MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 37-2017 (YEAR 1)
2017 ANNUAL REPORT

Title: Quest for an alternative, environment-friendly control method for charcoal rot fungus: mycoviruses

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BACKGROUND AND OBJECTIVES

Charcoal rot disease, caused by fungus *Macrophomina phaseolina*, causes serious economic damages to several crops worldwide including soybeans, where it may cause premature death of the affected plants. The disease is particularly severe on plants undergoing heat/drought stress and, under proper conditions, can affect entire fields, resulting in a total yield loss. Currently there are no effective chemical or biological methods for its control.

In this project we propose to look for innovative and environment-friendly methods to manage this disease. The project is based on the premise that certain mycoviruses (viruses infecting fungi) can seriously affect/reduce pathogenicity of their hosts - an effect termed "hypovirulence". These mild isolates of fungi are then used to outcompete more aggressive (pathogenic) isolates in certain environments (soils) and reduce effects of the disease. This strategy was successfully applied in the control of chestnut blight disease in Europe and can be potentially applied to other fungus.

This project is innovative and consists of two phases: **Phase 1**) identification of viruses affecting charcoal rot fungus (CRF) population in MS (this proposal – 2 years), to be followed by **Phase 2**) studying effect of selected viruses on CRF and further explorations by inoculating soybean plants.

The objective of this project (**Phase 1**) is to identify and characterize mycoviruses that naturally infect CRF population in Mississippi.

REPORT OF PROGRESS/ACTIVITY

Initial phase of the project was dedicated to collection of different charcoal rot fungus isolates. To this aim we scouted charcoal rot-affected fields in the second part of the growing season of 2017 (Figure 1).

We collected a total of 55 charcoal rot-infected samples that were used for fungus isolation in pure culture on PDA (potato-dextrose-agar) plates. Sampling included collection from different fields/geographic areas and, in few randomly selected sites, we also collected multiple plants from different areas of the same field in order to analyze an "in-field"

charcoal rot population structure using viruses as molecular markers.

Figure 1. General view of some of charcoal rot-affected fields used for sample collection (left) and a close-up of affected area.

Out of 55 collected samples we have successfully obtained pure cultures (deprived of contamination by other fungi or bacteria) of 38 CRF isolates. Pure cultures of CRF have been then maintained by several subcultures on the same substrate.

Cultures of some isolates clearly differed in morphology from the majority of others (Figure 2), which could have been the effect of infections by mycoviruses (certain mycoviruses can induce changes in mycelial color, robustness and growth rate, etc).

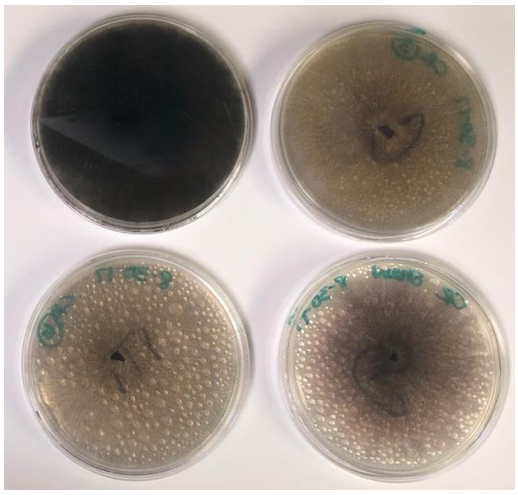

Figure 2. Morphology of some of *Macrophomina phaseolina* isolates obtained in this work.

All 38 CRF isolates have been grown individually by inoculating 250 ml of liquid media in a horizontal shaker at 28°C under a constant shaking of 100 rpm. Samples were harvested after 2 weeks by eliminating excess of liquid medium and then filtering through a filter paper and collecting the fungal tissue retained on the paper. Phenotypical differences have also been observed among filtrated fungi, as their color varied from light

grey to black (Figure 3).

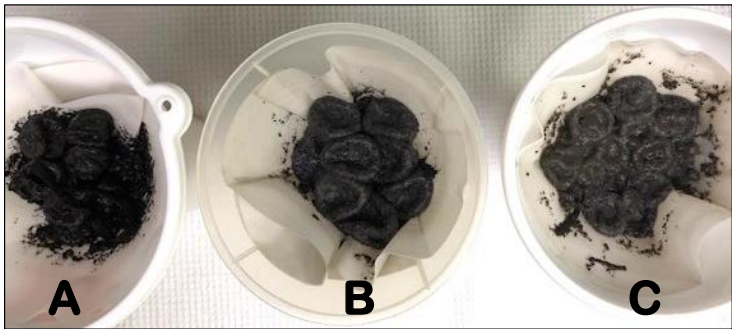

Figure 3. Some of CRF isolates collected from liquid media prior to extraction of dsRNAs. Please note morphological differences (size and color) of fungal tissues, which may be caused by virus infections.

Five grams of each isolate were submitted to extraction and purification of double-stranded RNAs (dsRNAs), performed by phenol-chloroform precipitation, followed by selective chromatography as explained in the Materials and Methods section of the proposal. Possible traces of host DNA and ribosomal RNAs were eliminated by further digestion by DNase A and RNase followed by precipitation with ethyl alcohol.

Presence/absence of dsRNAs, as well as their size (molecular weight), number and quality, were ascertained by electrophoresis in agarose gels (an example provided in Figure 4). As presented in Figure 4, the majority of charcoal rot fungus isolates tested in the Year 1 were infected by viruses. Some of them, for example isolates #1 and # 7, presented similar dsRNA patterns, suggesting that they might be infected by the same virus or combination of viruses.

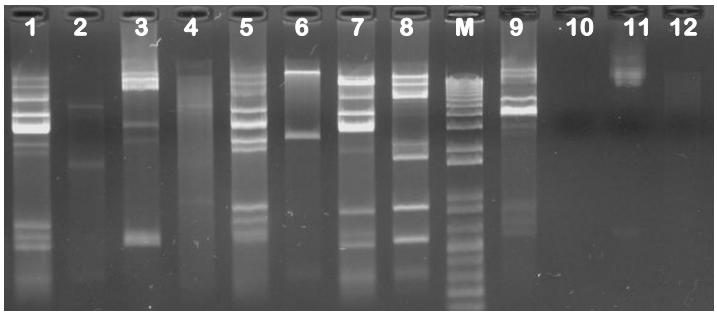

Figure 4. Agarose gel electrophoresis of dsRNAs isolated and purified from some isolates of charcoal rot fungus collected in 2017. Please note differences in number and size of dsRNA bands indicating differences in type of viruses causing infections.

All isolates with visible dsRNA content were used for further molecular characterization by cDNA synthesis, library production and custom-based High Throughput Sequencing by Illumina (as described in M&M in the original proposal) in order to identify what viruses are present in collected samples.

Along with sequencing of the above-mentioned isolates, we continuously transfer all fungal cultures (need to be done in certain time intervals in order to keep colonies alive).

Purified dsRNAs of 33 CRF isolates (five did not contain visible dsRNA bands) were custom-sequenced by High Throughput Sequencing performed on MySeq platform applying pair-end sequencing 2x250 nt. Raw sequence data provided between 2.5 and 4 million of short reads per isolate with high quality scores (Figure 5).

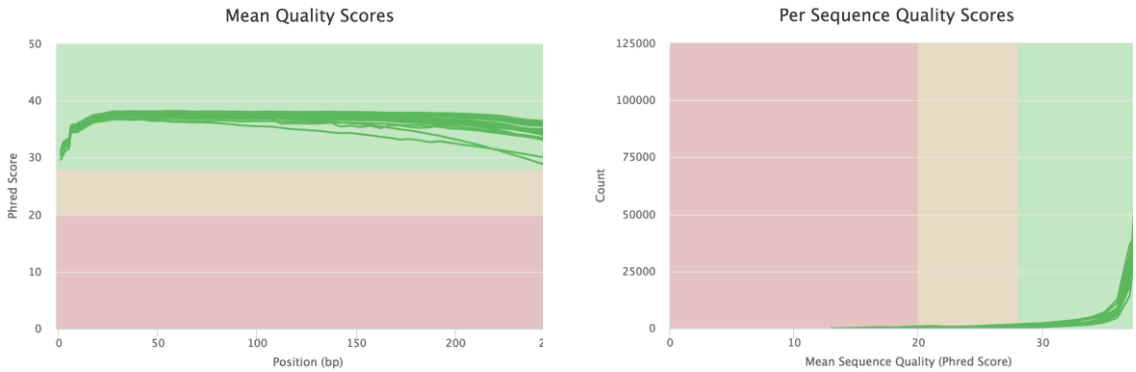

Figure 5. Representative results of Illumina pair-end sequencing generated MultiQC software, showing high quality of raw nucleotide sequence data. (Legend: green background fields indicate very good quality of "base calls")

Assembly of "raw" (short read) sequence data into longer contiguous reads ("contigs") was performed on a suite of biocomputing tools (various specialized software) as described in Materials & Methods (see original proposal). Once assembled, each individual contig was compared with genome sequences of known viruses available in the GenBank and NCBI.

Results of activities of Year 1 are complex and will be detailed in future proper peer-reviewed manuscripts. However, for the purpose of the Annual Report they can be summarized as follows:

- In the first year of this study we examined a total of 38 isolates that varied in terms of geographical location, morphology and size of colonies (see Figures 1-3).
- Majority (80%) of CRF isolates contained visible dsRNAs, often presented as complex patterns, suggesting ongoing infections by multiple viruses (Figure 4).
- Partially purified preparations from few examined isolates contained the putative virus particles of different size and morphology, including flexuous and rigid rods, as well as isometric particles (Figure 6).

- High throughput sequencing (HTS) resulted in high-quality raw data which (Figure 5) were then processed, assembled and analyzed by a pipeline for virus discovery and characterization routinely used in our lab.
- Assembly and analyses of HTS data showed a plethora of known and novel viruses with dsRNA, positive-sense RNA and negative-sense RNA genomes (totaling more than 137 distinct virus isolates) from diverse taxonomic allocations (Figures 6 and 7).
- Majority of Mp isolates were infected by multiple viruses (up to 14 different viruses – see Figure 7).
- Besides discovery of numerous novel RNA viruses (Figure 5 and not shown), in this work we extended and/or completed genome sequences of many recently partially characterized viruses by colleagues from USDA-ARS and University of Illinois (Marzano et al., 2017).
- The most prevalent virus in Mp isolates was Macrophomina phaseolina hypovirus 1 (MpHV1) - found in 16 isolates, followed by a "tobamo-like virus" detected in 11 isolates.
- Range of genome size of characterized viruses varied from 1.5 kbp to almost 20 kbp.
- Population of several viruses showed considerable intraspecific variation (i.e. isolates of MpHV1 varied up to 12% in overall polyprotein amino acid content).
- Five new viruses with negative-sense RNA genomes belonging to orders *Bunyavirales and Mononegavirales.* Curiously, one of the studied CRF isolates was infected by three distinct negative-sense RNA viruses.
- Multiple contigs of several kb in size could not be matched with a high confidence sequences currently available in NCBI/GenBank suggesting further expansion of the "mycovirome".

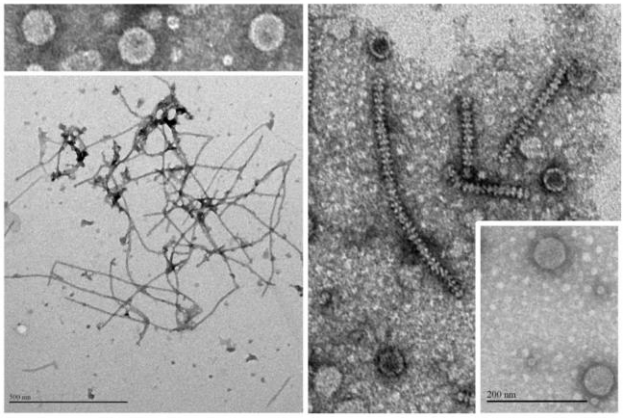

Figure 6. Negatively stained electron micrographs showing morphological variability of putative virions observed in partially purified preparations from mycelia of several CRF isolates.

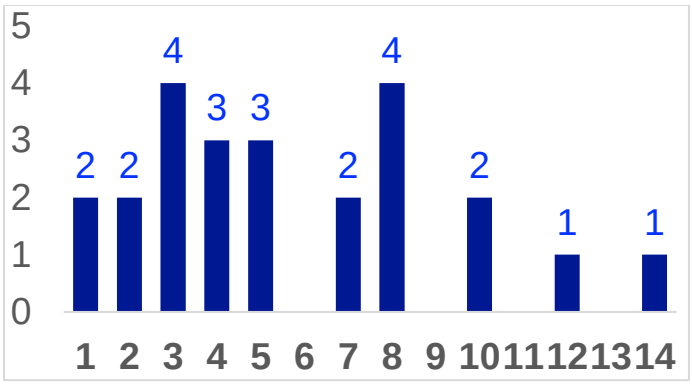

Figure 7. Number of viruses per fungal isolate and frequency.

The work on this project continues in 2018 by collecting additional isolates of the fungus for further virus discovery and analyses.

Additional information:

An abstract reporting preliminary data from this work has been prepared and was presented at the *2018 International Congress of Plant Pathology,* the most important scientific venue in the field of Plant Pathology, held in Boston at the end of July-beginning of August 2018.

Citation:

1. **Aboughanem-Sabanadzovic N,** P Deng, T Wilkerson, M Tomaso-Peterson, TW Allen, S **Sabanadzovic S**, 2018. RNA virome of two important phytopathogenic fungi. International Congress of Plant Pathology (ICPP) 2018: Plant Health in A Global Economy, Boston, MA, July 29-August 03, 2018. P-434.

Acknowledgements:

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