MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 37-2018
2018 ANNUAL REPORT

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

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ANNUAL REPORTING OF PROGRESS ACTIVITY

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 efficient chemical or biological methods for its control.

In this project we propose to look for innovative and environment-friendly methods. The project is based on the premise that certain mycoviruses (viruses infecting fungi) can seriously affect/reduce pathogenicity of their hosts - the 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 any other fungus.

This project is innovative and consists of two phases: **Phase 1**) identification of viruses affecting 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.

Objective: Project (**Phase 1**) is to identify and characterize mycoviruses that naturally infect charcoal rot fungus (CRF) population in Mississippi.

During the Year 2 of this Project we collected, isolated and analyzed new isolates of *Macrophomina phaseolina* (aka charcoal rot fungus – CRF) collected in soybean fields in Mississippi, as well as we proceeded with biocomputing analyses of the multitude of nucleotide sequences generated with Illumina sequencing during the previous year. Results of this project were presented at two scientific venues (11th International Congress of Plant Pathology in Boston, MA and at 46th Annual Meeting of Southern Soybean Disease Workers in Pensacola, FL) acknowledging the support by the Mississippi Soybean Promotion Board.

During 2018 we continue scouting soybean fields in order to collect additional samples and reach planned number of charcoal rot fungus (CRF) isolates Therefore, we from different parts of the State. Fungal isolation and culturing procedures were the same as adopted in 2017, consisting in initial isolation and subsequent series of sub-cultures in order to obtain pure cultures to work with later in the project on identifying viruses.

Preliminary visual observations revealed significant differences in the morphology among several isolates (color, robustness, growth rate of the fungal colonies), which could be associated with ongoing infections

with different viruses or virus combinations. In order to proceed with molecular analyses, 4-5 agar plugs containing mycelial growth were transferred to a potato dextrose broth and incubated for a week at 28C before harvesting fungal growth and storing at -80C until used.

Double stranded RNAs (dsRNAs) were extracted from 10 grams of each isolate by phenol-chloroform precipitation, and selective chromatography on cellulose (as described in the proposal). The dsRNAs were analyzed in 1 % TAE agarose gel electrophoresis.

Majority of analyzed CRF isolates contained visible amounts of dsRNA bands indicative of virus infections (Figure 1). While some isolates (for example, #4 and 19) contained single dsRNA band, numerous isolates (i.e samples in lanes 6,7,8, 12, 14,15,16) contained complex dsRNA patterns suggesting mixed infections by several viruses. Few samples (#1, 3, 5, 9, 10) did not contain any visible amounts of high-molecular-weight dsRNAs.

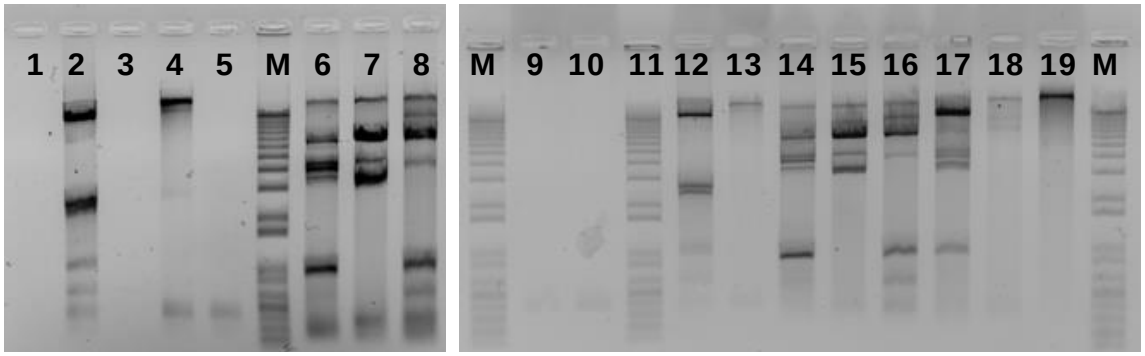

Figure 1. dsRNAs patterns observed in charcoal rot fungus isolates collected in 2018. Analyses were performed in 1% TAE agarose electrophoresis and visualized by GelRed staining and UV observations. M= DNA Ladder.

CRF isolates collected during the 2018 season (partially presented in Fig 1) were reverse-transcribed and libraries prepared for custom-based High Throughput Sequencing by Illumina 2x250nt pair-end sequencing methodology performed at the University of Illinois in May 2019.

The initial analyses of the quality of sequencing was evaluated by FastQC, a quality control tool for high throughput sequence data. Results of analyses showed very good “quality base calls” (green field), as presented in Figure 2 for multiple sequenced samples.

Figure 2. Graph showing very good mean quality of raw sequencing data as analyzed by FastQC software.

As high throughput sequencing was performed at the end of May 2019, results of specific in-depth analyses that are ongoing will be reported in reports related the 2019 Project.

Additionally, we have performed a lot of routine lab work in 2018/2019 in order to to keep CRF isolates clean and ready for further analyses. For that purpose, we regularly transferred all isolates of interest to fresh PDA plates (usually done each 5 weeks).

As mentioned, part of activities done in 2018/2019 was dedicated to biocomputing and analyses of data generated in 2017. In particular, in this period we carried out studies of population structure of several major viruses (i.e. viruses found in multiple CRF isolates) using a suite of proper software in order to understand degree of intra-species genetic structure with an ultimate aim to further overall knowledge on ecology of the virus, as well as to identify conserved genomic region for for diagnostic purposes, and/or for completing genome sequences by performing Rapid Amplification of cDNA Ends (RACE) experiments.

In order to give an example, we will illustrate work performed by presenting a representative "snapshot" of results generated using cases of MpHV1, one of the most widespread viruses found in CRF isolates from Mississippi

Surprisingly, the population of MpHV1 in CRF samples collected in Mississippi resulted genetically highly heterogeneous. Indeed, the identity of genome sequences of isolates from MS with reference isolate from Illinois (Mp2003b; published by Marzano et al., 2016) varied from virtual identity (99.34%) to a very distinct genotypes (with less than 83% nucleotide identity!). Comparable level of nucleotide sequence divergence was observed between isolates collected from MS (genetic identity varied from 82% to 98%).

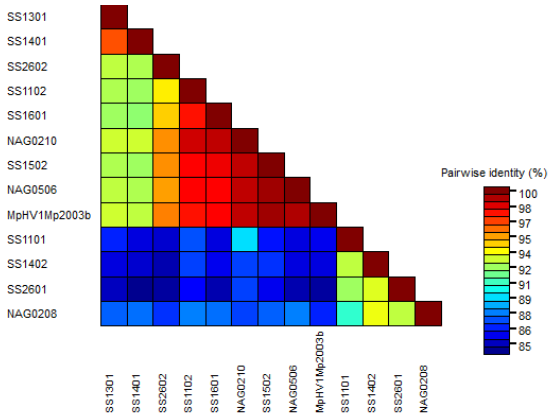

Figure 3. Genetic diversity among isolates of MpHV1 in Mississippi and their comparisons to the reference isolate Mp2003b from Illinois (Marzano et al., 2016).

Furthermore, evolutionary analyses suggested that several CRF isolates collected in MS were infected with two, genetically distinct, MpHV1 variants (Figure 4), which advances the knowledge of the ecology of this virus. The presence of more than one genetic variant of a given virus in the same fungal isolate suggest multiple/independent infection events in the past, most likely occurred through the phenomenon called anastomosis. During the anastomosis, two fungal isolates establish a "bridge", which leads to exchange of cellular contents between two organisms. If the fungi that are in contact are infected by different viruses, they may exchange viruses too. Therefore, our study, provided the first experimental evidence of mix infections of CRF isolates by more multiple genetic variants of the same virus (visually presented in Fig. 4).

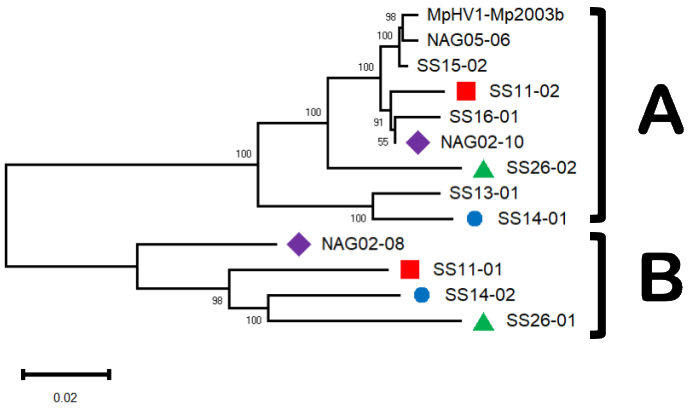

Figure 4. Phylogenetic analyses of some MpHV1 variants discovered and analyzed in this study. As visible from the tree, MpHV1 variants grouped into two major groups (A and B). Please notice that variants from the same CRF isolates, indicated with the same geometric figure (i.e. square, triangle, circle…), belonged to two different lineages.

Curiously, our data suggest that genetic variants of MpHV1 from different CRF isolates are more closely related to each other, than variants co-infecting the same fungal isolate (Figure 1).

Finally, MpHV1 isolates from MS resulted to have bigger genomes compared to the reference isolate Mp2003b recently reported from Illinois (Marzano et al., 2016). MpHV1-Mp2003b (GenBank Acc No KP900893). The Illinois isolate is 12,468 nt long, while the isolates from MS result to have genomes

exceeding 14,000 nt. Differences in size appear to be in the 5' and 3' ends of the genome (non-coding parts of the genome). Specific primers (total four sets: two sets for 5'end and two for 3'end) were designed on conserved regions of MpHV1 genome in order to perform RT-PCR to confirm that differences in genomes between isolates from IL and MS are real and not artifacts.

We also started performing re-sequencing of genomes of certain viruses to verify particular genomic features (i.e. presence of certain genes) and completing genome sequences by performing 5'/3' RACE experiments in order to progress with taxonomic allocation of newly discovered viruses.

Here, for the sake of brevity, we present only a specific case of a virus detected in the CRF isolate with the lab code NAG03 (aka "umbra-like virus"). Complete genome of this new mycovirus is 4,475 nt long and contains two in-frame open reading frames (ORFs; Figure 5) coding for proteins of an estimated molecular mass of 41.7 kDa (ORF1) and 59.2 kDa (ORF2). While the function of the ORF1-encoded protein is unknown, the 59K protein is RNA-dependent RNA polymerase (RdRp) involved in virus replication. BLASTp searches in GenBank revealed that ORF1 protein has limited identities with an ortholog encoded by few of newly discovered mycoviruses (i.e. Verticillium RNA virus). The same analyses showed that RdRp of this virus share conserved motifs with several viruses including Macrophomina phaseolina single stranded virus 3, Verticillium dahlia RNA virus, Magnaporthe oryzae RNA virus and, to lesser extent, with several viruses belonging to family *Tombusvididae*.

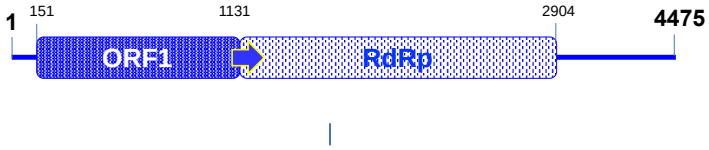

Figure 5. Genome organization of an "umbra-like" virus from CRF isolate NAG03 from Mississippi with major nucleotide coordinates.

The extent of similarity with known viruses shows that this virus represents a new species. In order to understand its relationships with currently known viruses, we performed evolutionary analyses applying Neighbor-Joining and Maximum Likelihood methods for estimation. Results of both analyses were congruent, strongly suggesting that virus from CRF isolate NAG03 belong to a new family of viruses (yet-to-be-established), as shown in Figure 6.

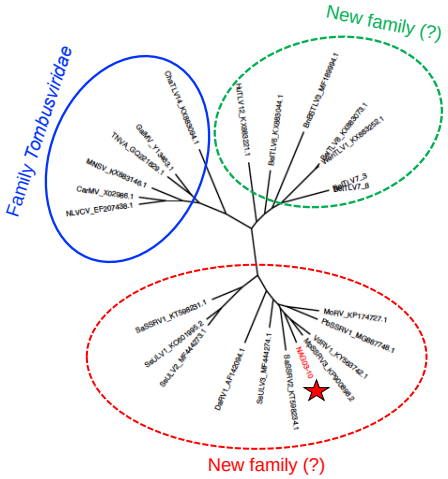

Figure 6. Neighbor-joining phylogenentic tree constructed on alignments of complete RdRp amino acid sequences of an umbra-like virus identified in CRF isolate NAG03 from

Mississippi and members of the plant-infecting family *Tombusviridae* and selected unclassified viruses. Position of the umbra-like virus is denoted by an asterisk. Analyses suggest that this virus belong to a new (yet-to-be established) family of viruses.

NOTE: The above-reported data are random-chosen examples used to give an idea about the type of work that was performed during Year 2. Similar analyses for numerous other viruses, many of which belong to new virus taxa.

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Published conference abstract in Year 2:

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

Aboughanem-Sabanadzovic N, T Wilkerson, TW Allen, S Sabanadzovic, 2019. Virome of Macrophomina phaseolina isolates collected from soybean fields in Mississippi. *Proceedings of the 46th Annual Meeting of Southern Soybean Disease Workers, Pensacola, FL, March 6-7, 2019. Page 9*

Abstracts submitted for presentation after Year 2:

Aboughanem-Sabanadzovic N, T Wilkerson, TW Allen, S Sabanadzovic, 2019. Virome of Macrophomina phaseolina from soybean fields in Mississippi. Annual Meeting of the American Phytopathological Society: *Plant Health 2019, August 3-7, 2019, Cleveland, OH*