculum for multiple years after they first appear, though they may heal if callus tissue develops (Byrde and Willetts 1977; Watson et al. 2002). Even with our small sample size, we detected a genotype that was present in a canker in one year and was found infecting a blossom in a nearby tree the next year. The retention of diversity in cankers also leads to the possibility for multiple generations of exposure to fungicide selection and increased risk of fungicide resistance development (Brent and Hollomon 1998).

Though cankers have been considered a source of *M. fructicola* inoculum for many years, focus on canker management within the orchard is generally ignored in commercial peach production (Tate and Wood 2000; Watson et al. 2002). However, management methods are relatively simple, including canker removal during pruning and application of fungicide active ingredients such as pyraclostrobin and boscalid, which are known to decrease canker sporulation (Burnett et al. 2010). Due to the added importance of cankers as a source for diverse inoculum, peach production methods in areas with high brown rot incidence should include canker management practices.

The results of this work may have application to other flower-infecting pathogens besides *M. fructicola*. For example, *Botrytis cinerea*, causal pathogen of gray mold, often progressively infects strawberry flowers and then vegetative tissue (Brannen et al. 2018). Recent research shows that multiple genotypes of *B. cinerea* may infect single strawberry flowers (Hu et al. 2018), and it is reasonable to assume that these genotypes transfer into vegetative tissue where they can produce diverse inoculum and sclerotia for infections throughout the season and even into future years in perennial production systems. This may be an explanation for the maintenance of high diversity in pathogens such as *B. cinerea* where sexual recombination is not considered a major part of the disease cycle.

In conclusion, we discovered that *M. fructicola* cankers can function as reservoirs of genetic diversity over a season and potentially over multiple years. Because cankers likely serve as a link between genetically diverse ascospore blossom infections in the spring and fruit infections in late spring and early summer, management by removal of cankers during pruning and using canker suppressive fungicide active ingredients are recommended. Further work is necessary to determine if diverse genotypes are transferred from infected flowers to vegetative tissue for other pathogens and hosts.

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