CAPTURING PATHOGENIC PLANT WATERBORNE VIRUSES: A NOVEL TOOL FOR AGRICULTURAL DIAGNOSTICS USING THREE MODEL VIRUSES

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Abstract: With continued growth of world population, a greater demand of natural resources is generated. Water, a natural resource used directly or indirectly by all is an essential resource capable of being contaminated with waterborne plant pathogens. For sustainable agriculture, growers take advantage of non-favorable cropping areas by pumping water from nearby lakes, rivers, wells, aquifers, or even runoff. These various water resources could be potential reservoirs for waterborne phytopathogens and act as a microbiome leading to their introduction in cropping systems. When considering large bodies of water and water dynamics, including dilution factors and volume, it becomes challenging to detect and identify potential pathogens, especially plant viruses, which are in very low numbers. Current plant pathogen detection tools are used retroactively by sampling various tissues or soils following disease symptoms. With highly virulent pathogens this can lead to unacceptable losses. There is a need to develop a system to monitor water sources for the presence of waterborne plant viruses to prevent accidental or intentional introduction from irrigation sources. The objective of this project is to develop a preemptive detection system that will readily sample water for plant waterborne viruses, develop laboratory protocols to process environmental water samples, and to establish biological significance of waterborne viruses. An inexpensive, scalable and robust water sampling device made from polyvinyl chloride (PVC) was developed. A protocol for elution, precipitation and RNA extraction of waterborne viruses from water samples was established. Plant based multiplex primers were adapted for nucleic acid based detection of three viral model viruses include *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) belonging to the genus *Potexvirus*, *Tombusvirus*, and *Tobamovirus*, respectively. All three viruses were recovered from water volumes up to 5 gal. Hydroponics test demonstrated movement of PepMV, TBSV, and PMMoV from inoculated plants to water and into healthy plants where they established diseases. Monitoring of microbial loads in agricultural irrigation systems, and other water sources, is essential for effective surveillance and disease prevention for plant and animal health.

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CHAPTER I

INTRODUCTION

Life on earth cannot exist without water. All plants and animals have a range of dependencies on water. We use water in a multitude of industries, including fabrication, cooling facilities, washing, paper mills, chemical plants, petroleum, food production, and many others. Every aspect of modern human activity involves water. Water quantity and quality is an absolute consideration for agriculture sustainability and continued growth of global communities [1]. Water sources include surface water or ground water from ponds, rivers, streams, canals, lakes, reservoirs, aquifers, rainwater, and municipals. A common practice for many agriculturalist is to establish ponds for water retention or to drill water wells. Within the United States, agriculture water used for irrigation, withdraws for livestock (feedlots and dairy), and aquaculture account for daily totals of 115,000 Mgal/day, 2,000 Mgal/day, and 9,420 Mgal/day; respectively [2]. These estimates by the United States Geological Survey totaled more than 126 Bgal/day in 2010 [2]. While water brings and maintains life, it can also act as a vessel for transporting disease causing agents. With a majority of the water sources not being treated or tested for plant pathogens, their use in agriculture could pose a risk for waterborne plant pathogen introductions. Additionally, the reliance on water in every aspect of our lives makes water one of the most valuable and vulnerable commodities that biosecurity agencies must address.

One of the most vulnerable industries in the United States is agriculture. Within the agriculture sector, there is even less if any, protection of aquatic systems [3]. Throughout the U.S., there are many towns and cities lacking the necessary security for protecting the nation's water resources. In addition to the lack of security, there are other considerations that serve as challenges in securing this resource, such as background microbial flora, that must be taken into account when screening for waterborne plant pathogens [4].

Within the forensics discipline it is critical for microbial forensic laboratories to focus on developing tools that strengthen national biosecurity. Many U.S. vulnerabilities were identified following the September 11, 2001 attacks on the World Trade Center, Pentagon, and subsequent anthrax mail attacks [5,6]. However, more work is needed, especially in regards to waterborne pathogens. With recent events including the Boston Marathon bombings on April 15, 2013; the November 13, 2015, coordinated attacks in Paris; and bombings in Turkey [7]; the willingness of individuals and groups to commit acts of terrorism has been demonstrated, which means the U.S. must consider vulnerabilities of agricultural water resources [8, 9]. An intentional effort seeking to compromise the U.S. water supply, either for human or agricultural consumption, would likely go undetected in the current environment until catastrophic consequences became obvious. Even then, our ability to correctly attribute the cause of such an action would be limited at best.

Scientists and officers in biosecurity need tools with discriminatory ability that meet a very high standard for use in forensics based investigations. A relevant gap in the U.S. agricultural biosecurity system is a lack of forensically based protocols that are readily accepted among the forensics community, be admissible in a courtroom, and easily explained to lay individuals that protect our water resources [10, 11].

Microorganisms including bacteria, viruses, protists, fungi, and algae are all found in lakes, rivers, streams and ponds. The vast majority of these microorganisms are beneficial to their respective

environments and have little, if any, ill effect on humans and crops [12]. However, there are some microorganisms that pose an increased risk to human health as well as pathogens capable of causing diseases in plants or animals. There is currently no established or adapted protocol for detection of waterborne plant pathogens within the agriculture sector. Development of such protocol is needed to proactively identify harmful pathogens on interests amongst the microflora and prevent pathogens from being introduced into cropping systems.

Current methods for recovering waterborne pathogens from water require a device and technique capable of sampling at high volumes from potable water and focus on enteric viruses [13, 14]. For research and diagnostics within agricultural disciplines, the numerous tests and water sources required, make the currently available approaches too costly. In addition to costs, the equipment, portability, and large sample volumes and greater number of samples makes current methods unfeasible for direct adaption to agriculture sampling [15]. Finding known and unknown pathogens is challenging due to extremely low virus titers and due to the volume, dilution factors, and dynamics of water [ ]. The properties of water and ultralow microorganism concentrations make working in this environment problematic. There is currently no device commonly used in research and routine diagnostics for irrigation water sampling. A method to sample large volumes of water and capture pathogens is needed along with a protocol for quick and proper processing of material.

The objectives of this research are to provide preliminary data on plant waterborne viruses identification and develop a device for capture of waterborne plant viruses in various aquatic ecosystems and to develop protocols for molecular analysis of samples following capture. To meet these objectives, the bioinformatic tools e-probe diagnostic nucleic acid analysis (EDNA) and MetaSim will be used for preliminary data analysis followed by protocol development and adaptation of plant-based reverse transcriptase multiplex polymerase chain reaction (RT-mPCR) for processing samples for detection/identification of waterborne viruses. After establishing molecular analysis, a

device for processing large volumes of water will be developed. Lastly, biological significance of waterborne viruses will be tested using hydroponics.

LITERATURE CITED

1. ICA, Intelligence Community Assessment. 2012. Global water security. Office of the Director of National Intelligence. The National Intelligence Council. 798325

2. Barber NL. 2014. Summary of estimated water use in the United States in 2010. In. Edited by Survey USG. Pubs Warehouse: National Water Use Program

3. de Grosbois D, Plummer R. 2015. Problematizing water vulnerability indices at a local level: a critical review and proposed solution. Water Resources Management 2015, 29(14):5015-5035

4. Sigee DC. 2005. Freshwater microbiology: biodeversity and dynamic interactions of microorganisms in the aquatic enviroment. J. Wiley Pub. ISBN0471485284

5. Comfort L, Kapucu N. 2006. Inter-organizational coordination in extreme events: The World Trade Center attacks, September 11, 2001. Nat Hazards 39:309-327

6. Flynn S. 2002. America the vulnerable. Council on Foreign Relations. Foreign Aff 81.60-74

7. Turquie. 2016. Une camion explose à Bingol, 5 morts. In. Conflits-FR

8. Comfort L, Kapucu N. 2006. Inter-organizational coordination in extreme events: The World Trade Center attacks, September 11, 2001. Nat Hazards. 39(2):309-327

9. Speckhard A. 2013. The Boston Marathon Bombers: the Lethal Cocktail that Turned Troubled Youth to Terrorism. Perspectives on Terrorism. 7(3)

10. Parker HS. 2003. Agricultural bioterrorism: a federal strategy to meet the threat. In: McNair Paper 65. Washington, D.C.: Institute for National Strategic Studies National Defense University

11. Waage JK, Mumford JD. 2008. Agricultural biosecurity. Philosophical Transactions of the Royal Society B: Biological Sciences. 363(1492):863-876

12. Llirós M, Inceoğlu Ö, García-Armisen T, Anzil A, Leporcq B, Pigneur L-M, Viroux L, Darchambeau F, Descy J-P, Servais P. 2014. Bacterial Community Composition in Three Freshwater Reservoirs of Different Alkalinity and Trophic Status. PLOS ONE. 9(12):e11614

13. Ramírez-Castillo FY, Loera-Muro A, Jacques M, Garneau P, Avelar-González FJ, Harel J, Guerrero-Barrera AL. 2015. Waterborne pathogens: detection methods and challenges. Pathogens. 4(2):307-334

14. Farrah SR, Gerba CP, Wallis C, Melnick JL. 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. Applied and Environmental Microbiology. 31(2):221-226

15. Lambertini E, Spencer SK, Bertz PD, Loge FJ, Kieke BA, Borchardt MA. 2008. Concentration of Enteroviruses, Adenoviruses, and Noroviruses from drinking water by use of glass wool filters. Applied and Environmental Microbiology. 74(10):2990-2996

CHAPTER II

REVIEW OF LITERATURE

Historical events

The intentional release or dissemination of biological agents to incite fear seeking religious and/or political objectives are considered acts of bioterrorism. The ultimate objective of bioterrorism may vary depending on who is committing these acts. Among the first documented uses of a biological agent for warfare is attributed to the Assyrians during the Sixth Century BC, who used the fungus *Claviceps purpurea*, the causal pathogen of ergot of rye, a plant disease [1]. In 590 BC, The Athenians reportedly used hellebore roots to poison aqueducts during the siege of Cirrha [2]. The Romans used clay pots filled with venomous snakes as well as bee hives and hornets nests to catapult over defensive walls during various battles between 300-100 BC [3]. The use of diseased and rotting human corpses against enemies has been reported by different militaries dating from 400 BC. The diseased bodies and feces were used to contaminate arrows. More recently, the Islamic State of Iraq and Syria (ISIS) documents discovered on a laptop computer exposed multiple plans detailing biological agent manufacturing, to the dumping of corpses by Malian militants in water wells [2, 4, 5].

Agroterrorism is defined as the deliberate introduction of disease causing animal or plant pathogens with the goal of generating fear, causing economic loss, and/or undermining social stability [6]. Agroterrorism has a long history; however, the specific focus and identification on this subset of terrorism primarily resulted from the September 11, 2001 attacks on the World Trade Centers and the acknowledgement of U.S. vulnerabilities [7,8]. Some of the early reports

identified as agroterrorism occurred during World War II, when the German secret service used *Burkholderia mallei*, the causal agent of Glanders disease, and *Bacillus anthracis*, the infection agent of anthrax, to target animals [9]. Also during WWII, Germany is accused of dropping boxes of the Colorado potato beetle on UK farms. Similar accusations were later made by East Germany claiming the U.S purposefully introduced the Colorado potato beetles into their farms in 1950 [10]. In 1984 members of a cult identified as Rajneeshee used the foodborne pathogen *Salmonella typhimurium* to contaminate various salad bars in Dalles, Oregon in an effort to sway a political outcome [11]. More recently, from 2003-2005 several regions in Italy had commercial drinking containers contaminated by an individual(s) that were radical anti-capitalist, environmentalist, or commercial saboteurs who used bleach, acetone, or ammonia [12].

Water, and the development of its infrastructure, is the most fundamental resource to the modernization and establishment of societies [86]. Early civilizations understood the importance of water and how it might be used to conquer others or defend their homelands. Dating back to 1790 BC, there are documented reports discussing irrigation systems and thefts of water [5]. Around 700 BC to 400 BC, the military use of water resources to defeat enemies either by preventing their use, destroying irrigation systems, or diverting rivers is reported [13]. It is also during this time that *Claviceps purpurea* the causal pathogen of ergot of rye, a plant disease, is used to poison the wells of Assyrian armies [1]. All of the above illustrates the early uses of biological agents added to water resources to defeat enemies with military intent. Historically, water has frequently been used as a means to wage war and sway political opinions, including the Saladin defeat of the Crusaders by denying them water in 1187, the destruction of New York water works in 1777 by Britain, and dumping of animal carcasses in ponds from 1860-1865 by Confederate soldiers [1, 14, 15]. Starting approximately in 1939 until present-day, the number of recorded incidences involving the direct attack or use of water resources has exponentially risen [16]. In 1945, German forces used sewage to pollute reservoirs in Bohemia, Czechoslovakia [17].

The Weathermen group, who opposed Vietnam War, attempted to obtain biological agents to contaminate U.S. water systems in 1970 [1]. Recently, in the state of Georgia, three men were arrested for planning terrorist attacks on water treatment plants and other water resources [18]. In 2012, 150 Afghan schoolgirls were poisoned after drinking contaminated water [19]. In the Central African Republic, Christian militants killed Muslim civilians and dumped their bodies in water wells [16]. There is a long history of targeting water resources and infrastructure to bring harm to individuals, groups, and/or governments for political, religious, and terror intent [13, 16]. This targeting of water resources is on the rise and merits special consideration to the various vulnerabilities and possible methods that might be implored to attack these vital systems. Not only is the intentional contamination of water resources a concern, but also unintentional contamination of water with heavy metals, as seen in Flint, Michigan, can be of serious concern. Starting in 2014 and nationally recognized in 2016, the Flint water crisis resulted from the failure of officials to apply corrosion inhibitors to the new water source. By not applying these inhibitors, elevated levels of heavy metals (lead) entered the publics' water supply and is reported by news agencies to be responsible for health problems in 6,000 to 12,000 children.

Biosecurity and agroterrorism

The definition of agricultural biosecurity has varying descriptions and many times is wrongly interchanged with biosafety. In this paper biosecurity is the combined package of science, policy and regulatory strategies used to protect a country's, state's, and/or locale's interests including food, agriculture, animal health, aquatic systems, and forestry associated with human health and interests [20]. Biosafety is similar in that it also uses science, policy, and regulatory strategies; however, its application is for the protection of personnel, equipment and environmental from exposure of potentially infectious agents or biohazards [20].

The agricultural sector has a need to strengthen biosecurity systems to continually update security and surveillance systems monitoring the vast acreage of land and multiple water systems making up this industry [21]. Current waterborne screening is primarily focused on detection of enteric pathogens in public water supplies [22, 23]. Developing better screening methods of plants and animals for presence of pathogens is critical in maintaining a robust biosecurity program [24, 25].

Due to the enormous volume and dilution factor of water, detecting pathogens is a challenge. The development of detection tools and protocols for waterborne plant viruses must consider water turbidity, which can lead to clogging of a filtering device, limiting the total water sampled [43]. Detection tools and protocols must also be able to use sorption to insure optimal capture and have a way to reverse the capture (elute), so that molecular tools can be used for confirmation. The most widely used molecular tools are immunological and nucleic acid (NA) based assays [26]. Immunological assays are based on antigen-antibody reactions and include a reporter label. These assays are inexpensive, quick, and allow for multiple samples to be screened at one time. Immunological assays are ideal for pre-screening the presence or absence of pathogens in a large number of samples, but have less specificity and sensitivity required in biosecurity applications [26]. Nucleic acid based assays offer the specificity and sensitivity used in biosecurity applications, but require previous sequence knowledge of the targeted pathogen. Nucleic acid based assays are also negative affected by inhibitors, which may limit or prevent detection [27]. Another option is next generation sequencing (NGS). NGS can be used to amplify the entire genome or transcriptome of a sample, thereby generating an entire profile of a sample. Bioinformatic tools can be used to analyze the data for confirmation of pathogens [28]. However, NGS based diagnostics are also limited by lower titer targets.

A preliminary requirement for any laboratory detection of waterborne pathogens is the creation of a sampling device to screen aquatic environments, which is critical for use in

biosecurity programs and water monitoring. By identifying waterborne pathogens in a grower's water resources, it may be possible to prevent the accidental introduction of pathogens, thereby limiting or eliminating the need for pesticides used in controlling disease.

Waterborne virus capture

Public drinking and recreational water sources are primary reservoirs for enteric viruses responsible for enteric illnesses worldwide. To put this in perspective, around 25% of the world population is exposed to fecal contaminated drinking water [22]. The primary enteric viruses responsible for human diseases from water are rotaviruses, adenoviruses, human caliciviruses, and astroviruses, which are dsRNA, dsDNA, ssRNA (+), and ssRNA (+) viruses, respectively. [29, 30]. For this reason, the detection and research of waterborne viruses is largely limited to enteric and animal viruses, with minimal research focused on plant viruses in water resources [31-36].

Sources of enteric waterborne disease agents include but are not limited to contaminated drinking supplies, consumption of infected crustaceans, infected agricultural crops, and recreational waters (lakes, rivers, and swimming pools) [31]. The primary contributors of enteric diseases to these water sources are humans, animals, and their feces and urine that enter the environment through urban sewage systems contributing to environmental pollutants [31, 36, 38, 39]. Chlorination, which is one of the most widely used disinfectants for water, is effective against bacterial pathogens; however, chlorination was shown to not always deactivate viruses [31]. In addition to human pathogens, plant pathogenic viruses are also excreted through natural plant decay and by humans and animals after leaving the gastrointestinal tract [39, 40].

In a comprehensive metagenomic study, Zhang et al. (2006), analyzed uncultured RNA viruses from healthy human feces and found 36,769 sequences closely related to plant RNA viruses [39]. Of these plant RNA viruses, in human fecal samples, a total of 35 individual plant

RNA viruses were identified to be *Pepper mild mottle virus* (PMMoV) as the dominating species [39]. In a separate study by Colson et al. (2010), PMMoV was isolated from human fecal material and Tabasco© sauce and used to inoculate *Nicotiana tabacum* L. for disease observation [41]. The ability of a plant virus to remain infections through the gastrointestinal tract or after processing for food products demonstrates the robust and stable nature of some plant viruses and their ability to persist harsh environmental challenges. Additionally, plant viruses *Tobacco mosaic virus, Tobacco necrosis virus,* and *Carnation mottle virus*, obtained from human feces, have been reported as being waterborne viruses [39, 40]. In a review by Mehle and Ravnikar (2012), plant waterborne viruses are found in the genera *Carmovirus*, *Cucumovirus*, *Dianthovirus*, *Tombusvirus*, *Potexvirus*, *Tobamovirus*, and *Tombusvirus*, which are all ssRNA (+) viruses [40]. Additional sources of plant viral pathogens and environmental pollutants include crop debris, floods, insect transmission, natural disasters, wildlife, and agricultural trade. Viral pathogens from all of these sources can enter water sheds. Some plant pathogenic viruses, like a few enteric viruses, are able to survive the harsh conditions of both the digestive track and environment, while remaining infectious to their respective hosts [30,34]. Adapting technologies for capturing enteric viruses from water systems will facilitate research in waterborne plant viruses. The identification of waterborne plant viruses will facilitate a proactive approach to managing potential disease outbreaks in agriculture.

Several technologies have been developed for capturing and concentrating human enteric viruses from water sources, include the 1-MDS Virosorb filters, Zeta-plus filters, NanoCerma, Filterite filters, and glass wool filters. The 1-MDS Virosorb, Zeta-plus, NanoCerma, and glass wool filters all have a net positive charge, while Filterite filters are negatively charged. The advantage of the electropositive filters is that there is no need for pre-conditioning with acids or polyvalent salts to enable virus capture, as observed with electronegative filters when sampling freshwater [31, 42, 43]. The pre-conditioning step can be impractical if sampling large volumes

of water. In the oceanic environment, where salt concentrations and turbidity are elevated, electronegative filters have an advantage over electropositive filters [42, 44].

In a comparative study by Rose et al. (1984), where the authors sampled sewage effluent, only 11 liters of water was able to be processed through the 1-MDS Virosorb due to clogging [42]. A range of 11-15 liters for the Zeta-plus could be processed before to clogging [42]. The Filterite filters processed the most with 19 liters of water before clogging [42]. Consideration of water turbidity is needed prior to sampling a particular source [42]. McMinn et al. (2016), reported that 100 – 1,600 liters of either drinking or ground water was required to be filtered before they could detect viruses [4]. Clogging is a limiting factor when sampling larger water sources like lakes, rivers, or streams. This research seeks to overcome this challenge by developing a water sampling device that is capable of processing large volumes of water to filter plant pathogenic waterborne viruses. Identifying plant viruses in watersheds used by agriculture will prevent accidental exposure of disease causing viruses to crops.

Glass wool filters are a cost effective and simple means for capturing waterborne plant viruses within various aquatic environments, to include: drinking water [34, 35, 45], aquifers [46], rivers [34, 36], lakes, ponds, and other systems used in greenhouse horticulture and/or agriculture farming [47, 48]. When compared with other virus filtering technologies, like the 1 MDS filters, cost saving by using glass wool virus capture are estimated around $340,000 for epidemiological based studies where 2,000 water samples may be required [33]. At the time of this paper, April 27th, 2016, 103 square feet of glass wool could be purchased for $29.68 from local hardware stores. Glass wool filter cartridges do not require the purchase of specialized housing units; rather, it is possible to construct such cartridges from polyvinyl chloride (PVC), a synthetic plastic polymer, which can be cut to any size, reused, and packed with glass wool at varying densities.

Virus capture by glass wool occurs due to the electropositive sites on the individual fibers of glass wool binding the negatively charged virus particles [33]. This relationship of glass wool fibers and virus coat protein is discussed in detail by Zerda et al. (1985) [49]. Briefly, it was determined that increasing the pH above a virus's isoelectric point (pI) influences the surface charge of the virus and enhance its absorption to the electropositive surface [49]. The pI is defined as the pH level that causes a switch in the viruses charge (-/+) in a given environment [50]. The pI can also be thought of as the point at which a virion is in an electrically neutral state. For non-enveloped viruses including *Potexvirus*, *Tobamovirus*, and *Tombusvirus*, the functional groups on the exterior of the coat protein primarily determine their surface charge. However, Langlet et al. (2008), found that in addition to the external functional groups, the interior RNA-protein binding will also influence pI [51]. Regardless, an environmental pH favoring a highly negative charge will allow greater capture to a charged surface or electropositive filter [49]. Work by Lukasik et al. (2000), found similar results by measuring indirect effects of adding salts or HCl, further determining that it was the change in pH that enhanced virus binding [52].

For optimal capture of waterborne plant viruses, it is necessary to know their respective pI so that alterations in source water can be made to facilitate the electronegative coat protein binding to the electropositive glass fibers. As previously discussed, the viral coat protein, internal RNA-protein binding, and environment all influence viral pI, which make it difficult or impossible to determine the exact pI. A detailed review by Michen and Graule (2010), found that pI of viruses was very scattered even when comparing the same species [50]. Differences in pI can be attributed to methods used in determining pI and to the purity of the sample. The pI for plant viruses ranged from 3.6 to 6.0 [50]. Even though exact pI at specific environmental conditions might not be known, having a range of pI provides opportunity to address this challenge. With the pI of plant viruses ranging from ~3.6 to 6.0 a majority of plant viruses could theoretically be captured so long as sampling pH remains above 6.

Following virus captured in electropositive glass wool, it is necessary to elute virions through a process termed desorption. One of the first reports demonstrating desorption of viruses bound to capture filters found that using 3% beef extract and sonic treatment yielded nearly complete recovery of three separate viruses [22]. Another comparison study of different elution buffers, Lee et al. (2011) found that 1.5% beef extract + 0.05 M glycine and 0.01% tween 80 provided the best elution buffer, which was also demonstrated in other work [49-51]. In a study comparing surfactant, dispersant, pH, and temperature influences on electropositive filter elusions, both higher pH (9) and the surfactant (Tween 80) consistently yielded higher virus recovery than other microorganisms [56]. In the same study the authors demonstrated that increasing the elution temperature to 37°C lowered recovery of *Salmonella* [56]. In the event that sampling various water sources yields elevated bacterial contamination, the possibility to reduce this error by increasing temperature during elution procedures remains likely. Collectively, there is no single method that elutes at 100% for all viruses; however, this limitation is partially overcome with improved sensitivity and selectivity of molecular diagnostics [26].

Following the elution step, it is necessary to further concentrate and remove the virions from the buffer. Polyethylene glycol (PEG) is shown to precipitate and recovery viruses from aqueous suspension [88, 89]. Polyethylene glycol is a polyether compound that is used in array of disciplines including medicine, industrial lubrications, rocket fuel, electrical insulating, toothpaste, preservation, DNA isolation, protein isolation, viral isolation, drug stabilization, and many others. Both PEG 6000 and PEG 8000 can be used to recover virus from solution; however, PEG 8000 had a higher recovery efficiency [53, 88, 89]. According to Colombet et al. (2007), PEG precipitation of viruses works by crystallizing virions within the interpolymer spaces between PEG molecules, a process termed pegylation [88]. In another study by Atha and Ingham (1981), demonstrate that PEG precipitations is not influence by pH, temperature, or ionic strength

[75]. Meaning; PEG is an ideal synthetic polymer for virus precipitation following elution with 1.5% to 3% beef extract + 0.05 M glycine.

Viruses used in this study

Tomato bushy stunt virus (TBSV) is the type member of the genus *Tombusvirus*, family *Tombusviridae*. The genome is ssRNA positive sense and lacks an envelope with a length of ~4.8 kb. There are five open reading frames that code for p33, p92, p41, p22 and p19, which code for viral replicase, coat protein, movement protein and gene-silencing suppressor, respectively. Infection starts once the virus enters the host cell and uncoats to begin translation on the 5' end to generate the viral replicase made up of p33 and p92. The generation of p92 occurs via stop codon readthrough [57]. The readthrough occurs as the result of a weak UAG stop codon to produce the p92 protein in different quantities as compared to p33, suggesting a regulator property [57-59]. Both p33 and p92 must be present for viral infection and replication to occur [60]. The remaining proteins, p41, p22 and p19, are transcribed as subgenomic mRNAs (sg mRNA). The first sg RNA transcribed is sg mRNA2 that codes the p22 movement protein and the p19 silencing suppressor [61]. The movement protein, p22, is the symptom determinate and required for cell to cell movement [61]. To ensure infection continues p19 acts on the host suppressor to silence it by recruiting small interfering RNAs, thereby stopping host antiviral mechanisms [62]. The final translated protein is p41, which originates from transcription of the sg mRNA1 to generate the coat protein. The coat protein is not required in cell to cell movement or systemic infection throughout the host, but is reported to contribute to virulence [63]. *Tomato bushy stunt virus* has a reported isoelectric point of 4.1 [64]. There are no reported insect or fungal vectors and transmission primarily occurs through seeds, grafting, and mechanical inoculation from contaminated tools. *Tomato bushy stunt virus* is reported in various waterways and was shown to be transmissible in indicator plants [65, 66]. These finding suggest TBSV is a candidate for

waterborne viral transmission, which poses a risk to agriculturalists who use various water sheds to irrigate crops.

Pepper mild mottle virus belongs to the genus *Tobamovirus*, family *Virgaviridae*. The genome is linear ssRNA positive sense with capsid that forms a rigid rod particle. *Capsicum* L. (peppers) including bell peppers, chili or "spice" peppers, and ornamental peppers are susceptible to a PMMoV, which has a worldwide distribution [66]. The infection cycle starts with entrance into the host cell, then uncoating and release of viral RNA. The viral RNA is translated to produce two proteins needed for replication and transcription. A dsRNA is made from the ssRNA (+) and transcribed and replicated producing ssRNA+. The capsid protein, movement protein and viral suppressor are generated from subgenomic mRNAs. All components are assembled in the cytoplasm, with the final virion moves to adjacent cells [68]. *Pepper mild mottle virus* causes leaf chlorosis, stunting, and malformed/mottled fruits with occasional necrotic spots and can have an infection rate near 100% [69]. Long distance dissemination of infectious PMMoV is thought to occur through human fecal matter where individuals carry the virus from one location to another [35]. *Pepper mild mottle virus* is also described as a seed-borne virus. In the soil environment, PMMoV will remain infections for long periods of time in plant debris, posing a risk to subsequent *Capsicum* crops [70]. Handling of infected and non-infected seeds is the primary means of spread, requiring proper sanitation and certified seeds, to ensure control.

Pepino mosaic virus (PepMV) is a member of the genus *Potexvirus*, family *Alphaflexiviridae*. The viral genome is ssRNA positive sense and with no envelope and a length of ~5.9-7 kb. The virions are flexuous rods. The genomic organization contains a 5' cap and 3' polyadenylated tail with five open reading frames (ORFs) that code for RNA dependent RNA polymerase (RdRp), three triple gene blocks (TGB1, TGB2, TGB3) and a coat protein (CP) [71, 72]. The region identified as the RdRp shares sequence homology with a methyltransferase, nucleoside triphosphate-binding, and polymerase motifs [71]. Open reading frames 2, 3, and 4,

code for TGB1, TGB2, and TGB3, which are multifunctional and responsible for viral movement [71]. The TGB1 protein contains conserved helicase motifs that assist in cell-to-cell movement along with the ability to increase plasmodesmata permeability [74-76]. Another critical role of TGB1 is the ability to act as a suppressor of host silencing during cell to cell movement, thereby aiding in systemic infections [76]. Both TGB2 and TGB3 contain transmembrane domains associated with the endoplasmic reticulum, which was shown to be necessary for viral movement [78, 79].

Justification

The volume, dilution factors, and dynamics of water makes identifying known and unknown biological pathogens challenging. Water can also act as a reservoir capable of transporting highly pathogenic pathogens long distances or maintaining them through recirculating systems as observed in horticulture, greenhouses, and hydroponics. The unique plant waterborne virus properties are magnified when considering environmental factors including droughts, floods, windblown droplets, and other viral dispersal mechanisms that can stress plants.

Water ecosystems contain pathogenic and nonpathogenic bacteria, viruses, protists, fungi, and algae. There is a need to catalog and to differentiate harmful agricultural pathogens and those that do not infect and damage crops and long term food sustainability. Modifying nucleic acid based approaches will offer the sensitivity and specificity needed to identify harmful pathogens of interests versus communal organisms. Currently, there are no routine technologies for concentration of plant waterborne viruses used in agriculture. Considering the dynamics of water, especially the dilution factors, there is a need for capturing and concentrating waterborne viruses from large volumes of water to facilitate detection by molecular analysis and/or immunocapture.

In a plant, viral titers can be high, especially when symptoms are present. Plant cells act as a replication sites for many plant viruses [80 - 82]. Once inside a susceptible host cell, most viruses will spread systemically and replicate in infected cells [72, 83, 84]. Plant viruses can only replicate in plant cells or insect vectors, resulting in higher titers compared to water, making detection relatively simple in plants [81, 85]. Plant viruses cannot freely replicate in aqueous environments. Viruses require host machinery for replication and movement to neighboring cells [86, 87]. Current water-based assay tools are used for human pathogen detection. There are no widely accepted protocols or devices that specifically concentrate and detect waterborne viruses in the agricultural sector. A method to capture plant viruses from large volumes of water would facilitate detection. This project is designed to address these technology gaps.

The limitations of detecting waterborne viruses must be overcome in order to protect and aid in agricultural sustainability. Developing a sampling device for waterborne plant virus detection is feasible. Additional modification of nucleic acid based technology will allow for discrimination of pathogens of interest. This technology can then be used to sample various aqueous environments and metagenomic analysis. A sensitive, mobile (field-ready), and inexpensive device for virus capture, detection, and metagenomic analysis will aid biosecurity agencies and diagnostic labs.

LITERATURE CITED

1. Eitzen EM, Takafuji, ET. 1997. Historical overview of biological warfare. Washington, D.C.: Office of the Surgeon General, Department of the Army

2. (TDoSH)Texas Department of State Health Services. 2015. History of Bioterrorism. In: http://www.dshs.texas.gov/preparedness/bt_public_history.shtm

3. Langford RE. 2004. Introduction to weapons of mass destruction: radiological, chemical, and biological. Hoboken, New York: John Wiley and Sons

4. Herald Doornbos JM. 2014. Found: The Islamic State's terror laptop of doom. In., vol. 2015: Foreign Policy

5. Hatami H, Gleick PH. 1993. Chronology of conflict over water in the legends, myths and history of the ancient middle east. Pacific Institute for Studies in Development, Environment and Security

6. Monke J. 2007. Agroterrorism: threats and preparedness. In: Edited by Analyst in Agricultural Policy Resources S, and Industry Division

7. Comfort L, Kapucu N. 2006. Inter-organizational coordination in extreme events: The World Trade Center attacks, September 11, 2001. Nat Hazards 39:309-327

8. Flynn S. 2002. America the vulnerable. Council on Foreign Relations. Foreign Aff 81.60-74

9. (CNS). 2009. Chronology of CBW incidents targeting agriculture 1915-2008. In: James Martin Center for Nonproliferation Studies (CNS) and Middlebury Institute of International Studies

10. (SIPRI) SIPRI. 1971. The problem of chemical and biological Warfare, vol. 1. New York: Humanities Press

11. Carus WS. 2000. Toxic terror: assessing terrorist use of chemical and biological weapons. Cambridge, Massachusetts: MIT Press

12. (CNS)b. 2009. Chronology of chemical and biological incidents targeting the food industry 1946-2006. In: James Martin Center for Nonproliferation Studies (CNS) and Middlebury Institute of International Studies

13. Gleick P. 2006. Water and terrorism. Pacific Institute 8(6)

14. Lockwood R. 2001. The batttle over the Crusades. In: Catholic League

15. Thatcher J. 1827. A military journal during the American Revolutionary War, from 1775 to 1783. Boston, Massachusetts: Cottons and Barnard

16. Water conflict chronology list. 2014. In: Water Conflict. Pacific Institute

17. (SIPRI)b SIPRI. 1971. The rise of CB Weapons: the problem of chemical and biological warfare, vol. 1. New York: Humanities Press

18. Office USAs. 2014. Three men charged with conspiracy to possess destructive devices. In: Atlanta, Northern District of Georgia: Federal Bureau of Investigation

19. Hamid M. 2012. Afghan schoolgirls poisoned in anti-education attack. In: Reuters; 2012

20. Ochoa-Corona F. 2011. Biosecurity, microbial forensics and plant pathology: education challenges, overlapping disciplines and research needs. Australasian Plant Pathol. 40(4):335-338

21. Harl NE. 2002. U.S. Agriculture, food production is threatened by bioterrorism attacks. In: Ag Lender

22. Gall AM, Mariñas BJ, Lu Y, Shisler JL. 2015. Waterborne viruses: a barrier to safe drinking Water. PLoS Pathog. 11(6):e1004867

23. Altintas Z, Gittens M, Pocock J, Tothill IE. 2015. Biosensors for waterborne viruses: detection and removal. Biochimie. 115:144-154

24. Bunn D, Beltran-Alcrudo D, Cardona C. 2011. Integrating surveillance and biosecurity activities to achieve efficiencies in national avian influenza programs. Prev Vet Med. 98(4):292-294

25. Fisher M, Henk D, Briggs C, Brownstein J, Madoff L, Gurr S. 2012. Emerging fungal threats to animal, plant and ecosystem health. In: Nature. vol. 484: Nature Publishing Group, a division of Macmillan Publishers Limited. 186-194

26. Ramírez-Castillo FY, Loera-Muro A, Jacques M, Garneau P, Avelar-González FJ, Harel J, Guerrero-Barrera AL. 2015. Waterborne pathogens: detection methods and challenges. Pathogens. 4(2):307-334

27. Rock C, Alum A, Abbaszadegan M. 2010. PCR inhibitor levels in concentrates of biosolid samples predicted by a new method based on excitation-emission matrix spectroscopy. Appl Environ Microb. 2010, 76(24):8102-8109

28. Stobbe AH, Daniels J, Espindola AS, Verma R, Melcher U, Ochoa-Corona F, Garzon C, Fletcher J, Schneider W. 2013. E-probe Diagnostic Nucleic acid Analysis (EDNA): A theoretical approach for handling of next generation sequencing data for diagnostics. Journal of Microb Meth. 94(3):356-366

29. Wang X, Liu F, Zhou G, Li XH, Li Z. 2006. Detection and molecular characterization of Pepper mild mottle virus in China. J Phytopath. 154(11-12):755-757

30. Guan H, Schulze-Makuch D, Schaffer S, Pillai SD. 2003. The effect of critical pH on virus fate and transport in saturated porous medium. Ground Water. 41(5):701-708

31. Hryniszyn A, Skonieczna M, Wiszniowski J. 2013. Methods for detection of viruses in water and wastewater. Advances in Microbiology. 3(5):442-449

32. Wommack KE, Hill RT, Colwell RR. 1995. A simple method for the concentration of viruses from natural water samples. J Microbiol Meth. 1995, 22(1):57-67

33. Lambertini E, Spencer SK, Bertz PD, Loge FJ, Kieke BA, Borchardt MA. 2008 Concentration of *Enteroviruses*, *Adenoviruses*, and *Noroviruses* from drinking water by use of glass wool filters. Appl Environ Microb. 2008, 74(10):2990-2996

34. van Heerden J, Ehlers MM, Heim A, Grabow WOK. 2005. Prevalence, quantification and typing of adenoviruses detected in river and treated drinking water in South Africa. J Appl Microbiol. 99(2):234-242

35. Gassilloud B, Duval M, Schwartzbrod L, Gantzer C. 2003. Recovery of feline calicivirus infectious particles and genome from water: comparison of two concentration techniques. Water Sci Technol. 47(3):97-101

36. Albinana-Gimenez N, Clemente-Casares P, Calgua B, Huguet JM, Courtois S, Girones R. 2009. Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. J Virol Methods. 158(1–2):104-109

37. Grabow WOK, Taylor, MB. 1993. New methods for the virological analysis of drinking water supplies. In: Department of Medical Virology. Edited by Pretoria Uo

38. Pina S, Puig M, Lucena F, Jofre J, Girones R. 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl Environ Microb. 64(9):3376-3382

39. Zhang T, Breitbart M, Lee WH, Run J-Q, Wei CL, Soh SWL, Hibberd ML, Liu ET, Rohwer F, Ruan Y. 2005. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol. 4(1):e3

40. Mehle N, Ravnikar M. 2012. Plant viruses in aqueous environment – Survival, water mediated transmission and detection. Water Res. 46(16):4902-4917

41. Colson P, Richet H, Desnues C, Balique F, Moal V, Grob J-J, Berbis P, Lecoq H, Harlé J-R, Berland Y, Raoult D. 2010. Pepper mild mottle mirus, a plant virus associated with specific immune responses, fever, abdominal pains, and pruritus in humans. PLoS ONE. 5(4):e1004

42. Gerba CP, Farrah SR, Goyal SM, Wallis C, Melnick JL. 1978. Concentration of enteroviruses from large volumes of tap water, treated sewage, and seawater. Appl Environ Microb. 35(3):540-548

43. Rose JB, Singh SN, Gerba CP, Kelley LM. 1984. Comparison of microporous filters for concentration of viruses from wastewater. Appl Environ Microb. 47(5):989-992

44. Katayama H, Shimasaki A, Ohgaki S. 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl Environ Microb. 68(3):1033-1039

45. Vivier JC, Ehlers MM, Grabow WOK. 2004. Detection of enteroviruses in treated drinking water. Water Res. 38(11):2699-2705

46. Powell KL, Taylor RG, Cronin AA, Barrett MH, Pedley S, Sellwood J, Trowsdale SA, Lerner DN. 2003. Microbial contamination of two urban sandstone aquifers in the UK. Water Res. 37(2):339-352

47. Millen HT, Gonnering JC, Berg RK, Spencer SK, Jokela WE, Pearce JM, Borchardt JS, Borchardt MA. 2012. Glass wool filters for concentrating waterborne viruses and agricultural zoonotic pathogens. JoVE. (61):3930

48. Corsi SR, Borchardt MA, Spencer SK, Hughes PE, Baldwin AK. 2014. Human and bovine viruses in the Milwaukee River watershed: hydrologically relevant representation and relations with environmental variables. Sci Total Environ. 490:849-860

49. Zerda KS, Gerba CP, Hou KC, Goyal SM. 1985. Adsorption of viruses to charge-modified silica. Appl Environ Microb. 49(1):91-95

50. Michen B, Graule T. 2010. Isoelectric points of viruses. J Appl Microbiol. 109(2):388-397

51. Langlet J, Gaboriaud F, Gantzer C, Duval JFL. 2008. Impact of chemical and structural anisotropy on the electrophoretic mobility of spherical soft multilayer particles: the case of bacteriophage MS2. Biophys J. 94(8):3293-3312

52. Lukasik J, Scott TM, Andryshak D, Farrah SR. 2000. Influence of salts on virus adsorption to microporous filters. Appl Environ Microb. 66(7):2914-2920

53. Lee H, Kim M, Paik S-Y, Lee CH, Jheong W-H, Kim J, Ko G. 2011. Evaluation of electropositive filtration for recovering norovirus in water. J Water Health. 9(1):27-36

54. Méndez J, Audicana A, Isern A, Llaneza J, Moreno B, Tarancón MaL, Jofre J, Lucena F. 2004. Standardised evaluation of the performance of a simple membrane filtration-elution method to concentrate bacteriophages from drinking water. J Virol Methods. 117(1):19-25

55. Sobsey MD, Glass JS. 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl Environ Microb. 40(2):201-210

56. Polaczyk AL, Roberts JM, Hill VR. 2007. Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. J Microbiol Meth. 68(2):260-266

57. Hearne PQ, Knorr DA, Hillman BI, Morris TJ. 1990. The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus. Virology. 177(1):141-151

58. Scholthof K-BG, Scholthof HB, Jackson AO. 1995. The Tomato bushy stunt virus replicase proteins are coordinately expressed and membrane associated. Virology. 208(1):365-369

59. Oster SK, Wu B, White KA. 1998. Uncoupled expression of p33 and p92 permits amplification of tomato bushy stunt virus RNAs. J Virol. 72(7):5845-5851

60. Panavien≐ Ž, Baker JM, Nagy PD. 2003. The overlapping RNA-binding domains of p33 and p92 replicase proteins are essential for tombusvirus replication. Virology. 308(1):191-205

61. Scholthof HB, Scholthof K-BG, Kikkert M, Jackson AO. 1995. Tomato bushy stunt virus spread is regulated by two nested genes that function in cell-to-cell movement and host-dependent systemic invasion. Virology. 213(2):425-438

62. Silhavy D, Molnár A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyán J. 2002. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. The EMBO Journal. 21(12):3070-3080

63. Scholthof HB, Morris, T. J., and Jackson, A. O. 1993. The capsid protein gene of tomato bushy stunt virus is dispensable for systemic movement and can be replaced for localized expression of foreign genes. Mol Plant-Microbe Interact. 6:309-322

64. Arthur Sproul McFarlane RAK. 1938. Physical propertiest of bushy stunt virus protein. Biochemical Journal. 32(9):1607-1613

65. Tomlinson JA, Faithfull EM. 1984. Studies on the occurrence of tomato bushy stunt virus in English rivers. Ann Appl Biol. 104(3):485-495

66. Buttner C, Nienhaus, F. 1989. Virus contamination of waters in two forest districts of the Rhineland area (FRG). Eur J Forest Pathol. 19(4):206-211

67. Antignus Y, Lachman O, Pearlsman M, Maslenin L, Rosner A. 2008. A new pathotype of Pepper mild mottle virus (PMMoV) overcomes the L4 resistance genotype of pepper cultivars. Plant Dis. 92(7):1033-1037

68. Tobamovirus [http://viralzone.expasy.org/viralzone/all_by_species/51.html]. Accessed 2016

69. Wetter CC, M. 1988. Pepper mild mottle virus: description of plant viruses. In

70. CABI: Pepper mild mottle virus (PMMV). 2015. In: Invasive Species. Centre for Agricultural Biosciences International

71. Aguilar JM, Hernández-Gallardo MD, Cenis JL, Lacasa A, Aranda MA. 2002 Complete sequence of the Pepino mosaic virus RNA genome. Arch Virol. 147(10):2009-2015

72. Cotillon AC, Girard M, Ducouret S. 2002. Complete nucleotide sequence of the genomic RNA of a French isolate of Pepino mosaic virus (PepMV). Arch Virol. 147(11):2231-2238

73. Verchot-Lubicz J. 2005. A new cell-to-cell transport model for Potexviruses. Mol Plant Microbe In. 18(4):283-290

74. Angell SM, Davies C, Baulcombe DC. 1996. Cell-to-cell movement of potato virus X is associated with a change in the size-exclusion limit of plasmodesmata in trichome cells of nicotiana clevelandii. Virology. 216(1):197-201

75. Lough TJ, Shash K, Xoconostle-Cázares B, Hofstra KR, Beck DL, Balmori E, Forster RLS, Lucas WJ. 1998. Molecular dissection of the mechanism by which Potexvirus triple gene block proteins mediate cell-to-cell transport of infectious RNA. Mol Plant Microbe In. 11(8):801-814

76. Kalinina NO, Rakitina DV, Solovyev AG, Schiemann J, Morozov SY. 2002. RNA helicase activity of the plant virus movement proteins encoded by the first gene of the triple gene block. Virology. 296(2):321-329

77. Senshu H, Yamaji Y, Minato N, Shiraishi T, Maejima K, Hashimoto M, Miura C, Neriya Y, Namba S. 2011. A dual strategy for the suppression of host antiviral silencing: two distinct suppressors for viral replication and viral movement encoded by Potato virus M. J Virol. 85(19):10269-10278

78. Krishnamurthy K, Heppler M, Mitra R, Blancaflor E, Payton M, Nelson RS, Verchot-Lubicz J. 2003. The Potato virus X TGBp3 protein associates with the ER network for virus cell-to-cell movement. Virology. 309(1):135-151

79. Mitra R, Krishnamurthy K, Blancaflor E, Payton M, Nelson RS, Verchot-Lubicz J. 2003 The potato virus x TGBp2 protein association with the endoplasmic reticulum plays a role in but is not sufficient for viral cell-to-cell movement. Virology. 312(1):35-48

80. Rose C. Gergerich VVD. 2006. Introduction to plant viruses, the invisible foe. APS

81. Alemu K: Detection of Diseases, Identification and Diversity of Viruses. 2015. A Review. Agriculture and Healthcare. 5(1):204-213

82. Tilsner J, Linnik O, Louveaux M, Roberts IM, Chapman SN, Oparka KJ. 2013. Replication and trafficking of a plant virus are coupled at the entrances of plasmodesmata. J Cell Biol. 201(7):981-995

83. White KA, Nagy PD: Advances in the Molecular Biology of Tombusviruses. 2004. Gene expression, genome replication, and recombination. In: Progress in Nucleic Acid Research and Molecular Biology. Academic Press. (78)187-226

84. Tsuda S, Kubota K, Kanda A, Ohki T, Meshi T. 2007. Pathogenicity of Pepper mild mottle virus is controlled by the RNA silencing suppression activity of its eeplication protein but not the viral accumulation. Phytopathology. 97(4):412-420

85. Naidu RA, JdAH. 2001. Methods for the detection of plant virus diseases. Ibadan, Nigeria: International Institute of Tropical Agriculture

86. Verchot J. 2012. Cellular chaperones and folding enzymes are vital contributors to membrane bound replication and movement complexes during plant RNA virus infection. Frontiers in Plant Science. 3:275

87. Nagy PD, Pogany J. 2008. Multiple roles of viral replication proteins in plant RNA virus replication. In: Plant Virology Protocols. Edited by Foster G, Johansen IE, Hong Y, Nagy P. Humana Press. (451)55-68

88. Colombet J, Robin A, Lavie L, Bettarel Y, Cauchie HM, Sime-Ngando T. 2007. Virioplankton 'pegylation': Use of PEG (polyethylene glycol) to concentrate and purify viruses in pelagic ecosystems. J Microbiol Meth. 71(3):212-219

89. El-Senousy WM, Costafreda MI, Pintó RM, Bosch A. 2013. Method validation for norovirus detection in naturally contaminated irrigation water and fresh produce. Int J Food Microbiol. 167(1):74-79

90. Sklivaniots M, Angelakis AN. 2006. Water for human consumption through history. In: Proc. 1st IWA Int. Symp. on Water and Wastewater Technologies in Ancient Civilizations, Iraklio, Greece

CHAPTER III

ESTABLISHING A PROTOCOL FOR PLANT WATERBORNE VIRUS IDENTIFICATION AND DETECTION BY NUCLEIC ACID AND E-PROBE DIAGNOSTIC NUCLEIC ACID ANALYSIS

Abstract

Agriculture and food security are the cornerstones of a productive society. It is critical to evaluate agricultural vulnerabilities and ways to resolve these potential problems before they occur to further support societal demands. One such vulnerability is irrigation water resources and the lack of surveillance for detection of plant waterborne pathogens. Screening protocol for detection of waterborne plant pathogens from environmental water sources do not currently exist. This research uses the waterborne plant viral pathogens *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) belonging to the genus *Potexvirus, Tombusvirus,* and *Tobamovirus*, respectively. The bioinformatic tools electronic probe nucleic acid analysis (EDNA) and MetaSim were used to provide preliminary data on plant waterborne virus recovery. Previously developed nucleic acid based primers were adapted to detect each virus and mixed viral populations of PepMV, TBSV, and PMMoV in a multiplex assay. Seeded reverse osmosis (RO) water was screened for virus recovery at volumes of 250 ml, 500 ml, and 1,000 ml. Individual viruses were recovered from seeded RO water at all volumes.

The bioinformatic tool, EDNA, provided evidence that waterborne viruses can be identified from environmental water resources. A quick and inexpensive protocol for processing up to 1,000 ml of sample was established. Results indicate that environmental water samples can be processed in a quick and inexpensive manner through nucleic acid and bioinformatic assays for the identification and detection of plant waterborne viruses. This work establishes an initial methodology that can be readily adapted by diagnostic laboratories.

Introduction

The presence of plant pathogenic waterborne viruses poses a significant threat to agriculture augmented by the lack of water screening tools and difficulty in controlling spread once infection and disease is established in in a crop production system. In fact, once a viral pathogen establishes infection in a susceptible plant host, there is no means to cure the plant. Cultural practices such as crop rotations with non-host, roguing, or deep tilling can all be used as a means to maintain economic sustainability, but ideally, prevention and use of resistant cultivars is the best approach [1]. When disease is observed in a cropping system, a diagnostic laboratory can test plant material to determine the causal agent. However, for diagnosticians, waterborne viruses are extremely difficult to detect due to the dilution factor of water [2]. In plants, viruses take advantage of host machinery for replication and movement throughout host cells. Plant viral replication takes place in cytoplasmic viral factories acting as assembly-lines forming numerous viral mRNAs for protein synthesis by host ribosomes [3]. The formation of virions within plant cells allows host tissues to reach high viral titers within a localized area, making virus detection in plant tissues less difficult than detection in aquatic environments. In water, where virions are free of host tissues, there are no viral factories or mechanisms for replication. Without host tissue for replication, waterborne viruses will not increase in titer, making it necessary to capture free-floating virions from environmental water sources to facilitate or enhance detection probabilities.

In order to provide proof of concept data on waterborne plant virus detection, e-probe diagnostic nucleic acid analysis (EDNA) can be used in combination with MetaSim, a next generation sequencing (NGS) simulator [4, 5]. The bioinformatic tool, EDNA, uses target specific electronic probes (e-probes) to query datasets for the presence of targeted pathogen signatures as determined by the individual user [6]. The NGS simulator MetaSim is capable of providing mock sample databases (MSD) for *in silico* simulations and analysis at no cost. MetaSim functions by loading single or multiple genomes into the simulator along with other datasets of interests and

assigning abundance values. The output file from MetaSim generates reads similar to the output file of an NGS run. This simulated output file can then be queried with e-probes for presence of pathogen signature sequences. Similar approaches have been used in studying pathogenic viruses [6] and foodborne human pathogens [7].

There is a risk of false negatives due to virion loss at multiple stages of plant waterborne virus filtering including the initial sampling, processing, and RNA extraction, all of which are required for identification and detection. First, during the plant waterborne virus capture, there currently is no sampling method demonstrating a 100% recovery in every sampling scenario. However, glass wool recovery of virions was demonstrated and shows promise to recover waterborne viruses allowing molecular detection [4-6]. Second, it is possible for incomplete desorption of virions, which remain adsorbed to the glass wool fibers. Finally, even after a successful capture and elution, if virions cannot adequately be recovered from the high volume of elution buffer, there will be a potential possibility for a false negative. The total amount of extraction buffer used to elute virions obtained from water samples using electropositive filters or by flocculation is demonstrated for volumes of 100 ml [7], 200 ml [5, 6], 300 ml [8],and 330 ml [9]. For larger filters or where more material is present it may be necessary to elute at higher volumes than 330 ml. This can be demonstrated by using reverse osmosis water, which does not contain inhibitors that may interfere with virus recovery, in volumes up to 1,000 ml.

Several methods have described the use of salts or lower pH to cause flocculation of viruses with suspended proteins; however, this additional chemistry is not necessary when using polyethylene glycol (PEG) for viral concentration from aqueous chemistries [8-11]. Polyethylene glycol works by trapping viruses suspended in water or from elution with beef extract into the interpolymer spaces between PEG molecules [12]. This binding protects and stabilizes the virus and occurs in acidic, basic, and neutral conditions [12]. Lewis and Metcalf (1988) demonstrated that PEG with a molecular weight (mw) of 6,000 was more efficient than organic flocculation for

recovering viruses [14]. Similar findings were also reported by Colombet et al. (2007), where PEG with a mw of 6,000 or greater had better recovery than other concentration methods, like ultracentrifugation [12]. Aside from protecting the virion from lysozymes and environmental damage, PEG is also safe and inexpensive, two features that are important to plant diagnostic laboratories.

Plant diagnostics laboratories use immunological and nucleic acid based assays for detection and identification of plant pathogens. Immunoassays such as enzyme-linked immunosorbent (ELISA) and lateral flow immunoassays, offer inexpensive and rapid screenings of many samples, but lack discriminatory specificity if attempting to identify at strain-level. In contrast, polymerase chain reaction (PCR), is a nucleic acid based method that provides specificity and sensitivity for species and strain-level discrimination. Like ELISA, PCR can also be used for broader detection at genus-level. For PCR, this is achieved through primer design where organisms of a genus are aligned to find common forward and reverse sequence. Both technologies offer the ability to detect and identify plant pathogens and can be used for the detection of waterborne plant viruses. The limiting factor for both technologies is acquisition of enough protein or nucleic acid material to run diagnostics. However, PCR based assays, which have a greater degree of sensitivity is highly desirable for detection of waterborne viruses.

The objective of this project is to develop a protocol for processing environmental aquatic samples for nucleic acid based analysis for waterborne virus detection. This will be done by testing previously developed plant-based primers for broad detection of three plant virus genera; *Potexvirus*, *Tombusvirus*, and *Tobamovirus*, in particular *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV), respectively (Ochoa Corona, unpublished). When using glass wool for waterborne virus capture it is necessary to elute adsorbed virions from the glass wool via desorption. This project will test seeded water volumes up to 1,000 ml for the ability to recover each virus using PEG. Polyethylene glycol facilitates

virus recovery following elution. Successful recovery of viruses from seeded water will indicate the total volume of elution buffer that can be used during desorption and subsequent recovery.

Materials and Methods

E-probe design

Development of e-probes was done according to Stobbe et al., (2013). Briefly, target viral sequences were downloaded for *Potexvirus, Tombusvirus,* and *Tobamovirus* (Table 1). First, target sequences were aligned and compared with non-target sequences. For example: *Potexvirus* sequences were compared to *Tombusvirus,* and *Tobamovirus* sequences. All sequences with similarity were removed leaving unique sequences to the target viral genus. Secondly, these unique sequences were queried against the NCBI nonredundant nucleotide (nt) database to ensure specificity to the target genus. All e-probes with an e-value of 1×10^{-9} or lower and those that were assigned to non-targets were removed. The e-probe length was limited to 20 nt and 80 nt. As controls, decoy e-probes were generated by taking the reverse sequence of each final e-probe and performing the identical analysis as the non-decoy e-probes.

Mock sample database construction and query

To test e-probes, a mock sample database (MSD) was produced using metagenomic data of soil obtained from 100 year farmland as host background [20] and 27 *Tobamovirus* genomes (Table 1). Simulation of the 454 pyrosequencing was performed using MetaSim software [5]. The MSD was formatted and queried via BLASTn algorithm with e-probes for *Potexvirus, Tombusvirus,* and *Tobamovirus* as described in Stobbe et al. (2013). The data was then parsed at an e-value of 1×10^{-9}. The e-probes that aligned with the MSD were termed a match.

Statistical analysis of EDNA mock database screening

The Pearson goodness of fit statistic (chi-square) with one degree of freedom and a 5% significance level was performed on all positive e-probe matches compared to the decoy e-probe matches to assess statistical difference as described in Blagden et al. (2016). Chi-square statistics were converted to p-values by using R-programing in Linux with significance value of 0.05. A significant difference indicates an elevated confidence that the pathogen is present in the queried dataset.

Total RNA extraction

Lyophilized plant viruses used as positive controls for *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV), belonging to the genera *Potexvirus, Tombusvirus,* and *Tobamovirus*, respectively, were obtained from Agdia (Agdia Inc., Elkhart, IN). A single vial of lyophilized virus was used for individual total RNA extraction followed by multiplex reverse transcriptase-polymerase chain reaction (RT-mPCR). Total RNA was obtained using RNeasy Plant mini kits (Qiagen, Valencia, CA) with modifications to the Quick-Start Protocol. Briefly, 1 ml of RLT buffer was combined with 10 µl β-mercaptoethanol (β-ME) and added to a single lyophilized virus vial, followed by vortexing until contents were reconstituted. Contents were divided between two Qiagen shredder columns and procedure continued following the manufacturer's recommended protocol with a final elution of 30 µl. This procedure was repeated for each lyophilized virus vial.

Complementary DNA (cDNA) preparation

Extracted RNAs from each lyophilized virus vial were used to generate cDNA libraries. These libraries were prepared using the following protocol. First, a reaction mix with a volume of 14.7 µl per sample was prepared containing 9.5 µl of diethyl dicarbonate (DEPC) water, 1 µl total deoxynucleotides (dNTPs) at 10 mM, 0.2 µl of random hexamer primers at 0.5 µg/µl, and 4

µl sample RNA. Each sample reaction mix was heated to 70°C +/- 3°C for 5 min and placed immediately on ice for 2 min. Next, to each sample 0.5 µl of RNasin Plus (Promega, Madison, WI), 4 µl 5X buffer M-MLV RT, and 0.8 µl 200 U/µl M-MLV RT enzyme was added for a final volume of 20 µl. Each sample was incubated for 90 min at 37°C +/- 2.5°C and proceeded directly to multiplex polymerase chain reaction.

Serial dilutions

Each individual virus (PepMV, PMMoV, and TBSV), obtained from lyophilized material, had cDNA concentrations verified on a NanoDrop (Thermo Scientific, Wilmington, DE) and were diluted to 1 ng/µl with molecular grade water (Hardy Diagnostics, Santa Maria, CA). Ten-fold serial dilutions of cDNA were prepared from 1 ng/µl to 1 fg/µl to test primer sensitivity.

Hot start multiplex-PCR (mPCR)

Hot start multiplex polymerase chain reaction (mPCR) samples were prepared in 25 µl total volume reactions to include a total cDNA volume of 4 µl. The mPCR master mix included 3.43 µl molecular grade water, 12.5 µl Hot Start Taq 2X master mix (New England BioLabs Inc., Ipswich, MA), 0.88 µl magnesium chloride at 50 mM, 0.1 µl forward and 0.1 µl reverse *Tombusvirus* primers at 25 µM each, 1 µl forward and 1 µl reverse *Tobamovirus* primers at 25 µM each, and 1 µl forward and 1 µl reverse *Potexvirus* primers at 25 µM each. The mPCR cycling conditions were as follows: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 52°C for 80 sec, and 72°C for 1 min, with a final incubation of 72°C for 2 min. All mPCR reactions were carried out on a TProfessonal (Biometra, Göttingen, Germany).

Recovering plant waterborne viruses from varying volumes of water with polyethylene glycol (PEG)

Three vials of each lyophilized virus PepMV, TBSV, and PMMoV, were reconstituted using 1 ml of reverse osmosis (RO) water. For each target virus, one vial was added to each of the three test water volumes of 249 ml, 499 ml, and 999 ml in an appropriately sized Erlenmeyer flask (250 ml, 500 ml, and 1,000 ml). A separate test was conducted by reconstituting lyophilized PepMV, TBSV, and PMMoV in 1 ml of RO water each and mixing the three virus species to 247 ml, 497 ml, and 997 ml in an Erlenmeyer flask for a total of 250 ml, 500 ml, and 1,000 ml, respectively. Viruses were tested individually and in mixtures for recovery at all three volumes. A 7% concentration of polyethylene glycol (PEG) (G-Biosciences, St. Louis, MO) with a molecular weight (mw) of 8,000 and density of 1.0845 g/ml (70°C) was used for each of the three volumes. Each volume of 250 ml, 500 ml, and 1,000 ml of water containing the viral sample and PEG was stirred for a minimum of 2 hr on ice. Following this cold incubation, a maximum of eight 30 ml Nalgene Oak Ridge High-Speed Centrifuge Tubes (ThermoScientific, Wilmington, DE) were used and reused per sample until all of the sample was processed. Samples were centrifuged at 10,000 rpm using the J-17 rotor while chilled at 4°C in a centrifuge (Beckman J2-21M/E, Brea, CA). After centrifugation, the water was decanted, leaving the pellet undisturbed. In one of the eight tubes, the pellet was resuspended with 1 ml of RLT buffer containing 10 µl β-mercaptoethanol (BME). This tube was vortexed for 15-20 sec and the contents decanted into subsequent tubes. Vortexing and decanting into subsequent tubes was repeated for all remaining seven tubes (Figure 1).

After centrifugation and pellet resuspension, the mixture in the final tube was used for RNA extraction using a Qiagen RNeasy Plant mini kit (Valencia, CA) following the manufacture's Quick-Start Protocol with modifications. Briefly, two Qiagen shredder columns were used to aliquot the resuspended pellet and centrifuged for 2 min at 14,000 rpm. The

supernatant was transferred, without disturbing the pellet, to two individual 2 ml microcentrifuge tubes, one for each shredder column. This process was repeated until all of the sample was filtered and collected in the 2 ml microcentrifuge tubes. The eluate from the shredder column was mixed with 0.5 volumes 100% EtOH and transferred to a single capture column. This was repeated until all eluate was processed through the single capture column. The remaining steps proceed according to the Qiagen RNeasy Plant mini kit protocol with a final elution volume of 30 µl.

Results

A total of 1,386, 635, and 1,209 e-probes were generated for *Potexvirus, Tombusvirus,* and *Tobamovirus*, respectively. The MSD had a total of 10k reads generated at varying lengths of approximately 500 ± 30 nt. The average abundance of each *Tobamovirus* viral genomic sequences was 0.14% with a total viral abundance of 3.76% (Table 2). The MSD was formatted and queried with the e-probe sets and decoys using the BLASTn algorithm followed by parsing at an e-value of 1×10^{-6} as describe in Stobbe et al. (2013). There were 343 matches in the viral spiked MSD with a p-value of 0.05 or lower identifying all 27 *Tobamoviruses* (Tables 1 and 3). The non-spiked MSD had a single match with Odontoglossum ringspot virus, a ssRNA (+) *Tobamovirus* (Table 3).

Lyophilized plant viruses, from reference positive controls had total RNA concentrations of 99.4 ng/µl for PepMV, 72.5 ng/µl for TBSV, and 62.5 ng/µl for PMMoV. The cDNA of each virus sample was diluted to 1 ng/µl with starting concentrations of 752 ng/µl for PepMV, 740.1 ng/µl for TBSV, and 689.9 ng/µl for PMMoV. Dilutions were done at 10-fold intervals from 1 ng/µl to 1 fg/µl. Detection of all three viruses in a multiplex was not observed. Both TBSV and PMMoV were detected in the multiplex with a sensitivity limit of 0.01 ng/µl (Figures 2, 3, and 4).

Dilution series of individual viruses following cDNA indicated a single reaction PCR sensitivity of 0.1 ng/µl for PepMV (Figure 5), TBSV (Figure 6), and PMMoV (Figure 7).

Three vials of lyophilized virus PepMV were reconstituted using 1 ml of water and distributed to volumes of 249 ml, 499 ml, and 999 ml were mixed with PEG 8000 at 7%. All three volumes of PepMV seeded in RO water were successfully detected (Table 4, Figures 8 and 9). Similar results were observed for TBSV and PMMoV (Table 4). For each volume and each experimental condition, no more than eight 30 ml centrifuge tubes were used and reused (Figure 1). In a multiplex test six vials of lyophilized PepMV, TBSV, and PMMoV were reconstituted in 1 ml of RO water each and added to 247 ml, 497 ml, and 997 ml in an Erlenmeyer flask for total volumes of 250 ml, 500 ml, and 1,000 ml, respectively. The multiplex test did not achieve consistent detection of all three viruses (Figure 10 and 11). Only TBSV and PMMoV were successfully detected at all volumes; however, PepMV was only detected once in the 1,000 ml RO water seeded replicas.

Discussion

The research of waterborne agricultural viruses remains largely unexplored. As a result, there are no established tools or protocols for researchers or diagnostic laboratories. The bioinformatic pipeline EDNA and NGS simulator MetaSim provide preliminary and theoretical data on plant waterborne virus detection [4, 6]. To simulate plant viral dilutions, which would be observed when sampling water sources, the primary populating metagenomic data was obtained from a study of a 100-year agriculture field [20]. The large metagenomic dataset overpopulates the MSD when combined with 27 Tobamoviruses, thereby limiting total viral sequences of each plant virus (Tables 2 and 3). The successful recovery of all 27 Tobamoviruses at abundance values at or below 0.14% suggest that plant viruses can be detected from water even at very low concentrations. Additionally, the identification of the unreported plant virus Odontoglossum

ringspot virus in the metagenomic data suggest EDNA can assist traditional molecular based diagnostics; however, statistically there was no significance. Regardless, the bioinformatic analysis by using e-probes to screen for multiple viruses in a single assay provides promising screening to aid traditional analysis of waterborne virus discovery.

Prior to developing a standardized method for detection of plant waterborne viruses, water samples were gathered from a river located 25 miles south of Oklahoma State University campus. These samples were processed and amplified using previously developed primers as [22,]. Sequencing of the PCR product yielded products of 555 bp and 525 bp with no significant results when queried against the NCBI nr/nt database. However, by using the same EDNA e-probes used to screen MSD datasets (Tables 1, 2, and 3), there were identified viral signature sequences for three Potexviruses and two Tobamoviruses. The viral signature sequences matched with Potato aucuba mosaic virus, Schlumbergera virus X, and Narcissus mosaic virus all belonging to the genus Potexvirus, and Bell pepper mottle virus and Cactus mild mottle virus belonging to the genus Tobamovirus (Table 5). The findings suggest EDNA's bioinformatic analysis could identify unknowns obtained through traditional nucleic acid based analysis and provide relevant information for diagnostics and research.

The use of lyophilized plant viruses PepMV, TBSV, and PMMoV provided adequate positive controls for experimentation purposes. Personal communications with Agdia indicated that lyophilized viruses sold by them to be used as positive controls come from infected plant tissues and can remain infectious after reconstitution. Viruses obtained in this way more closely mimic environmental sampling where host (plant tissue) nucleic acid will also be present in water versus a purified virus sample. By reconstituting lyophilized viruses in RO water and performing nucleic acid extractions, this represented what might be observed during environmental water sampling and testing. The original plant-based primers could be tested in this way to ensure they

worked well as demonstrated when using RNA obtained directly from infected plant material (data not shown).

The initial primers obtained from literature for use in screening environmental water samples for the presence of waterborne viruses cannot be adapted for use in a multiplex assay. It was necessary to use novel primer sets developed in-house for multiplex assays. After testing previously developed plant-based primers for broad detection of *Potexvirus*, *Tombusvirus*, and *Tobamovirus*, using *Pepino mosaic virus* (PepMV), T*omato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV), it was evident that the multiplex requires additional optimization. Initial mPCR experiments did not amplify the target PepMV. Further investigation revealed that the recommended forward primer was labeled incorrectly. Using the proper forward and reverse primers did amplify each virus; however, inconsistencies remained with the mPCR. These inconsistencies are likely the result of thermodynamics where each primer set has an optimal temperature slightly different than the others [21]. Another possibility is that the primers are interacting with each another or binding to non-target regions [21]. While *in silico* data indicates primers should work optimally, applying them to environmental samples produced inconsistent results. In order for these primers sets to be used in a multiplex it is necessary for them to undergo further optimization. Alternatively, considering the likelihood that any plant waterborne virus detection effort will need to be able to detect low titers of the target, these assays could successfully be used as singleplex assays, where some of the complexities of a competitive PCR regime are minimized.

Using a capture cartridge to adsorb viruses from a water source provides researchers and/or diagnostic laboratories the ability to screen large volumes of water without needing to retain large volumes of water. Processing samples, which contain plant viruses, following elution from a capture cartridge is critical to ensure recovery. This work established an initial protocol that could be used by diagnostic laboratories to process samples following elution in a quick and

inexpensive method. Testing detection of lyophilized viruses from RO water by using 7% PEG provided conditions that lacked potential inhibitors [15]. Results obtained from this data indicate that viruses can be detected using volumes up to 1,000 ml. This is shown for PepMV (Figure 8), TBSV (Figure 8), and PMMoV (Figure 9). Detecting all three viruses from 250 ml, 500 ml, and 1,000 ml was unsuccessful. This would indicate that the primer sets for each virus should be used to screen in a single reaction PCR assay versus a multiplex.

The results support that an environmental sample that has been obtained and brought to the laboratory can be processed with a quick and inexpensive protocol as outlined in this paper. The above method fills a technology gap as there are currently no routine diagnostic assays for such an application. This protocol also provides a first attempt at establishing a diagnostics and research tool that can be readily adapted by laboratories at minimal cost, labor, and without the need for specialized equipment.

REFERENCES

1. Maloy OC. 2005. Plant disease management. The plant health instructor. APS. DOI: 10.1094/PHI-I-2005-0202-01

2. Hong CX, Moorman GW. 2005. Plant pathogens in irrigation water: challenges and opportunities. Critical Reviews in Plant Sciences. 24(3):189-208

3. Dolnik O, Stevermann L, Kolesnikova L, Becker S. 2015. Marburg virus inclusions: a virus-induced microcompartment and interface to multivesicular bodies and the late endosomal compartment. European J Cell Biol. 94(7–9):323-331

4. Stobbe AH, Daniels J, Espindola AS, Verma R, Melcher U, Ochoa-Corona F, Garzon C, Fletcher J, Schneider W. 2013. E-probe Diagnostic Nucleic acid Analysis (EDNA): A theoretical approach for handling of next generation sequencing data for diagnostics. J Microb Meth. 94(3):356-366

5. Richter DC, Ott F, Auch AF, Schmid R, Huson DH. 2008. MetaSim - Sequencing simulator for genomics and metagenomics, PLoS ONE 3(10): e3373. doi:10.1371/journal.pone.0003373

6. Stobbe AH, Schneider WL, Hoyt PR, Melcher U. 2008. Screening metagenomic data for viruses using the e-probe diagnostic nucleic acid assay. Phytopathology. 104(10):1125-1129

7. Blagden T, Schneider W, Melcher U, Daniels J, Fletcher J. 2016. Adaptation and validation of e-probe diagnostic nucleic acid analysis for detection of *Escherichia coli* O157:H7 in metagenomic data from complex food matrices. J Food Protection. 79(4):574-581

8. Lambertini E, Spencer SK, Bertz PD, Loge FJ, Kieke BA, Borchardt MA. 2008. Concentration of Enteroviruses, Adenoviruses, and Noroviruses from drinking water by use of glass wool filters. Appl Environ Microb. 74(10):2990-2996

9. Albinana-Gimenez N, Clemente-Casares P, Calgua B, Huguet JM, Courtois S, Girones R. 2009. Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. J Virol Methods. 158(1–2):104-109

10. Millen HT, Gonnering JC, Berg RK, Spencer SK, Jokela WE, Pearce JM, Borchardt JS, Borchardt MA. 2012. Glass wool filters for concentrating waterborne viruses and agricultural zoonotic pathogens. JoVE. (61):3930

11. van Heerden J, Ehlers MM, Heim A, Grabow WOK. 2005. Prevalence, quantification and typing of adenoviruses detected in river and treated drinking water in South Africa. J Appl Microbiol. 99(2):234-242

12. Katzenelson E, Fattal B, Hostovesky T. 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl Environ Microb. 32(4):638-639

13. Safferman RS, Rohr ME, Goyke T. 1988. Assessment of recovery efficiency of beef extract reagents for concentrating viruses from municipal wastewater sludge solids by the organic flocculation procedure. Appl Environ Microb. 54(2):309-316

14. Berman D, Berg G, Safferman RS. 1981. A method for recovering viruses from sludges. J Virol Methods. 3(5):283-291

15. Hurst CJ, Farrah SR, Gerba CP, Melnick JL. 1978. Development of quantitative methods for the detection of enteroviruses in sewage sludges during activation and following land disposal. Appl Environ Microb. 36(1):81-89

16. Colombet J, Robin A, Lavie L, Bettarel Y, Cauchie HM, Sime-Ngando T. 2007. Virioplankton 'pegylation': Use of PEG (polyethylene glycol) to concentrate and purify viruses in pelagic ecosystems. J Microbiol Meth. 71(3):212-219

17. Atha DH, Ingham KC. 1981. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. J Biol Chem. 256(23):12108-12117

18. Lewis GD, Metcalf TG. 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl Environ Microb. 54(8):1983-1988

19. Rock C, Alum A, Abbaszadegan M. 2010. PCR inhibitor levels in concentrates of biosolid samples predicted by a new method based on excitation-emission matrix spectroscopy. Appl Environ Microb. 2010, 76(24):8102-8109

20. Wang BZ, Zhang CX, Liu JL, Zeng XW, Li FR, Wu YC, Lin XG, Xiong ZQ, Xu J, Jia ZJ. 2012. Microbial community changes along a land-use gradient of desert soil origin. pedosphere. 22(5):593-603

21. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. 1997. Multiplex PCR: Critical parameters and step-by-step protocol. Research Reports. BioTechniques. 23:504-511

22. van der Vlugt RAA, Berendsen M. 2002. Development of a General Potexvirus Detection Method. European Journal of Plant Pathology 2002, 108(4):367-371

TABLES

Table 1. Electronic probes were generated from genomes of *Potexvirus*, *Tombusvirus*, and *Tobamovirus*. List includes individual viruses with the National Center for Biotechnology Information accession number used.

	Virus	Accession number	Virus	Accession number
Potexvirus	Tamus red mosaic virus	NC_016003	Pepino mosaic virus	NC_004067
	Allium virus X	NC_012211	Scallion virus X	NC_003400
	Schlumbergera virus X	NC_011659	Plantago asiatica mosaic virus	NC_003849
	Hosta virus X	NC_01154	White clover mosaic virus	NC_003820
	Lettuce virus X	NC_010832	Strawberry mild yellow edge virus	NC_003794
	Asparagus virus 3	NC_010416	Cactus virus X	NC_002815
	Phaius virus X	NC_010295	Opuntia virus X	NC_006060
	Malva mosaic virus	NC_008251	Zygocactus virus X	NC_006059
	Alternanthera mosaic virus	NC_007731	Tulip virus X	NC_004322
	Nerine virus X	NC_007679	Potato aucuba mosaic virus	NC_003632
	Alstroemeria virus X	NC_007408	Bamboo mosaic virus	NC_001642
	Potato virus X	NC_011620	Cassava common mosaic virus	NC_001658
	Lily virus X	NC_007192	Papaya mosaic virus	NC_001748
	Cymbidium mosaic virus	NC_001812	Narcissus mosaic virus	NC_001441
	Mint virus X	NC_006948	Clover yellow mosaic virus	NC_001753
	Hydrangea ringspot virus	NC_006943	Foxtail mosaic virus	NC_001483
Tombusvirus	Artichoke mottled crinkle virus	NC_001339	Grapevine Algerian latent virus	NC_011535
	Carnation Italian ringspot virus	NC_003500	Eggplant mottled crinkle virus	NC_023339
	Cucumber Bulgarian latent virus	NC_004725	Pelargonium necrotic spot virus	NC_005285
	Cucumber necrosis virus	NC_001469	Pothos latent virus	NC_000939
	Cymbidium ringspot virus	NC_003532	Milk vetch dwarf C2 alphasatellite	NC_003639
	Eggplant mottled crinkle virus	NC_007983	Carnation mottle virus	NC_001265
	Tomato bushy stunt virus	NC_001554	Tobacco necrosis virus A	NC_001777
	Maize necrotic streak virus	NC_007729	Panicum mosaic virus	NC_002598
	Moroccan pepper virus	NC_020073	Maize chlorotic mottle virus	NC_003627
Tobamovirus	Clitoria yellow mottle virus	NC_016519	Pepper mild mottle virus	NC_003630
	Bell pepper mottle virus	NC_009642	Kyuri green mottle mosaic virus	NC_003610
	Wasabi mottle virus	NC_003355	Tobacco mosaic virus	NC_001367
	Rattail cactus necrosis associated virus	NC_016442	Ribgrass mosaic virus	NC_002792.2
	Passion fruit mosaic virus	NC_015552	Paprika mild mottle virus	NC_004106
	Cactus mild mottle virus	NC_011803	Tomato mosaic virus	NC_002692
	Brugmansia mild mottle virus	NC_010944	Youcai mosaic virus	NC_004422
	Rehmannia mosaic virus	NC_009041	Obuda pepper virus	NC_003852
	Maracuja mosaic virus	NC_008716	Cucumber fruit mottle mosaic virus	NC_002633
	Cucumber mottle virus	NC_008614	Turnip vein-clearing virus	NC_001873
	Streptocarpus flower break virus	NC_008365	Cucumber green mottle mosaic virus	NC_001801
	Hibiscus latent Singapore virus	NC_008310	Tobacco mild green mosaic virus	NC_001556
	Odontoglossum ringspot virus	NC_001728	Frangipani mosaic virus	NC_014546
	Zucchini green mottle mosaic virus	NC_003878		

Table 2. A total of 27 Tobamoviruses were used in a simulated 454 pyrosequencing run as pathogen targets for mock database generation using MetaSim. The *in silico* detection of viruses using e-probes is indicated with a (+).

Virus	Total reads in simulation out of 10k	Percent of total reads in simulation	Positive/negative detection
Tobacco mosaic virus	12	0.12	+
Tobacco mild green mosaic virus	11	0.11	+
Odontoglossum ringspot virus	16	0.16	+
Cucumber green mottle mosaic virus	15	0.15	+
Turnip vein clearing virus	17	0.17	+
Cucumber fruit mottle mosaic virus	17	0.17	+
Tomato mosaic virus	13	0.13	+
Ribgrass mosaic virus	16	0.16	+
Wasabi mottle virus	14	0.14	+
Kyuri green mottle mosaic virus	10	0.1	+
Pepper mild mottle virus	22	0.22	+
Obuda pepper virus	11	0.11	+
Zucchini green mottle mosaic virus	11	0.11	+
Paprika mild mottle virus	17	0.17	+
Youcai mosaic virus	16	0.16	+
Hibiscus latent Singapore virus	15	0.15	+
Streptocarpus flowaer break virus	3	0.03	+
Cucumber mottle virus	15	0.15	+
Maracuja mosaic virus	12	0.12	+
Rehmannia mosaic virus	10	0.1	+
Bell pepper mottle virus	21	0.21	+
Brugmansia mild mottle virus	15	0.15	+
Cactus mild mottle virus	12	0.12	+
Frangipani mosaic virus	14	0.14	+
Passion fruit mosaic virus	12	0.12	+
Rattail cactusnecrosis associated virus	13	0.13	+
Clitoria yellow mottle virus	16	0.16	+
Average viral load		0.139259259	
Total virus load		3.76%	

+ indicates a p-value of 0.05 or less

Table 3. MetaSim was used to simulate 454 pyrosequencing runs that were analyzed *in silico* with e-probes generated for virus genera *Potexvirus*, *Tombusvirus*, and *Tobamovirus*.

MetaSim next generation sequencing simulator	Total # of *Tobamovirus* genomes	Avgerage individual virus load	Total viral load	Total # of matches	Pathogenic virus present yes/no
MetaSim (spiked dataset)	27	0.14%	3.76%	343	Yes
NGS simulator (non-spiked dataset)	Unknown	Unknown	Unknown	1	Yes
Data was obtained in 2012					

Table 4. Summary of reverse-transcriptase multiplex polymerase chain reaction (RT-mPCR) detection assays from lyophilized viruses reconstituted in different volumes of reverse osmosis water. Total numbers of positive detections out of three replicates for individual viruses. Total number of positive detections for all three viruses (PepMV, TBSV, and PMMoV) out of six replications.

Virus	250 ml	500 ml	1,000 ml
***Pepino mosaic virus* (PepMV)**	3/3	3/3	3/3
***Tomato bushy stunt virus* (TBSV)**	3/3	3/3	2/3
***Pepper mild mottle virus* (PMMoV)**	3/3	3/3	3/3
PepMV + TBSV + PMMoV	0/6	0/6	2/6

Table 5. Electronic probes (e-probes) were developed for bioinformatic detection of waterborne plant viruses in the genus Potexvirus, Tombusvirus, and Tobamovirus. The unique e-probe that matched with plant viral signature sequences from environmental samples is listed with NCBI accession number.

E-probe	Virus (Species)	Genus	Accession #
CAAATTGCCGTTGAAGTAAC	Potato aucuba mosaic potexvirus	Potexvirus	S73580
AGGAGGGTAGGATTTCCATC	Schlumbergera virus X	Potexvirus	AY366207
TTTCATAGTGCAGAAAGCCA	Narcissus mosaic virus	Potexvirus	D13747
CTCTCGTCCGCTTGGGCTGA	Bell pepper mottle virus	Tobamovirus	DQ355023
AGAAGATGCGATTTCTCCAA	Cactus mild mottle virus	Tobamovirus	EU043335
GCCGACTCGGTGGGGTCTTG	Cactus mild mottle virus	Tobamovirus	EU043335

FIGURES

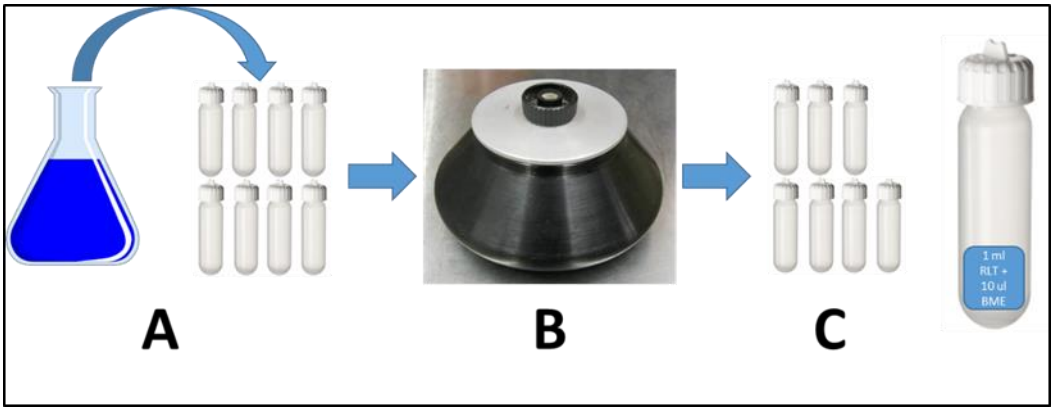


Figure 1. Reverse osmosis (RO) water seeded with individual viruses or a mixture of viruses *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), or *Pepper mild mottle virus* (PMMoV) at varying volumes were brought to a 7% polyethylene glycol (PEG) concentration and distributed to eight 30 ml centrifuge tube (A). Tubes were centrifuged for 30 min at 10,000 rpm at 4°C (B). Liquid was decanted and discarded. Then 1 ml of RLT buffer + 10 µl of beta mercaptoethanol was added to a single tube, vortexed and used to resuspend the pellet in subsequent tubes (C).

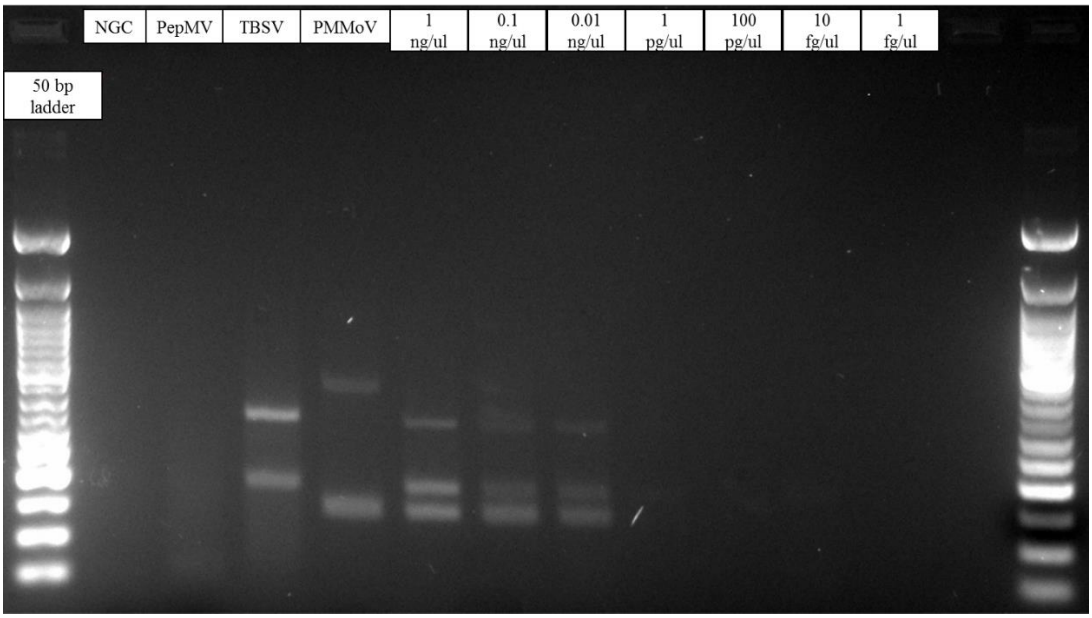


Figure 2. Sensitivity assay to determine detection limits of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) using multiplex-PCR in all lanes. NGC – negative control.

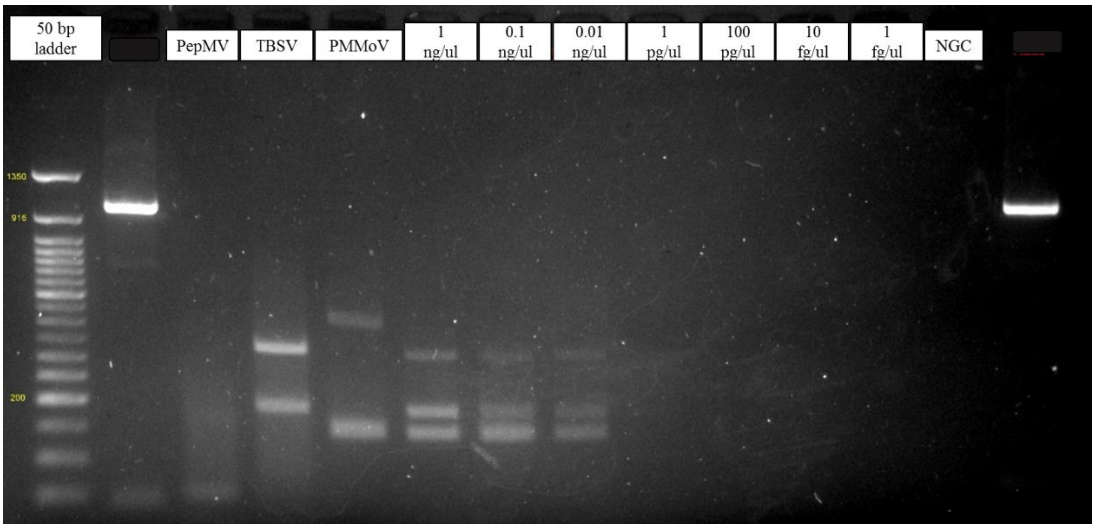


Figure 3. Sensitivity assay to determine detection limits of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) using multiple-PCR. NGC – negative control.

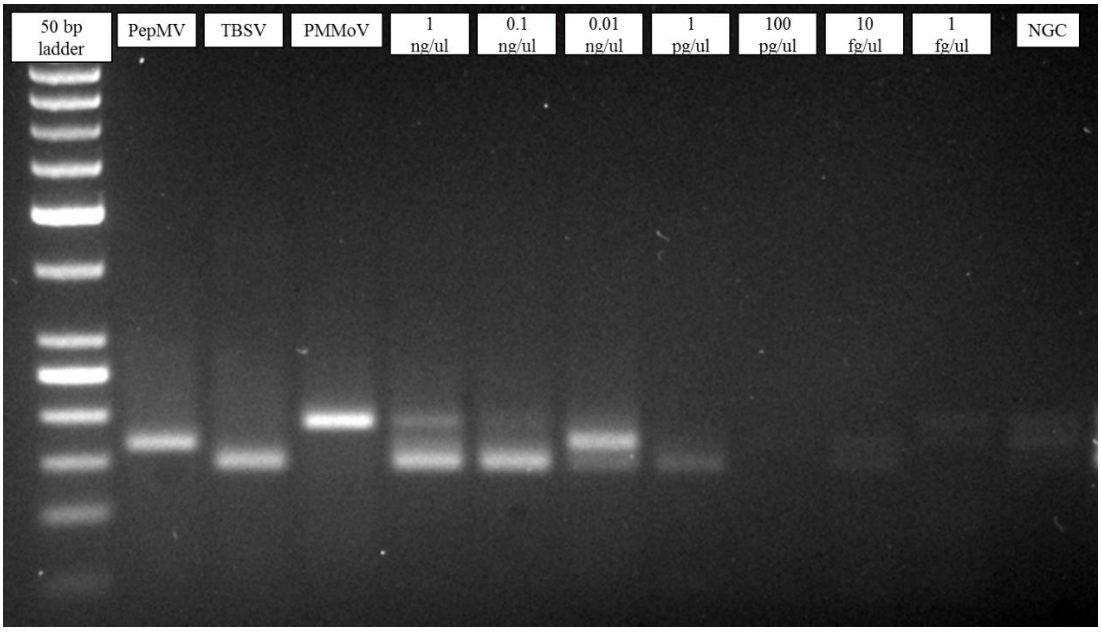


Figure 4. Results of a sensitivity assay to determine detection limits of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) using multiple-PCR. Consistent detection of all three viruses was not observed. NGC – negative control.

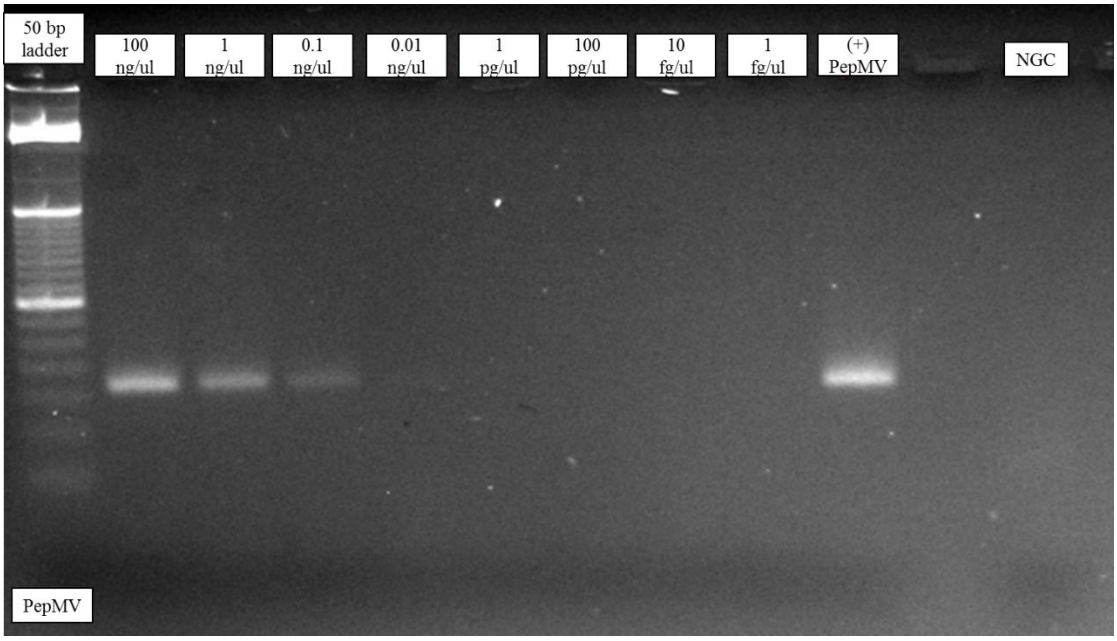


Figure 5. Sensitivity assay for a multiplex primer set used for broad detection of *Potexvirus*. *Pepino mosaic virus* was used as the model virus for the sensitivity assay with 0.1 ng/µl being the limit of detection. NGC – negative control.

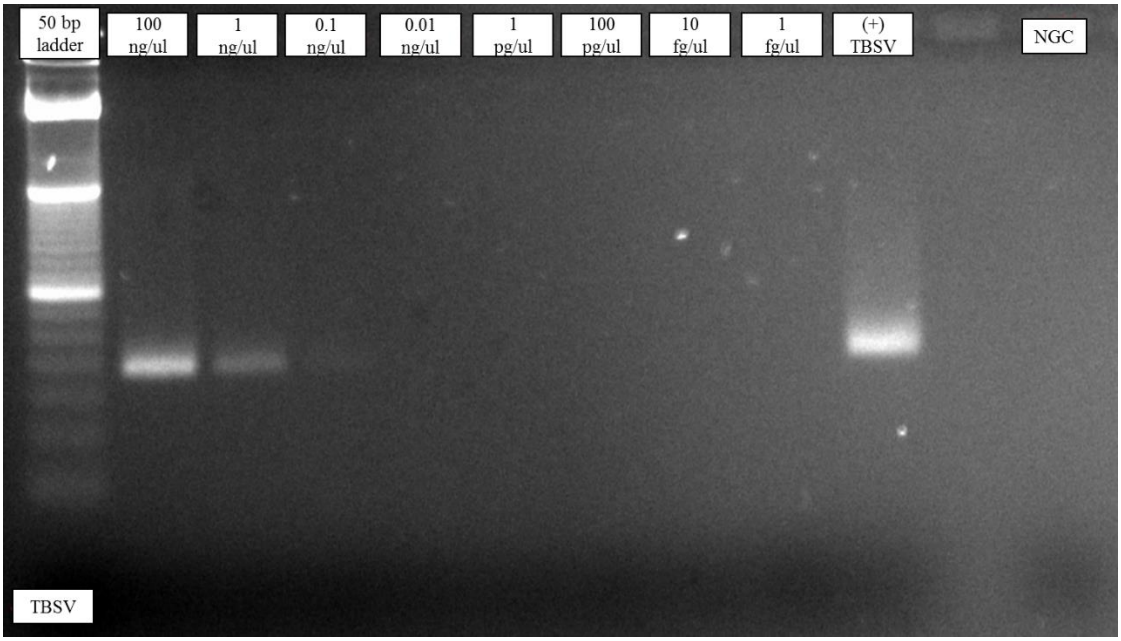


Figure 6. Sensitivity assay for a multiplex primer set used for broad detection of *Tombusvirus*. *Tomato bushy stunt virus* was used as the model virus for the sensitivity assay with 0.1 ng/µl being the limit of detection. NGC – negative control.

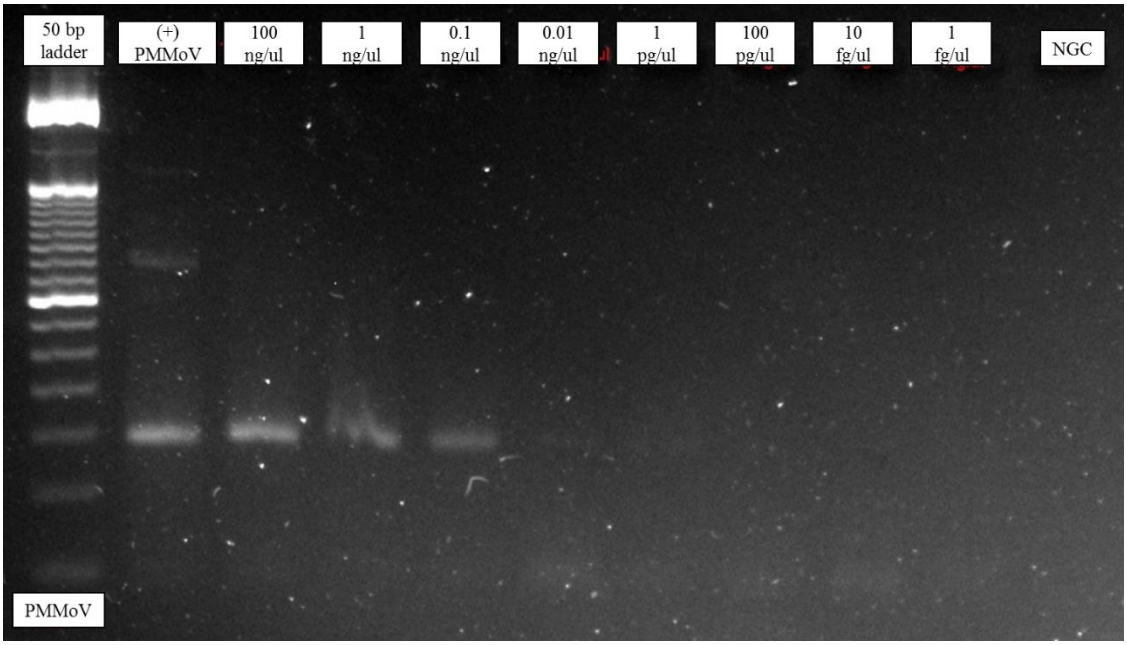


Figure 7. Sensitivity assay for a multiplex primer set used for broad detection of *Tobamovirus*. *Pepper mild mottle virus* was used as the model virus for the sensitivity assay with 0.1 ng/µl being the limit of detection. NGC – negative control.

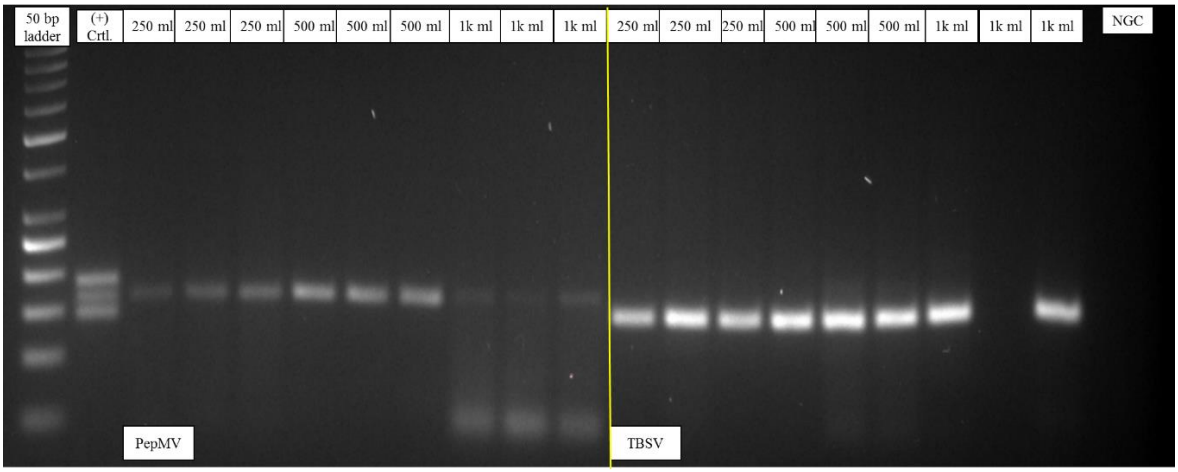


Figure 8. Recovery and detection on individual viruses from reverse osmosis (RO) water seeded with *Pepino mosaic virus* (PepMV) or *Tomato bushy stunt virus* (TBSV) at total volumes of 250 ml, 500 ml, and 1,000 ml each. NGC – negative control.

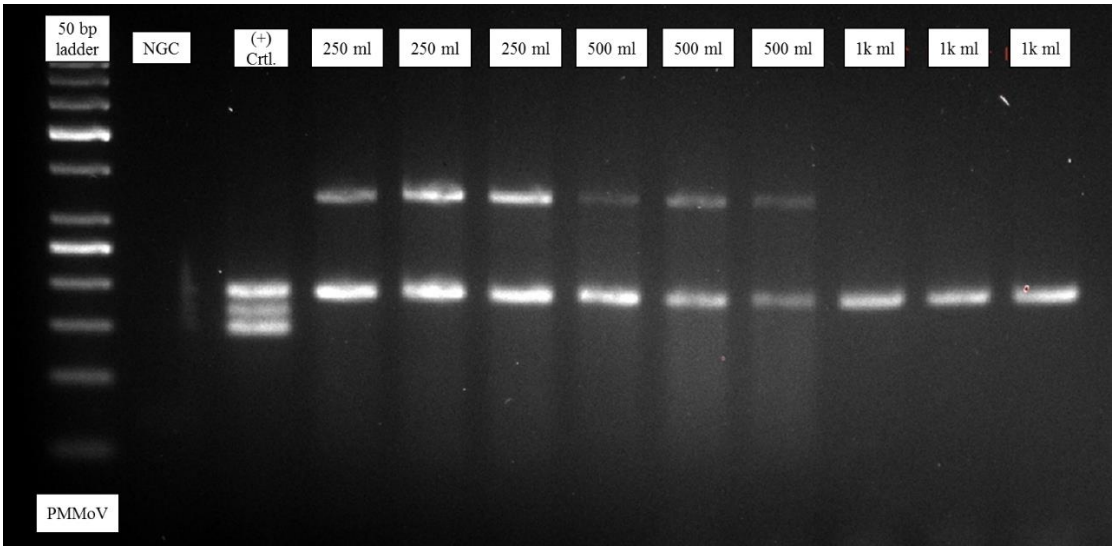


Figure 9. Recovery and detection of *Tomato bushy stunt virus* (TBSV) from seeded reverse osmosis (RO) water at total volumes of 250 ml, 500 ml, and 1,000 ml each. NGC – negative control.

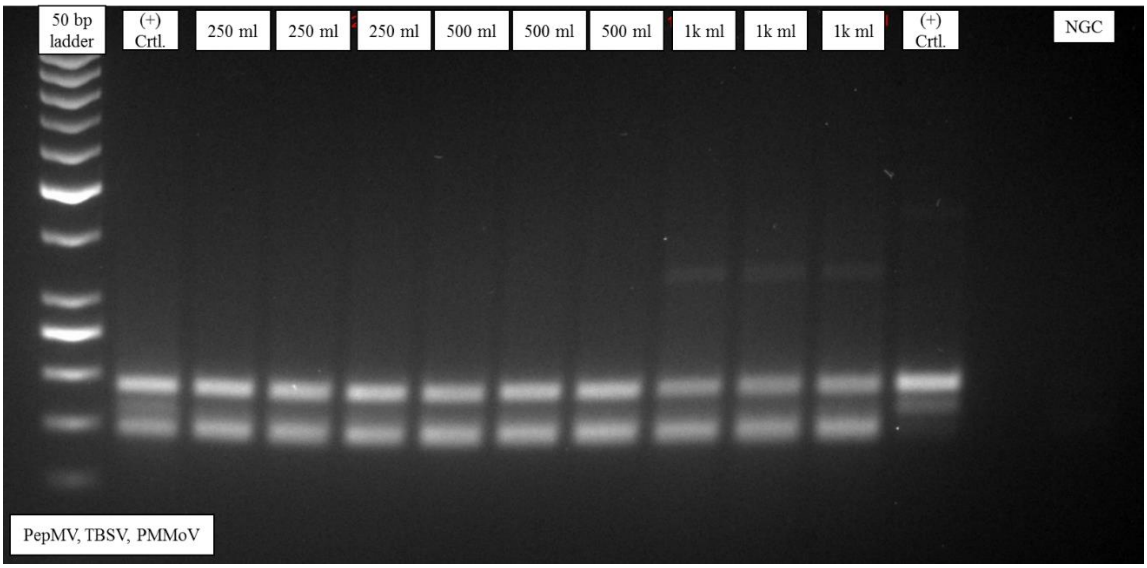


Figure 10. Reverse osmosis (RO) water seed with *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) at total volumes of 250 ml, 500 ml, and 1,000 ml did not yield detection of all three viruses. NGC – negative control.

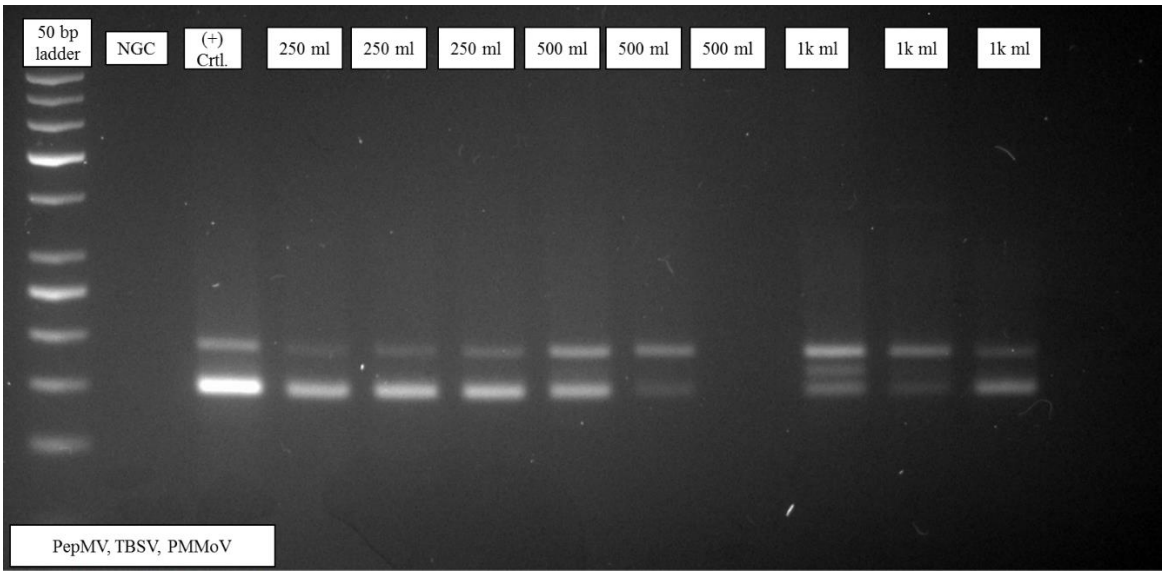


Figure 11. Reverse osmosis (RO) water seeded with *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) at total volumes of 250 ml, 500 ml, and 1,000 ml did not yield detection of all three viruses. NGC – negative control.

CHAPTER IV

DEVELOPMENT OF A WATER SAMPLING DEVICE FOR CAPTURING WATERBORNE PLANT PATHOGENIC VIRUSES

Abstract

The discovery of waterborne plant and animal pathogens is not novel. Considerable research has been devoted to studying and detecting waterborne human viruses in potable water. In contrast, the study of waterborne plant viruses has been limited. Currently, there is no tool used by diagnosticians or researchers for studying waterborne plant viruses in irrigation waters. This project seeks to address this gap. Successful development of a polyvinyl chloride (PVC) device that is scalable, robust, lightweight, field deployable, and inexpensive was completed. Three individual waterborne viruses include *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) were successfully recovered from large volume seeded dilutions. This research provides the initial tools and methods for diagnostics and research laboratories to screen and study agricultural irrigation water for the presence/absence of waterborne plant viruses.

Introduction

Considering the ~126 Bgal of water used daily by the agriculture sector, there is a high probability of unintentional waterborne plant pathogen introduction to crops. Water is well documented as a source of enteric pathogens [1, 2-4]. In addition to enteric pathogens many plant pathogens have been discovered in water [5, 6, 7]. A major challenge for plant-based diagnostics in detecting waterborne pathogens in water is the dilution factor and the limited tools for high volume water sampling. In addition to dilution and volume, suspended sediment in agricultural water resources can also negatively influence sampling as well as pH, location, personnel, equipment size, and the ability for reproducible and consistent data. For this reason, there is little research conducted on methods developed in capturing and analyzing agriculture waterborne plant pathogens.

Current filters used in concentrating viruses from water fall into two main categories: electronegative and electropositive. Electronegative filters include Millipore HA (nitrocellulose), Filterite filter, and Whatman cellulose filter. Along with a negative charge, these filters tend to have a very small pore size causing them to clog quickly, which limits the total volume of water to be sampled. Additionally, electronegative filters require amendments with salts like NaCl, $MgCl_2$, or Al^{3+} for optimal virus recovery. With viruses having an electronegative charge in the outer protein coat, the addition of salts prior to filtration promotes the hydrophobic interactions and was found to limit the electrostatic attractions between the virus and filter media [8]. In addition to promoting hydrophobic interactions, the addition of salts also lowers the pH and promotes flocculation. The forming of flocs or larger clumps of viruses allows for enhanced recovery by the electronegatively charged filters, as these lager clumps are mechanically captured. Electropositive filters are capable of sampling larger volumes of water with higher turbidity levels, which is important when sampling water sources used in agriculture [9-12]. In addition to greater sampling volumes, these filters are also capable of capturing different

waterborne pathogens including viruses, bacteria, and parasites [11, 13]. The ability to trap a multitude of plant pathogens provides a greater utility for screening and offers additional cost-savings. As reported by Millen et al. (2012), oiled sodocalcic glass wool or unfaced fiberglass insulation was able to filter 1000+ liters of water and was effective for recovering waterborne enteroviruses, *Salmonella enterica*, *Cryptosporidium parvum*, and avian influenza virus [11, 14]. Similar findings are also reported for the 3M Zeta Plus 1MDS [13]. Glass wool electropositive filters offer a promising and cost-efficient alternative to the 3M Zeta Plus 1MDS and the NanoCeram. Johns Manville (Denver, CO) R-19 fiberglass attic blown-in insulation can be purchased at $29.68 for 103 ft^2 (2016 pricing). At this price, numerous filters can be assembled very little cost. Several studies have used glass wool for waterborne human virus capture, indicating its promising use for agriculture [9, 10, 15].

To understand virus adsorption to glass wool, there must first be an understanding of the viral coat protein (CP) and the charge it holds. All viruses have an electronegative charge surrounding their CP along with hydrophobic properties. This negative charge is influenced by pH [16], salts [8], and can be manipulated outside the aquatic environment by an electrostatic particle collector [17]. When a virus CP transitions from negative to positive, or reaches a state that is neither negative nor positive, this state is considered the isoelectric point (pI). When pH is near or above the virus pI, adsorption to a positively charged surface is enhanced [8, 16, 18]. Knowing the pI is an important component to capturing plant waterborne viruses. In a paper by Michen and Graule, (2010), they found the pI of plant viruses to range from 3.6 for *Turnip yellow mosaic virus* to 6.3 for *Belladonna mottle virus* [19]. Considering the pI for plant viruses is below 7.0, sampling above or near 7.0 would be optimal for electrostatic and hydrophobic attraction to the positively charged glass wool fibers.

Once virus has adsorbed to the glass wool fiber, it is necessary to create an environment that favors the desorption process. The use of beef extract as an elution media was first described

by Berg et al. (1971) [20]. Berg and colleagues screened multiple eluants in an effort to make conditions favorable for desorption from a 0.45 µm porous membrane and found that beef extract was the common ingredient among the working eluants [20]. This finding was also confirmed and shown that it does not allow reabsorption, which is critical when recovering viruses from glass wool that favors electrostatic attraction [21]. The overall concentration of beef extract has been tested from 3% to 15% with a 3% concentration described as an optimal concentration [22]. As recommended by the United State Environmental Protection Agency (USEPA) and shown in recent studies, elution from glass wool using 3% beef extract and 0.05 M glycine [23] provided efficient recoveries [11, 24].

Water for human consumption requires testing by certified laboratories, and ideally, a certified plant disease diagnostic lab should have tools and protocols available for screening various quantities of water used in agricultural settings. Such tools and protocols do not yet exist. What is needed is a device and protocol that can overcome current challenges of cost, equipment, portability, and large sample volume. This research seeks to overcome these challenges by developing a water sampling device for use in agriculture. The device must be economical, reusable, robust, field deployable, and user friendly so that the learning curve is kept to a minimum. The device must be able to process water with a lot of suspended sediment, as observed in ponds, rivers, streams, and during floods. The device must also not retain a lot of water, as an ideal tool will remove microorganisms and allow sampled water to return to the original source. Additionally, it will be important to make the device scalable so that users can construct and replicate it to meet their respective sampling needs.

The objective of this study is to address the need of a robust, inexpensive, and field deployable sampling device for use in sampling agricultural water resources. The present study expands on previous studies [11, 25-27] to provide an entire system, which can be purchased and

assembled, at minimal expense and with minimal equipment. This will be done by assembling multiple devices from PVC and development of a protocol for various volumes of water analysis.

Materials and Methods

Construction of a device for waterborne virus capture

Multiple polyvinyl chloride (PVC) devices derived from previous work by Millen et al. (2013) were constructed for aquatic sampling and capturing of waterborne viruses [11]. Briefly, 1.905 cm PVC pipes were used as the main lines throughout the device. An in-line pre-filter 25.4 cm sediment removal canister (Whirlpool, Benton Harbor, MI) was used prior to the cartridge housing the glass wool. An in-line hose with attached peristaltic pump was added, as needed, to adjust pH (6.1-7.5) following the pre-filter. Several 5.08 cm x 15.24 cm PVC cartridges were constructed to house the glass wool (Figures 1 and 2). All PVC fittings were chemically welded with Oatey PVC primer and cement (Cleveland, OH).

Glass wool preparation for virus capture

Preparation of glass wool was performed as described by Lambertini et al. (2008) [9]. Briefly, Johns Manville Pro R60 fiberglass blown-in insulation (Denver, CO) was rinsed for 15 min with reverse osmosis (RO) water. Following rinse, 1 M HCl was used to soak the glass wool for 15 min, then drained. A second soaking and draining was done using 1 M NaOH for 15 min followed by an RO rinse until glass wool fibers were at pH of 7. Glass wool was stored at 4°C in phosphate-buffered saline (PBS) until use. Glass wool was packed into the 5.08 cm x 15.24 cm PVC capture cartridges. All PVC threads were covered in thread seal tape (PVC tape). All components, except the 5.08 cm x 15.24 cm PVC capture cartridges, were assembled just prior to sampling.

To test for virus capture and recovery from glass wool, lyophilized *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) were obtained from Agdia (Elkhart, IN) and reconstituted in three separate 5 gal buckets of RO water. Seeded water containing the individual virus was pumped through the device housing the glass wool cartridge. A total of three separate devices were constructed for this purpose.

Capture cartridge elution

Elution of virions from glass wool was done according to Lambertini et al. (2008) [9] with modifications. Briefly, glass wool was removed from the cartridge and placed into a 500 ml beaker. A 3% beef extract (wt/vol) containing 0.05 M glycine (pH 10) elution buffer was prepared by dissolving 60 g beef extract powder and 7.5 g of glycine in 1.5 L of RO water. For adjusting the pH, 5 M NaOH was added until the elution buffer had a final volume of 2 L with a pH of 10. A total volume of 180 ml of elution buffer was added to the beaker containing the glass wool and placed on a shaker table at 150 rpm for 15 min. Following the 15 min shake, all liquid was decanted into a separate 500 ml Erlenmeyer flask. Autoclaved cheese cloth and a large funnel were used to squeeze any remaining liquid from within the glass wool. All liquid was processed as described below.

Polyethylene glycol (PEG) virus concentration from elution buffer

Polyethylene glycol (PEG) (G-Biosciences, St. Louis, MO) with a molecular weight (mw) of 8000 and density of 1.0845 g/ml (70°C) was added to each sample to obtain a 7% concentration of PEG to sample volume. Each sample of elution buffer containing the viral sample and PEG was stirred for a minimum of 2 hr on ice. Following this incubation, a maximum of eight 30 ml Nalgene Oak Ridge High-Speed Centrifuge Tubes (ThermoScientific, Wilmington, DE) were used and reused until all of the sample is processed. Samples were centrifuged at 10,000 rpm using the J-17 rotor while chilled at 4°C in a Beckman J2-21M/E Centrifuge (Brea,

CA). After centrifugation, the eluate was decanted from each of the eight repetition, leaving the pellet undisturbed. In one of the eight tubes, the pellet was resuspended with 1 ml of RLT buffer containing 10 µl β-mercaptoethanol (BME). This tube was vortexed for 15-20 sec and the contents decanted into subsequent tubes. Vortexing and decanting into subsequent tubes was repeated for all remaining seven tubes (Figure 3).

Obtaining RNA for molecular analysis

Following centrifugation and pellet resuspension, the liquid in the final tube was processed using a Qiagen RNeasy plant mini kit (Valencia, CA) following the manufacture's Quick-Start Protocol with modifications. Briefly, two Qiagen shredder columns were used to aliquot the resuspended pellet and centrifuged for 2 min at 14,000 rpm. Eluate was transferred, without disrupting the pellet, to two individual 2 ml microcentrifuge tubes, one for each shredder column. This process was repeated until all of the sample was filtered and collected in the 2 ml microcentrifuge tubes. The eluate from the shredder column was mixed with 0.5 volumes 100% EtOH and transferred to a single capture column. This was repeated until all eluate was processed through the single capture column. Remaining steps proceed according to the Qiagen RNeasy Plant mini kit protocol with a final elution volume of 30 µl.

Complementary DNA (cDNA) preparation

Extracted RNAs from each sample was used to generate cDNA libraries. These libraries were prepared using the following protocol. First, a reaction mix with a volume of 14.7 µl per sample was prepared containing 9.5 µl of diethyl dicarbonate (DEPC) water, 1 µl total deoxynucleotides (dNTPs) at 10 mM, 0.2 µl of random hexamer primers at 0.5 µg/µl, and 4 µl RNA. Each sample reaction mix was heated to 70°C +/- 3°C for 5 min and placed immediately on ice for 2 min. Next, to each sample 0.5 µl of RNAsin Plus, 4 µl 5X buffer M-MLV RT, and 0.8 µl

200 U/µl M-MLV RT enzyme was added for a final volume of 20 µl. Each sample was incubated for 90 min at 37°C +/- 2.5°C.

Hot start RT-mPCR

The hot start mPCR, samples were prepared in 25 µl total volume reaction that included a total cDNA volume of 4 µl. The mPCR master mix included 3.43 µl molecular grade water, 12.5 µl Hot Start Taq 2X master mix (New England BioLabs Inc., Ipswich, MA), 0.88 µl magnesium chloride at 50 mM, 0.1 µl forward and 0.1 µl reverse *Tombusvirus* primers at 25 µM each, 1 µl forward and 1 µl reverse *Tobamovirus* primers at 25 µM each, and 1 µl forward and 1 µl reverse *Potexvirus* primers at 25 µM each forward and reverse primers. Primers for each virus were previously designed plant-based and unpublished. The mPCR cycling conditions were as follows: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 52°C for 80 sec, and 72°C for 1 min, with a final incubation of 72°C for 2 min. All mPCR reactions were carried out on a Biometra TProfessonal (Göttingen, Germany).

Environmental sampling of different aquatic ecosystems

Aquatic samples were obtained from different nurseries across the state of Oklahoma including Marcum's Nursery (35.349231:-97.548872), Myriad Garden (35.465075:-97.51969), Total Environment (35.635491:-97.640490), Deep Fork Tree Farm (35.656241:-97.331507), and Theta Pond (36.119974:-97.070730). Samples were obtained using PVC device sampler, described above, by filtering water for 30 min at each location. Once a sample was obtained, it was stored on ice or at 4°C for no longer than 24 hr. Elution of virions from glass wool cartridges and total RNA extraction were done as described above.

Results

Construction of a PVC device for water sampling of environmental samples was completed (Figures 1 and 2). The basic construction of the sampling device involves an inlet containing a valve that allows the user to adjust flow. A pre-filter that removes suspended sediment to prevent clogging of capture cartridge. An optional peristaltic pump to add an acid or base allowing for pH adjustment to the water prior to its entering the capture cartridge. After the peristaltic pump is the capture cartridge housing electropositive glass wool used to capture waterborne plant viruses. The final piece to the device is a water flow meter, allowing the user to measure how much water has passed through the capture cartridge. Once sampling is complete, the glass wool cartridge can be unscrewed and placed in a cooler for transportation to laboratory processing and testing. All remaining parts can be disassembled and placed in a bin for cleaning and reuse.

Glass wool cartridges were effective for capturing PepMV, TBSV, and PMMoV viruses in individual RO water samples. Three separate five gallon buckets of RO water seeded with one of the three viruses was pumped through the device housing the glass wool cartridge for a total of three fully constructed devices. The use of 3% beef extract (wt/vol) containing 0.05 M glycine (pH 10) elution buffer followed by PEG concentration provided adequate recovery for detection of each virus (Figure 4).

Extracted RNAs were obtained from sampling and processing of three separate 5 gal buckets. Each bucket contained a single virus including PepMV, TBSV, or PMMoV seeded in RO water. Once centrifugation, pellet resuspension, and RNA extraction was completed, total RNA concentrations were 5.2 ng/µl for PepMV, 2.2 ng/µl for TBSV, and 1.6 ng/µl for PMMoV. RNAs from these three samples were used to generate cDNA libraries. These libraries had cDNA concentrations of 441.2 ng/µl for PepMV, 312.1 ng/µl for TBSV, and 300.1 ng/µl for PMMoV.

All three viruses were successfully captured, eluted, and amplified from 5 gal seeded RO water using the collecting device (Figure 4).

Samples obtained from environmental water sources were performed in duplicates and processed as described above with the exception of elution. During elution, the buffer was not allowed to cool to room temperature and was used immediately after finishing the autoclave cycle. None of the samples were positive (Figure 5).

Discussion

Existing technologies and methods used in screening water for the presence/absence of enteric pathogens in potable water as recommended by the United States Environmental Protection Agency (EPA) are referred to as the Virus Adsorption-Elution (VIRADEL) technique [24]. The VIRADEL technique is not feasible for use in agricultural systems due to cost, limited sampling volumes, and limited field compatibility [29]. An alternative approach would be one that allows users to build an inexpensive customizable sampling device. This device would require a pre-filter to adequately reduce sediment prior to glass wool cartridge capture (Figure 1). By adding a pre-filter, diagnostic laboratories and/or research groups would be able to sample greater volumes and screen both the pre- and capture-filters for waterborne pathogens. The capture filter will need media that is equally as inexpensive as the device and demonstrated to work. Following capture, there needs to be a simple and quick elution with molecular analysis. This work provides such tools and approaches.

The use of polyvinyl chloride (PVC) for constructing a device is ideal. Previous work by Millen et al. (2012), describes using PVC for constructing a cartridge to house glass wool used in concentrating waterborne viruses. Polyvinyl chloride is a material used for residential and commercial development for plumbing, doors, and windows. The automobile industry and medical industry commonly use PVC for a variety of applications, some of which require sterile

environments. Polyvinyl chloride has a melting temperature 212°C. Other properties of PVC include its being lightweight, very resistance to acids, alkalines, oil/grease, and flammable liquids. Additionally, PVC acts as an insulator for electrical applications. In addition to these properties, PVC is inexpensive, easy to work with, and found globally. Together, these properties make PVC an ideal material for constructing a water sampling device as compared to other devices currently on the market.

There are currently several types of electropositive filters used. The United States Environmental Protection Agency recommends the 3M Zeta Plus 1MDS (SunSource, North America) filter for use in virus concentration from tap water. The 3M Zeta Plus 1MDS filter cost $278.46 (08/25/2016), which is too expensive for research and/or routine field sampling for agricultural pathogens. Other electropositive filters include NanoCeram (Argonide Advanced Filtration Systems, Sanford, FL), which are made of a matrix of microglass fibers and cellulose with nanoalumina fibers containing a positive charge. At a quoted price of $49.50/filter (01/07/2016), the NanoCeram is more cost-effective than the 3M Zeta Plus 1MDS, but still impractical for use in research and routine diagnostics where a large number of filters will be required. The use of glass wool as a capture media offers a promising alternative due to its low cost and availability across the globe.

The use of fiber glass or glass wool fibers for virus capture has been previously demonstrated [9-11, 15]. The primary property of glass wool that makes it suitable for use in virus capture is its electropositive charge, which attracts and captures negatively charged virions. The viral electronegative charge can be influenced by pH [16] and salts [8]. Sampling above the virus's isoelectric point (pI), which is when the coat protein contains a neutral charge, was shown to enhance adsorption to glass wool [8, 16, 18]. When environmental pH is lowered below the viral pI, viral adsorption to a positively charged surface decreases and flocculation can occur [23]. If using glass wool, there is an important consideration of pH dropping below the plant virus pI,

creating an environment that favors a positive charge, virus adsorption to a positively charged surface (glass wool) decreases. However, as mentioned previously, conditions below the pI (low pH) favor flocculation [23], which can aid in mechanical viral removal using porous filters due to the formation of larger viral clumps. In an agriculture field setting, obtaining viruses by lowering pH is problematic. First, this requires the water be removed and then treated with an acid to lower the pH to ≤3 (below the pI). Secondly, conditions are created that no longer favor electrostatic attraction; rather, an environment of repulsion is established [8, 16]. Third, with the increased amount of suspended material in agriculture water sources, there would be a large amount of unwanted material requiring additional processing. This material would also cause larger clumps that would limit filtration due to clogging. For these reasons, environmental sampling with glass wool as a capture media should be conducted above plant virus pI.

Following sampling, elution of captured virions is necessary to release viruses from the positively charged filter or glass wool. Elution is achieved by creating an environment favoring desorption. Different buffers have been shown to elute viruses from electropositive capture include: 3% beef extract with sonic treatment [20], 1.5% beef extract with 0.05 M glycine at pH 9.5 [12], 1.5% beef extract with 0.05 M glycine plus 0.01% Tween 80 at pH 9.5 [28], and 3% beef extract in 0.05 M glycine at pH 9.5 [11]. Warming of the eluent to 37°C was previously tested and shown to not enhance recovery of viruses but did significantly decrease the recovery of bacteria previously captured in glass wool [13]. This reduction of bacteria recovery by heating would limit detection of other potential plant pathogens of concern within water samples and is problematic for metagenomic sampling. Considering the slight variations in elution buffers, this work settled on using a 3% beef extract with 0.05 M glycine; although, further analysis on optimal elution buffer is warranted. Glycine has been shown to limit the PCR inhibitor effects of proteins in the beef extract [34]. Elution and recovery were successful in this study. However, additional research is required to optimize and standardize the protocol.

Following elution from capture media, there is a need to precipitate suspended virions to further concentrate in preparation for RNA extraction. Several methods describe the use of salts or lower pH to cause flocculation of viruses with suspended proteins. This process was ideal when testing drinking water; however, when screening water used in agriculture, this process is cumbersome due to the requirement to pre-treat the water and an increased clogging rate from formation of flocs and from suspended sediments. Additionally, this added chemistry was shown to be unnecessary when using PEG for viral concentration [21, 22, 29, 30]. Polyethylene glycol was previously demonstrated to enhance concentration of viruses and found to produce greater recovery than organic flocculation [28, 31-33]. Both PEG 6,000 [31] and PEG 8,000 [28] were used to recover viruses; however, PEG 8,000 had greater viral recovery efficiencies from beef extract with 0.05 M glycine than PEG 6,000 [28]. In addition to recovery efficiencies, PEG's properties of precipitating in acidic, basic, and neutral conditions and PEG's ability to stabilize virions makes it an ideal polymer for virus concentration from water samples eluted with beef extract buffer.

The results support that an inexpensive and customizable sampling device can readily be made by research and diagnostic laboratories for screening environmental water sources for the presence of waterborne viruses. This device offers an inexpensive and robust option by using materials found globally. The protocol as outlined in this paper establishes a baseline for processing samples; however, additional optimization and testing is warranted. This fulfills a necessary gap where there currently are no routine diagnostic assays for such an application. This device provided a first attempt at establishing a diagnostic tool that can be readily adapted by any laboratory at minimal financial cost, labor, and with minimal equipment.

REFERENCES

1. Hryniszyn A, Skonieczna M, Wiszniowski J. 2013. Methods for detection of viruses in water and wastewater. Advances in Microbiology. 3(5):442-449

2. Graczyk TK, Girouard AS, Tamang L, Nappier SP, Schwab KJ. 2006. Recovery, bioaccumulation, and inactivation of human waterborne pathogens by the Chesapeake Bay nonnative oyster, *Crassostrea ariakensis*. Appl Environ Microb. 72(5):3390-3395

3. (WHO) WHO. 2004. Waterborne zoonoses : identification, causes, and control: London : IWA Publishing

4. El-Senousy WM, Costafreda MI, Pintó RM, Bosch A. 2013. Method validation for norovirus detection in naturally contaminated irrigation water and fresh produce. Int J Food Microbiol. 167(1):74-79

5. Mehle N, Ravnikar M. 2012. Plant viruses in aqueous environment – Survival, water mediated transmission and detection. Water Res. 46(16):4902-4917

6. Raudales RE, Parke JL, Guy CL, Fisher PR. 2014. Control of waterborne microbes in irrigation: A review. Agricultural Water Management. 143(0):9-28

7. Kulakov LA, McAlister MB, Ogden KL, Larkin MJ, O'Hanlon JF. 2002. Analysis of bacteria contaminating ultrapure water in industrial systems. Appl Environ Microb. 68(4):1548-1555

8. Lukasik J, Scott TM, Andryshak D, Farrah SR. 2000. Influence of salts on virus adsorption to microporous filters. Appl Environ Microb. 66(7):2914-2920

9. Lambertini E, Spencer SK, Bertz PD, Loge FJ, Kieke BA, Borchardt MA. 2008. Concentration of Enteroviruses, Adenoviruses, and Noroviruses from drinking water by use of glass wool filters. Appl Environ Microb. 74(10):2990-2996

10. Albinana-Gimenez N, Clemente-Casares P, Calgua B, Huguet JM, Courtois S, Girones R. 2009. Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. J Virol Methods. 158(1–2):104-109

11. Millen HT, Gonnering JC, Berg RK, Spencer SK, Jokela WE, Pearce JM, Borchardt JS, Borchardt MA. 2012. Glass wool filters for concentrating waterborne viruses and agricultural zoonotic pathogens. JoVE. (61):3930

12. Sobsey MD, Glass JS. 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl Environ Microb. 40(2):201-210

13. Polaczyk AL, Roberts JM, Hill VR. 2007. Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. J Microbiol Meth. 68(2):260-266

14. Abd-Elmaksoud S, Spencer SK, Gerba CP, Tamimi AH, Jokela WE, Borchardt MA. 2014. Simultaneous concentration of Bovine viruses and agricultural zoonotic bacteria from water using sodocalcic glass wool filters. Food and Environmental Virology. 6(4):253-259

15. Grabow WOK, Taylor, MB. 1993. New methods for the virological analysis of drinking water supplies. In: Department of Medical Virology. Edited by Pretoria Uo

16. Taylor DH, Moore RS, Sturman LS. 1981. Influence of pH and electrolyte composition on adsorption of poliovirus by soils and minerals. Appl Environ Microb. 42(6):976-984

17. Kettleson EM, Ramaswami B, Hogan CJ, Lee M-H, Statyukha GA, Biswas P, Angenent LT. 2009. Airborne virus capture and inactivation by an electrostatic particle collector. Environmental Science and Technology. 43(15):5940-5946

18. Zerda KS, Gerba CP, Hou KC, Goyal SM. 1985. Adsorption of viruses to charge-modified silica. Appl Environ Microb. 49(1):91-95

19. Michen B, Graule T. 2010. Isoelectric points of viruses. J Appl Microbiol. 109(2):388-397

20. Gerald Berg DRD, Adonald Berman. 1971. Recovery of small quantities of viruses from clean waters on cellulose nitrate membrane filters. Amer Soc Microb. 22(4):608-614

21. Katzenelson E, Fattal B, Hostovesky T. 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl Environ Microb. 32(4):638-639

22. Berman D, Berg G, Safferman RS. 1981. A method for recovering viruses from sludges. J Virol Methods. 3(5):283-291

23. Farrah SR, Gerba CP, Wallis C, Melnick JL. 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. Appl Environ Microb. 31(2):221-226

24. Safferman RS. 1988. Indentifiaction of enteroviruses. In. Edited by Agency USEP. US EPA New England Region 1 Library: Enviromental Protection Agency

25. Corsi SR, Borchardt MA, Spencer SK, Hughes PE, Baldwin AK. 2014. Human and bovine viruses in the Milwaukee River watershed: hydrologically relevant representation and relations with environmental variables. Sci Total Environ. 490:849-860

26. Liu OC, Brashear DA, Seraichekas HR, Barnick JA, Metcalf TG. 1971. Virus in water: I. A Preliminary study on a flow-through gauze sampler for recovering virus from waters. Applied Microbiology. 21(3):405-410

27. Fout GS, Cashdollar JL, Varughese EA, Parshionikar SU, Grimm AC. 2015. EPA Method 1615. Measurement of enterovirus and norovirus occurrence in water by culture and RT-qPCR. I. Collection of Virus Samples. (97):e52067

28. Lee H, Kim M, Paik S-Y, Lee CH, Jheong W-H, Kim J, Ko G. 2011. Evaluation of electropositive filtration for recovering norovirus in water. J Water Health. 9(1):27-36

29. Safferman RS, Rohr ME, Goyke T. 1988. Assessment of recovery efficiency of beef extract reagents for concentrating viruses from municipal wastewater sludge solids by the organic flocculation procedure. Appl Environ Microb. 54(2):309-316

30. Hurst CJ, Farrah SR, Gerba CP, Melnick JL. 1978. Development of quantitative methods for the detection of enteroviruses in sewage sludges during activation and following land disposal. Appl Environ Microb. 36(1):81-89

31. Lewis GD, Metcalf TG. 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl Environ Microb. 54(8): 1983-1988

32. Atha DH, Ingham KC. 1981. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. J Biol Chem. 256(23): 12108-12117

33. Colombet J, Robin A, Lavie L, Bettarel Y, Cauchie HM, Sime-Ngando T. 2007. Virioplankton 'pegylation': Use of PEG (polyethylene glycol) to concentrate and purify viruses in pelagic ecosystems. J Microbiol Meth. 71(3):212-219

34. Rock C, Alum A, Abbaszadegan M. 2010. PCR inhibitor levels in concentrates of biosolid samples predicted by a new method based on excitation-emission matrix spectroscopy. Appl Environ Microb. 76(24):8102-8109

TABLES

Table 1. Bill of materials list for constructing filter cartridge device. Totals equal the initial cost for materials when purchased in bulk quantities. Adjusted totals were calculated based on remaining materials purchased during the initial investment.

Part	Item description	Quantity	Pricing*	Totals	Adjusted totals
363403	Mueller Streamline 3/4 in PVC Sch 40 Female In-Line Ball Valve (Flow adjustment)	1	$4.38	$4.38	$4.38
188214	LASCO 3/4 in Dia PVC Sch 40 Adapter	1	$0.85	$0.85	$0.85
23856	LASCO 3/4 in Dia PVC Sch 40 Adapter	4	$0.31	$1.24	$1.24
89374	Opaque Whole-House Pre-Filtration Housing (Pre-filter)	1	$22.00	$22.00	$0.00
412007	LASCO 3/4 in Dia PVC Sch 40 Adapter Elbow	2	$0.53	$1.06	$1.06
23833	Charlotte Pipe 2 in x 5 ft 280-PSI Schedule 40 PVC Pipe	1	$5.56	$5.56	$0.00
23906	LASCO 2 in Dia PVC Sch 40 Adapter with female threads	2	$1.53	$3.06	$3.06
23904	LASCO 2 in Dia PVC Sch 40 Adapter with male threads	2	$1.32	$2.64	$2.64
51047	LASCO 2 in Dia x 3/4 in Dia PVC Sch 40 Bushing	2	$1.90	$3.80	$3.80
351144	3/4 in x 2 ft PVC pipe	1	$1.15	$1.15	$0.00
149008	Replacement Filter (2 pack)	1	$9.97	$9.97	$4.98
47670	Adjustable metal clamp	1	$0.76	$0.76	$0.76
456833	Plumbing tape	1	$1.38	$1.38	$0.00
452381	PVC primer combo for chemical welding	1	$6.46	$6.46	$0.00
213143	Glass wool	1	$29.68	$29.68	$0.00
***Pricing as of October 2016.**				**$93.99**	**$22.77**

FIGURES

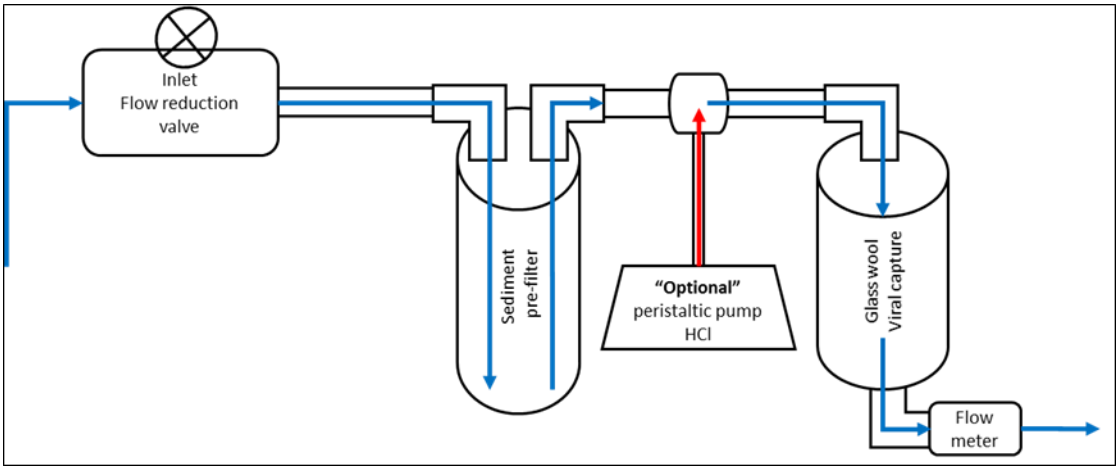


Figure 1. Diagram of the waterborne plant pathogen capture device. Blue arrows indicate water flow. An inlet flow reduction valve can be adjusted to increase or decrease the flow of water. Following the inlet is a sediment pre-filter to reduce suspended sediment. The optional peristaltic pump can be used to amend the sampled water prior to entering the glass wool capture cartridge. The glass wool viral capture cartridge houses the glass wool for sample collection. A final out-flow meter is used to measure the total volume sampled.

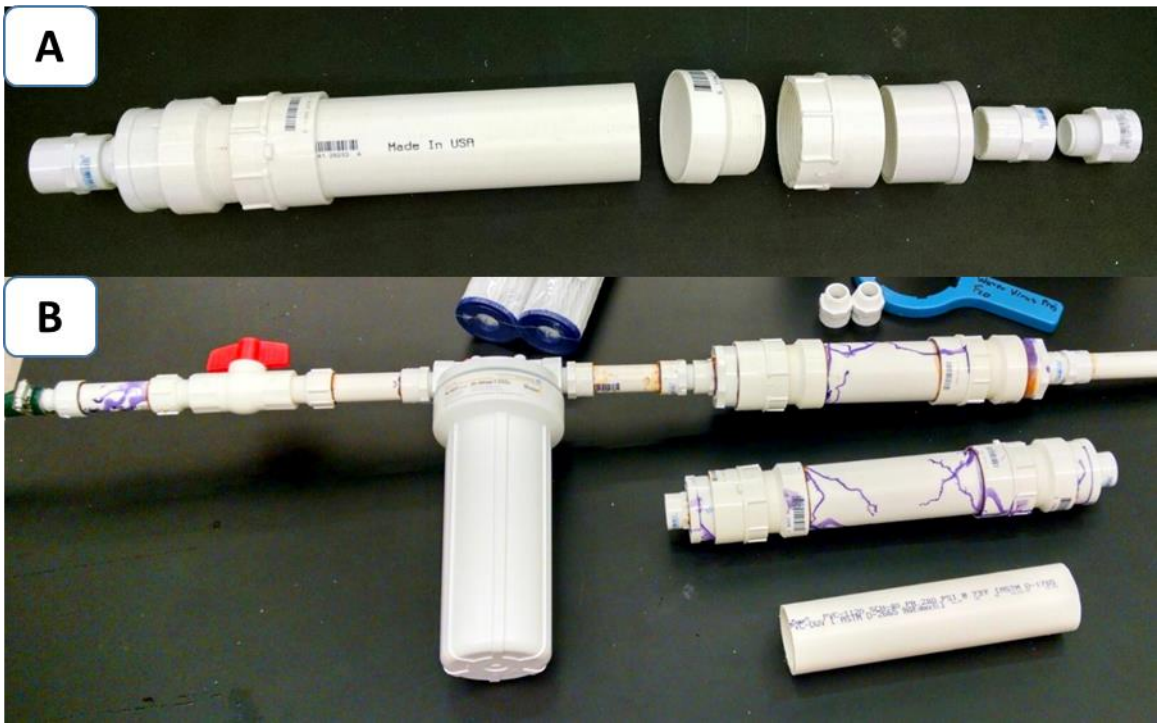


Figure 2. A capture cartridge partially assembled to show the different components (A). A fully assembled waterborne virus capture device with inlet valve (red handle), initial sediment filtration cartridge, and capture cartridge housing the glass wool (B).

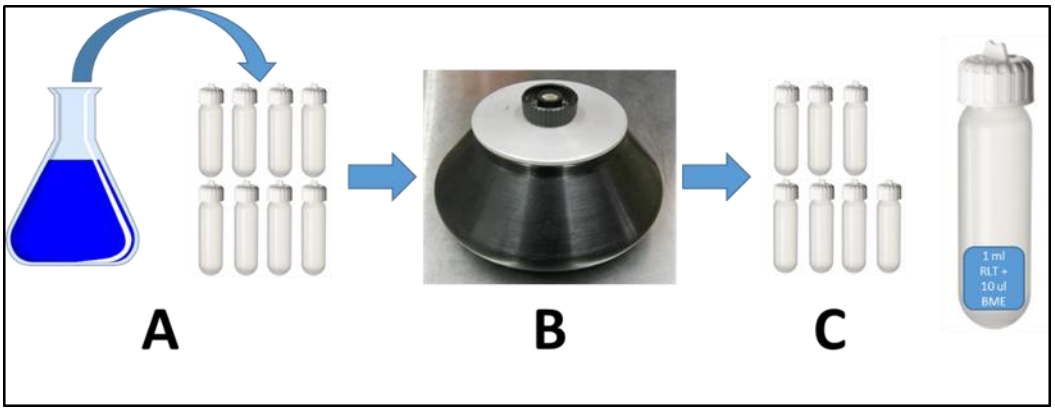


Figure 3. Reverse osmosis (RO) water seeded with individual viruses or a mixture of viruses *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), or *Pepper mild mottle virus* (PMMoV) at varying volumes were brought to a 7% polyethylene glycol (PEG) concentration and distributed to eight 30 ml centrifuge tube (A). Tubes were centrifuged for 30 min at 10,000 rpm at 4°C (B). Liquid was decanted and discarded. Then 1 ml of RLT buffer + 10 µl of beta mercaptoethanol was added to a single tube, vortexed and used to resuspend the pellet in subsequent tubes (C).

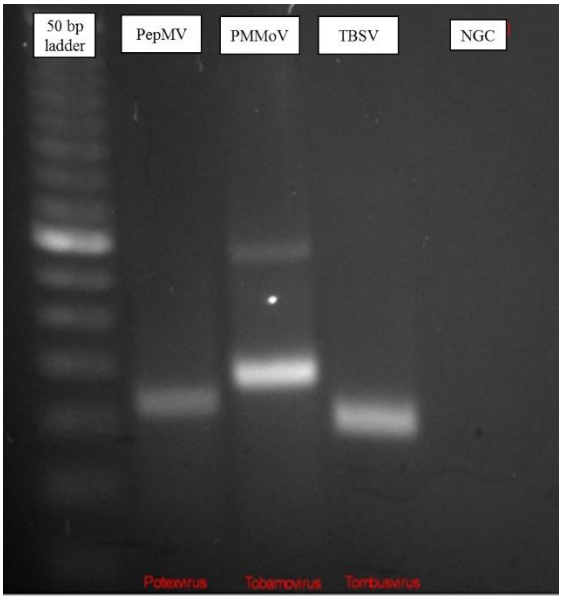


Figure 4. Gel image showing individual detection of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) using capture device. Three separate 5 gal buckets were filled with osmosis water and seeded individually with one of the three viruses using lyophilized viruses obtained from Agdia Inc. NGC – negative control.

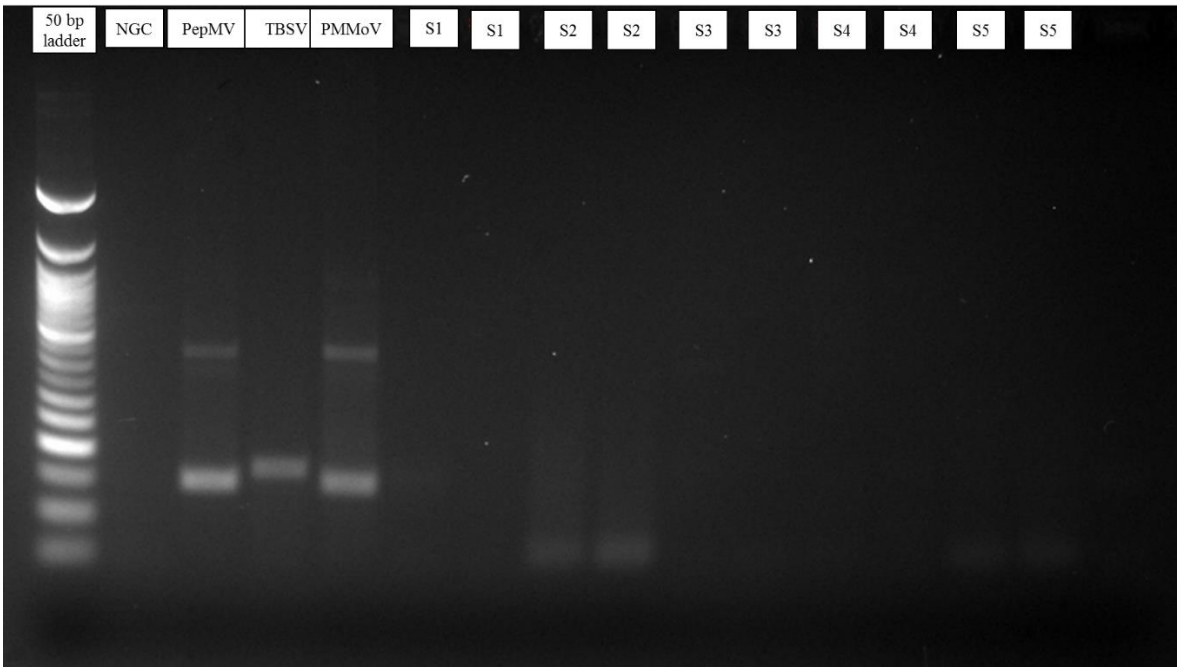


Figure 5. Environmental samples were collected and processed in duplicates from locations across the state of Oklahoma. Sample locations were Marcum's Nursery (S1), Myriad Garden (S2), Total Environment (S3), Deep Fork Tree Farm (S4), and Theta Pond (S5). NGC – negative control.

CHAPTER V

INFECTIVITY AND MOVEMENT OF PepMV, TBSV, AND PMMoV PLANT VIRUSES IN WATER

Abstract

Plant waterborne viruses originate from infected decaying plant material, sewage, and animal excrement. These plant viruses can enter irrigation water through runoff, sewage drainage, and as the result of natural events like floods and hurricanes. Viruses that are waterborne can pose a serious threat if the irrigation or recirculated nutrient waters provides a viable means of host to host transmission. The objective of this study was to assess infectivity and movement of waterborne plant viruses from infected tomato or pepper plants to healthy tomato or pepper plants in a hydroponics system. Three model viruses were used for this purpose include *Pepino mosaic virus* (PepMV, *Potexvirus*), *Tomato bushy stunt virus* (TBSV, *Tombusvirus*), and *Pepper mild mottle virus* (PMMoV, *Tobamovirus*). Using hydroponics ebb and flow flood tables clearly demonstrated that viruses were able to move from infected plant to uninfected plants through recirculated waters and cause nearly 100% infection. To assess potential environmental risk, glass aquariums were set up and contaminated with infected plant material. Results of aquarium studies were inconclusive and likely the result of time restrictions. The biosafety and biosecurity risk associated with waterborne plant viruses was demonstrated as well as the need for cost effective sampling tools and frequent monitoring of irrigation waters.

Introduction

The primary focus in waterborne pathogen research has been studying microorganisms that are causal agents of enteric diseases of humans found in drinking water. These pathogens are mainly viruses and include rotavirus, norovirus, adenovirus, and hepatitis A, [1-4]. Interestingly, these waterborne viruses are not solely acquired from drinking water but also from consumption of infected crustaceans, contaminated food crops, and from swimming in contaminated reservoirs [1]. Unlike bacteria and other larger macro-/micro-organisms, viruses can be very difficult to remove or attenuate in water due to their extremely small size and ability to flocculate to other material, thereby protecting them from sterilizing agents [5, 6]. Equally as important as human enteric viruses are plant waterborne viruses. One of the earliest reports of water as a source of plant pathogens was by Bewley and Buddin, (1921) [7]. For both human and plant waterborne viruses, the primary means of contamination is through sewage and runoff. Unfortunately, there remains little research that focuses on plant waterborne viruses and their significant risk factor to agricultural commodities to date.

Few studies have been conducted on plant viruses outside their hosts. In a review by Mehle and Ravnikar (2012), they discuss sources of waterborne plant virus originating from infected decaying plant material, sewage, and animal excrement. There are reportedly high numbers of plant viruses in human waste that survive the alimentary canal [13]. Plant viruses enter agricultural irrigation water resources via drainage systems and sewage runoff [12]. There remain relatively few studies of waterborne plant pathogenic viruses due to the lack of standardized collection and recovery methods and extensive experimentation [12]. However, even with the relatively few studies, there are reports that virus transmission in soilless systems can quickly cause economic losses [12, 14, 15].

In a detailed review of waterborne plant pathogens by Hong and Moorman (2005), a list of multiple taxa includes 17 species of *Phytophora*, 26 *Pythium*, 27 other fungi, 8 bacterial species, 13 nematode species, and 10 viruses was mentioned [8]. Sources of these waterborne pathogens include ponds, rivers, canals, streams, lakes, runoff, nutrient solutions, wells, watersheds, water reservoirs, recirculating systems, holding tanks, and hydroponics systems [8]. Current agricultural practices continue to supplement and recirculate irrigation waters due to limited rainfall, water availability, and in some countries due to regulations. When irrigation waters are contaminated with waterborne pathogens these microorganisms can quickly be disseminated leading to crop loss from disease [9].

As weather conditions change and urbanization of fertile cropping areas continues to increase, hydroponic farming offers a promising alternative to traditional soil-based cropping. In the United States, hydroponic farming is predicted to produce $847.8 million in revenue for 2016 with a projected value of $879.8 million by 2021 [10]. Greenhouse hydroponics provides several advantages over soil-based production. These benefits include year-round growing, complete control over nutrient and pH, water use reduction, higher crop yields, capacity to grow in non-favorable environments, greater control of pests, and ability to isolate crops if needed. Environmentally, hydroponics also offers benefits in that fewer pesticide are needed, topsoil erosion is not a concern, protection from extreme weather conditions, and considerably less water is required due to nutrient recirculating [11]. Disadvantages to hydroponics includes initial cost when setting up a system, demand for continual monitoring, and production limits. In a quantitative assessment of lettuce grown in hydroponics versus traditional soil-based agriculture, hydroponics was shown to grow yields of 11 ± 1.7 times higher than soil but required 82 ± 11 times more energy [11]. This suggest that hydroponics is most economical in conditions that are less favorable for soil-based agriculture but have renewable energy sources like solar and wind power. The greatest threat to this industry is waterborne pathogens. Hydroponic systems are

typically setup on a recirculating system where nutrient solution is recycled through a loop. If any plant within the loop is contaminated with a pathogen, there is a high likelihood that the entire loop will become infected leading to tremendous loss [21].

In hydroponic systems, waterborne viruses in the genera of *Potexvirus* and *Tobamovirus* were shown to remain infections in nutrient waters from three weeks to six months, respectively [14, 15]. Considering the lengthy growing time and ability of viruses to remain infections for extended periods, this highlights a biosecurity risk especially in areas that require soilless agriculture.

Three viruses used in this study include *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) belonging to the genera *Potexvirus*, *Tombusvirus*, and *Tobamovirus*, respectively. The waterborne plant virus PepMV is a serious problem for greenhouse production across the globe and is considered an ideal model viral pathogen for research studies and should be considered a serious threat by biosecurity agencies [22-24]. PepMV is well established as a major pathogen of tomatoes [23] but not of peppers [25] where it is reported to show few to no symptoms with all isolates except the US isolate [25]. Like PepMV, the waterborne plant virus TBSV is also transmissible to tomato and pepper plants through mechanical damage [26]. Normally TBSV infections tend to remain localized; however, systemic infections can occur depending on host which makes this plant virus a model organism for studying virus-plant interactions [27, 28]. Interestingly, TBSV is also considered seed-borne [26, 28]. The plant virus PMMoV has a global distribution and is reported as a major pathogen in hydroponics [29, 30]. PMMoV is one of the few viral plant pathogens that has been proposed as an indicator of human fecal contamination in potable water, demonstrating the robust propriety of remaining viable through the alimentary canal [31]. A commonality among PepMV, TBSV, and PMMoV is that all are reported as being waterborne and causing significant damage to crops, especially when grown in hydroponics [31-33].

The fact that plant viruses can be detected in environmental and agricultural water systems does not present a clear picture of the risk presented by these viruses. Plant viruses in agricultural water systems only present significant threats if the irrigation itself provides a viable means of host to host transmission. The objective of this research is to assess infectivity and movement of waterborne plant viruses from infected tomato or pepper plants to healthy tomato or pepper plants in a hydroponics system. To achieve this objective, we used PepMV, TBSV, and PMMoV were used as model plant viruses from three different families.

Materials and Methods

Hydroponics Setup

Ebb and flow flood tables setups

Three separate hydroponics ebb and flow flood tables measuring 149.86 cm x 182.88 cm x 10.16 cm (L x W x H) were constructed to monitor the movement of plant viruses from plant to plant within the aqueous environment of a hydroponic system. Tables were tilted at 10° to allow water to flow towards a drain, which emptied into a 60 gal tank (Figure 1). Each table was covered in a black 4 mil heavy duty plastic sheet to prevent leakage. Drain holes with a 19.05 mm fitting were covered with Silicone II Window and Door (General Electric Company, Huntersville, NC). Each tank held a nutrient solution containing 60 gal tap water and one pound 10N-20P-10K (nitrogen - phosphorus – potassium) nutrient mix (J.R. Peters Inc., Allentown, PA) with a measured total electrical conductivity of 3 dS/m. To circulate nutrient solution, each tank contained a Wayne GFU110 120V, 2.5-amp utility pump (Wayne Water Systems, Harrison, OH) with an attached 19.05 mm hose. The 19.05 mm hose contained a compression clamp to limit water flow, thereby preventing an overflow of each table. The hose was secured at the top of each table allowing nutrient water to run from top to bottom, preventing stagnant pooling of nutrient water (Figure 1).

Floating plants

To float and hold plants in place within the nutrient solution, R5 unfaced Polystyrene foam board insulation 12.7 mm thick measuring 2.4384 m x 1.2192 m (Dow Chemical Company, North America) was bored with 7.62 cm holes distributed 40.64 cm apart from side to side and top to bottom. A 76.2 mm plastic netted pot (Greentrees Hydroponics, Vista, CA) was placed in each hole for a total of 20 pots per table. Rows were labeled A – E with columns numbered 1-4 (Figure 2).

Germinating plants for hydroponics

A total of 75 *Solanum lycopersicum* L. (tomato, black sea man) or *Capsicum annuum* L. (pepper, poblano) were germinated in Grodan A-OK 38.1 mm starter plugs (Milton, ON, Canada), which was used as a soilless seed bed, and watered with tap water. At 2 to 3 weeks post emergence, starter plugs were added to hydroponics flood tables containing nutrient mixture of 10N-20P-10K (Figure 3). Temperature was set at 21℃/18℃ day/night with a photosynthetic photon flux density (PPFD) range of 600 to 1,200 μmoles/m^2/s^{-1} at 12 hr. Prior to inoculation all plants were screened at 1 week post introduction of hydroponics tables for presence of PepMV, TBSV, and PMMoV by DAS ELISA.

Inoculating plants and cutting roots in hydroponic systems

Table 1 served as a negative control. Row A on each of tables 2 and 3 had all four plants inoculated with a viral mixture of PepMV, TBSV, and PMMoV at unknown viral titers (Figures 2 and 3). Virus inoculations were performed by rubbing carborundum-dusted cotyledons of young leaves with unpurified virions in virus cocktail containing PepMV, TBSV, and PMMoV in Phosphate-buffered saline (PBS) at pH 7. All plants in table 3 had roots trimmed at weeks 1, 2, 3, and 4 post viral mixture inoculations providing an increased likelihood of viral release into the surrounding water. Table 2 served as the test tank with roots remaining intact. Leaves were

gathered from all plants from each of the three tables at 1, 2, 3, and 4-week post inoculation and screened via DAS ELISA for presence of PepMV, TBSV, and PMMoV.

Double Antibody Sandwich (DAS) ELISA

Double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) was obtained for PepMV, TBSV, and PMMoV (Agdia, Elkhart, IN). DAS ELISA was performed according to manufacturer's protocol. Briefly, a total of 100 µl capture antibody was added to each test well and left for overnight incubation at 4°C in a humid box. Following incubation, plates were left at room temperature while samples were prepared. Using Agdia extraction buffer, each sample was ground at a 1:10 ratio (tissue weight to buffer volume). Prior to adding test samples, all 96 wells were washed with PBST. A total of 100 µl of prepared sample was added to each test well and left for overnight incubation a 4°C in a humid box. Following overnight incubation of test material, all 96 wells were washed. An enzyme conjugate was added at a volume of 100 µl/ per well and left to incubate in a humid box for 2 hr at room temperature. Following two-hour incubation, all 96 wells were washed. A total of 100 µl PNP solution was added to each sample well. The absorbance at 405 nm was measured after 1 hr and at 1.25 hr of incubation with the substrate using a spectrophotometric BioTek Instruments microplate reader (Winooski, VT). The threshold for a positive call was two times the mean of healthy controls with the mean buffer values removed.

Glass aquariums

Glass aquarium setups

A designed screening test to evaluate virions movement from diseased plant material to the aqueous environment was set up using two 20 gal glass aquariums. Each aquarium contained 9.07 kg of sand substrate and 9.07 kg of rock substrate. In addition to the substrates, each aquarium had two pieces of natural drift wood and a circulating pump that moves 200 gal/hr of

water (Figure 4). One aquarium served as the negative control tank with the second aquarium serving as the test tank. Both aquarium pumps had the carbon-based filter and bio-wheel removed during testing.

Testing of material in aquarium

A separate experiment was designed to assess viral stability and leaching of viral particles from plant debris in water. The initial screen of virus stability was performed by reconstituting three lyophilized vials each of PepMV, TBSV, and PMMoV (Agdia, Elkhart, IN) with 1 ml aquarium water from the test tank. A 200 ml sample was collected at 24 hr, 72 hr, and weeks 2, 3, and 4 post introduction. Each 200 ml sample was processed for molecular analysis. Additional testing of viral leaching from infected plant material was performed by inoculating tomato plants at one-week post emergence with a viral mixture of PepMV, TBSV, and PMMoV at unknown concentrations. At three weeks post viral inoculations, tomato plant leaves weighing 40.48 g and stem 43.08 g were quartered and placed in test aquarium. A 200 ml sample was collected at 24 hr, 72 hr, and weeks 2, 3, and 4 post introduction. Glass aquariums were set up and allowed to run for 3 months prior to experimentation. This allowed for establishment of natural microbial flora.

Polyethylene glycol (PEG) virus concentration from aquariums

A total of 14 g of polyethylene glycol (PEG) (G-Biosciences, St. Louis, MO) with a molecular weight (mw) of 8,000 and density of 1.0845 g/ml (70°C) was added to each 200 ml sample to obtain a 7% concentration of PEG to sample volume. Each sample was stirred for a minimum of 2 hr on ice. Following this incubation, a maximum of seven 30 ml Nalgene Oak Ridge High-Speed Centrifuge Tubes (ThermoScientific, Wilmington, DE) were used. Samples were centrifuged at 10,000 rpm using the J-17 rotor while chilled at 4°C in a Beckman J2-21M/E Centrifuge (Brea, CA). After centrifugation, the eluate was decanted, leaving the pellet

undisturbed. In one of the seven tubes, the pellet was resuspended with 1 ml of RLT buffer containing 10 µl β-mercaptoethanol (BME). This tube was vortexed for 15-20 sec and the contents decanted into subsequent tubes. Vortexing and decanting into subsequent tubes was repeated for all remaining six tubes (Figure 5).

RNA extraction

Once centrifugation and pellet resuspension was completed, the liquid in the final tube was processed using a Qiagen RNeasy plant mini kit (Valencia, CA) following the manufacture's Quick-Start Protocol with modifications. Briefly, two Qiagen shredder columns were used to aliquot the resuspended pellet and centrifuged for 2 min at 14,000 rpm. Eluate was transferred, without disrupting the pellet, to two individual 2 ml microcentrifuge tubes, one for each shredder column. This process was repeated until all of the sample was filtered and collected in the 2 ml microcentrifuge tubes. The eluate from the shredder column was mixed with 0.5 volumes 100% EtOH and transferred to a single capture column. This was repeated until all eluate was processed through the single capture column. Remaining steps proceed according to the Qiagen RNeasy Plant mini kit protocol with a final elution volume of 30 µl.

Complementary DNA (cDNA) preparation

Extracted RNAs from each sample was used to generate cDNA libraries. These libraries were prepared using the following protocol. First, a reaction mix with a volume of 14.7 µl per sample was prepared containing 9.5 µl of diethyl dicarbonate (DEPC) water, 1 µl total deoxynucleotides (dNTPs) at 10 mM, 0.2 µl of random hexamer primers at 0.5 µg/µl, and 4 µl RNA. For a total reaction volume of. Each sample reaction mix was heated to 70°C +/- 3°C for 5 min and placed immediately on ice for 2 min. Next, to each sample 0.5 µl of RNAsin Plus, 4 µl 5X buffer M-MLV RT, and 0.8 µl 200 U/µl M-MLV RT enzyme was added for a final volume of 20 µl. Each sample was incubated for 90 min at 37°C +/- 2.5°C.

Hot start RT-mPCR

The hot start mPCR, samples were prepared in 25 µl total volume reactions to include a total cDNA volume of 4 µl. The mPCR master mix included 3.43 µl molecular grade water, 12.5 µl Hot Start Taq 2X master mix (New England BioLabs Inc., Ipswich, MA), 0.88 µl magnesium chloride at 50 mM, 0.1 µl forward and 0.1 µl reverse *Tombusvirus* primers at 25 µM each, 1 µl forward and 1 µl reverse *Tobamovirus* primers at 25 µM each, and 1 µl forward and 1 µl reverse *Potexvirus* primers at 25 µM each forward and reverse primers (unpublished). The mPCR cycling conditions were as follows: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 52°C for 80 sec, and 72°C for 1 min, with a final incubation of 72°C for 2 min. All mPCR reactions were carried out on a Biometra TProfessonal (Göttingen, Germany).

Results

Hydroponics

A total of 75 tomato and 75 pepper seeds were germinated successfully for two separate experiments. All successfully germinated plants were added to the hydroponics system. At one-week post introduction to hydroponics table, 10 tomato plants and eight pepper plants died. Dead plants were replaced with living plants from original germination. Of these, 20 were used per table per experiment, for a total of 60 tomato plants and 60 pepper plants.

Visual symptoms of yellowing, stunting, leaf distortion, scorching, and necrosis (Figure 6) were observed in all plants in tables two and three for both tomato and pepper experiments. At one week post hydroponic introduction all tomato plants were negative for PepMV, TBSV, and PMMoV. At one week post hydroponic introduction of pepper plants, a total of 4, 4, and 5 from tables one, two, and three, respectively, tested positive for TBSV. Confirmation of virus incidence was based on positive DAS-ELISA.

Throughout the testing period there were a total of 12, 3, and 8 viral incidence of PepMV in tomato plants for tables one, two and three, respectively (Table 1). There were a total of 2, 0, and 5 incidence of TBSV in tomatoes for tables one, two, and three, respectively (Table 1). For PMMoV there were a total of 1, 4, and 1 incidence in tomatoes in tables one, two, and three, respectively (Table 1). Mixed infections (same plant) of PepMV + TBSV were observed in two tomato plants in table three only. A mixed infection of PepMV + PMMoV was observed in a single tomato plant in table two and a single plant in table three (Table 1). Results indicated a single tomato plant in table three with a mixed infection of TBSV + PMMoV. Table three was also the only table to observed a mixed infection of PepMV + TBSV + PMMoV in a single tomato plant (Table 1).

Throughout the testing period there were a total of 14, 13, and 11 viral incidence for PepMV in pepper plants in tables one, two, and three, respectively (Table2). For TBSV there were a total of 9, 15, and 18 incidence in pepper plants in table one, two, and three, respectively (Table 2). For PMMoV the total incidence was 10, 10, and 13 in pepper plants in tables one, two and three, respectively. Mixed viral infection incidence of PepMV + TBSV were 7, 9, and 11 in tables one, two, and three, respectively (Table 2). For PepMV + PMMoV mixed infection incidence were 5, 7, and 9 in table one, two, and three, respectively (Table 2). For TBSV + PMMoV the total incidence were 5, 7, and 13 in table one, two, and three, respectively (Table 2). Incidence of mixed infections of PepMV + TBSV + PMMoV in pepper plants were 5, 5, and 9 in tables one, two, and three, respectively (Table 2).

Roots were only sampled during the final sampling period. All tomato plant roots were positive for PepMV (Table 3). For TBSV there were a total of 1, 1, and 0 incidence of tomato plant root in tables one, two, and three, respectively (Table 3). Mixed viral infection incidence of PepMV + TBSV in tomato roots were 1, 1, and 0 in tables one, two, and three, respectively (Table 3). Like tomato plant roots, pepper plant roots were only sampled during the final week.

There were a total of 18, 13, and 19 incidents in pepper roots for tables one, two and three, respectively (Table 4). For TBSV, there were a total of 2, 2, 1 incidence in pepper plant roots for tables one, two and three, respectively (Table 4). There were a total of 3, 10, and 19 incidence of PMMoV in pepper roots for tables one, two and three, respectively (Table 4). Mixed viral infection incidence of PepMV + TBSV in pepper roots were 2, 1, and 1 in tables one, two, and three, respectively (Table 4). For PepMV + PMMoV mixed infection incidence were 3, 8, and 19 in table one, two, and three, respectively (Table 2). For TBSV + PMMoV the total incidence were 0, 2, and 1 in pepper roots for table one, two, and three, respectively (Table 4). Incidence of mixed infections of PepMV + TBSV + PMMoV in pepper plant roots were 0, 1, and 1 in tables one, two, and three, respectively (Table 4).

Testing of material in aquarium

Total RNA concentrations obtained from the aquarium were 6.5 ng/µl at 24 hr, 5.9 ng/µl at 72 hr, 7.1 ng/µl at week 2, 13.1 ng/µl at week 3, and 8.2 ng/µl at week 4. The cDNA concentrations were 690.1 ng/µl, 701.2 ng/µl, 685 ng/µl, 989.8 ng/µl, and 801.2 ng/µl at 24 hr, 72 hr, week 2, week 3, and week 4, respectively. Faint bands following the hot start mPCR were observed for water samples following the introduction of tomato plants infected with PepMV, TBSV, and PMMoV at sampling periods of 24 hr, 72 hr, and weeks 1, and 3 post introduction. Faint bands were also observed week 3 in the control tank (Figure 5). Sequencing of bands aligned with uncultured freshwater eukaryotic gene (NCBI sequence ID: AB721079.1) and *Mus musculus* L. BAC clone (NCBI sequence ID: AC154842.2). Testing virus recovery from glass aquarium yielded very faint bands for experiments using plant material and no bands for experiment using three vials each of lyophilized PepMV, TBSV, PMMoV from 200 ml aquarium water.

Discussion

To meet the demands on food production and urban expansion, agriculturalists and municipals take advantage of non-favorable cropping and/or development areas where fresh water is pumped from nearby lakes, rivers, wells, aquifers, or even runoff. This re-distribution of water moves microbiomes creating new localized ecosystems. Increasing demands for fresh produce throughout the entire year has created a strong demand for modified approaches to agriculture. The demand for fresh fruit and vegetables throughout both summer and winter has driven the advancement of hydroponics, allowing for the rapid growth of fresh market produce in controlled conditions year round. Currently, the majority of fresh market cucumbers, tomatoes and peppers sold in the U.S. come from hydroponic production systems. In all likelihood, this demand will continue to increase.

For hydroponics, where nutrient waters are recirculated through a system, dissemination of waterborne plant pathogens can be even more devastating and lead to total loss due to there being no buffer, as provided by soil-based growing systems. The pathogens most commonly associated with hydroponic agriculture are oomycetes, which feature a motile swimming form as a part of their life cycle. Bacteria, which also have the capacity for self-controlled movement, also are a readily recognized threat for hydroponic growth regimes. In contrast, less attention is paid to viral pathogens, even though these viruses have been identified in numerous water environments [22-24, 26, 29-33]. Part of the reason for the discrepancy is the fact that little is known about the biological impacts of plant viruses in water. After all, there is a distinct possibility that the viruses are simply present as artifacts after being shed from their typical plant environment. For example, plant viruses, like PMMoV, have been found in high concentrations in human fecal waste and consumer products like pepper sauces, where there are undoubtedly remnants of digested or processed plant material rather than infectious pathogens of humans [31].

In order to clearly establish the epidemiological significance of plant viruses in hydroponic and irrigation water there was a necessity to determine the potential for plant to plant transmission via water. The hydroponic table experiment demonstrated transfer of PepMV and TBSV, but very limited movement of PMMoV from infected to non-infected tomato plants. For pepper plants, PepMV, TBSV, and PMMoV all moved from infected to non-infected plants. It should be noted that the experiments were conducted for a relatively limited amount of time compared to typical hydroponic production systems due to nearly complete loss of both tomato and pepper plants in the experimental tables 2 and 3 (Figures 7 and 8). Additionally, the positive detection of PepMV in the roots of both tomato and pepper plants of the healthy control would likely produce symptomatic fruits given longer time (Tables 3 and 4). This result highlights the biosafety and biosecurity risk associated with waterborne viruses. At the very least this experiment demonstrated the water transmissibility of PepMV, TBSV, and PMMoV, suggesting that viruses belonging to the families *Potexvirus*, *Tombusvirus*, and *Tobamovirus* are suited to plant to plant transmission via water.

Results of the current study using pepper plants differ in that there was a mixed infection of PepMV, TBSV, and PMMoV; however, symptoms of all three viruses were present (Figure 6). It is unknown if these symptoms are solely the result of a single virus infection or the result of co-infection with all three plant viruses (PepMV, TBSV, and PMMoV). DAS ELISA data confirmed 15-60% of tomato plants were positive for PepMV and 35-70% of pepper plants as positive for PepMV (Tables 1 and 2). Interestingly, pepper plants grown hydroponically had an elevated percentage of virus incidence versus tomatoes grown under the same conditions. This may be the result of co-infection and multiple viruses synergistically causing higher titer infections in peppers versus tomato plants, or it could be that the pepper variety used is more susceptible. Unlike PepMV, the total number of incidence of TBSV infections varied greatly from tomato to pepper plants. In tomato plants, the total percentage of incidence was 0-25% and in peppers the

total percentage of incidence was 45-90%. Also observed was 13 pepper plants testing positive for TBSV prior to mixed viral inoculations. This suggests seeds were contaminated with TBSV. The relatively low number of incidence of TBSV in tomato plants versus the higher number of incidence in pepper plants could be an indication of localized infection in tomatoes versus systemic spread in pepper plants. Plant leaves were sampled based on appearance of symptoms. For a localized infection, the same leaf would need to be sampled each week. In contrast, in a systemic infection there is a greater likelihood of capturing the detecting the virus in other leaves. Similar to TBSV, the plant virus PMMoV also had low incidence in tomato plants and high incidence in pepper plants. The range of percentage of incidence of PMMoV in tomatoes was 5-20% and in peppers was 50-65%. The lower incidence of TBSV and PMMoV versus PepMV in tomato plants can be attributed to the highly virulent and infectious nature of PepMV where it outcompetes for plant resources or resistance genes in the host plants.

Results of DAS ELISA screening of plant roots determined that 100% of the tomato plant roots were positive in all three tables. Similar findings are observed with pepper plants where 65-95% of roots are infected with PepMV. This finding is supported by previous studies by Schwarz et al. (2010), which detected PepMV in roots but had only occasional detection in older leaves [34]. Interestingly, in pepper plants, table two, which was subjected to infected plants, fewer incidence of viruses in the roots were observed, while table three under the same conditions except roots were cut had 95%. This variability of root infection is likely the result of major damage to roots allowing for greater entry and establishment by the virions. Additionally, this difference could be due to root density of both tomato and pepper plants in tables two and three compared to tables one in both tomato and pepper plants (Figure 9).

The aquarium experiment was designed to assess the general environmental risks of infected plant debris in water. Culling of infected plants is a generally accepted practice, so there is a likelihood that infected plant material would end up in streams, lakes and reservoirs.

Interestingly, when purified virus stocks were added to the aquarium system no virus was detected by RT-mPCR from 200 ml aliquots. When infected plant material was added to the aquarium system amplified products were detected from 200 ml aliquots, but sequence analysis indicated that the amplified product was not viral (Figures 10 and 11). Based on these results, it would appear that the risk of viral shedding from culled material into environmental water is limited. However, it should be noted that again these experiments were conducted for limited amounts of time with a discrete amount of spiked virus or virus infected plant material. Increasing the amount of virus or decreasing the amount of water would most likely dramatically increase the likelihood of viral recovery.

In conclusion, the risks of waterborne plant viruses cannot be discounted, particularly in hydroponic growing systems. This work demonstrates the effective water transmission of PepMV and TBSV in tomato plants and PepMV, TBSV, and PMMoV in pepper plants, in a limited amount of time and inoculum, suggesting that water is a viable means of viral pathogen spread. This further demonstrates the need for effective tools for both concentration and sampling of irrigation and hydroponic water as well as highly sensitive diagnostics for virus detection. Additional studies on plant waterborne virus epidemiology, biology, and metagenomes is needed to fully understand the biosecurity risk associated with plant waterborne viruses.

REFERENCES

1. Hryniszyn A, Skonieczna M, Wiszniowski J. A, MSaJW. 2013. Methods for detection of viruses in water and wastewater. Adv Microb. 3(5):442-449

2. Lee H, Kim M, Paik S-Y, Lee CH, Jheong W-H, Kim J, Ko G. 2011. Evaluation of electropositive filtration for recovering norovirus in water. J Water Health. 9(1):27-36

3. El-Senousy WM, Costafreda MI, Pintó RM, Bosch A. 2013. Method validation for norovirus detection in naturally contaminated irrigation water and fresh produce. Int J Food Microbiol. 167(1):74-79

4. McMinn BR, Korajkic A, Grimm AC. 2016. Optimization and evaluation of a method to detect adenoviruses in river water. J Virol Methods. 231:8-13

5. Blatchley ERG, Woei-Long A, James ER, Huffman JB, Otaki DE, Masahiro, Lisle JT. 2007. Effects of wastewater disinfection on waterborne bacteria and viruses. Water Enviromental Research. 79(1):81-92

6. Kukkula M, Maunula L, Silvennoinen E, von Bonsdorff C-H. 1999. Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like viruses. J Infect Dis. 180(6):1771-1776

7. Bewley WF, Buddin W. 1921 Ont the fungus flora of glasshouse water supplies in relation to Plant Dis.. Ann Appl Biol. 8(1):10-19

8. Hong CX, Moorman GW. 2005. Plant pathogens in irrigation water: challenges and opportunities. Critical Reviews in Plant Sciences. 24(3):189-208

9. Ghimire SR, Richardson PA, Kong P, Hu J, Lea-Cox JD, Ross DS, Moorman GW, Hong C. 2011. Distribution and diversity of phytophthora species in nursery irrigation reservoir adopting water recycling system during winter months. J Phytopathol. 159(11-12):713-719

10. Industry at a glance [http://clients1.ibisworld.com/reports/us/industry/ataglance.aspx?entid=4012] Accessed 2016

11. Barbosa LG, Almeida Gadelha FD, Kublik N, Proctor A, Reichelm L, Weissinger E, Wohlleb GM, Halden RU. 2015. Comparison of land, water, and energy requirements of lettuce grown using hydroponic vs. conventional agricultural methods. In J Envir Res and Public Health. 12(6):6879-6891

12. Mehle N, Ravnikar M. 2012. Plant viruses in aqueous environment – Survival, water mediated transmission and detection. Water Res.. 46(16):4902-4917

13. Zhang T, Breitbart M, Lee WH, Run J-Q, Wei CL, Soh SWL, Hibberd ML, Liu ET, Rohwer F, Ruan Y. 2005. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol 2005, 4(1):e3

14. Pares RD, Gunn LV, Cresswell GC. 1992. Tomato mosaic virus infection in a recirculating nutrient solution. Journal of Phytopathology. 135(3):192-198

15. Schwarz D, Beuch U, Bandte M, Fakhro A, Büttner C, Obermeier C. 2010. Spread and interaction of Pepino mosaic virus (PepMV) and Pythium aphanidermatum in a closed nutrient solution recirculation system: effects on tomato growth and yield. Plant Pathology 59(3):443-452

16. Raudales RE, Parke JL, Guy CL, Fisher PR. 2014. Control of waterborne microbes in irrigation: A review. Agricultural Water Management. 143(0):9-28

17. Deborde M, von Gunten U. 2008. Reactions of chlorine with inorganic and organic compounds during water treatment—Kinetics and mechanisms: A critical review. Water Res. 42(1–2): 13-51

18. Cayanan DF, Dixon M, Zheng Y, Llewellyn J.2009.Response of container-grown nursery plants to chlorine used to disinfest irrigation water. HortScience 44(1)164–167

19. Newman, S. 2004. Disinfecting irrigation water for disease management. In: 20th Annual Conference on pest management of ornamentals. Society of American Florists, San Jose. 1-10

20. Bandte M, Rodriguez MH, Schuch I, Schmidt U, Buettner C. 2016. Plant viruses in irrigation water: reduced dispersal of viruses using sensor-based disinfection. Irrigation Science. 34(3):221-229

21. Schwarz D, Beuch U, Bandte M, Fakhro A, Büttner C, Obermeier C. 2010. Spread and interaction of *Pepino mosaic virus* (PepMV) and Pythium aphanidermatum in a closed nutrient solution recirculation system: effects on tomato growth and yield. Plant Pathology. 59(3):443-452

22. Welter S, Dölle S, Lehmann K, Schwarz D, Weckwerth W, Worm M, Franken P. 2013. Pepino mosaic virus infection of tomato affects allergen expression, but not the allergenic potential of fruits. PLoS ONE. 8(6):e65116

23. Hanssen IM, Thomma BPHJ. 2010. Pepino mosaic virus: a successful pathogen that rapidly evolved from emerging to endemic in tomato crops. Mol Plant Pathol.. 11(2):179-189

24. Gómez P, Sempere RN, Elena SF, Aranda MA. 2009. Mixed Infections of Pepino mosaic virus strains modulate the evolutionary dynamics of this emergent virus. J Virol. 83(23):12378-12387

25. Blystad D-R, van der Vlugt R, Alfaro-Fernández A, del Carmen Córdoba M, Bese G, Hristova D, Pospieszny H, Mehle N, Ravnikar M, Tomassoli L, Varveri C, Nielsen SL. 2015. Host range and symptomatology of Pepino mosaic virus strains occurring in Europe. European J Plant Pathol. 143(1):43-56

26. Tomlinson JA, Faithfull EM. 1984. Studies on the occurrence of tomato bushy stunt virus in English rivers. Ann Appl Biol. 104(3):485-495

27. Yamamura Y, Scholthof HB. 2005. Tomato bushy stunt virus: a resilient model system to study virus–plant interactions. Mol Plant Pathol. 6(5):491-502

28. Hafiz Husnain Nawaz MU, Sadia Bano, Anam Usmani, Memoona Naseer. 2014. A research review on Tomato bushy stunt virus disease complex. Journal of Natural Sciences Research. 4(5)

29. Choi GS, Kim JH, Kim JS, Kim HR. 2004. Tobamoviruses of green peppers growing on hydroponic systems. Res Plant Dis. 10(3):194-197

30. Antignus Y, Lachman O, Pearlsman M, Maslenin L, Rosner A. 2008. A new pathotype of Pepper mild mottle virus (PMMoV) overcomes the L4 resistance genotype of pepper cultivars. Plant Dis. 92(7):1033-1037

31. Haramoto E, Kitajima M, Kishida N, Konno Y, Katayama H, Asami M, Akiba M. 2013. Occurrence of Pepper mild mottle virus in drinking water sources in Japan. Appl Environ Microb. 79(23):7413-7418

32. Mehle N, Ravnikar M. 2012. Plant viruses in aqueous environment – Survival, water mediated transmission and detection. Water Res. 46(16):4902-4917

33. Hong CX, Moorman GW. 2005. Plant pathogens in irrigation water: challenges and opportunities. Crit Rev Plant Sci. 24(3):189-208

34. Schwarz D, Beuch U, Bandte M, Fakhro A, Büttner C, Obermeier C. 2010. Spread and interaction of Pepino mosaic virus (PepMV) and Pythium aphanidermatum in a closed nutrient solution recirculation system: effects on tomato growth and yield. Plant Pathology. 59(3):443-452

TABLES

Table 1. Three separate hydroponics ebb and flow flood tables were set up with 20 tomato plants in each system. Table 1 was setup as a healthy control. Table 2 had four plants inoculated with a mixture of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) with roots not being cut. Table 3 had four plants inoculated with a mixture of PepMV, TBSV, and PMMoV and roots cut after each sampling. DAS-ELISA kit obtained from Agdia was used for detection of PepMV, TBSV, and PMMoV. Total experiment time was five weeks following 1-week post introduction to hydroponics tables.

Virus incidence in tomato plants growing in hydroponics							
Tomato plants hydroponics		Table 1 Undisturbed		Table 2 Uncut roots innoculated		Table 3 Cut roots innoculated	
		Incidence	Percentage	Incidence	Percentage	Incidence	Percentage
Single infection	PepMV	12/20	60%	3/20	15%	8/20	40%
	TBSV	2/20	10%	0/20	0%	5/20	25%
	PMMoV	1/20	5%	4/20	20%	1/20	5%
Mixed Infection	PepMV + TBSV	0/20	0%	0/20	0%	2/20	10%
	PepMV + PMMoV	0/20	0%	1/20	5%	1/20	5%
	TBSV + PMMoV	0/20	0%	0/20	0%	1/20	5%
	PepMV + TBSV + PMMoV	0/20	0%	0/20	0%	1/20	5%

Table 2. Three separate hydroponics ebb and flow flood tables were set up with 20 pepper plants in each system. Table 1 was setup as a healthy control. Table 2 had four plants inoculated with a mixture of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) with roots not being cut. Table 3 had four plants inoculated with a mixture of PepMV, TBSV, and PMMoV and roots cut after each sampling. DAS-ELISA kit obtained from Agdia was used for detection of PepMV, TBSV, and PMMoV. Total experiment time was five weeks following 1-week post introduction to hydroponics tables.

Virus incidence in pepper plants growing in hydroponics							
Pepper plants hydroponics		Table 1 Undisturbed		Table 2 Uncut roots innoculated		Table 3 Cut roots innoculated	
		Incidence	Percentage	Incidence	Percentage	Incidence	Percentage
Single infection	PepMV	14/20	70%	13/20	35%	11/20	55%
	TBSV	9/20	45%	15/20	75%	18/20	90%
	PMMoV	10/20	50%	10/20	50%	13/20	65%
Mixed Infection	PepMV + TBSV	7/20	35%	9/20	45%	11/20	55%
	PepMV + PMMoV	5/20	25%	7/20	35%	9/20	45%
	TBSV + PMMoV	5/20	25%	7/20	35%	13/20	65%
	PepMV + TBSV + PMMoV	5/20	25%	5/20	25%	9/20	45%

Table 3. Three separate hydroponics ebb and flow flood tables were set up with 20 tomato plants in each system. Table 1 was setup as a healthy control. Table 2 had four plants inoculated with a mixture of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) with roots not being cut. Table 3 had four plants inoculated with a mixture of PepMV, TBSV, and PMMoV and roots cut after each sampling. DAS-ELISA kit obtained from Agdia was used for detection of PepMV, TBSV, and PMMoV. Total experiment time was five weeks following 1-week post introduction to hydroponics tables.

Virus incidence in roots of tomato plants growing in hydroponics							
Tomato plant roots hydroponics		Table 1 Undisturbed		Table 2 Uncut roots innoculated		Table 3 Cut roots innoculated	
		Incidence	Percentage	Incidence	Percentage	Incidence	Percentage
Single infection	PepMV	20/20	100%	20/20	100%	20/20	100%
	TBSV	1/20	5%	1/20	5%	0/20	0%
	PMMoV	n/a	n/a	n/a	n/a	n/a	n/a
Mixed Infection	PepMV + TBSV	1/20	5%	1/20	5%	0/20	0%
	PepMV + PMMoV	n/a	n/a	n/a	n/a	n/a	n/a
	TBSV + PMMoV	n/a	n/a	n/a	n/a	n/a	n/a
	PepMV + TBSV + PMMoV	n/a	n/a	n/a	n/a	n/a	n/a

n/a = not tested or dead plants

Table 4. Three separate hydroponics ebb and flow flood tables were set up with 20 pepper plants in each system. Table 1 was setup as a healthy control. Table 2 had four plants inoculated with a mixture of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) with roots not being cut. Table 3 had four plants inoculated with a mixture of PepMV, TBSV, and PMMoV and roots cut after each sampling. DAS-ELISA kit obtained from Agdia was used for detection of PepMV, TBSV, and PMMoV. Total experiment time was five weeks following 1-week post introduction to hydroponics tables.

Virus incidence in roots pepper plants growing in hydroponics							
Pepper plant roots hydroponics		Table 1 Undisturbed		Table 2 Uncut roots innoculated		Table 3 Cut roots innoculated	
		Incidence	Percentage	Incidence	Percentage	Incidence	Percentage
Single infection	PepMV	18/20	90%	13/20	65%	19/20	95%
	TBSV	2/20	10%	2/20	10%	1/20	5%
	PMMoV	3/20	15%	10/20	50%	19/20	95%
Mixed Infection	PepMV + TBSV	2/20	10%	1/20	5%	1/20	5%
	PepMV + PMMoV	3/20	15%	8/20	40%	19/20	95%
	TBSV + PMMoV	0/20	0%	2/20	10%	1/20	5%
	PepMV + TBSV + PMMoV	0/20	0%	1/20	5%	1/20	5%

FIGURES

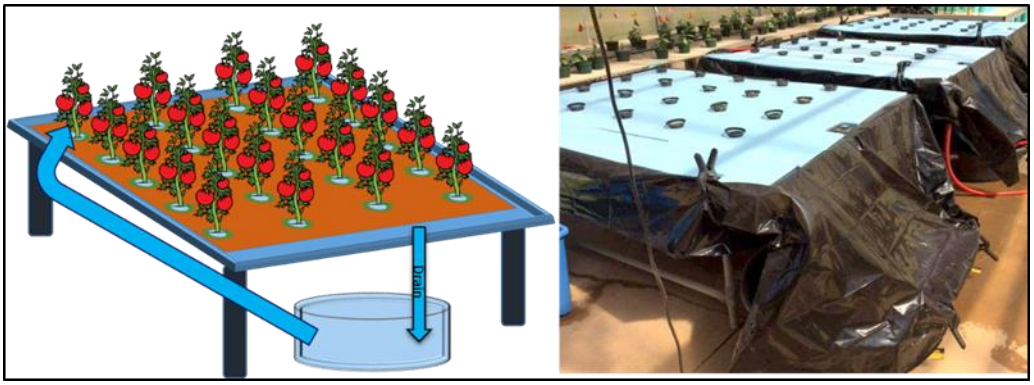


Figure 1. Diagram of a single hydroponics flood table set up (left). Three separate hydroponics tables with black tarp covering table and nutrient tank, Styrofoam used to hold plants, and netted 76.2 mm pots (right).

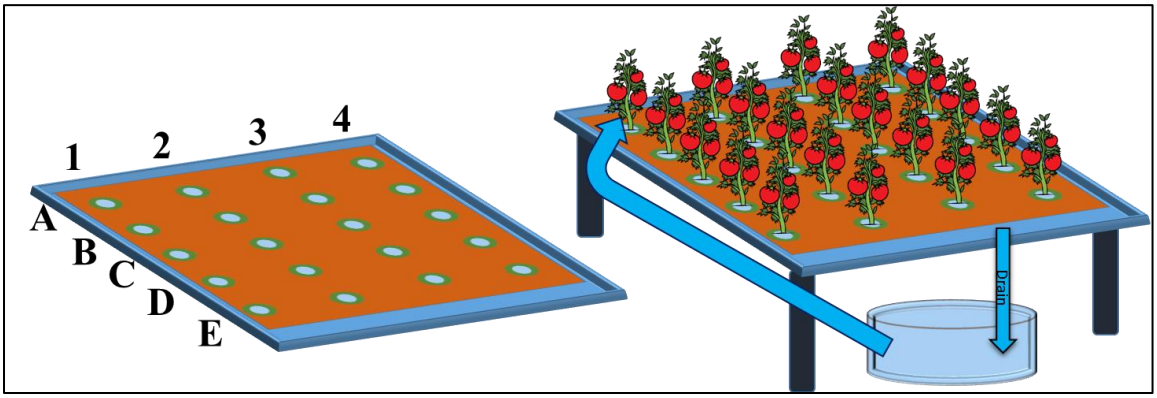


Figure 2. Hydroponics ebb and flow flood tables were set up with a a total of 20 pots per table. Rows were labeled A – E with columns numbered 1-4 (Left). Individual plants were added to each well (Right).

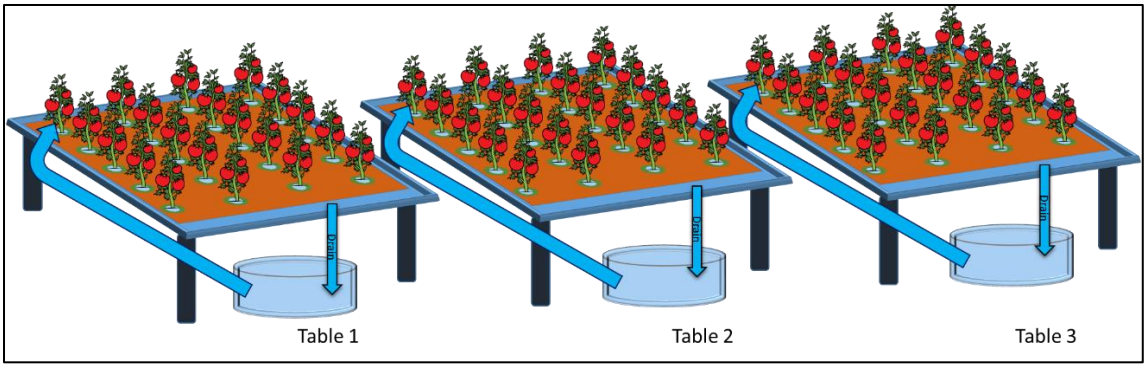


Figure 3. Hydroponics ebb and flow flood table setup showing nutrient water flow from tank to top of table where it is discharged and allowed to drain back to the 60 gal nutrient holding tank. Undisturbed healthy control (Table 1). Test table with four plants at top row were inoculated with a viral cocktail of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) and roots were left uncut (Table 2). Test table with four plants at top row were inoculated with a viral cocktail of PepMV, TBSV, and PMMoV and roots were cut (Table 3).

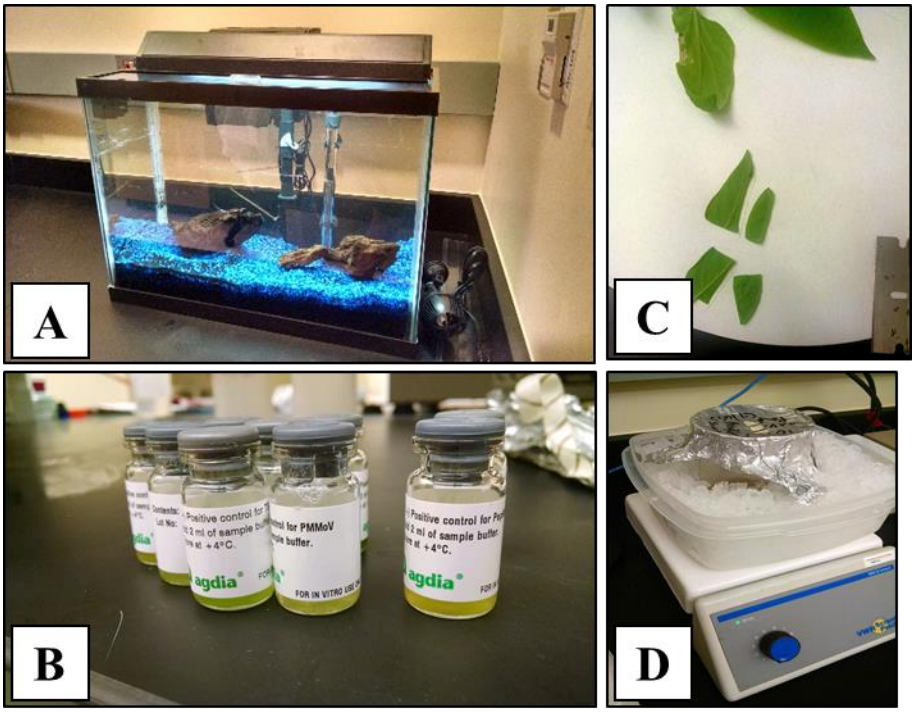


Figure 4. Experiments were conducted in glass aquariums to test movement of waterborne viruses from infected plant material to the water. Two separate glass aquariums were setup with one acting as a control and the second as the test (A). Three vials each of lyophilized *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV) were added to the test tank and sampling was conducted over three weeks (B). A separate experiment used infected tomato plant leaves and stems that were quarter and added to the test tank (C). All sample volumes were 200 ml and were immediately brought to a 7% polyethylene glycol concentration and stirred on ice for 2 hr prior to RNA extraction (D).

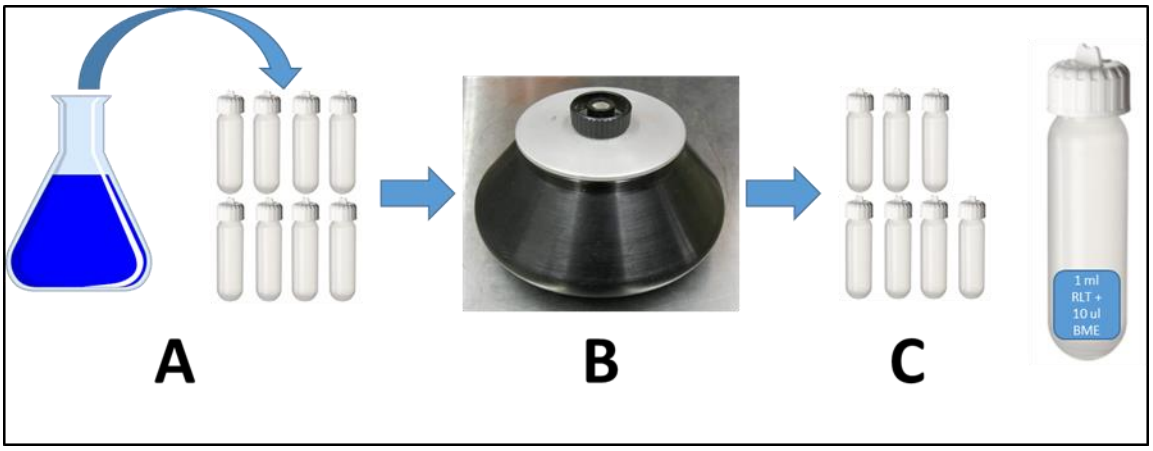


Figure 5. Reverse osmosis (RO) water seeded with individual viruses or a mixture of viruses *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), or *Pepper mild mottle virus* (PMMoV) at varying volumes were brought to a 7% polyethylene glycol (PEG) concentration and distributed to eight 30 ml centrifuge tube (A). Tubes were centrifuged for 30 min at 10,000 rpm at 4°C (B). Liquid was decanted and discarded. Then 1 ml of RLT buffer + 10 µl of beta mercaptoethanol was added to a single tube, vortexed and used to resuspend the pellet in subsequent tubes (C).

Figure 6. Symptoms of viral infection: nettle head, yellowing, stunting, leaf distortion, scorching, and necrosis are observed in both tomato (A) and pepper (B) plants. Red arrow indicates healthy tomato plant leaf (A).

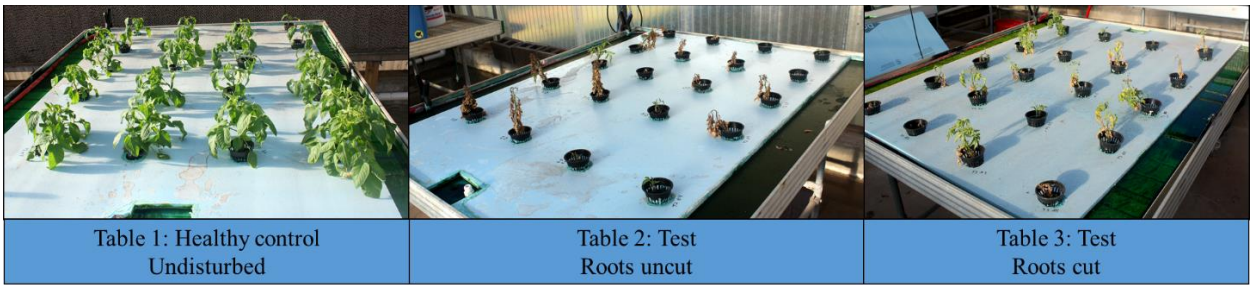


Figure 7. Tomato plants grown in hydroponics at week 5. Table 1 contains tomato plants that were undisturbed and used as healthy controls. There were no symptoms of viral infection in table one plants. Table 2 had a single row of plants inoculated with a virus cocktail of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV). In table 3 a single row of plants was inoculated with a virus cocktail of PepMV, TBSV, and PMMoV. No plants survived in table 2 and only four were still alive in table 3.

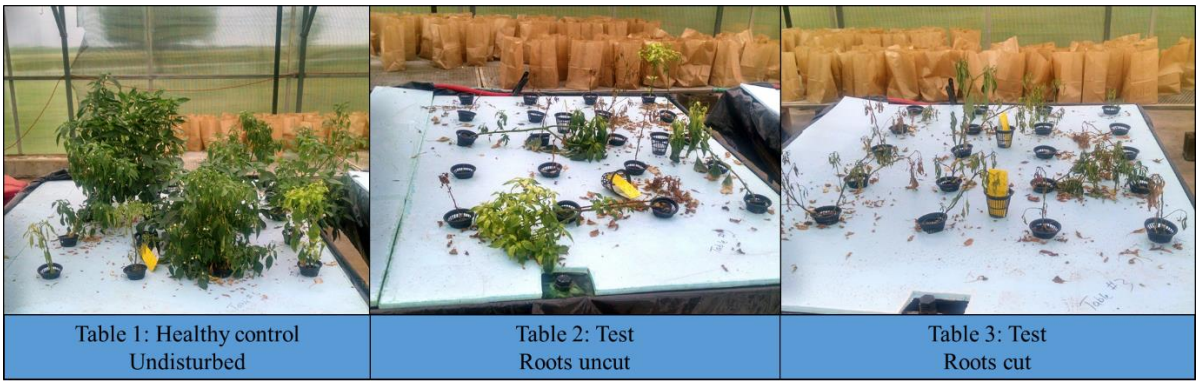


Figure 8. Pepper plants grown in hydroponics at week five. Table 1 contains pepper plants that were undisturbed and used as healthy controls. Viral symptoms were present in all plants and in all tables. Table 2 had a single row of plants inoculated with a virus cocktail of *Pepino mosaic virus* (PepMV), *Tomato bushy stunt virus* (TBSV), and *Pepper mild mottle virus* (PMMoV). In table 3 a single row of plants was inoculated with a virus cocktail of PepMV, TBSV, and PMMoV. While a few plants did have leaves in tables 2 and 3, none of the plants survived. Table 1 had three plants die by week five and all remaining were symptomatic.

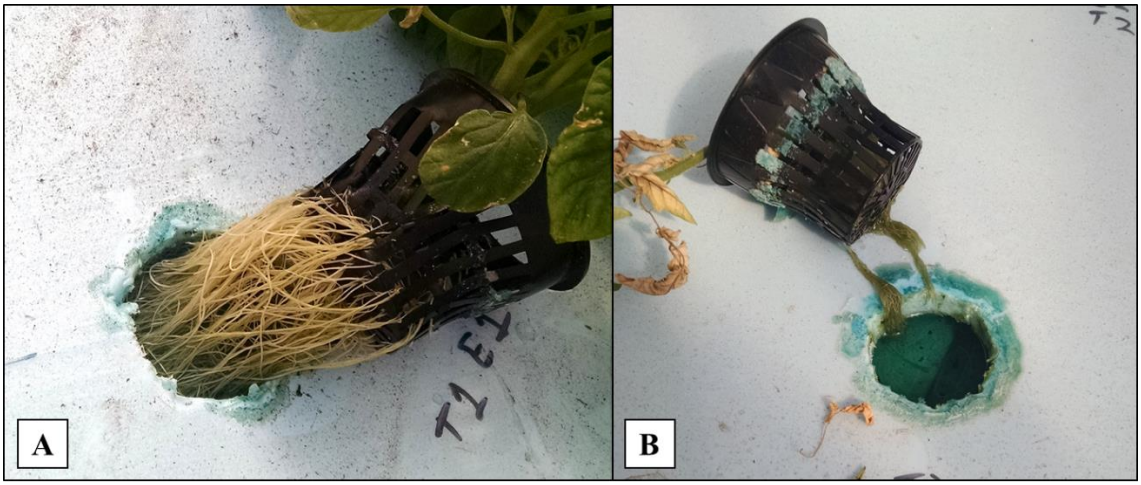


Figure 9. Roots during the final week of sampling. Table 1 was the healthy control (A). Table two was a test table containing four plants inoculated with a virus mixture of *Pepino mosaic virus*, *Tomato bushy stunt virus*, and *Pepper mild mottle virus* (B).

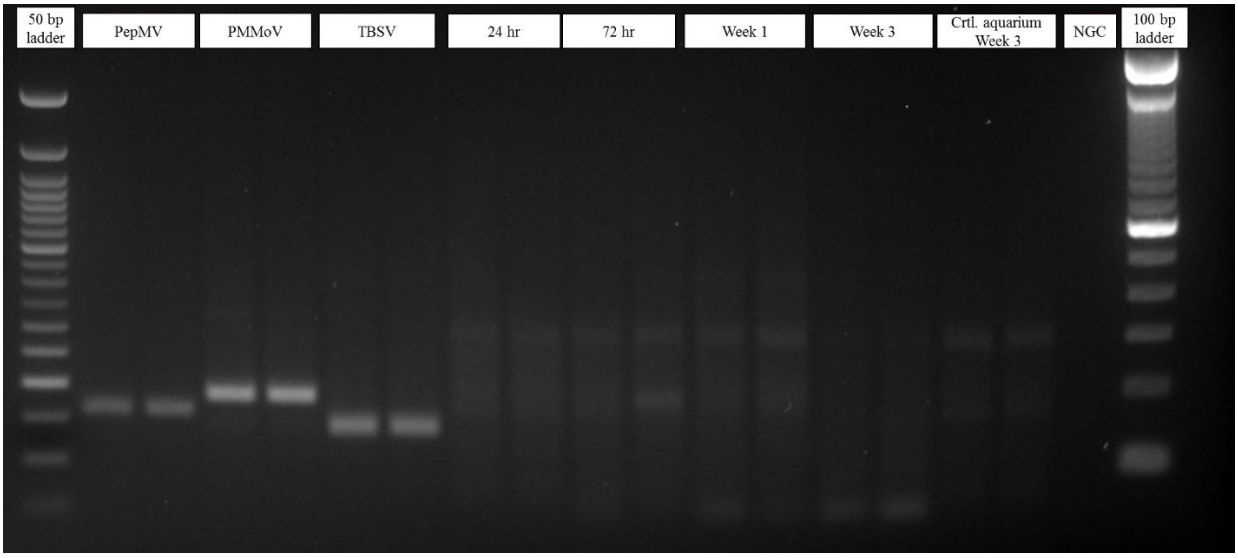


Figure 10. Only positive detections obtained from sampling test aquarium contaminated with infected tomato plant tissues were re-run in duplicates to obtain material for sequencing. Control tank week 3 had faint bands that was re-run in duplicate to obtain enough DNA for sequencing. NGC – negative control.

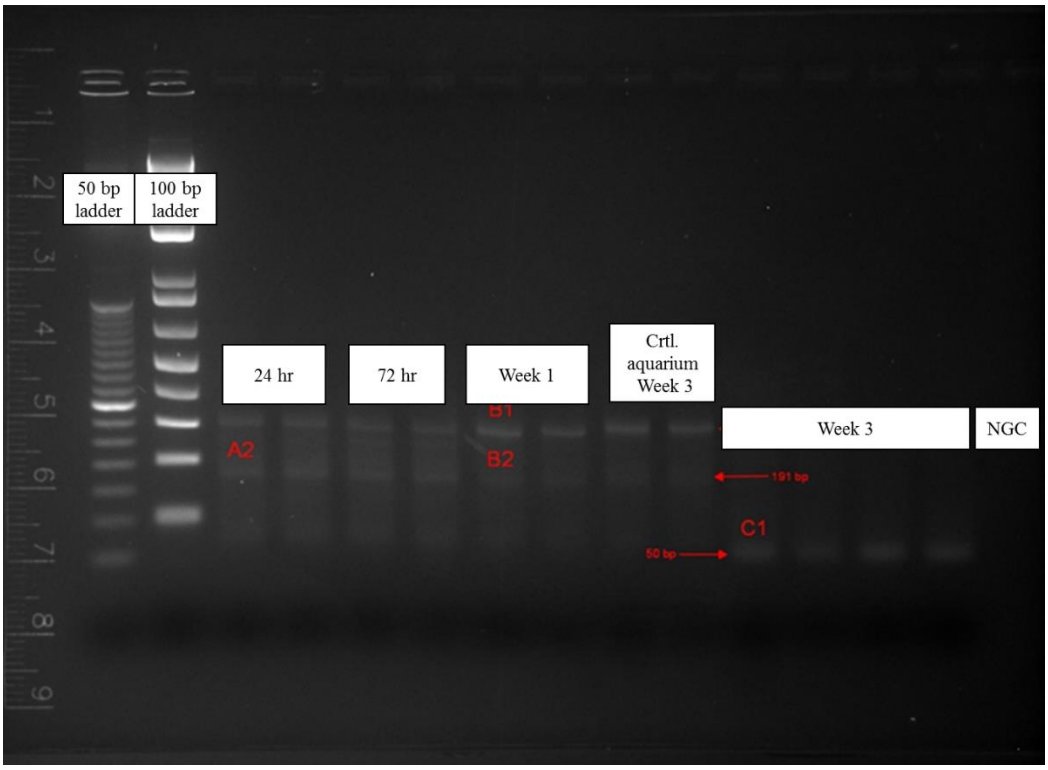
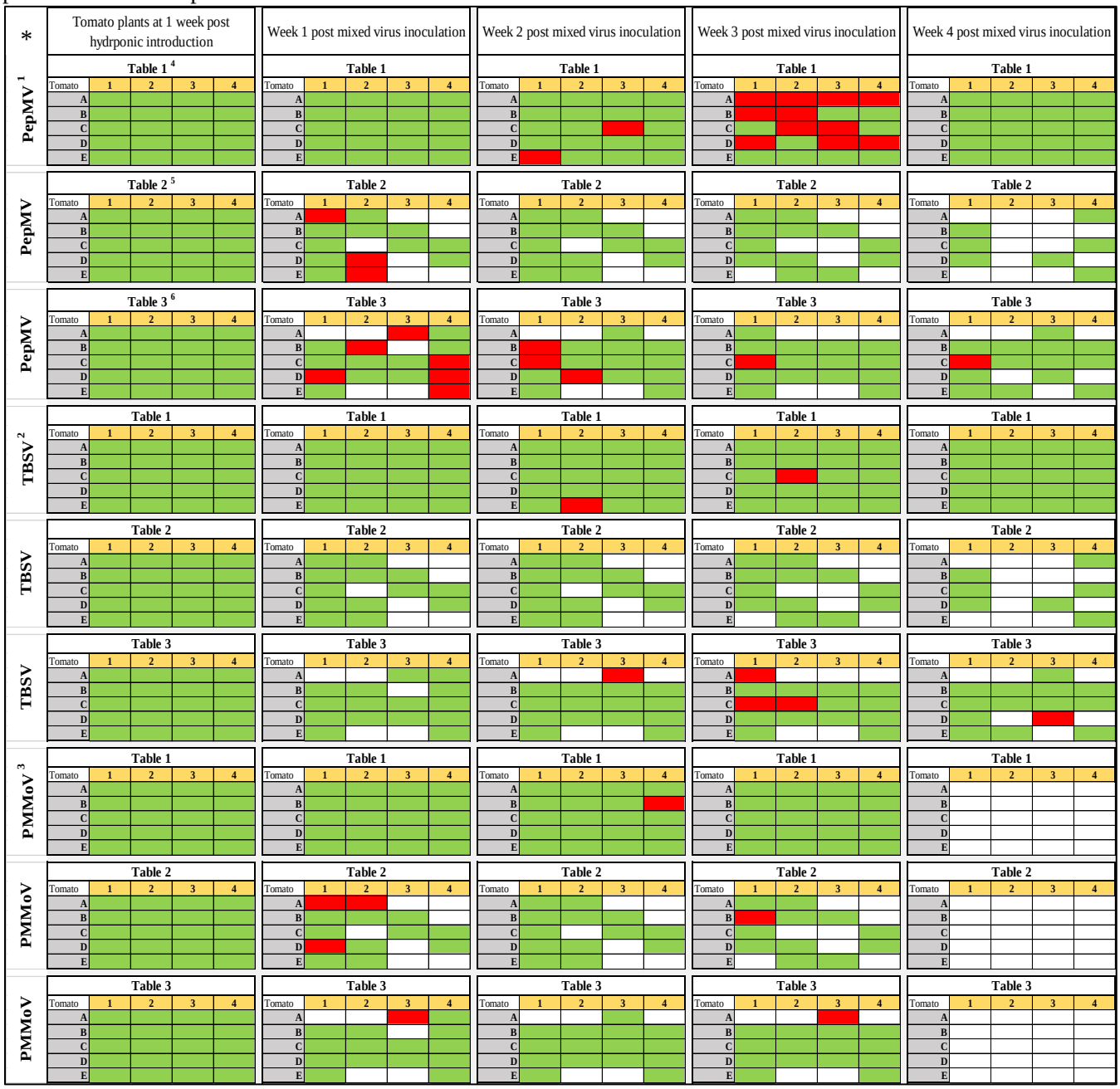


Figure 11. Polymerase chain reaction products obtained from previous PCR amplification of positive test and control aquarium samples were re-amplified to obtain greater concentrations of nucleic acid for sequencing. NGC – negative control.

APPENDICES

Table 1. Tomato plants grown hydroponically in ebb and flow flood tables with DAS ELISA test performed on each plant over the course of five weeks.



* Results of DAS ELISA: Green (negative), red (positive), and white (non-tested)

[1]*Pepino mosaic virus* DAS ELISA assay

[2]*Tomato bushy stunt virus* DAS ELISA assay

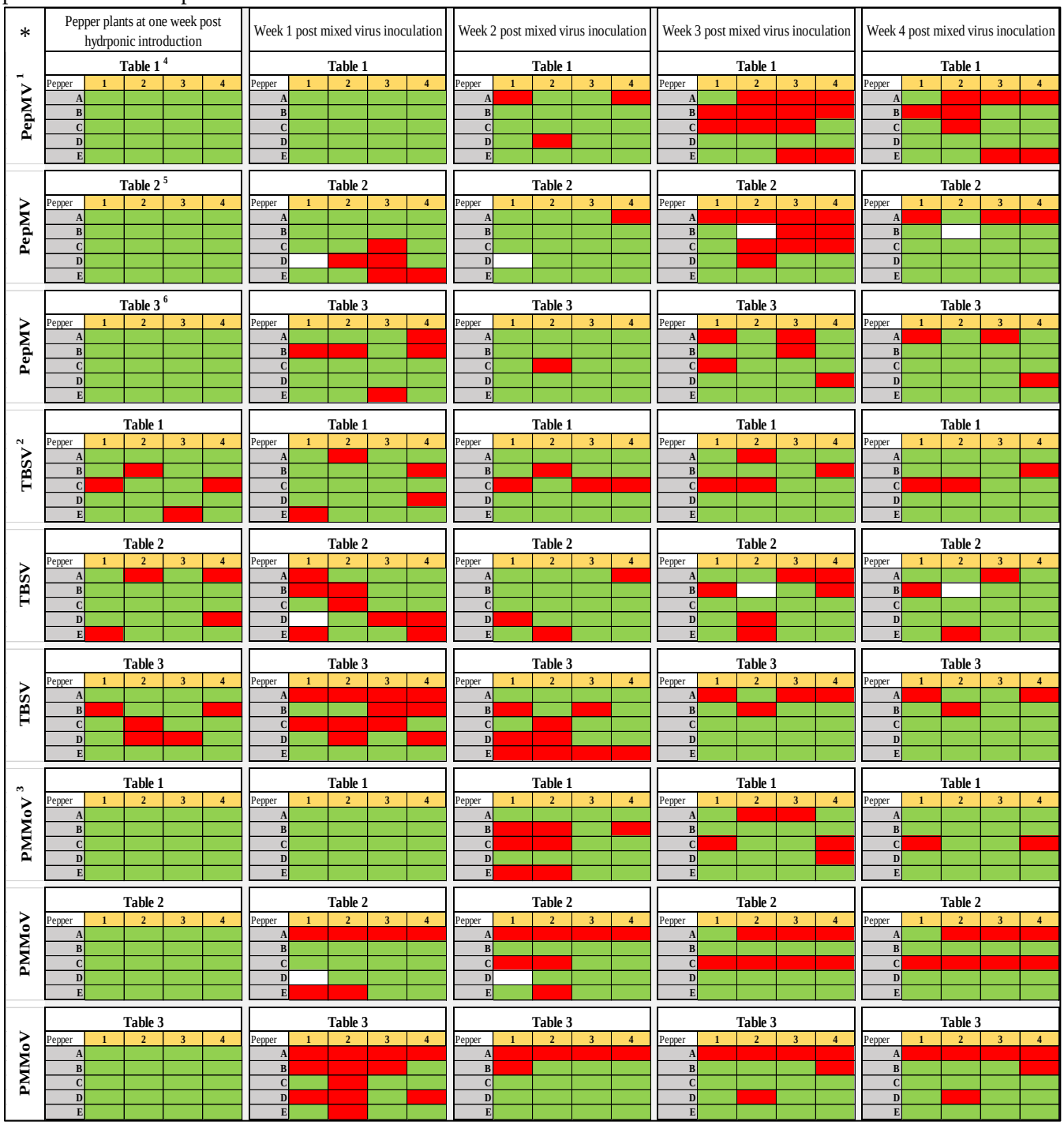
[3]*Pepper mild mottle virus* DAS ELISA assay

[4]Table 1 (healthy control)

[5]Table 2 (test: roots uncut)

[6]Table 3 (test: roots cut)

Table 2. Pepper plants grown hydroponically in ebb and flow flood tables with DAS ELISA test performed on each plant over the course of five weeks.



* Results of DAS ELISA: Green (negative), red (positive), and white (non-tested)

[1]*Pepino mosaic virus* DAS ELISA assay

[2]*Tomato bushy stunt virus* DAS ELISA assay

[3]*Pepper mild mottle virus* DAS ELISA assay

[4]Table 1 (healthy control)

[5]Table 2 (test: roots uncut)

[6]Table 3 (test: roots cut)

Table 3. Data presented as DAS ELISA optical density (OD) values that are normalized for Table 1 (healthy controls).

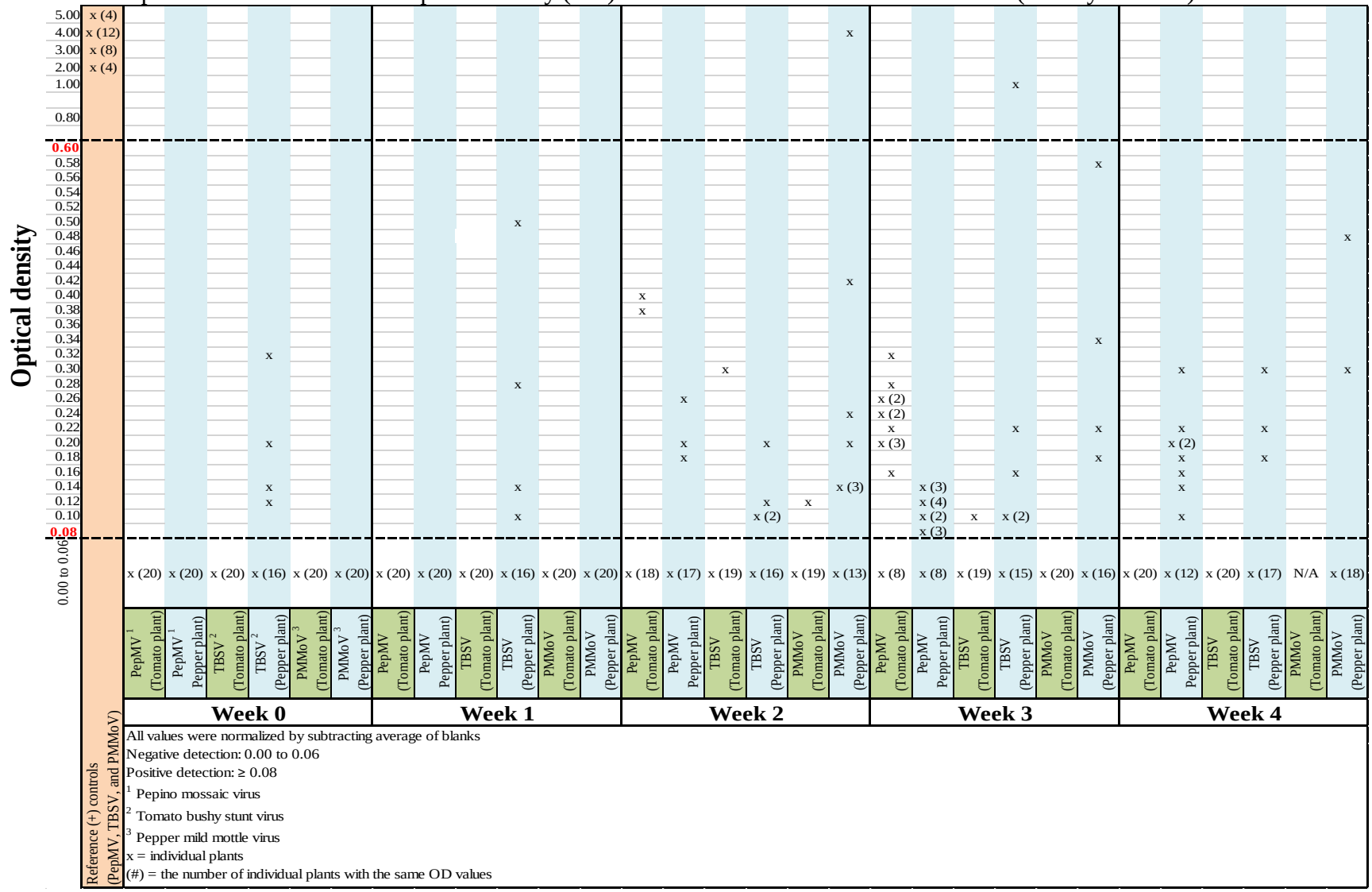


Table 4. Data presented as DAS ELISA optical density (OD) values that are normalized for Table 2 (roots uncut).

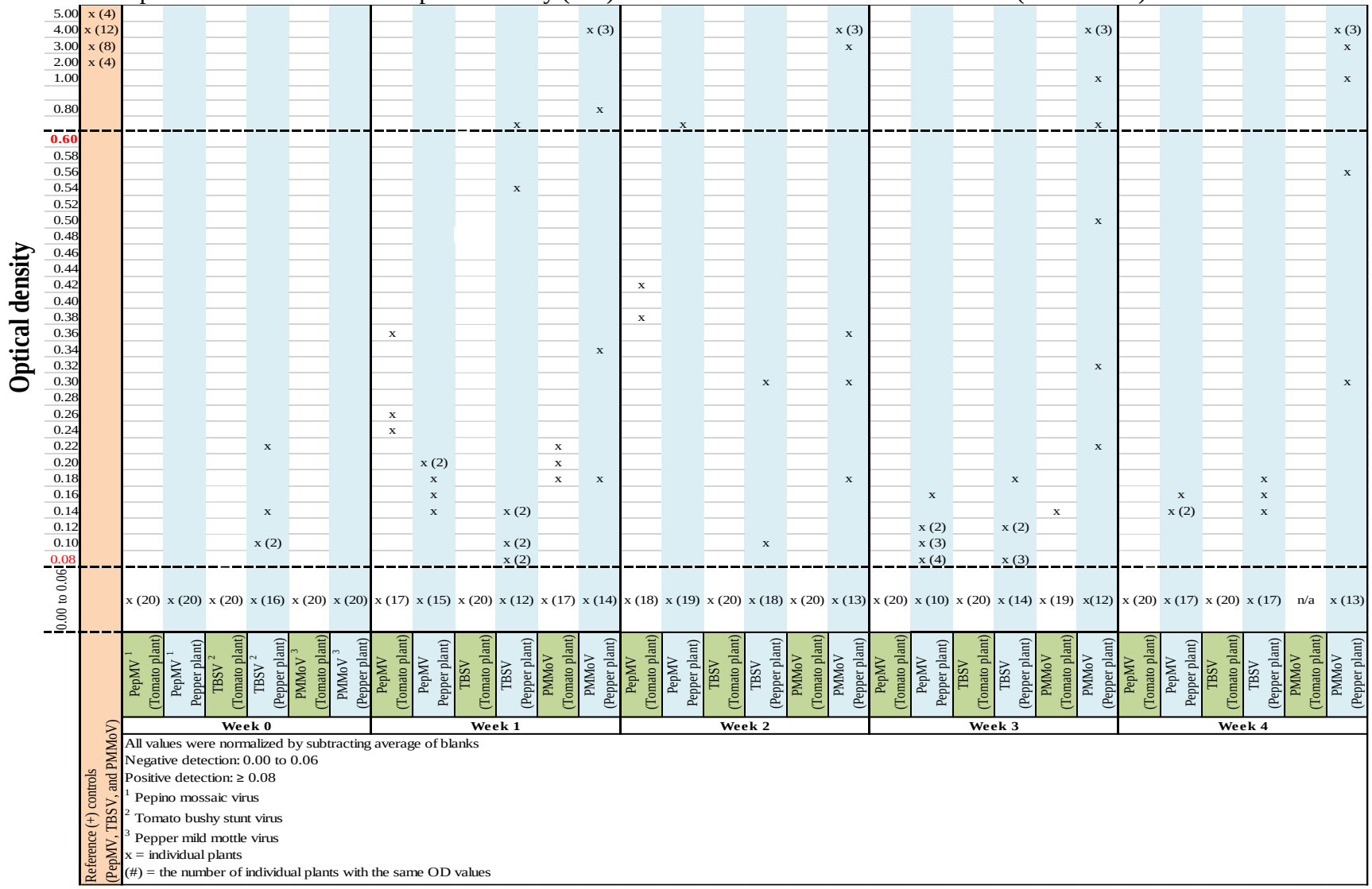
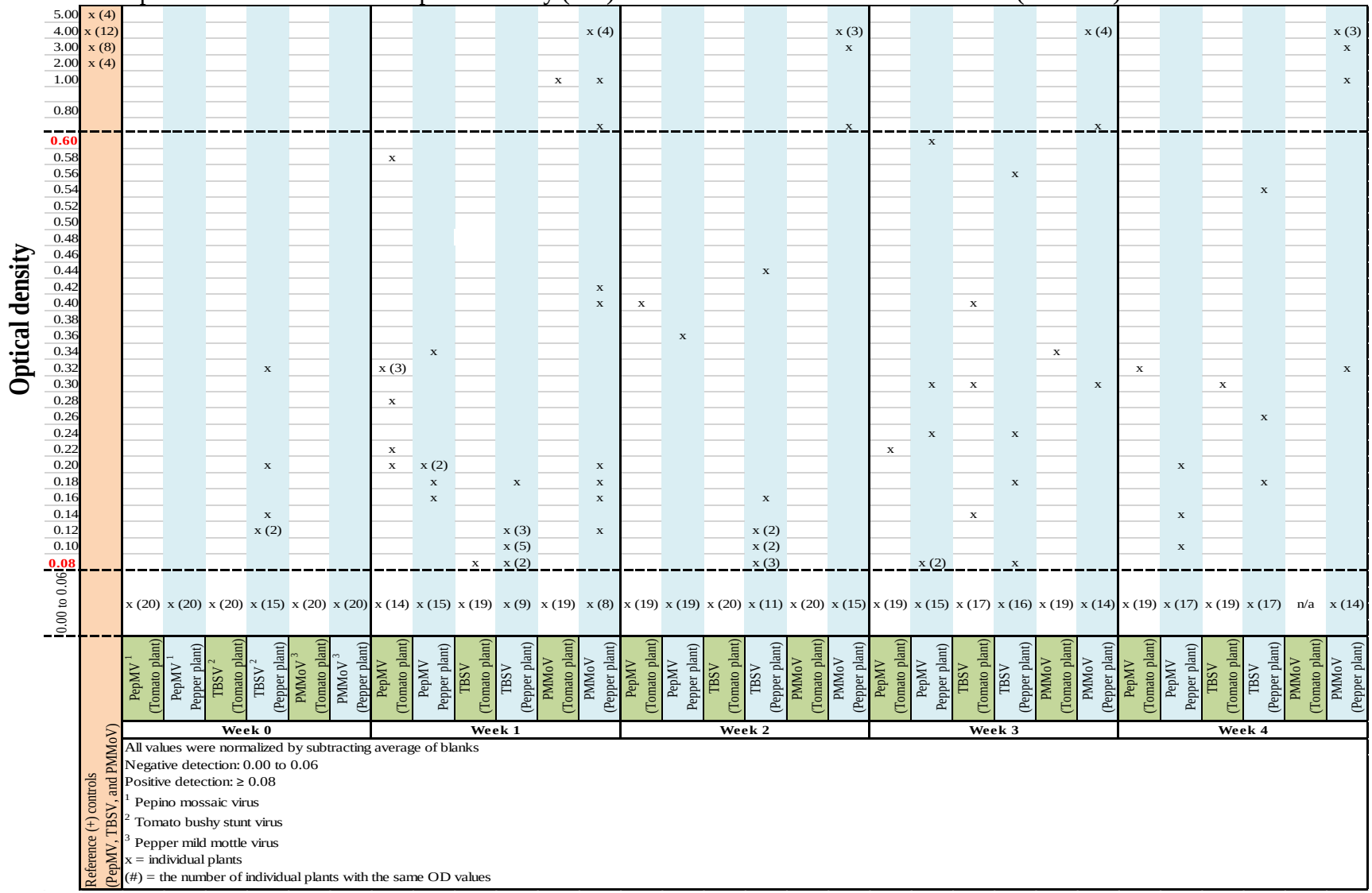


Table 5. Data presented as DAS ELISA optical density (OD) values that are normalized for Table 3 (roots cut).



VITA

Jon Michael Daniels

Candidate for the Degree of

Doctor of Philosophy

Thesis: CAPTURING WATERBORNE PLANT PATHOGENIC VIRUSES: A NOVEL TOOL FOR AGRICULTURAL DIAGNOSTICS USING THREE MODEL VIRUSES

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Biographical:

Education:

Completed the requirements for the Doctor of Philosophy/Education in Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in December, 2016.

Completed the requirements for the Master of Science in Entomology and Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in December in 2013.

Completed the requirements for the Bachelor of Science in Biology at Roger's State University, Claremore, Oklahoma in 2009.

Experience:

For my Master of Science in Entomology and Plant Pathology, I researched the use of next generation sequencing (NGS) and bioinformatics as a biosecurity tool to detect highly consequential plant pathogens. I inoculated plants with select agents and performed total nucleic acid extractions and 454 pyrosequencing. I then developed a computer pipeline that would generate electronic probes (e-probes) capable of screening NGS data sets for high-threat pathogens with minimal computations demand. My work on this project has been cited by multiple authors and is being taught through workshops. For my Doctoral degree in Plant Pathology, I developed a new water sampling device and protocol to screen irrigation waters for the presence/absence of plant waterborne viruses. This cost-effective, portable, and robust device provides scientists and diagnosticians the means to sample great volumes of irrigation waters or other water resources without having to retain a large amount of water.

Professional Memberships:

2010 – current: American Phytopathological Society (APS)
2010 – current: American Society for Microbiology