MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 37-2019
FINAL REPORT

PROJECT TITLE: Completion of molecular characterization charcoal rot fungus mycoviruses

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EXECUTIVE SUMMARY

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 had a single objective: to identify and characterize viruses infecting charcoal rot fungus (CRF) population in Mississippi in order to make a baseline knowledge on mycoviruses with RNA genomes that could eventually be explored as biological control tool in future studies.

During the project a total of 64 CRF isolates from different locations in Mississippi have been cultured, of which 50 were grown in pure culture and used for further study. All 50 isolates were analyzed for the presence of dsRNA molecules (a fingerprint for ongoing infections by RNA viruses) and 38 CRF isolates with visible amount of dsRNAs were chosen for further studies by applying cutting edge technologies (high-throughput sequencing of viral genomes with Illumina platforms). Hundreds of millions of raw sequence data were processed, meticulously controlled for quality and assembled by biocomputing involving several advanced software. Assembled sequences were compared with publicly available data on virus genomes deposited in the National Center for Biotechnology Information (NCBI) database of the National Institute for Health (NIH).

In total we were able to identify and obtain/assemble genome sequences of a total of 217 viruses from 36 CRF isolates. These viruses could be divided roughly in three groups according their type of genome. Most numerous viruses were those with positive-sense RNA genomes (+ssRNA), followed by those with with double-stranded RNA (dsRNA) genomes and, finally, negative-sense RNA viruses (-ssRNA).

Number of viruses per fungal isolate varied from one to 14. Indeed, unexpectedly, the majority of studied

fungal isolates were infected by multiple viruses. Initial genome sequences obtained by high-throughput sequencing were completed for at least one isolate per virus by applying specific lab methodologies.

Final analyses showed that 217 viruses discovered in this study belong to more than 50 different taxonomic species, many of which are yet to be classified. While majority of these viruses could be classified in already established genera and families, several of them undoubtfully will represent new taxa of viruses. Therefore, this study made a fundamental contribution to virus taxonomy and overall knowledge on mycovirus diversity and, indirectly, sheds the light on virus evolution in general.

Viruses identified in this study varied in their incidence: few viruses were widespread in studied fungal isolates, while others were detected only in single or a few CRF isolates. Observed phenotypical differences among different CRF isolates from MS could be attributed to infections by different viruses. However, the impact of specific single viruses on fungal phenotype was impossible to evaluate in this study and was originally planned to be the target of future studies ("Phase II").

In conclusion, results of this study exceeded the original expectations. a fundamental contribution to the knowledge of viruses present in population of charcoal rot fungus infecting soybean in MS. Genomic data generated in this project provides much needed base for further, sophisticated studies on possible use of viruses as tool for biological control by synthesizing infectious clones for those of particular interest.

BACKGROUND

Charcoal rot disease is an endemic root disease in southern states of the USA, as well as problem in the central Midwest. Furthermore, charcoal rot outbreaks have also been recently reported from other states as Illinois, Indiana, Iowa, Minnesota, North Dakota and Wisconsin (http://www.planthealth.info/charcoal_basics.htm).

The disease is caused by a soil-inhabiting fungus Macrophomina phaseolina, or charcoal rot fungus (CRF). CFR has a broad host range and can infect more than 500 plants, including soybeans. Soybean plants of any age are susceptible to the fungus, but serious damages such as premature death of infected plants usually occur under the dry and hot weather conditions (90-95°F). Sometimes, entire fields can be affected by this disease resulting in a total yield loss. At present, there are no efficient chemical or biological methods for control of this pathogen/disease.

One of alternatives to use of chemicals is the employment of biological means for the pathogen control. Viruses that infect fungi (called "*mycoviruses*") are relatively widespread in studied fungi. Some of them can change morphological and/or biological characteristics and reduce pathogenicity of the fungus ("*hypovirulence*"). Hypovirulence has been already exploited as biological tool for some pathogenic fungi and it was successfully applied to control chestnut blight pathogen *Cryphonectria parasitica* in Europe. Virus-induced hypovirulence has been recently described for several other plant pathogenic fungi (i.e *Helminthospotium victoriae*, *Sclerotinia sclerotiorum*, etc). Therefore, virus-induced hypovirulence may be much more widespread in nature than thought.

Our preliminary data of research on other plant pathogenic fungi (i.e. *Rhizoctonia, Sclerotium,* etc), sponsored by MAFES-MSU, allowed to generate some scientifically valid preliminary data. Therefore, in this study we aimed to expand this research and to characterize population of mycoviruses present in a representative number of isolates of charcoal rot fungus (*M. phaseolina*) collected in soybean fields in Mississippi as an initial phase to further studies on this topic.

The study was carried out over the period 2017-2020.

MATERIALS & METHODS

Initial phase of the project was dedicated to collection of different charcoal rot fungus isolates. To this aim we scouted a charcoal rot-affected fields in the second part of the growing season of 2017 (and continued in 2018 - Figure 1).

We collected a total of 64 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.

In order to proceed with molecular analyses, 4-5 agar plugs with 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 five grams of each isolate by phenol-chloroform precipitation, and selective chromatography on cellulose. 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 1% TAE agarose gels.

All isolates with visible dsRNA content were used for further molecular characterization by cDNA synthesis, library production and custom-based High Throughput Sequencing (HTS) by Illumina in order to identify what viruses are present in collected samples. Sequencing was performed on MySeq platform applying pair-end sequencing 2x250 nt. Raw sequence data provided millions of short reads for each isolate that were initially evaluated for the quality by FastQC, a quality control tool for high throughput sequence data.

Further analyses were performed by a suite of software and involved assembly of raw sequencing data, their mapping, comparison with available virus genome data in public databases (GenBank/NCBI) and phylogenetic analyses. Genomes of selected isolates of viruses of interest were completed by applying 5'/3' RACE experiments on total RNAs extracted by specific commercial kits slightly modifying manufacturer's

procedures. Products of RACE experiments were cloned, sequenced and sequence data assembled together with those generated by HTS.

Morphological traits of viruses infecting CFR isolates were studied by virus purification involving clarification with organic solvents and series of specific differential centrifugations. Virus preparations were finally resuspended in small volume of phosphate buffer (low molarity). Virus particles were observed with transmission electron microscope of negatively stained preparations (2% aqueous solution of uranyl acetate).

Taxonomic position of each virus was determined according to an updated Virus Taxonomy (2020).

RESULTS

Out of 64 collected samples we have successfully obtained pure cultures (deprived of contamination by other fungi or bacteria) of 50 charcoal rot fungus (CRF) isolates. Pure cultures of CRF have been then maintained by regular subculturimg on the same type of substrate.

Cultures of several isolates differed in morphology, color, robustness and growth rate (Figure 2) . Such differences could be effect of infections by mycoviruses.

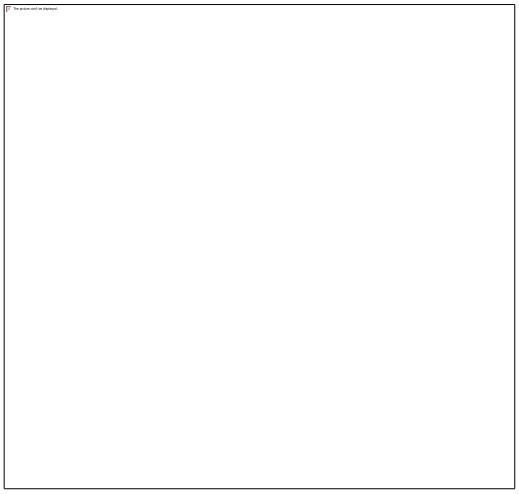

Figure 2. Morphological differences among four *Macrophomina phaseolina* isolates obtained in this work. The isolates were plated on the same day and kept under identical environmental conditions.

Majority (76%) of analyzed CRF isolates contained visible amounts of dsRNA bands indicative of virus infections (Figures 3 and 4). Number of dsRNA bands and their patterns varied among the CRF isolates suggesting presence of different viruses and/or combination of viruses. While few CRF isolates (for example, #4 and 19) contained a single dsRNA band, majority of samples (i.e samples in lanes 6-8, 12, 14-17) contained complex dsRNA patterns suggesting mixed infections by several viruses. A total of 12 out of 50 tested samples (for example, see#1, 3, 5, 9, 10) did not contain any visible amounts of high-molecular-weight dsRNAs.

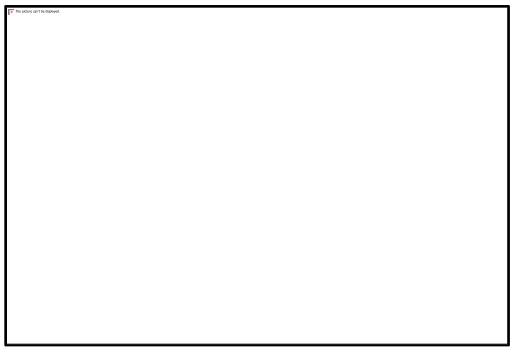

Figure 3. Ratio between isolates with and without visible dsRNAs.

Figure 4. An example of dsRNA patterns observed in extracts of charcoal rot fungus isolates collected during the project Analyses were performed in 1% TAE agarose electrophoresis and visualized by GelRed staining and UV observations. M= DNA Ladder.

Partially purified preparations from selected CFR isolates contained putative virus particles of different size and morphology, including flexuous and rigid rods, as well as isometric virions (Figure 5). A mixture of virus particles with different morphology was present in several examined samples.

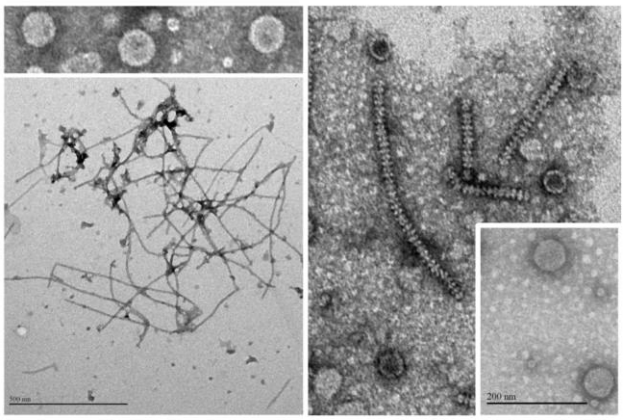

Figure 5. Negatively stained electron micrographs showing morphological variability of putative virions observed in partially purified preparations from mycelia of several CRF isolates.

All CFR isolates with visible dsRNA content were selected for further molecular characterization by cDNA synthesis, library production and custom-based High Throughput Sequencing by Illumina in order to identify which specific viruses are present in collected samples.

Purified dsRNAs of 38 CRF isolates (12 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 5 millions of short reads per isolate with high quality scores (an example provided in Figure 6).

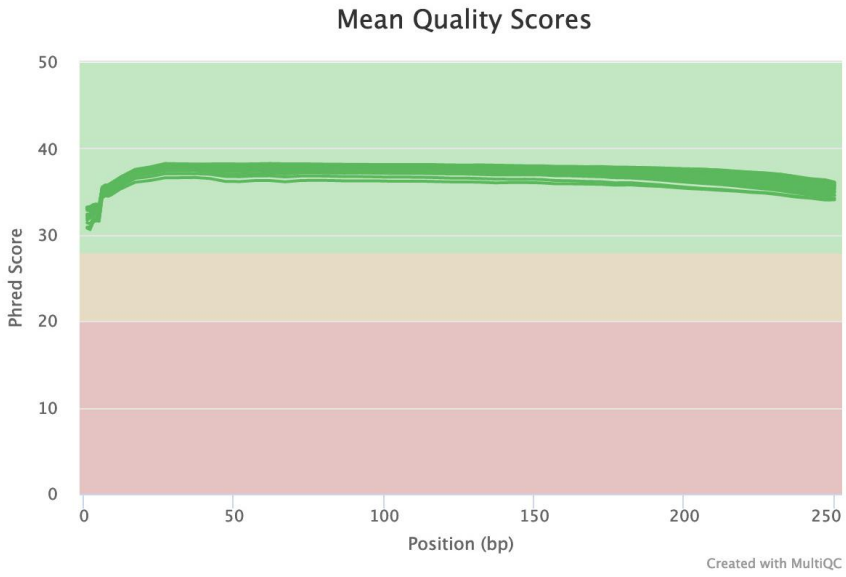

Figure 6. Graph showing very good mean quality of raw sequence data as analyzed by FastQC software.

Assembly and analyzes of HTS data showed a plethora of known and novel viruses. In total we were able to assemble genome sequences of a total of 217 viruses from 36 CRF isolates (surprisingly, two isolates did not produce virus sequences despite visible dsRNAs). Range of genome size of these viruses varied from 1.5 kb to almost 15 kb. Viruses identified could be divided roughly in three groups according their type of genome. Most numerous viruses were those with positive-sense RNA genomes (+ssRNA) making more than a half of identified viruses (53%), followed by those with double-stranded RNA (dsRNA) genomes (35%) and, finally, negative-sense RNA viruses (-ssRNA) representing 12% (Figure 7).

Figure 7. Repartition of viruses identified and characterized in this project by their genome type.

As it could be deduced from variable dsRNA patterns, sequencing results confirmed great variation in number of viruses per fungal isolate, ranging from one to 14. Only two fungal isolates were infected by single viruses, while most frequent case was co-infection by 8 viruses found in 6 fungal isolates (Figure 8). Initial genome sequences obtained by high-throughput sequencing were completed for at least one isolate per virus by applying specific lab procedures (5'/3' RACE experiments).

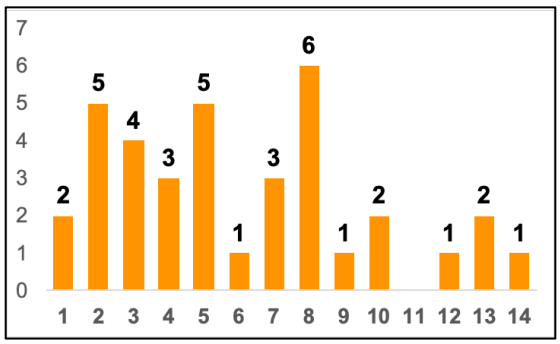

Figure 8. Number of viruses per fungal isolate and frequency.

Final analyses showed that 217 viruses discovered in this study belong to more than 50 distinct taxonomic species, many of which are yet to be classified. Interestingly, part of these viruses could be classified in already established genera and families, but several others do not fit established taxonomy and, accordingly, represent new taxa of viruses. Few examples of new viruses that will likely be part of new families or genera are given in Figure 9

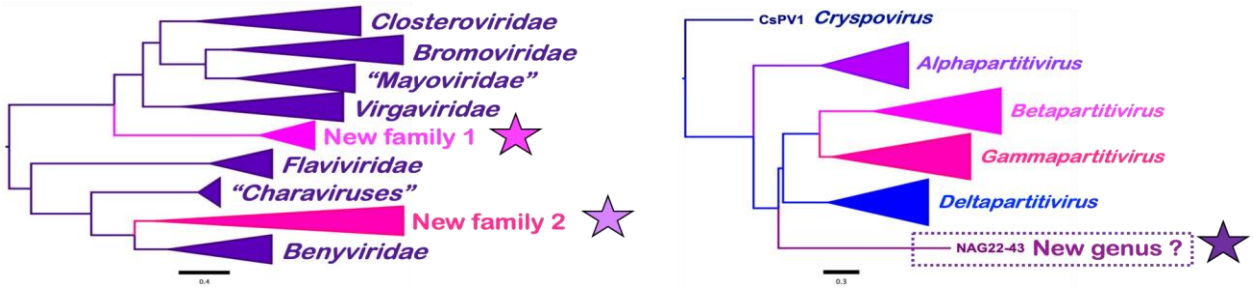

Figure 9. Simplified depiction of taxonomic position of new families and genera (labeled by asterisks) represented by some of new viruses discovered in this work.

Besides discovery of numerous novel RNA viruses, in this work we also completed genome sequences of several partially characterized viruses in a recent study by colleagues from USDA-ARS and University of Illinois (Marzano et al., 2016). Viruses identified in this study varied in their incidence: few viruses were widespread in studied fungal isolates, while others were detected only in single or a few CRF isolates. Most widespread viruses were a hypovirus MpHV1and a so called "tobamo-like virus"

Part of study was also dedicated to analyses of population structure of several major viruses (i.e. viruses found in multiple CRF isolates) 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 diagnostic purposes.

As an example, we briefly illustrate work performed on MpHV1. The population of this virus in in Mississippi is genetically heterogeneous with nucleotide differences up to 18% among MS isolates. Comparable level of nucleotide sequence divergence was observed when compared with an isolate from Illinois (Mp2003b; published by Marzano et al., 2016) .

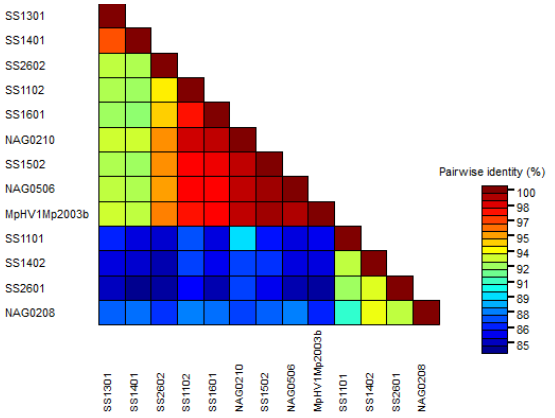

Figure 10. Genetic diversity among isolates of MpHV1 in Mississippi and their comparisons to the isolate Mp2003b from Illinois (Marzano et al., 2016).

Furthermore, evolutionary analyses suggested that several CRF isolates collected in MS were co-infected with two, genetically distinct, variants of MpHV1 variants (Figure 11), 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. 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 Figure 11).

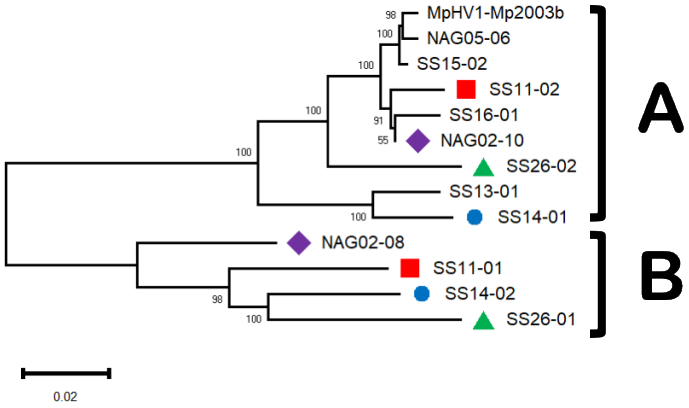

Figure 11. Phylogenetic analyses of some MpHV1 variants discovered and analyzed in this study. As visible from the tree, MpHV1 variants grouped into two major groups (A and B). Please notice that variants from the same CRF isolates, indicated with the same geometric figure (i.e. square, triangle, circle…), 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 11).

Finally, we designed primers and developed one-step RT-PCR test for specific detection of two most frequent viruses (MpHV1 and "tobamo-like virus") which allows their quick and reliable detection in fungal isolates.

Project impact on Mississippi Soybean Production and science

Management strategies for charcoal rot of soybean are very limited, and they do not provide complete protection against *M. phaseolina*. Additionally, resistant cultivars are not available to growers too. Therefore, identification and complete characterization of viruses that reduce pathogenicity of certain isolates of charcoal rot fungus could lead to the development of sustainable, environment-friendly disease management methods based on use of hypovirulent isolates. Identification and characterization of virome ("community of viruses") in charcoal rot fungus in Mississippi represent an initial and indispensable step that will facilitate further studies in the state and elsewhere (for example, by engineering infectious clones of viruses discovered in this study and in-depth research on their impact on fungal pathogeneicity).

Furthermore, characterization of new viruses of *Macrophomina phaseolina* furthered knowledge of virus biodiversity, ecology and evolution and contributed to overall advancement of virus taxonomy.

End Products

Results of this project were presented at several national and international scientific venues in form of oral/poster presentations (for example, 11th International Congress of Plant Pathology, Annual Meetings of the American Phytopathological Society, as well as at Annual Meeting of Southern Soybean Disease Workers).

Additionally, major outcome of the project are complete genome sequences of a number of mycoviruses for further use in various studies (i.e. infectious clone synthesis and use). Genome sequences of all viruses will be deposited in Public Databases (NCBI/GenBank). Furthermore, technical protocols for RT-PCR detection of some of discovered during the project will be made available to the other scientists.

We also anticipate several peer-reviewed papers reporting complete genomes of various mycoviruses to be published, each acknowledging the multi-annual support from MSPB (two manuscripts under preparation).

Finally, we plan preparing and submitting multiple Taxonomic Proposals to the International Committee on Taxonomy of Viruses for creation of new taxa of viruses (species, genera and families) based upon results of this research.

Conference Presentations with published abstracts:

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* infecting soybean in Mississippi. *Proceedings of the 46th Annual Meeting of Southern Soybean Disease Workers, Pensacola, FL, March 6-7, 2019. Page 9*

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*

Manuscript under review or in preparation:

Aboughanem-Sabanadzovic N, S Sabanadzovic, 2021.Characterization of a tobamo-like virus widespread among isolates of *Macrophomina phaseolina* in Mississippi. Archives of Virology (in preparation)

Aboughanem-Sabanadzovic N, T Wilkerson, TW Allen, S Sabanadzovic, 2021. Virome of *Macrophomina phaseolina* isolates collected from soybean production fields in Mississippi. Virus Research (in preparation)

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