Coupling Spore Traps and Quantitative PCR Assays for Detection and Quantification of Airborne Spores of Antrodia sinuosa in Lemon Orchards of Yuma - 2018[1]

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Introduction

Growers in southwestern Arizona are increasingly concerned about a disease of lemon, brown wood rots (BWR). This disease is characterized by broken or dying branches, light brown fractured infected wood with or without presence of white fungal mycelium, decayed stump, and ultimate death of the tree. In the past, 20-year-old trees and older were likely to become infested, today trees younger than 20-year-old are increasingly likely to be infested with the wood rots. This is likely because the spore inoculum pressure in the orchard is increasing. Increasing inoculum pressure may be because of the large infested branches that remain on the floor, because the hedged prunings are infested, or because application of Bordeaux is not sufficient to reduce spores. Some growers have responded by spraying the trees with fungicides, spraying the prunings on the orchard floor, and by more aggressively removing larger, infested branches.

Several fungal pathogens have been implicated in BWR disease complex. *Coniophora eremophila*, a wood rotting fungus, was first reported in lemons in 1992 (Matheron et. al. 1992). Another species, *Antrodia sinuosa* was found to be infesting lemons, and was isolated in 1997 (Bigelow et. al., 1998). Further research found that *Coniophora* has been found sporulating on desert plants, but not on lemon wood (Bigelow *et al.*, 1996), while *Antrodia* has been found sporulating on decaying fallen wood within lemon groves (Matheron and Porchas, 2006). Furthermore, the optimum temperature range for growth of *Antrodia* and *Coniophora* in the wood was determined to be 30-35ºC (86-95ºF), and the rate of decay for *Coniophora* and *Antrodia* in Lisbon lemon is higher than that for orange, tangelo and grapefruit trees. Finally, wood decay experiments suggest that *Antrodia* is a greater threat to lemon trees than is *Coniophora*.

Early and accurate warning of the increases in airborne inoculum of plant pathogen can help manage disease outbreak and is recognized as a major grower priority. The ability to detect and quantify airborne spores may not only reduce costs associated with the number and frequency of fungicide applications, but also contribute to reduced or delayed fungicide resistance development. Currently, little is known about the epidemiology and mode of inoculum dispersal in lemon orchards. Knowledge of inoculum availability during timing of inoculum dispersal, as well as the environmental factors that favor spore release, is critical to determine periods of low infection risk and, therefore, potential preventative measures, including appropriate timing for pruning and pruning wound treatments as well as infected debris removal from orchard floor.

The objectives of this study are to: (1) develop a fast and sensitive real-time quantitative PCR test for the presence of *A. sinuosa* independently of the presence of disease symptoms, (2) quantify airborne spore concentrations at infested lemon orchards with spore trap and a real-time PCR assay.

Materials and Methods

Spore traps. In July 2018, purchase order of two Burkard Multi-Vial Cyclone Samplers were sent to Burkard Manufacturing Co Ltd, England. The spore samplers were shipped by manufacturer in March 2019 and were placed in two lemon orchards on the Yuma Mesa in May 2019 (Figure 1). The spore traps were powered by solar panels and batteries and routinely maintained by Dr. Wright's field crew. Spore samples are being collected on daily basis and stored at -20ºC until further analysis.

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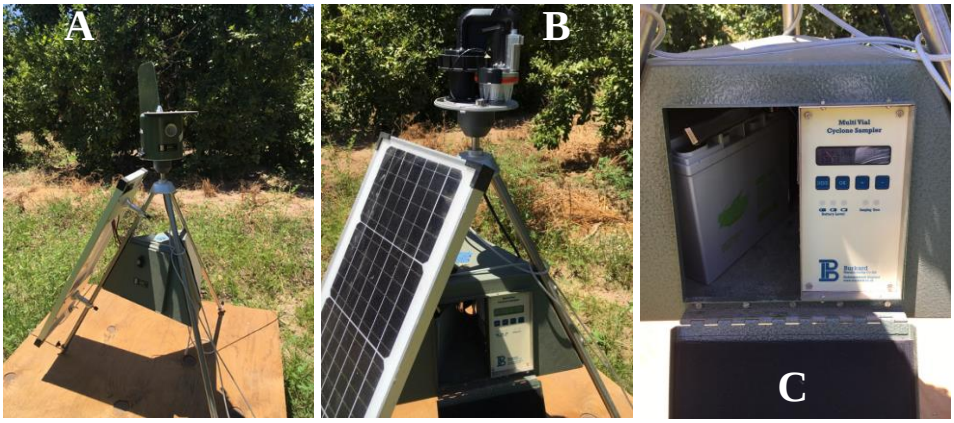

Figure 1. Burkard Multi-Vial Cyclone Samplers placed in Mesa lemon orchards: (A) Multi-Vial Cyclone Samplers, (B) solar panel, (C) control unit

Spore enumerations. Semi-selective media for Basidiomycetes were developed to count spores in a spore sample. One milliliter of distilled water was added to the Eppendorf tube and one hundred microliter was transferred to media and three plates were replicated for each sample. 10-fold or 100-fold dilution were made if necessary. The inoculated plates were incubated in the dark at room temperature. Spore enumeration will be corroborated by real-time qPCR assay developed in this study.

Isolation and characterization of fungal pathogens associated with brown wood rot. Symptomatic branch samples were collected from July 2018 to August 2019. Samples were taken from lemon trees in commercial citrus orchards. Isolations were made from symptomatic tissues, and the resulting fungi were identified morphologically and further characterized using molecular methods. Two gene loci, internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and a portion of the translation elongation factor 1-α (EF1-α) gene and second largest (RPB2) subunits of RNA polymerase, were used for molecular phylogenetics to confirm the identities of these fungi. To determine the pathogenicity of selected fungi, three branches of "Lisbon" lemon trees (*Citrus limon* "Lisbon") were wound inoculated in both field and greenhouse using Matheron's method (Matheron et al. 2006). The inoculum were grown on sterilized sorghum seeds. Disease was assessed approximately 3 months later by removing inoculated branches, splitting them in half, and measuring the length of resultant decay columns. Control trees received sorghum grains that did not contain a pathogen.

qPCR development. Twenty-five isolates of *Fomitopsis meliae* were used to design primers that is specific to *F. meliae*. Isolates were cultured on PDA and DNA was isolated using CTAB method. Universal primers were used to amplify ITS, EF1-α, and RPB2. Sequencing was performed by Eton Bioscience Co, CA. Phylogenetic analysis was performed using Geneious Software. Specific primers for F. meliae were designed with primer3 software on the basis of the alignment of the genes. The specificity of the primers used to amplify F. meliae DNA was tested with a PCR comparison of other fungi associated with citrus. Real-time PCRs were carried out with SYBR-Green (Bio-rad Laboratories).

Results and Discussion

Isolation and characterization of fungal pathogens associated with brown wood rot. Basidiomycete *Fomitopsis meliae*, not *Antrodia sinuosa*, was found to be causal agent for brown wood rot of lemon. More than 100 isolates of *F. meliae* were isolated from infected lemon trees. *F. meliae* was the only fungal pathogen detected from numerous symptomatic lemon tree branches (Figure 2). All of these isolates have white fluffy mycelium and may produce spores on PDA culture (Figure 3). Phylogenetic analysis supports our morphological identification and confirms that the species *F. meliae* causes the disease known as brown wood rot (Figures 3 and 4).

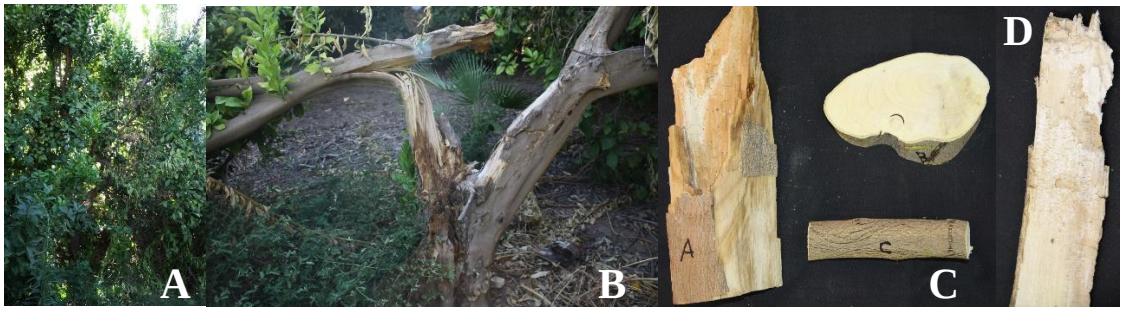

Figure 2. Photographs of various symptoms of lemon brown wood rot: (A) branch dieback, (B) broken branch, (C) light brown fractured infected wood with presence of white fungal mycelium, (D) fractured wood with white fungal mycelium


Figure 3. Sample pictures of 1-week-old isolates of *F. meliae* growing on PDA plates in the laboratory.

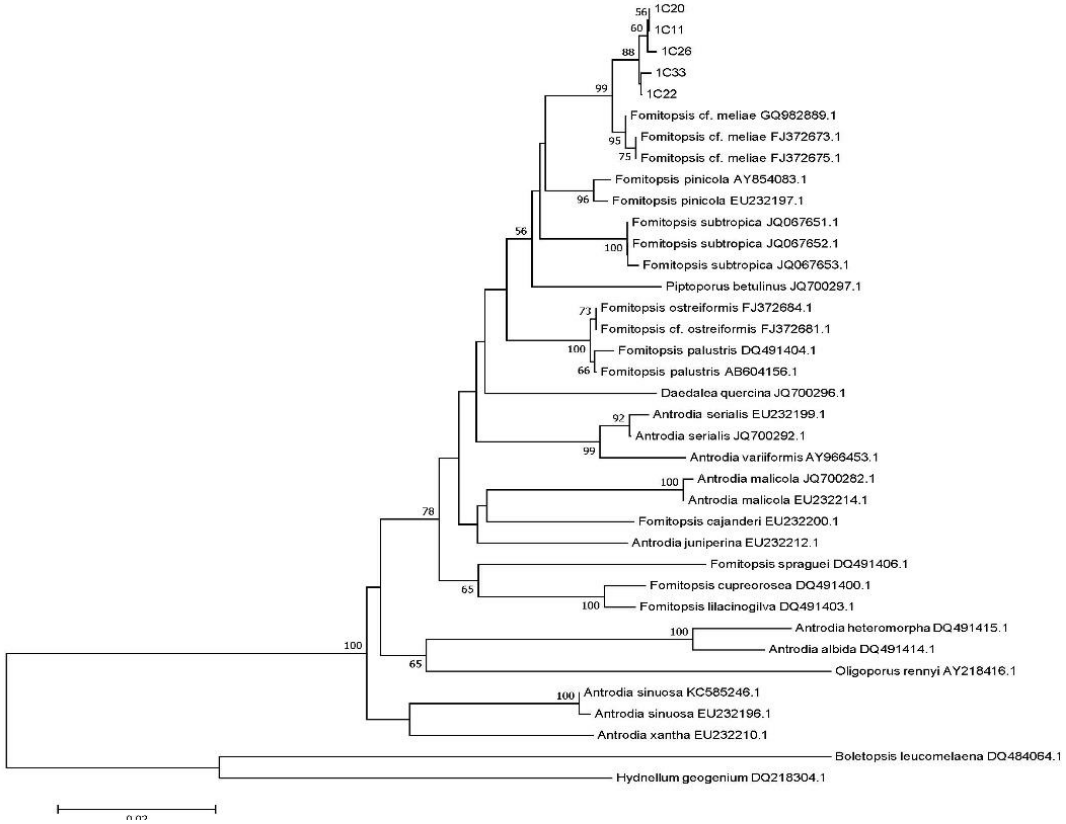

Figure 4. Phylogenetic tree of five isolates of *F. meliae* based on parsimony analysis based on ITS, RPB2, and EF1-α

Figure 5. Pathogenicity test with lemon trees in greenhouse and fields: (A) *F. meliae* colonized sorghum grains, (B) dying main stem due to infections by *F. meliae*, (C) lesions on 30-year-old lemon trees.

Based on the results from pathogenicity assay, *F. meliae* are pathogenic on lemon as it is able to produce lesions and could be isolated from inoculated branches (Figure 5). The length of wood decay column on inoculated branch ranged from 3.9 cm to 4.9 cm (Table 1).

Table 1. 2018 and 2019 Lemon tree height, canopy volume and health rating.

Isolates	Length of wood decay column on inoculated branch (cm)
1C70	4.89 ± 1.67
1C26	4.18 ± 1.96
1C6	3.88 ± 1.53

qPCR development. Ninety sequences were obtained from ITS, EF1-α, and RPB2. A dozen primer pairs specific for *F. meliae* were designed with the primer3 software on the basis of alignment. The specificity of the primers was checked by PCR analyses of closely related fungi and other fungi commonly associated with citrus trees in desert regions. Two primer pairs were found highly specific to *F. meliae*. Standard curves were calculated for one primer pair targeting EF1-α using DNA extracted from mycelium and from spores. With this primer set, the correlation between Ct and DNA content in a 10-fold dilution series was high for both mycelium and spores and was linear from 10^3 to 10^5 spores/ml.

Spore enumerations. *F. meliae* colonies were distinct in morphology on semi-selective media. It often took 5-7 days for the colony to emerge on the plate. *F. meliae* can be easily distinguished from other fungal colonies (Figure 6). Colonies of *F. meliae* are white, cottony, and fluffy in appearance.

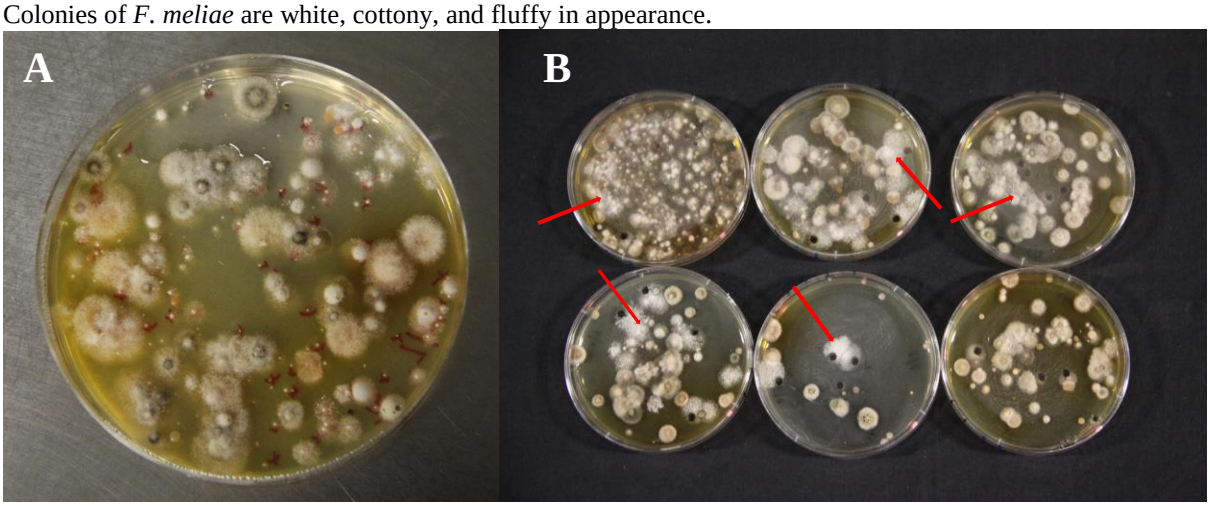

Figure 6. Spore germination assay: (A) various colonies on semi-selective media, (B) *F. meliae* on semi-selective media

The spore count data for two spore samplers were summarized in charts (Figure 7). Clearly, spore counts varied greatly from May to July. The average spore counts for *F. meliae* were in the range of several hundreds to a few thousands spores per day. The spore counts for *F. meliae* peaked on Jun 30 at the site 1 and on July 20 at the site 2. The spore count trends were different at two sites. We continue to monitor spore counts at different

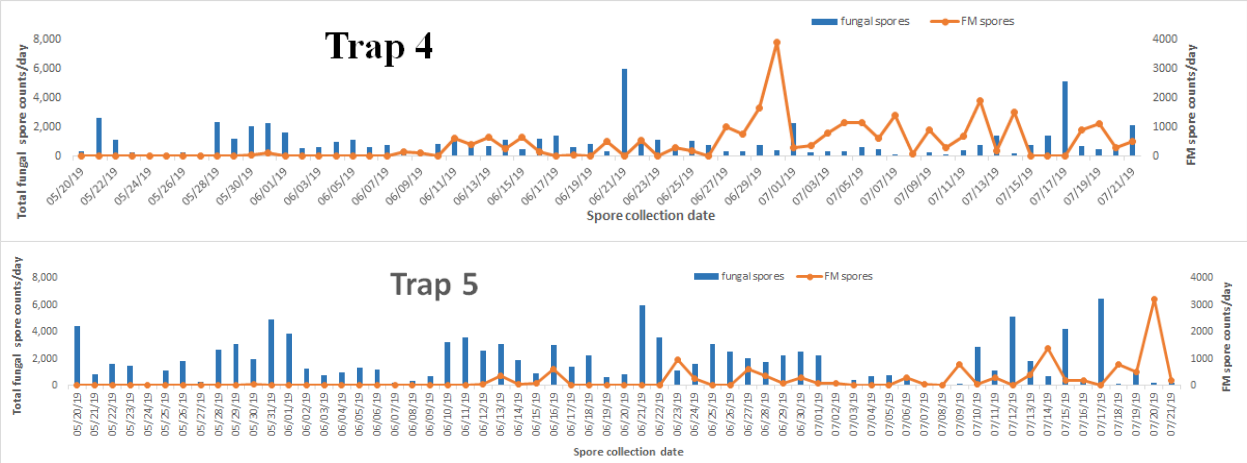

Figure 7. Temporal spore distribution of *F. meliae* in lemon orchards in Yuma.

Conclusions

1. *Fomitopsis meliae* is the causal agent for brown wood rot of lemon in Yuma. Given the fact that other fungi such as *Coniophora eremophila* and *Nodulisporium* sp. were detected in BWR-infested trees, a survey is needed to determine the frequency and importance of all fungal pathogens associated with BWR.
2. A real-time qPCR assay has been developed to quantify spores of *F. meliae*. In addition, a semi-selective media were made to enumerate alive spores of *F. meliae* in the orchards. The qPCR assay are being optimized to provide robust and accurate estimate of *F. meliae* spores.
3. *F. meliae* spores appeared to fluctuate greatly from day to day, week to week, and month to month. Spore counts at different sites seemed not to follow the same pattern, indicating the importance of local inoculum source and influence of different orchard management practices.

References

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