Evaluation of the Population Structure of *Macrophomina phaseolina* and Optimization of Quantification Assays

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**Summary**

*Macrophomina phaseolina* has become a damaging and widespread disease problem in California strawberry production, causing serious losses in all production districts. Population analysis using simple sequence repeat (SSR) markers of 490 isolates recovered from different hosts in California combined with results previously reported on east coast isolates recovered from a variety of hosts identified a single clade containing over 98% of the strawberry isolates. Pathogenicity tests revealed isolates from this genotype were lethal to strawberry in a matter of weeks while isolates recovered from other hosts representing a different genotype didn’t cause disease on strawberry, or rarely low levels of disease. Virulence tests are also under way to quantify different levels of aggressiveness observed among genotypes, with the main strawberry genotype much more aggressive than another genotype. Illumina sequence data were generated.
for a number of isolates and using a comparative genomics approach a genotype specific TaqMan assay for detection and soil quantification of the main strawberry genotype of *M. phaseolina* has been developed and validated with field samples (soil quantification accurate to 3 microsclerotia/g soil). A soil plating assay was developed to use as a reference for validating the ability of the TaqMan assay to quantify the pathogen in the soil. This TaqMan assay has been transferred to the isothermal recombinant polymerase amplification (RPA) platform so plant assays can be completed rapidly in the lab or directly in the field. The RPA assay has been fully validated with field samples and is currently used to test plant samples by the UCCE lab in Salinas, CA. To serve as a reference for additional work with the pathogen the nuclear genome of an isolate representing the main strawberry genotype and another genotype have been assembled and are currently being annotated. Similar research is in progress with *Fusarium oxysporum* f. sp. *fragariae*; comparative genomics has been used to identify a unique conserved region in all isolates of this pathogen, but which is absent from other *F. oxysporum* isolates. This region has been used to develop a TaqMan assay that is in the final stages of field validation for specificity.

**INTRODUCTION**

Beginning at least as early as 2005 and continuing through 2017, the industry has seen a significant increase of charcoal rot caused by *Macrophomina phaseolina*. The problem was initially confined to two counties, Orange and Ventura. However, by 2014 the pathogen had been confirmed by researchers in all the major strawberry producing regions as well as in other parts of the state that have smaller acreage. Confirmed counties are the following: San Diego, Orange, Ventura, Santa Barbara, San Luis Obispo, Monterey, Santa Cruz, San Benito, Santa Clara, Alameda, Sacramento and Fresno. In addition to the increasing list of counties reported to have diseased strawberries, for most counties the number of infected fields likewise increases each season.

Symptoms consist of wilting of foliage, plant stunting, and drying and death of older leaves, though the central youngest leaves often remain green and alive. Plants can eventually collapse and die. When plant crowns are cut open, internal vascular and cortex tissues are dark to orange brown. In locations where the disease has occurred for more than one season, the patches can be quite large and appear to have spread from the initial problem area. In Ventura, Santa Barbara, and Monterey counties *M. phaseolina* and *F. oxysporum* together cause significant plant decline. It is noteworthy that in these cases we have rarely isolated other important, well known soilborne pathogens such as *Phytophthora* or *Verticillium*.

In California, there is a strong correlation between fields most seriously affected by these plant collapse problems and the use of pre-plant, bed-applied alternatives to the previous methyl bromide + chloropicrin standard. Because of the political/regulatory trend away from available, effective fumigants that are used by the strawberry industry, this disease situation is a critical threat to the long-term health of the strawberry industry. The 12-year spread of *Macrophomina* to previously uninfested parts of California indicates that charcoal rot is currently a significant threat to the industry which at present does not have satisfactory plant resistance with which to combat the pathogen.

*Macrophomina phaseolina* has a broad host range encompassing over 100 plant families and 500 species on a worldwide basis, though our work indicates that strawberry isolates have a host preference to strawberry. The pathogen forms resting structures called microsclerotia that are capable of surviving in the soil for long periods of time, hence the need for effective management strategies for preventing pathogen establishment and disease outbreaks. There are reports in the literature of some level of host specificity of field isolates and of grouping of isolates from particular hosts or geographic regions based on molecular criteria (reviewed below). With this in mind, it would be important to know more about the population structure of the isolates in California causing disease problems on strawberry. This would clarify if specific genotypes of the pathogen are responsible for the current disease problem. This information in turn would be useful for developing soil assays for the pathogen that would focus only on the genotypes virulent on strawberry as well
as provide important background information on strains of the pathogen that should be used in strawberry resistance screening programs.

**Background on the Pathogen**

*Host specificity and population structure of M. phaseolina*

There have been a number of reports in the literature suggesting host specialization of isolates of the pathogen. For example, Pearson et al., (1987) observed that isolates recovered from soil that corn was growing in exhibited different growth morphologies when grown on medium amended with chlorate, yet isolates recovered from corn roots recovered from the same soil exhibited a single morphology type; there was no differential colonization of isolates on soybean roots in the same soil. When looking at fields that were monocropped to corn, cotton, sorghum and soybean for 15 years, Su et al., (2001) reported some level of host specialization for isolates recovered from corn and that all isolates grouped according to host when DNA fingerprint analysis was done using random amplified polymorphic DNA (RAPD) analysis. Interestingly, isolates exhibited variation in their growth morphology on chlorate medium that reflected differential grouping of isolates in RAPD analysis. Zveibil et al., (2012) reported that isolates of *M. phaseolina* from other hosts in Israel were equally virulent on strawberry.

A number of other studies examining population structures of isolates using various techniques have been reported. Similar groupings of isolates from different hosts, and in some cases geographic origin, have also been reported in other studies using RAPD primers (reviewed in Das et al., 2008), amplified fragment length polymorphism analysis (AFLP; reviewed in Reyes-Franco et al., 2006), simple sequence repeat analysis (SSR; Jana et al., 2005b) and primers developed from repetitive sequences of rice (Jana et al., 2005a). However, other studies did not find a consistent correlation between molecular grouping and location of isolate recovery or virulence on a particular host (Reyes-Franco et al., 2006). Two additional SSR studies reporting on new primer sets have recently been published and in both cases the grouping of genotypes was correlated with the host of recovery. Baird et al., (2010) used 12 SSR loci to evaluate 109 isolates of the pathogen recovered from different geographic regions and hosts and observed that some genotype clustering by host and geographic region was observed. However, strawberry isolates that were included in the analysis (1 from CA and 3 from FL) were identical and were located on a branch separate from most isolates from other hosts. Arias et al., (2011) reported on new primer pairs for 182 SSR loci and examined 24 isolates recovered from a range of hosts and observed grouping of genotypes for isolates recovered from the same host (isolates from snap bean, pumpkin, sunflower and corn each had their own clade while isolates from soybean, cotton, and sorghum grouped together in a larger clade).

**OBJECTIVES**

The specific objectives of this project were:

- Develop an agar plate detection method for quantifying *M. phaseolina* in soil.
- Evaluate the population structure of *M. phaseolina* in strawberry production areas of California to see if there is any genetic grouping of isolates that attack or are more virulent on strawberry.
- Evaluate real time PCR molecular diagnostic technique for detection of *M. phaseolina* on strawberry. The focus will be on detection from plants as well as soil quantification.
**Materials and Methods**

**Culture Collection**

Through the CSC-supported diagnostic lab program (operated by UC Cooperative Extension in Monterey County), growers from Ventura, Santa Barbara, Monterey, Santa Clara, and Santa Cruz regularly submitted diseased strawberry samples for analysis. These cultures are forwarded to the Martin lab for storage and genotyping. Additional pathogenicity tests on strawberry and other reported hosts of *M. phaseolina* with these isolates are in progress. Collaborations were established with researchers in Florida, Spain, Israel and Argentina to obtain DNA from their strawberry isolates so we could include them in our SSR analysis.

**Soil Plating Assays**

A soil plating assay that parallels the assay currently used for quantification of *Verticillium dahliae* was found to work well for quantification of total *M. phaseolina*. Over 20 strawberry soils naturally infested with *M. phaseolina* were plated to determine pathogen inoculum density. Samples were also processed for DNA extraction using the techniques of Bilodeau et al., (2012) with slight modifications so comparisons could be made between plate counts and the results of the TaqMan real time PCR assay.

**Isolate Genotyping**

Simple sequence repeats (SSRs or Microsatellites) analysis as described by Arias et al., (2011) was used to genotype isolates in this project. A collaboration has been established with Renee Arias to use the same 182 SSR loci she used in her previous analysis of the pathogen to identify the optimal loci to use; 24 were selected. Also, the SSR data for strawberry isolates that have been collected thus far were generated on the same genotyping unit that she used to collect her data (Arias et al., 2011), so the datasets are able to be combined for a larger scale analysis comparing the populations from strawberry to isolates collected from other hosts.

**Molecular Detection of *Macrophomina phaseolina***

Thirteen isolates were sequenced by Illumina sequencing technology at the UC Davis Genome Center and comparative genomics used to identify sequence unique only to isolates in the main strawberry genotype. PCR primers were designed to amplify this region and over 80 isolates representing all genotypes and hosts in the culture collection were tested for amplification; an amplified product was recovered only from isolates in the main strawberry genotype. A TaqMan real time PCR assay was developed and the validation assay with the culture collection, as well as a range of other commonly encountered soil fungi was done; the assay was specific. The marker system was validated with field plants where the presence of the pathogen was verified by culturing.

The primers and probe were modified so they would work in an isothermal RPA assay. Specificity and field validations were completed the same way the TaqMan assay was validated and the assay found to be specific for the strawberry main genotype.

**Molecular Detection of *Fusarium oxysporum* f. sp. *fragariae***

Using the same comparative genomics approach with isolates of the pathogen provided by Tom Gordon’s lab or that we collected and tested for pathogenicity, we identified several unique loci that were present in all isolates of the pathogen but absent from other formae speciales or saprophytic isolates. One locus was found to work well in initial testing and has been the focus of development of a TaqMan real time PCR and RPA assay for *F. oxysporum* f. sp. *fragariae*. We are currently in the final stages of validation with field samples.
RESULTS

Develop an agar plate detection method by evaluating differential media for *M. phaseolina* in soil.

Experiments conducted using previously published techniques for quantification of inoculum were not satisfactory and were discontinued. In running assays for *V. dahliae*, Steve Koike noticed that *M. phaseolina* was growing on the plates so this assay was evaluated for the utility of quantifying *M. phaseolina* in the soil. From plating multiple soils, it was found to take several weeks to complete the assay, but worked well for pathogen quantification.

**Establish a culture collection of *M. phaseolina* representing production areas in California**

In 2012 through 2016, purified isolates from these samples were sent to the Martin lab for storage and inclusion in the genetic grouping analysis. Isolates from other hosts have also been collected and added to the culture collection to facilitate evaluation for host specificity. Over 490 isolates have had DNA extracted and been genotyped. DNA from isolates of *M. phaseolina* recovered from strawberry grown in Israel, Spain, Argentina and Florida was provided by collaborators and included in the SSR analysis.

**SSR Analysis of Culture Collection**

SSR analysis has been completed on 494 isolates with 24 loci (Figure 1) with the results indicating:

- Approximately 98% of the California isolates recovered from strawberry are placed on the main strawberry clade.
- The out of state isolates of the pathogen grouped as follows:
  - Israel - all isolates were not in the main CA strawberry clade.
  - Spain - six isolates from strawberry grouped in a clade closely related and basal to the main CA strawberry clade with several remaining isolates placed in other clades.
  - Argentina - two isolates from strawberry grouped in a clade closely related and basal to the main CA strawberry clade with nine remaining strawberry isolates placed in clades with isolates recovered from other hosts.
  - Florida - 10 isolates from strawberry grouped in the main California strawberry clade or a clade closely related and basal to the main CA strawberry clade with several remaining isolates placed in clades with isolates recovered from other hosts.

**Pathogenicity tests**

Steve Koike has been conducting pathogenicity tests (funded from CSC (Gordon) and other sources) using two different techniques: soil infestation with the pathogen or toothpick inoculations into the stem; both techniques provided similar results. Using this approach strawberry isolates from the main SSR genotype were highly aggressive on strawberry whereas the strawberry isolate from Sacramento that grouped in another genotype with isolates from other hosts caused little disease on strawberry. When tested on other hosts (snap bean, corn, soybean, sunflower and cantaloupe) by a toothpick/stem inoculation the strawberry isolates caused limited disease on cantaloupe but none of the other plants. A number of isolates have been screened on strawberry using the toothpick inoculation and members of the main strawberry genotype were found to be aggressive on strawberry, while limited pathogenicity on strawberry was observed for isolates not in the main strawberry genotype (most isolates had no effect on strawberry while a few caused limited disease).
Experiments with a range of defined inoculum levels added to soil are in progress to evaluate differences in virulence. In one trial with California strawberry isolate 11-12 (in the main strawberry clade) and 11-16 (recovered from strawberry but not in the main California clade), if 11-12 was present at an inoculum level of approximately 20% of 11-16 all the strawberry plants were killed whereas only one plant was killed for 11-16. Similar results were obtained in subsequent trials.

**Molecular detection of *Macrophomina phaseolina***

It is important to make sure we know what pathogen genotypes are responsible for causing disease in California production fields so any soil quantification assays that are developed accurately reflect the risk of disease. Additional pathogenicity tests evaluating isolates recovered from other hosts on strawberry are in progress, but results obtained thus far suggest the focus should be on detection of the clade that contains 98% of the strawberry isolates genotyped to date. In an effort to identify unique sequences in these isolates that can be used for designing genotype specific markers, genomic DNA from strawberry isolates (from the main California strawberry genotype) and isolates from other hosts (13 isolates total) were sequenced by Illumina and comparative genomics used to identify unique regions in the strawberry isolates that were highly conserved. A TaqMan real time PCR diagnostic assay has been developed based on one locus that is highly specific only for isolates representing the main California strawberry genotype; other *M. phaseolina* genotypes or soil fungi were not detected. Soil quantification assays revealed an excellent correlation between pathogen inoculum density from soil plating and results of the TaqMan real time PCR assay (Figure 1).

**Figure 1.** Correlation between soil plate counts of *Macrophomina phaseolina* (log microsclerotia/g soil) and results of the strawberry genotype specific TaqMan real time PCR assay of DNA extracted from the same soil (Ct as cycle threshold).
Molecular detection of *Fusarium oxysporum* f. sp. *fragariae*

A conventional PCR detection assay for *F. oxysporum* f. sp. *fragariae* was described by Suga et al., (2013); however, a small percentage of isolates are not amplified by this marker (Henry et al., (2017). We redesigned this marker for a TaqMan real time PCR assay but found this assay was not capable of detecting the isolates that were not detected by the conventional PCR assay with the same target. A TaqMan and RPA assay for *F. oxysporum* f. sp. *fragariae* TaqMan assay has been developed using the same approach with genome assemblies/comparative genomics as described above for *M. phaseolina*. It is currently in the later stages of validation (detects all isolates, including the ones the Suga marker misses with no background detection of other *F. oxysporum* isolates). Once validation has been completed the TaqMan will be evaluated with soil naturally infested with different levels of the pathogen to characterize the relationship between soil inoculum counts and results of the TaqMan assay.

NOTE - support from a CDFA Specialty Crops Block Grant that expired June 2017 has allowed us to leverage CSC funding to complete the following:

- The TaqMan assay for *V. dahliae* was converted into an isothermal RPA assay and validated; it has been turned over to the UCCE in Salinas and is currently being used.
- To provide a reference for additional work with this pathogen, a comprehensive assembly of the nuclear genome of an isolate from the main California strawberry clade and another isolate outside this clade was completed using Pacific Biosystems long sequencing reads (nearly chromosome sized contigs with 97-99% of the genome represented by 13 contigs). Annotation is currently in progress. This reference genome will allow us to better evaluate sequence variation among isolates differing in host range as well as variation among strawberry isolates at a molecular level.
- The nuclear genomes for additional isolates of *M. phaseolina* representing a wide range of genotypes have been sequenced/assembled and will provide molecular resources for further studies with this pathogen (identification of genes associated with host preference to strawberry).

**DISCUSSION**

The development of a soil plating assay for *M. phaseolina* has provided a means for enumeration of the pathogen in field soil, but it is not able to specifically identify isolates of the main strawberry genotype and takes weeks to complete. However, the TaqMan assay for quantification of the main strawberry genotype of the pathogen can accurately detect inoculum levels down to approximately 3 microsclerotia/g soil within days and should provide a more time efficient and accurate means for pathogen enumeration. Both these assays have been useful in virulence assessments of isolates in the main strawberry clade compared to other genotypes found in California. The results thus far indicate isolates of the main strawberry genotype are much more virulent on strawberry than isolates of other genotypes (some isolates will not cause infection).

The comparative genomics approach that was used for identification of genotype specific sequences in the *M. phaseolina* assay was also effective for identification of highly conserved sequences in all isolates of *F. oxysporum* f. sp. *fragariae*. This marker has been tested on a range of isolates and found to be specific; evaluations with field samples are currently in progress. Once the RPA assay has been completely validated it will be provided to UCCE in Salinas for their use. Once the TaqMan real time PCR assay has been fully validated it will be tested for the ability to accurately quantify the pathogen in soil using the same approach that was used for *M. phaseolina*. 
REFERENCES


