Title: Integrating a field deployable water filtration system with bioinformatics and pyrosequencing for effective monitoring and survey of water-borne plant viruses.

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Start Date: (07/01/2015)

End Date: (06/30/2017)

Descriptors: Water-borne plant viruses, water filtration, next generation sequencing, bioinformatics, EDNA.

Students:

Student's Current Status	#	Institute-Department	Was the student working for experience or towards a degree?
Undergraduate	3	NIMFFAB-EPP	Undergraduate research dissertations
M.Sc.	1	NIMFFAB-EPP	Toward a M.Sc. program
Ph.D.	1	NIMFFAB-EPP	Toward a Ph.D. program
Total	5		

NIMFFAB = National Institute for Microbial Forensics & Food and Agricultural biosecurity. **EPP =** Department of Entomology and Plant Pathology.

Problem and Research Objectives:

Problem. Plant viruses serve as convenient research models for studying water-borne pathogens as they are non-infectious to humans and are more easily manipulated than viruses that cause human disease. Plant viruses can be carried in water and may pollute waterways and reservoirs naturally or be introduced intentionally by some hoping to cause harm to agriculture and the economy. Detection of such viruses is complex due to water dynamics and dilution factors, therefore species detection and a maximum sensitivity are two serious considerations for developing reliable detection and remediation systems for water forensics and water biosecurity.

Research Objectives. Develop a water monitoring system for detection of water-borne viruses combining statistically validated sampling, filtering method for mid-size volumes of surface water using glass wool filters and bioinformatics to allow viruses to be captured and identified. We concentrate the virus nucleic acids for detection using reverse transcription polymerase chain reaction (RT-PCR) and Electronic Diagnosis of Nucleic acids Analysis (EDNA). The obtained results will be applicable to human and animal viruses as well.

Importance of Project and Findings:

Importance. Detection of plant viruses in water sources common to agriculture and environment offer solutions for effective pathogen monitoring and rapid decision making, improving prevention, alerting, networking, emergency management, recovery and restoration. Implementation of this broad detection and discrimination method will allow accurate monitoring of irrigation water at plant production units, hydroponic farms, and water treatment plants. All of which increases the U.S. capability to detect viral contamination in small and large bodies of water, thereby increasing detection rates, speeding response time, and reducing the risks of unwanted introductions that can occur during management, and trading.

This project also offered a great opportunity to integrate education and training of undergraduate and graduate students. I mentored two graduate research assistants (GRA) and three undergraduate students. These five students became ambassadors of the Oklahoma Water Resources Center and CASNR. Moreover, OSU graduate and undergraduate students had the opportunity to develop professionally, leadership skills making presentations about microbial water quality to specialists. Students attended national and international meetings and participated in conversations with the OK water resource community addressing team-based solutions to critical OK water issues.

Meetings attended and publications were:

- 2016 American Phytopathological Society (APS). Annual Meeting Abstracts of Special Session Presentations July 30–August 3, 2016, Tampa, Florida
- CIBB III: 2016 International Congress of Biotechnology and Biodiversity. (Congreso Internacional de Biotecnología y Biodiversidad. 2016.). Investigación y competitividad, claves para la producción. Libro de memorias. Guayaquil. Ecuador. October 10—13, 2016. Eds. Centro de Investigaciones Biotecnológicas del Ecuador (CIBE); Escuela Superior Politécnica del Litoral (ESPOL). 206 pp.
- 2016 Water Research symposium October 11-12, in conjunction with the 2016 Oklahoma Governor's Water Conference.

Publications.

Scientific articles are in preparation, follows published articles presented in scientific meetings.

- F. Ochoa-Corona, Y. Carrillo Tarazona. 2016. Validation of RT-PCR High Resolution Melting as a detection method of *Tombusvirus.* (Abstr.) Phytopathology 106:S4.117.
- J. Garcia Suarez, S. Dobhal, F. Ochoa-Corona. 2016. Virus detection of *Tobamovirus* with wide spectrum degenerate oligonucleotides by TD RT-PCR High Resolution Melting. (Abstr.) Phytopathology 106:S4.118.

- Olmedo-Velarde, F. Ochoa-Corona. 2016. Discriminating *Potexvirus* species by RT-PCR coupled to High Resolution Melting. (Abstr.) Phytopathology 106:S4.118.
- J. Daniel, B. Gallucci Mazziero, B. Dunn , F. Ochoa-Corona. 2016. A preemptive detection system to screen water for the presence of plant viruses. (Abstr.) Phytopathology 106:S4.118.
- B. Gallucci Mazziero. 2016. Waterborne plant virus sampling and detection in aqueous environment. (Abstr.) Phytopathology 106:S4.118.

Findings.

1. Water volumes varying from 10 to 100 liters were sampled. These water volumes were filtrated through self-made interchangeable glass wool filters using *Pepino mosaic virus* (PepMV), a model waterborne plant virus. It was determined filtering 50-80 gallons of water allows detection of PepMV in very low concentrations (approximately 5 nanograms in 50 gallons.
2. Most water sources in OK are often calcareous (alkaline), pH 8-9, and water pH adjustment toward acidity may need adjustment before filtration.
3. Plant waterborne viruses were eluted from interchangeable glass wool filters and further concentrated for RNA extraction and molecular detection.
4. A reference RT-mPCR system for simultaneous detection and discrimination of three major waterborne plant virus genera (*Tombusvirus, Tobamovirus* and *Potexvirus*) tested and compared successfully as expected and therefore used as reference detection method.
5. Additional detection-discrimination molecular methods were developed: Three Reverse Transcription Polymerase Chain Reaction coupled to High Resolution Melting (RT-PCR+HRM). The new methods not only detect viruses within each of the three selected genera *Tombusvirus, Tobamovirus and Potexvirus*, it also discriminate the sampled virus into species.
6. A next generation sequencing EDNA database for three waterborne plant virus genera, to include electronic probes was developed.

Changes to the Project since Implementation:

No changes were made.

Methodology:

- Water filtration systems were assembled and tested in the laboratory, glasshouse and field. Assembly of our filtration method considered previous descriptions by Darling and Wright 1986 (Applied and Environmental Microbiology 51 (6): 1326-1331), Vilaginès et al. 1993 (Wat. Sci. Tech. 27: (3-4): 299-306) and, Millen et al. 2012 (*Journal of visualized experiments*, 3930), all with modifications.
- Water was collected at the source using a submergible pump. Volumes from 50 to 80 gallons were tested. The water was pumped into a polystyrene container and the pH determined using a portable pH meter. Acidity of the sampled water was adjusted to pH 6.5-7 using 1M HCL to ensure virus filtration. Then the

sample of water was pumped back to the source while filtered through glass wool filter. Glass wool filters were maintained in ice during transportation and refrigerated until virus elution within 24-48 hours.

- Glass wool filters were washed at neutral pH or higher and virus eluates were centrifuged with 7% polyethylene glycol (PEG) for virus precipitation. Nucleic acids (RNA) were extracted from the obtained pellet with a Plant Extraction RNeasy Mini Kit from QIAGEN or Tryzol as per the manufactures protocol.
- A genus-specific and discriminatory multiplex RT-PCR for three genera of plant waterborne viruses developed in our laboratory (before starting this project) was used as reference method to confirm virus presence within three genera (*Tombusvirus, Tobamovirus and Potexvirus*) by RT-mPCR and to optimize the water filtration system.
- The obtained virus pellet is transcribed, enriched and sequenced using next generation sequencing.
- The output database was submitted to EDNA (bioinformatics tool) and virus presence in the water sample analyzed using specific discriminatory electronic probes (e-probes) for *Tombusvirus, Tobamovirus and Potexvirus species.*
- Depending on the waterborne virus species and genera detected, a confirmatory assay based on RT-PCR+HRM allowed confirmation and discrimination of virus species within the three genera (Fig 1).

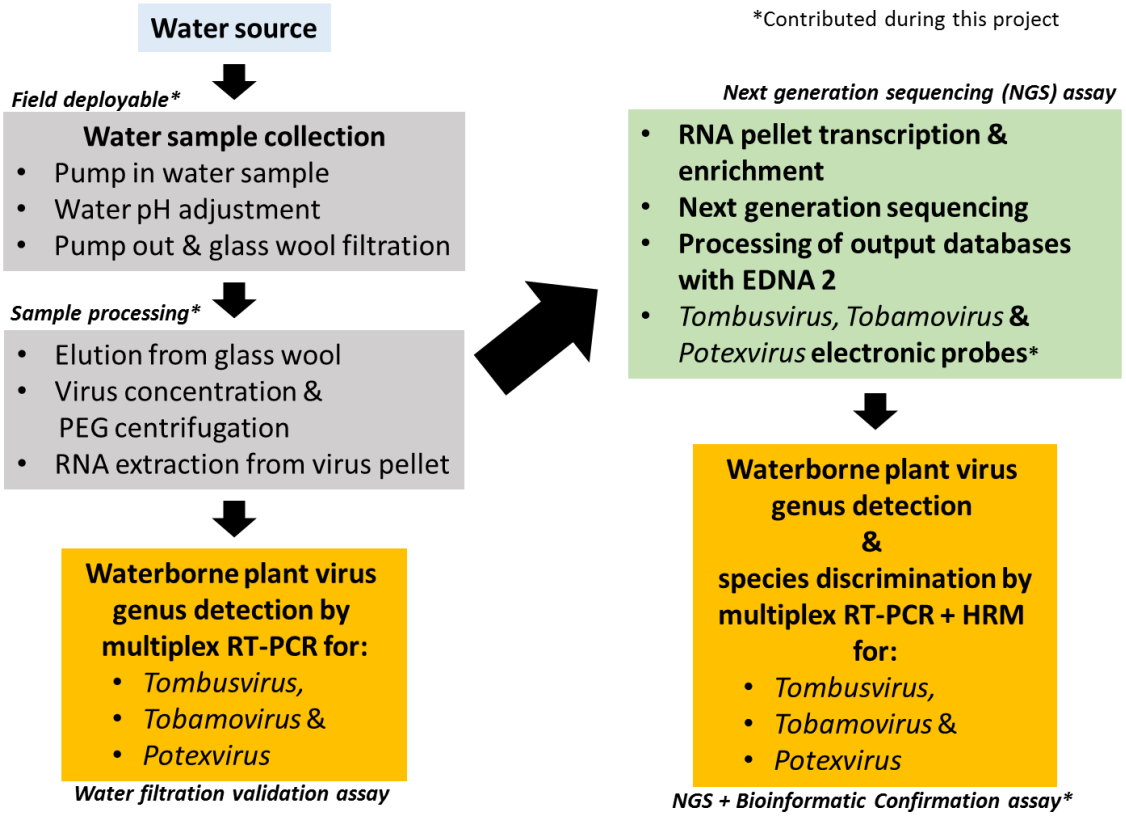

Fig.1. Work flow of methods developed for a field deployable water filtration system based in pH dependent glass wool filtration, virus elution, concentration, and sequencing using NGS followed by a confirmation assay based on RT-mPCR+HRM. Methods contributed with funding support of this project are highlighted with *.

Fig.2. Graduate students Jon Daniels (Doctoral) and Beatriz Mazziero (Master) performs water filtration at Coffee creek, OK

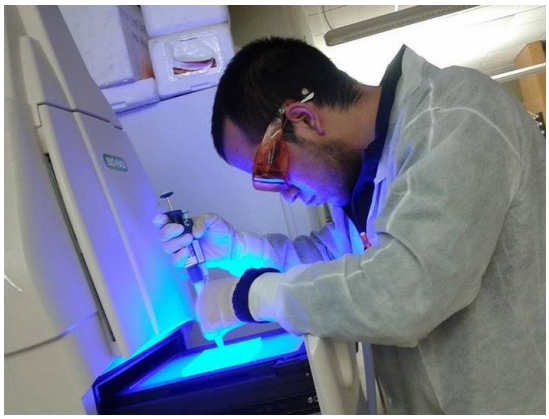

Fig.3. Undergraduate student, Alejandro Olmedo is shown excising RT-mPCR amplified DNA from electrophoresed gels for sequencing.

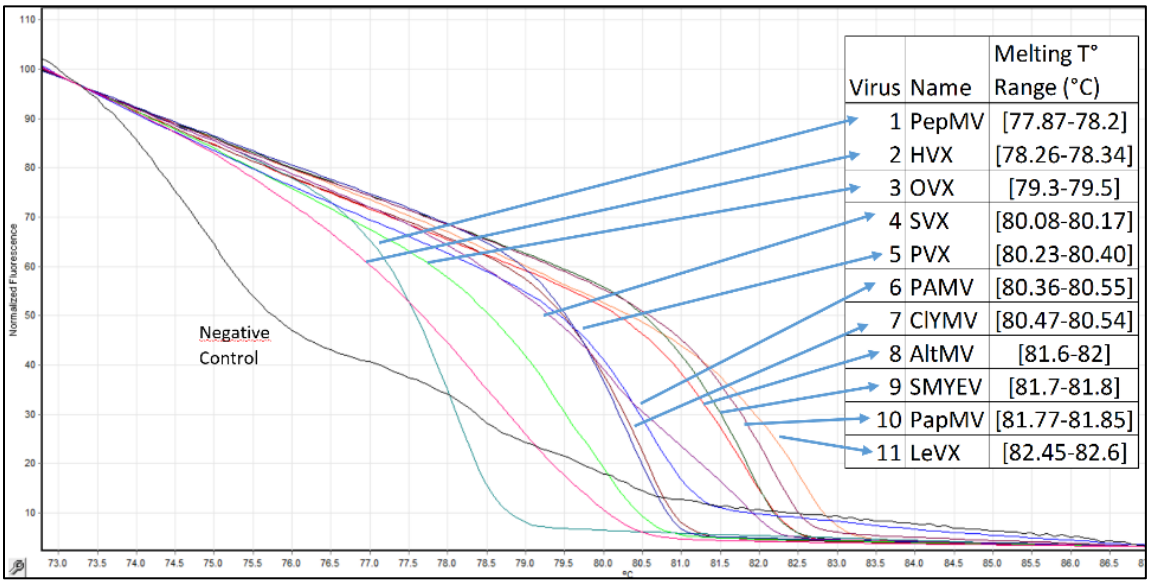

Fig.4. *Potexvirus* RT-mPCR+High Resolution Melting analysis showing discrimination of 11 viruses within the genera. Each line represent a different virus. The test is consistently repeatable.

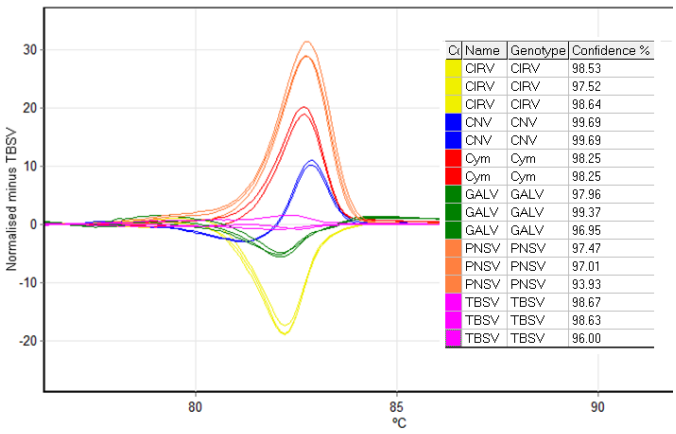

Fig.5. Six *Tombusvirus* species discrimination derived from normalized data minus TBSV

Principal Findings and Significance:

- This project overcame initial high risk expectations and contributed methods that set the basis for further research and automation of water quality monitoring systems based on pH dependent filtration, nucleic acid analysis and NGS.
- This research project also contributed data useful during the optimization of procedures for water sampling. At present most filtration systems mainly focus on filtration of bacteria, fungi and parasites all of which are comparatively high size relative to viruses.
- Moreover, the project contributed new molecular detection methods based on RT-PCR+HRM for detection of three major virus groups (genera) reported to be waterborne plant viruses. These three methods also allow discrimination of the detected virus to species level of 10 *Tombusvirus*, 11 *Tobamovirus*, and 11 Potexvirus. A total of 32 viruses. Named**: *Tombusvirus*:** *Carnation italian ringspot virus* (CIRV), *Cucumber necrosis virus* (CNV), *Cymbidium ringspot virus* (CymRSV), *Grapevine Algerian latent virus* (GALV), *Neckar river virus* (NRV), *Pelargonium leaf curl virus* (PLCV), *Pelargonium necrotic spot virus* (PNSV), *Petunia asteroid mosaic virus* (PetAMV), *Tomato bushy stunt virus* (TBSV) and *Pelargonium line pattern virus* (PLPV). ***Tobamovirus*:** *Bell pepper mottle virus (*BPeMV), *Pepper mild mottle virus* (PMMoV), *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Paprika mild mottle virus* (PaMMV), *Tobacco mild green mosaic virus* (TMGMV), *Streptocarpus flower break virus* (SFBV), *Ribgrass mosaic virus* (RMV), *Odontoglossum ringspot virus* (ORSV), *Cucumber green mottle mosaic virus* (CGMMV), *Sunn hemp mosaic virus* (SHMV). ***Potexvirus:**Alternanthera mosaic virus* (AltMV), *Clover yellow mosaic virus* (ClYMV), *Lettuce virus X* (LeVX), *Opuntia virus X* (OpVX), *Papaya mosaic virus* (PapMV), *Pepino mosaic virus* (PepMV), *Potato aucuba mosaic virus* (PAMV), *Potato virus X* (PVX), *Schlumbergera virus X* (SchVX), *Strawberry mild yellow edge virus* (SMYEV) and *Hosta virus X* (HVX),
- Detailed protocols are in preparation for transferring this technology to the OSU Plant Disease and Insect Diagnostic Laboratory (PDIDL) for implementation and assessment and scientific manuscript publication.

Next Steps:

Future objectives are the miniaturization of the filtering system for ease of handling for agriculture, remote sensing and/or drone deployable filtration. Additionally we propose to expand the e-probe databases of waterborne viruses and publish the data that we have generated.