# PREVALENCE AND LONGITUDINAL STUDY OF HUMAN AND PLANT PATHOGENS IN NEW YORK SURFACE IRRIGATION WATER, UV SURFACE WATER TREATMENT, AND GENOME ASSISTED DIAGNOSTIC PROTOCOL DEVELOPMENT FOR VIRULENT FIELD STRAINS OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO*

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# PREVALENCE AND LONGITUDINAL STUDY HUMAN AND PLANT PATHOGENS IN NEW YORK SURFACE IRRIGATION WATER, UV WATER TREATMENT, AND GENOME ASSISTED DIAGNOSTIC PROTOCOL DEVELOPMENT FOR VIRULENT FIELD STRAINS OF *PSEUDOMONAS SYRINAGE* PV. *TOMATO*

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Human and plant pathogenic microorganisms can be spread through water sources. Understanding the risks associated with using surface water for irrigation can better aid growers in the production of fruit and vegetables. A surface irrigation water survey was conducted during the growing seasons of 2010 and 2011 to characterize the presence of the potential human pathogens, *Escherichia coli* and *Salmonella* spp., and the oomycete plant pathogens. Water quality parameters, irrigation site properties, and precipitation data were collected for all water samples and statistical analyses were performed to determine if any correlations existed with pathogen prevalence or concentrations. Prevalence for *E. coli* and *Salmonella* spp. in the 123 samples analyzed were 34% and 47% positive, respectively. No correlations were found for the prevalence or concentration of *E. coli* in irrigation water. *Salmonella* spp. were found to be correlated to precipitation. A sample was more likely to be positive if <0.64 cm precipitation was recorded 3 days prior to sampling. The highest concentrations of *Salmonella* spp. were most likely to occur if precipitation was between 0.38 cm and 0.64 cm 3 days prior to sampling. For oomycetes, 88% of the 210 samples analyzed were positive. Irrigation source type was found to be correlated to the concentration of oomycetes. Creeks had on average 19 CFU/L, while ponds

have an average of 11 CFU/L. Turbidity was also correlated to oomycete concentration; higher turbidity levels were associated with higher oomycete concentrations.

Ultraviolet light was examined for its treatment efficacy of surface water contaminated with human and plant pathogens. Water from two surface water irrigation sources was collected and inoculated with a bacterial or oomycete pathogens and treated with UV light. The pathogens tested were; *E. coli, S. enterica, Listeria monocytogenes, Pseudomonas syringae* pv. *tomato, Clavibacter michiganensis* subsp. *michiganensis* and *Phytophthora capsici*. Water samples were inoculated with $1 \times 10^8$ to $1 \times 10^{10}$ CFU/L bacteria or $5 \times 10^4$ to or $5 \times 10^5$ oomycete zoospores/L. In all cases, 99.9% or greater inactivation was achieved with UV treatment. Log reduction values ranged from 10.0 to 6.1 and from 5.0 to 4.2 for bacterial pathogens and *Ph. capsici*, respectively.

A genome assisted diagnostic protocol was developed for virulent field strains of *P. syringae* pv. *tomato* (*Pto*). A draft genome of a New York strain of *Pto* 09150 was generated and used in genomic comparison studies to select targets that would distinguish more virulent strains of *Pto* from less virulent strains of *Pto* and other bacterial tomato pathogens that can produce symptoms similar to those produced by *Pto*. Targets found in the genome of *Pto* 09150 that could be responsible for high levels of virulence were, genes for coronatine biosynthesis, a flg11-28 allelic difference, and a large repertoire of type III effectors (T3Es). Primers were developed for the T3Es *avrA, hopW1, hopN1*, and *hopR1* and were used successfully to distinguish more virulent *Pto* strains from less virulent strains.

# BIOLOGICAL SKETCH

Lisa A. Jones was born in Royal Oak, MI and grew up in the towns of Napoleon and Rives Junction, MI. Lisa graduated from Napoleon High School as valedictorian. During high school Lisa attended many classes at Jackson Community College and accrued many credits that transferred to Cornell University where she attended as an undergraduate and majored in plant biology. After earning a Bachelor of Science degree she worked at an Environmental Laboratory in Gainesville, FL for three years, analyzing water and soil samples for chemical and microbial properties. Next, she acquired a position with the Division of Plant Industry in Gainesville, FL working for the Cooperative Agriculture Pest Survey (CAPS). During her employment with the CAPS program, Lisa worked of the diagnostics of new and emerging plant pathogens and pests threatening Florida agriculture. In the fall of 2009, Lisa started as a graduate student in the laboratory of Dr. Christine Smart. As a graduate student, Lisa focused on translational research with oomycete and bacterial plant pathogens to benefit fruit and vegetable growers. Outcomes from this research help us better understand the risk of using surface irrigation water and the efficacy of UV treatment for surface water. Also, this research provides a diagnostic protocol for the detection of virulent field strains of *Pseudomonas syringae* pv. *tomato* which can be used to fine tune pest management strategies.

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## INTRODUCTION

Fruit and vegetable growers face management pressures from both human and plant pathogenic microorganisms. One avenue that pathogenic organisms are introduced into a produce growing environment is through agricultural water such irrigation water. Irrigation water comes from three sources, municipal, groundwater, or surface water. Municipal water is treated to remove potential pathogenic microorganisms and is at very low risk for pathogen contamination. Groundwater is at low risk for pathogen contamination because the water is filtered through many layers of soil and sediment. Surface water is open to many routes of contamination and is considered high risk for pathogenic microorganism contamination. Even though surface water is the irrigation source associated with the highest risk for pathogen contamination, it is the most widely used. According to the United States Geological Survey's report on the estimated use of water in the US, about 60% of water used for irrigation is drawn from a surface water source (Kenny 2009).

Human and plant pathogenic microorganisms have been known to spread through contaminated surface water sources for decades (Fair & Morrison 1967; Bewley & Buddin 1921) but only recently have growers been pressed to monitor their microbial irrigation water quality. In 1998, the Food and Drug Administration (FDA) released the "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" (U.S. Dept. of Health and Human Services 1998). In this guide, monitoring for pathogens in irrigation sources was only suggested to growers if they were concerned about their water quality. In 2013, the FDA passed the Food Safety Modernization Act (FSMA) to ensure the safety of the American food supply. To ensure the safety of produce FSMA focuses on the prevention of contamination in the produce growing

environment. Under the act, agricultural water, including irrigation water, many growers will be required to monitor their water for the fecal indicator *Escherichia coli.* Thresholds for surface irrigation water quality under FSMA are >236 CFU/100 ml generic *E. coli* in one sample or a mean over 5 samples of >126 CFU/100 ml. If irrigation water quality exceeds thresholds, growers would have to discontinue use of the irrigation source unless it is treated and further testing results are below threshold levels (US FDA 2013).

Regulations only exist for human pathogens in irrigation water sources because they are a public health concern. Plant pathogens in irrigation water sources are not regulated but are of great concerns to growers. Oomycete plant pathogens, also known as water-molds, are a group of organisms that are well suited to spread through water sources and have been frequently found in surface water sources (Hong & Moorman 2005; Ouedemans 1999; Zappia 2012). Many oomycete species in the genera *Phytophthora* and *Pythium* are plant-pathogenic and produce abundant numbers of motile zoospores that are capable of contaminating surface water sources (Erwin & Ribeiro 1996). If contaminated water is used for irrigation, plant disease could occur and result in yield and economic loses.

Some growers are in need of suitable surface water treatment for food safety and plant health applications. Many different irrigation situations exist and one treatment option will not be adequate for all. Some current treatment methods include; chlorination, ozone, heat, filtration, and use of surfactants. Ultraviolet light treatment of water has been successfully used for the treatment of drinking water, but not for surface water because of its variability in water quality parameters such as turbidity and solids content. Components of turbidity and particulates in water can shield or absorb UV light and make treatment less effective (Spellman 2004). Recent

improvements in UV water treatment technology, such as those developed the CiderSure company (FPE Inc., Rochester, NY), have allowed for the successful treatment of apple cider, a liquid with variable turbidity and high solids content. Due to the similar properties of apple cider and surface water UV treatment of surface water for irrigation should be explored. One advantage of UV treatment is that the process is pH independent (Basaran et al. 2004). Some chemical water treatments, such as chlorination, are pH dependent. Chlorination is an effective treatment option only if the pH of the water is below neutral (White and Black & Veatch 2010). The pH of many surface waters can be outside the range for effective chlorine treatment (see Chapter 1). Chlorination is also known to produce harmful disinfection byproducts that can be harmful to human health (Karanfil 2008). Ultraviolet light treatment does not produce harmful disinfection byproducts (Environmental Protection Agency 2013). Another advantage for UV treatment of surface water is that the method can be utilized by both organic and conventional growers.

Detection of microorganisms from water sources can be challenging. Surface water environments are ecosystems filled with many microorganisms that could complicate detection strategies for specific pathogens. In the bacterial genus *Pseudomonas* there are almost 200 described species with new species still being discovered (Tribedi et al. 2012; Arasu et al. 2012; De Jonghe et al. 2012). Pseudomonads are known for their diversity and their ability to live in a wide range of environmental niches (Madigan & Martinko 2006). Traditionally, detection of *P. syringae* pathovars was based on phenotypic properties after the bacterium was isolated from a symptomatic host plant. Within the species *Pseudomonas syringae* there are over 60 plant pathogenic subspecific pathovar designations based on host range, many with overlapping host ranges (Cai et al. 2011; Young 2010). Isolation of a pseudomonad from a known host may not

be sufficient or timely for identification of a pathovar. In some cases, host information may not be available if one is trying to detect pathogens from an environmental source such as water or soil. Many pseudomonads, such as *P. putida* and *P. fulva* form endophytic relationships with plants and can confound culture based identification methods (Ryan et al. 2008; Pokojska-Burdziej et al. 2004). Other pseudomonads, such as *P. fluorescens*, can colonize plant surfaces and live successful epiphytic lifestyles (Stockwell et al. 2013). These epiphytic pseudomonads can also complicate pathogen detection methods. Detection based on DNA sequences has largely replaced culture based methods (Young 2010). Most DNA-based detection methods are developed from a gene or loci, for example the internal transcribed spacer region between ribosomal subunits or the gene for a ribosomal subunit itself, which all strains of a species have in their genomes. Sometimes, these genomic regions do not provide sufficient resolution down to the species level. More than one pathovar, for example, *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* can cause disease on tomato. In some cases, resolution is necessary to a subspecies level. Resolution within the same *P. syringae* pathovar may be necessary due to large differences in virulence among individual strains.

Within this study, I try to gain a better understanding of risk associated with using surface water in agriculture production to aid growers in their continual efforts to improve food safety and plant health. I also test a UV treatment system for its utility to treat surface water with pathogens that threaten agricultural production. Finally, I use a genome assisted approach to develop a detection protocol for a plant pathogen with sub-pathovar resolution. All of this work was done for the improvement of agricultural production of fruits and vegetables.

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# CHAPTER ONE

## Prevalence and longitudinal study of *Escherichia coli, Salmonella* spp. and plant pathogenic oomycetes in surface irrigation water sources in New York State

**ABSTRACT**

In the United States, surface water is commonly used to irrigate a variety of produce crops and can harbor pathogens responsible for foodborne illnesses and plant diseases. Understanding when pathogens infest water sources is valuable information for produce growers to improve the food safety and production of these crops. In this study, prevalence data along with regression trees analyses were used to correlate water quality parameters (pH, temperature, turbidity), irrigation site properties (source, livestock or fowl nearby) and precipitation data to the presence and concentrations of *Escherichia coli*, *Salmonella* spp., and hymexazol-insensitive (HIS) oomycetes (*Phytophthora* and *Pythium* spp.) in New York surface waters. A total of 123 samples from 18 sites across New York State were tested for *E. coli* and *Salmonella* spp., 36% and 46% were positive, respectively. Additionally, 210 samples from 38 sites were tested for HIS oomycetes and 88% were found to be positive, with 10 species of *Phytophthora* and 11 species of *Pythium* identified from samples. Regression analysis found no strong correlations between water quality parameters, site factors, or precipitation, to the presence or concentration of *E. coli* in irrigation sources. For *Salmonella*, precipitation (≤0.64cm) three days before sampling, was correlated to both the presence and highest counts. Analyses for oomycetes found creeks to have higher average counts than ponds and higher turbidity levels were associated with higher oomycete counts. Overall, information gathered from this study can be used to better understand the food safety and plant pathogen risks of using surface water for irrigation.

## INTRODUCTION

Water is necessary for crop production and used for irrigation, pesticide applications, freeze protection and other agricultural purposes. Many produce growers in the United States use surface water for crop production from a variety of sources, which may include ponds, creeks, streams, lakes, and canal ways. A recent study of NY fruit and vegetable growers responding to a survey found that 57 % use surface water for irrigation and 18% use surface water for mixing of topical/pesticide sprays (Bihn et al. 2013). Surface water sources are considered high-risk for pathogen contamination because they are open to many routes by which both human foodborne illness and plant disease causing microorganisms can enter. Human pathogenic bacteria are believed to enter surface waters mainly through contamination from fecal material, from wildlife, and livestock, directly or indirectly via contaminated water, soil, or debris. Plant pathogens can enter surface water sources through many routes including infested soil, water and debris, cull piles, and field drainage tiles. The types and frequency of disease causing microorganisms can differ from one geographic location to the next, due to climate, available hosts, and other environmental characteristics.

There has been increasing scrutiny of agricultural water with respect to food safety, especially for fruit and vegetable growers whose produce is likely to be consumed raw. Two bacteria that are frequently associated with fresh produce foodborne illnesses are pathogenic *Escherichia coli* and *Salmonella*. Both of these bacteria have been reported from surface water sources and could be introduced into the fruit and vegetable growing environment through irrigation (Strawn et al. 2013). Once introduced, these bacteria have the potential to cause foodborne illness due to the consumption of contaminated produce. *Escherichia coli* and *Salmonella* are have been shown to

be capable of surviving on plant surfaces and persisting in the soil for long periods of time (Poza-Carrion et al. 2013; Wright et al. 2013; Zheng et al. 2013; Barak et al. 2011; Habteselassie et al. 2010; Barak et al. 2008; Ibekwe et al. 2007; Brandl et al. 2002). There is even evidence that these bacteria, whose main hosts are mammalian digestive tracts, when exposed to surface water before introduction to a plant surface, are better able to survive and persist on plant surfaces due to a stress response that causes the bacteria to produce structures that mediate bacterial attachment and exopolysaccharides that protect them from desiccation and UV exposure (White et al. 2006; Jeter et al. 2005; Lapidot et al. 2009; Xicohtencatl-Cortes et al. 2009). Foodborne illness can lead to deaths, and great financial losses can result if an outbreak is associated with a farm or type of produce. The recent Food Safety Modernization Act (FSMA), passed by the Food and Drug Administration, focuses on the prevention of contamination in the growing environment and require many growers to monitor their irrigation water for generic *E. coli*, an indicator of fecal contamination, and discontinue use if levels exceed >235 CFU/100 ml or an average over 5 samples of 126 CFU/100 ml.

All major groups of plant pathogens, which include bacteria, viruses, fungi, nematodes, and oomycetes, have been found in irrigation water. In a 2005 review of plant pathogens in irrigation water, Hong and Moorman listed 8 species of bacteria, 43 species of oomycetes, 27 species of fungi, 10 viruses, and 13 species of nematodes that have been recovered from irrigation water sources, many of which were from surface water (Hong & Moorman 2005). Among the plant pathogens, the oomycetes present the biggest water borne threat for fruit and vegetable growers. In particular, members of the genera *Phytophthora* and *Pythium* are pathogenic to plants are well-suited to be spread through surface water and are commonly referred to as water-molds. Many members of the *Phytophthora* and *Pythium* genera produce abundant numbers of

biflagellate, asexual zoospores that are suited to move through water towards potential plant hosts. Zoospores are believed to be primarily responsible for water infestations, but other structures such and sporangia, oospores, and mycelia may also infest water sources. Zoospores have been reported to remain viable in surface waters for hours to weeks, depending on the species and environmental factors (Porter et al. 2004; Zan et al. 1962). *Phytophthora capsici* is the causal agent of Phytophthora Blight of many vegetables including, tomato, pepper, cucurbits, snap and lima beans, and has many weed hosts (Erwin & Ribeiro 1996; Gevens et al. 2008; Davidson et al. 2002; Tian et al. 2004). *Phytophthora capsici* has been found in surface waters in several agricultural regions of the United States, but it's presence in NY surface waters was previously unknown (Gevens et al. 2007; Ivey 2011; Wang et al. 2009). This study confirms the presence of *Ph. capsici* in NY surface water sources and suggests the potential for spreading through NY surface water sources.

This study was designed to develop a better understanding of the prevalence and population levels of generic *E. coli, Salmonella* spp., and HIS oomycete plant pathogens in surface waters used for irrigation of fruit and vegetable crops in New York State. Water quality parameters, irrigation site properties, and precipitation data were collected and analyzed for correlations with the presence and levels of microorganisms in surface water irrigation sources. This study adds to the growing body of information regarding foodborne and plant pathogen risks associated with surface irrigation water.

## MATERIALS & METHODS

**Water sampling and analysis.** During the growing seasons of 2010 and 2011, a pathogen survey was conducted in actively used surface water irrigation reservoirs throughout vegetable

growing regions of New York (Figure 1.1). Monthly water samples were collected between May and October (Table 1) near the surface where water was drawn for irrigation. Two liters were were collected and collected from each sampling event in sterile 1L bottles (Nalgene, Penfield, NY) using a telescopic swing sampler (Nasco, Fort Atkinson, WI). Water temperature was measured using a pond thermometer (Lifeguard Aquatics, Cerritos, CA), and samples were stored in a cooler for transport back to the laboratory where they were processed within 24 hrs. The pH (Hanna HI 2211 pH/ORP meter, Woonsocket, RI) and turbidity (HACH 2100P portable turbidimeter, Loveland, CO) measurements were taken for all samples collected in 2011. To test for the presence generic *E. coli* and *Salmonella* spp., 100 ml of water was filtered using 47 mm, 0.45 μm pore size filters (Thermo Fisher Scientific, Waltham, MA) and placed onto Violet Red Bile Agar (VRBA, Hardy Diagnostics, Santa Maria, CA) + 4-Methylumbelliferyl-fl-D-Glucuronide (MUG, HACH, Loveland, CO) to test for *E. coli* and on bismuth sulfite agar (Criterion, Santa Maria, CA) to test for *Salmonella* spp. Bacterial samples were incubated at 37˚C for 18 h. Eighteen sites were assayed for both *E. coli* and *Salmonella* spp. (Table 1.1). Identification of *E. coli* was determined by characteristic appearance on VRBA + MUG agar and fluorescence under longwave UV (365 nm) light. Identification of *Salmonella* spp. was based on diagnostic appearance (dark brown to black colonies with or without a metallic sheen) on bismuth sulfite agar.

To assay for oomycetes, 1L of water was filtered using a sterile magnetic filter funnel (PALL, Port Washington, NY) and 47 mm, 5.0 μm pore size filters (EMD Millipore, Billerica, MA). One or more filters were used per 1L sample to prevent clogging. The filters were inverted onto PARPH agar and incubated at 25˚C for up to five days, PARPH agar is selective for hymexazol-insensitive (HIS) oomycetes that includes most *Phytophthora* spp. some *Pythium* spp. (Jeffers

Figure 1.1 Map of irrigation water sampling sites in New York

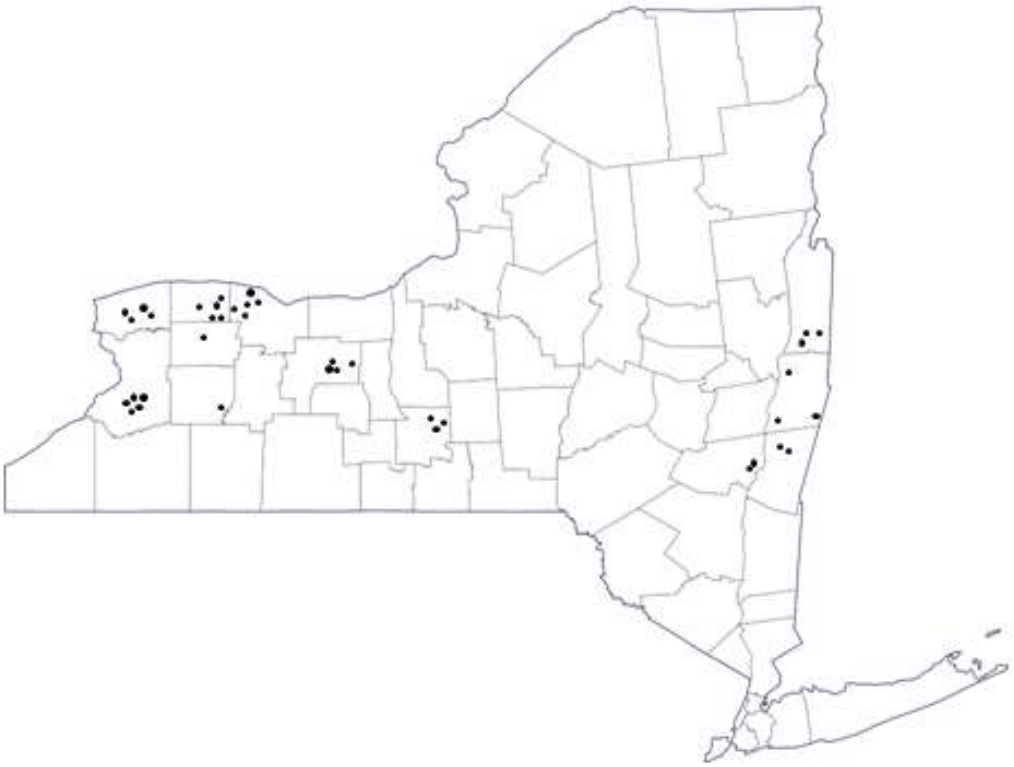

Table 1.1 Surface water irrigation site sampled and water quality parameters

| Site | County | Source | Bait Trap | 2010 | 2011 | pH[a] | Turbidity NTU[a] | Temp-erature ˚C[b] |
|---|---|---|---|---|---|---|---|---|
| A | Genesee | Pond | Yes | May-Sept | NT[c] | 8.20 | NT | NT |
| B[d] | Monroe | Canal | No | May-Oct | Aug-Oct | 8.33 | 7.93 | 18.9-26.7 |
| C | Orleans | Creek | No | May-Sept | NT | 8.31 | NT | NT |
| D | Orleans | Creek | Yes | July-Sept | NT | 8.05 | NT | NT |
| E | Monroe | Creek | Yes | May-Sept | NT | 8.37 | NT | NT |
| F | Columbia | Pond | Yes | June-Sept | NT | 9.59 | NT | NT |
| G[d] | Columbia | Pond | Yes | June-Sept | June-Oct | 9.15 | 58.27 | 18.9-26.7 |
| H[d] | Rensselaer | Pond | Yes | June-Sept | June-Oct | 8.43 | 4.09 | 18.9-26.7 |
| I[d] | Rensselaer | Creek | Yes | June-Sept | June-Oct | 8.11 | 2.34 | 16.11-23.9 |
| J[d] | Rensselaer | Pond | Yes | June-Sept | June-Oct | 8.92 | 3.75 | 16.7-26.7 |
| K | Greene | Pond | Yes | June-Sept | NT | 8.24 | NT | NT |
| L | Greene | Pond | No | June-Sept | NT | 8.10 | NT | NT |
| M[d] | Erie | Pond | Yes | June-Sept | June-Oct | 8.22 | 3.41 | 18.9-27.8 |
| N[d] | Erie | Pond | Yes | June-Sept | June-Oct | 8.17 | 3.40 | 18.9-26.7 |
| O[d] | Erie | Pond | Yes | June-Sept | June-Oct | 8.03 | 0.75 | 18.9-26.7 |
| P[d] | Erie | Pond | Yes | June-Sept | June-Oct | 8.17 | 7.23 | 17.8-26.7 |
| Q[d] | Erie | Pond | Yes | June-Sept | June-Oct | 8.97 | 5.44 | 18.9-26.7 |
| R[d] | Niagara | Pond | Yes | June-Sept | June-Oct | 7.71 | 15.46 | 19.4-26.7 |
| S[d] | Niagara | Pond | No | June-Oct | June-Oct | 8.20 | 2.08 | 18.9-27.8 |
| T | Monroe | Pond | Yes | July-Sept | NT | 8.29 | NT | NT |
| U[d] | Ontario | Pond | Yes | NT | May-Oct | 8.74 | 16.52 | 13-27.8 |
| V[d] | Ontario | Pond | Yes | NT | May-Oct | 9.32 | 9.70 | 18.9-27.8 |
| W[d] | Tompkins | Pond | No | NT | May-Oct | 8.31 | 2.85 | 18.9-26.7 |

continued

Table 1.1 (continued) Surface water irrigation site sampled and water quality parameters

| Site | County | Source | Bait Trap | 2010 | 2011 | pH[a] | Turbidity NTU[a] | Temp-erature °C[b] |
|---|---|---|---|---|---|---|---|---|
| X[d] | Tompkins | Creek | No | NT | May-Oct | 8.38 | 3.20 | 16.7-24.4 |
| Y[d] | Tompkins | Creek | No | NT | June-Oct | 8.32 | 2.67 | 18.9-24.4 |
| Z[d] | Niagara | Pond | Yes | NT | June-Oct | 8.58 | 5.73 | 18.9-27.8 |
| AA | Niagara | Pond | No | NT | June-Oct | 8.18 | 3.91 | 20-25.6 |
| BB | Orleans | Creek | No | NT | Aug-Oct | 8.39 | 7.72 | 18.9-25.6 |
| CC | Orleans | Creek | No | NT | Aug-Oct | 8.42 | 3.39 | 18.9-25.6 |
| DD | Monroe | Creek | No | NT | Aug-Oct | 8.49 | 3.76 | 20.6-25.6 |
| EE | Monroe | Creek | No | NT | Aug-Oct | 8.32 | 2.44 | 18.9-25.6 |
| FF | Wyoming | Creek | No | May | NT | 8.77 | NT | NT |
| GG | Ontario | Pond | No | NT | June | 8.67 | 6.88 | 27.8 |
| HH | Orleans | Creek | No | Aug | NS | 8.61 | NT | NT |
| JJ | Ontario | Creek | No | Oct | June | 7.91 | 1.55 | 17.8 |
| KK | Washington | Pond | Yes | NT | Oct | 8.17 | 2.06 | 18.9 |
| LL | Washington | Pond | No | NT | Oct | 7.08 | 7.35 | 18.9 |
| MM | Washington | Pond | No | NT | Oct | 7.79 | 4.15 | 18.9 |

[a] These values represent averages

[b] These values represent ranges

[c] Not Tested

[d] Sites assayed for *E. coli* and *Salmonella* spp. (All sites were assayed for oomycetes)

& Martin1986; Ferguson & Jeffers 1999). Single isolates were transferred to new PARPH agar as growth occurred. All sites were assayed for oomycetes.

**Oomycete baiting in water.** Oomycetes were assayed directly from water samples, and also with the use of pear, cucumber and lemon leaf baits. Green pears (Bartlett) were collected and placed in cold storage (4 ˚C) until needed. Cucumbers were purchased from a local grocery store as needed. Lemon (*Citrus limon*) trees were maintained in a greenhouse and leaves were collected as needed. Before use, all baits were surfaced sterilized in 10% bleach for 1 min and rinsed with distilled water. Baits (2 cucumbers, 2 pears, and 2 lemon leaves) were placed in single door rigid live traps (EDMBG, Fort Wayne, IN), trap mechanisms removed, with cylindrical pieces of polyethylene foam (Gladon, Oak Creek, WI) attached to the sides to keep traps upright and floating (Figure 1.2). A landscaping brick attached to wire was used as an anchor to keep traps from washing ashore. Baited traps were floated in irrigation sources for 7 days, baits were then collected and transported back to the laboratory in a cooler and processed within 24 hrs. Baits were rinsed with sterile distilled water. Tissue was excised from single lesions, placed onto PARPH agar, and incubated at 25˚C for up to five days (Figure 1.3). Baits still in good condition (few or no lesions) were placed in a moist chamber for up to 5 days at 25˚C for further lesion development and were checked and isolated from daily. As a negative control, baits were routinely checked for native oomycete infections.

**DNA extraction, PCR, and identification of oomycetes.** Oomycete isolates were grown in 15% clarified V8 broth for DNA extraction. Mycelium was rinsed with distilled water and DNA was extracted according to a CTAB based method developed by Keb-Llanes et al. (2000) with modifications (polyvinylpyrrolidone and β- mercaptoethanol were omitted from


Figure 1.2. Baiting for oomycete plant pathogens. A: Bait trap containing lemon leaves, cucumbers and pears.

B: Bait trap floating in irrigation pond.

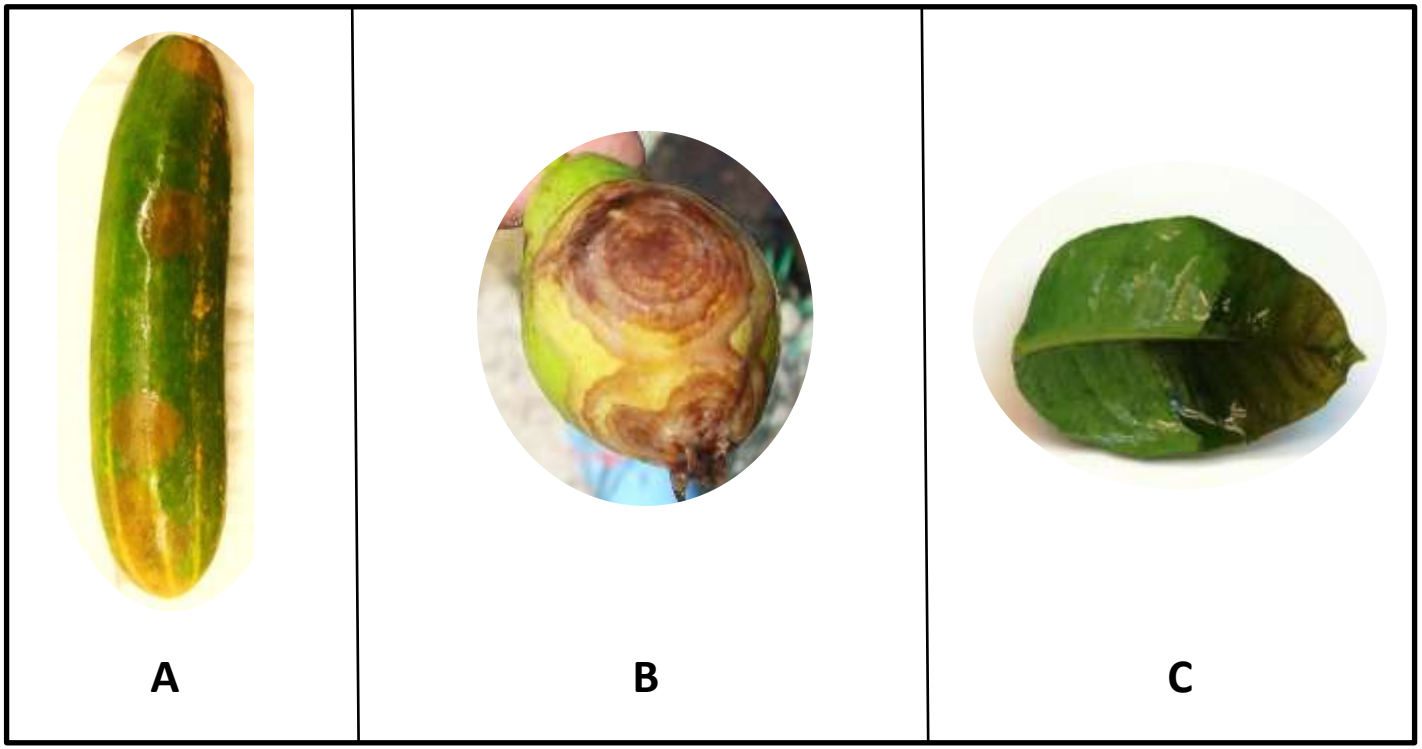


Figure 1.3. Infected baits from irrigation sources. A: Cucumber; B: Pear; C: Lemon leaf.

extraction buffer A). The 5.8S *rRNA* gene and internal transcribed spacer regions (ITS1 and ITS2) were amplified using 50 µl PCR reactions with primers ITS4 and ITS5 as previously described (White et al. 1990) under the following conditions; initial denaturation of 95 ˚C for 5 min, followed by 35 cycles at 95 ˚C for 1 min, 56 ˚C for 1 min, 72 ˚C for 1 min, and a final extension

step of 72 ˚C for 10 min using the Eppendorf Mastercyler gradient thermocycler (Eppendorf, Hauppauge, NY.) Amplicons were sequenced at Cornell University's Biotechnology Resource Center (Ithaca, NY). Identification was based on sequence comparison to previously identified oomycetes in GenBank (Benson et al. 2006) and the Phytophthora Database (www.phytophthoradb.org).

**Calculations and statistics.** Total prevalence of *E. coli*, *Salmonella*, or oomycetes was calculated as the number of positive (≥ 1 CFU/100 ml for *E. coli* or *Salmonella*, ≥1 CFU/L for oomycetes) samples divided by the total number of samples assayed for the particular organism. For each organism, prevalence was also calculated for samples within each water source type (pond, creek, or canal). A paired t-test was conducted in JMP Pro 11 (SAS Institute Inc., Cary, NC) using average CFU/100 ml *Salmonella* and average CFU/100 ml *E. coli* per site to determine if the average level of one bacterium is greater than the other. The hypothesized mean value was set at zero.

Parameters recorded from water samples used in statistical analyses as possible predictor variables were: site, pH, turbidity, water temperature, presence of livestock near irrigation site, presence of fowl in or near site, precipitation amounts for the day of sampling, and precipitation 3 days prior to sampling. Presence or absence and concentration of each study organism were

used as response variables. Precipitation data was collected using the National Climate Data Center Climate Data Online mapping tool selecting from the nearest weather station with complete precipitation data available (all stations used were within 15 km of a site), (http://www.ncdc.noaa.gov/cdo-web/).

All regression trees were built using Random Effect – Estimation Methods (RE-EM) (Sela & Simonoff 2012). Regression trees were computed using the REEMtree package (version 2.14.2) in R (R Core Team 2013), where irrigation site was specified as the random effect. This method was chosen because it was designed for analysis of longitudinal data where data collected from repeated sampling of the same subjects or sites are not independent. Each response variable for qualitative and quantitative measures of study organisms was analyzed in a correlation matrix, using JMP Pro 11, with possible predictor variables, those variables with the greatest correlation coefficients were used for building regression trees (Tables 1.2, 1.3 and 1.4). A correlation matrix was used because the units of measure between variables differ. Each regression tree was run with a 10-fold cross validation.

Cluster analyses were used to study the relationship between oomycete counts and turbidity because turbidity was the variable with the greatest correlation to oomycete counts. The cluster analyses were computed for each irrigation source separately because source and oomycete counts were correlated. All cluster analyses (k-mean method) were performed using JMP Pro 11.

Table 1.2 Variable names and descriptions

| Response Variable | Description |
|---|---|
| EcoliPA | water sample positive for *E. coli*, yes or no |
| Ecoli | *E. coli* CFU/100 ml |
| SalmoPA | water sample positive for *Salmonella* spp., yes or no |
| Salmo | *Salmonella* spp. CFU/100 ml |
| OomPA | sample positive for oomycetes, yes or no |
| Oom | oomycetes CFU/L |
| **Predictor Variable** | **Description** |
| SiteID | unique identifier for each irrigation source |
| Source | irrigation water source type, pond, creek, or canal |
| pH | pH of water sample |
| Turbidity | turbidity (NTU) of water sample |
| Temp | water temperature (˚C) at time of sampling |
| Livestock | was livestock present within 100 m of irrigation source, yes or no |
| Fowl | were fowl present in irrigation source at time of sampling |
| PrecipYN | was cumulative precipitation 3 days prior to sampling ≥ 0.64 cm, yes or no |
| PrecipDay | precipitation (cm) on day of sampling |
| Precip3Days | cumulative precipitation (cm) 3 days prior to sampling |

Table 1.3. Correlation matrix for *E. coli* and *Salmonella* regression tree development

| | EcoliPA | Ecoli | SalmoPA | Salmo | pH | Source | Temp |
|---|---|---|---|---|---|---|---|
| EcoliPA | 1 | 0.329 | 0.1568 | 0.1512 | -0.0686 | 0.1451 | 0.0124 |
| Ecoli | 0.329 | 1 | 0.0943 | 0.2262 | -0.0314 | 0.0765 | 0.0058 |
| SalmoPA | 0.1568 | 0.0943 | 1 | 0.6071 | -0.0092 | 0.1415 | 0.0387 |
| Salmo | 0.1512 | 0.2262 | 0.6071 | 1 | -0.0359 | 0.1167 | 0.1145 |
| pH | -0.0686 | -0.0314 | -0.0092 | -0.0359 | 1 | 0.1032 | 0.1374 |
| Source | 0.1451 | 0.0765 | 0.1415 | 0.1167 | 0.1032 | 1 | 0.2368 |
| Temp | 0.0124 | 0.0058 | 0.0387 | 0.1145 | 0.1374 | 0.2368 | 1 |
| Turbidity | 0.0182 | 0.0012 | -0.1036 | -0.1146 | 0.3536 | 0.159 | 0.106 |
| Livestock | -0.0007 | 0.179 | -0.0379 | -0.0161 | 0.1551 | -0.053 | -0.0956 |
| PrecipYN | 0.0707 | 0.2242 | -0.1878 | -0.1242 | -0.075 | 0.0303 | -0.5581 |
| PrecipDay | -0.0488 | 0.0013 | -0.1449 | -0.0732 | 0.098 | -0.1485 | -0.1696 |
| Precip3Days | 0.1245 | 0.2524 | -0.2421 | -0.1651 | -0.0259 | -0.0609 | -0.2313 |
| Fowl | -0.0414 | 0.1023 | 0.0597 | 0.1361 | -0.0231 | 0.2591 | 0.1329 |

| | Turbidity | Livestock | PrecipYN | PrecipDay | Precip3Days | Fowl |
|---|---|---|---|---|---|---|
| EcoliPA | 0.0182 | -0.0007 | 0.0707 | -0.0488 | 0.1245 | -0.0414 |
| Ecoli | 0.0012 | 0.179 | 0.2242 | 0.0013 | 0.2524 | 0.1023 |
| SalmoPA | -0.1036 | -0.0379 | -0.1878 | -0.1449 | -0.2421 | 0.0597 |
| Salmo | -0.1146 | -0.0161 | -0.1242 | -0.0732 | -0.1651 | 0.1361 |
| pH | 0.3536 | 0.1551 | -0.075 | 0.098 | -0.0259 | -0.0231 |
| Source | 0.159 | -0.053 | 0.0303 | -0.1485 | -0.0609 | 0.2591 |
| Temp | 0.106 | -0.0956 | -0.5581 | -0.1696 | -0.2313 | 0.1329 |
| Turbidity | 1 | -0.0194 | -0.0342 | -0.0894 | 0.0576 | -0.2337 |
| Livestock | -0.0194 | 1 | 0.1406 | -0.0335 | 0.1576 | 0.1077 |
| PrecipYN | -0.0342 | 0.1406 | 1 | 0.112 | 0.7406 | -0.0688 |
| PrecipDay | -0.0894 | -0.0335 | 0.112 | 1 | 0.1493 | 0.0198 |
| Precip3Days | 0.0576 | 0.1576 | 0.7406 | 0.1493 | 1 | -0.1256 |
| Fowl | -0.2337 | 0.1077 | -0.0688 | 0.0198 | -0.1256 | 1 |

Table 1.4. Correlation matrix for oomycete regression tree development and cluster analyses

| | OomPA | Oom | Temp | pH | Turbidity | PrecipYN | PrecipDay |
|---|---|---|---|---|---|---|---|
| OomPA | 1 | 0.3513 | 0.0262 | -0.0465 | 0.0689 | -0.0558 | 0.0768 |
| Oom | 0.3513 | 1 | 0.014 | 0.0212 | 0.3605 | 0.0769 | 0.1073 |
| Temp | 0.0262 | 0.014 | 1 | 0.2819 | 0.1953 | -0.3766 | -0.1836 |
| pH | -0.0465 | 0.0212 | 0.2819 | 1 | 0.3304 | -0.1507 | 0.0584 |
| Turbidity | 0.0689 | 0.3605 | 0.1953 | 0.3304 | 1 | 0.0111 | -0.0546 |
| PrecipYN | -0.0558 | 0.0769 | -0.3766 | -0.1507 | 0.0111 | 1 | 0.1239 |
| PrecipDay | 0.0768 | 0.1073 | -0.1836 | 0.0584 | -0.0546 | 0.1239 | 1 |
| Precip3Day | 0.0313 | 0.045 | -0.1207 | -0.0235 | 0.0575 | 0.7232 | 0.1282 |
| Source2 | 0.1804 | 0.2787 | -0.1385 | -0.1105 | -0.1676 | 0.0693 | 0.2367 |

| | Precip3Day | Source |
|---|---|---|
| OomPA | 0.0313 | 0.1804 |
| Oom | 0.045 | 0.2787 |
| Temp | -0.1207 | -0.1385 |
| pH | -0.0235 | -0.1105 |
| Turbidity | 0.0575 | -0.1676 |
| PrecipYN | 0.7232 | 0.0693 |
| PrecipDay | 0.1282 | 0.2367 |
| Precip3Day | 1 | 0.0431 |
| Source2 | 0.0431 | 1 |

Table 1.5. Frequency of samples and sites positive for *E. coli*, *Salmonella*, and oomycetes in irrigation water sources

| Organism<br>Sites | Frequency[a] | | | |
|---|---|---|---|---|
| | Overall | Pond | Creek | Canal |
| | | | | |
| *E. coli* | 44(123) | 38(99) | 6(19) | 0(5) |
| Sites | 16(18) | 13(14) | 3(3) | 0(1) |
| | | | | |
| *Salmonella* | 57(123) | 49(99) | 6(19) | 2(5) |
| Sites | 17(18) | 13(14) | 3(3) | 1(1) |
| | | | | |
| Oomycetes | 184(210) | 131(155) | 47(47) | 6(8) |
| Sites | 38(38) | 24(24) | 13(13) | 1(1) |

[a]Frequency data is shown as number of samples or sites positive (total number of samples or number of sites).

## RESULTS

**Prevalence of *E. coli*, *Salmonella* spp. and oomycetes.** Across all *E. coli* samples (n=123), 36% were positive. A total of 18 sites were assayed for *E. coli* and 16 of these sites were positive at least once during the survey (Table 1.5). The two sites where *E. coli* was not detected during the survey were the canal site (B) and one pond in Tompkins County (W). For each water source the prevalence of *E. coli* positive samples was 39%, 32%, and 0%, in ponds, creeks, and canal, respectively (Table 1.5). *Escherichia coli* positive samples ranged from 1- >300 CFU/100 ml. Three samples, from two different pond sites (H, P), had >300 *E. coli* CFU/100 ml.

Across all *Salmonella* samples (n=123), 46% were positive (≥1 CFU/100 ml). A total of 18 sites were assayed for *Salmonella* and 17 of the sites were positive at least once during the survey (Table 1.5). The single site where *Salmonella* was not detected during the survey was a pond site (W) in Tompkins County. For each water source, the prevalence of *Salmonella* positive samples was 49%, 32%, and 40%, in ponds, creeks, and canal, respectively (Table 1.5). Prevalence was equal to or higher for *Salmonella* than observed for *E. coli* in each water source with more frequent *Salmonella* counts exceeding 300 CFU/100 ml. *Salmonella* positive samples ranged from 1->300 CFU/100 ml with 23 samples, from 14 different sites (B, H, G, J, M, N, O, P, Q, R, S, U, V, Z) producing *Salmonella* counts of >300 CFU/100 ml. The average CFU/100 ml for *Salmonella* for each site was generally higher than the level CFU/100 ml of *E. coli* (Table 1.6). The results of a t-test constructed on the differences of the average *Salmonella* and *E.coli* levels for each site showed that differences were significantly different than zero (t-Test Prob > t < 0.0001).

Table 1.5. Frequency of samples and sites positive for *E. coli*, *Salmonella*, and oomycetes in irrigation water sources

| Organism<br>Sites | Frequency[a]<br>Overall | Pond | Creek | Canal |
|---|---|---|---|---|
| | | | | |
| *E. coli* | 44(123) | 38(99) | 6(19) | 0(5) |
| Sites | 16(18) | 13(14) | 3(3) | 0(1) |
| | | | | |
| *Salmonella* | 57(123) | 49(99) | 6(19) | 2(5) |
| Sites | 17(18) | 13(14) | 3(3) | 1(1) |
| | | | | |
| Oomycetes | 184(210) | 131(155) | 47(47) | 6(8) |
| Sites | 38(38) | 24(24) | 13(13) | 1(1) |

[a]Frequency data is shown as number of samples or sites positive (total number of samples or number of sites).

Across all oomycete samples (n=210), 88% were positive (≥1CFU/L), and all sites (n=38) assayed for HIS *Phytophthora* and *Pythium* were positive for one or more samples during the survey (Tables 1.5). A total of 1093 oomycetes were isolated, including 10 species of *Phytophthora* and 11 species of *Pythium* that were identified during the survey (Table 1.7 and 1.8). The most prevalent (present in ≥ 10% of samples) oomycetes were *Ph. lacustris*, *Ph. hydropathica*, *Ph. irrigata*, and *Py. litorale*. Many isolates (n=150) of both *Phytophthora* and *Pythium* could not be identified to the species level because their ITS DNA sequences did not clearly align with sequences of known species available in GenBank or the Phytophthora Database. Further investigation would be necessary to determine the identification of these isolates. Frequency of oomycetes ranged from 0-200 CFU/L of irrigation water. One-hundred percent of samples from creeks were positive while 85% of samples from ponds and 75% of samples from the canal were positive.

**Longitudinal study of *E. coli* .** Regression trees were computed for *E. coli* positives using the highest correlative variables for *E. coli* positive samples including; presence of *Salmonella*, irrigation source, and precipitation 3 days prior to sampling. The tree for *E. coli* positive samples resulted in a root node only suggesting that the variables used did not have strong predictive power for distinguishing between positive and negative samples. A regression tree was also computed for *E. coli* counts (CFU/100 ml) using variables for *Salmonella* counts and precipitation 3 days prior to sampling. The tree for *E. coli* counts resulted in a root node only, suggesting that the variables used did not have strong predictive power for characterizing *E. coli* CFU/100 ml. No parameters in the study were strongly correlated to the presence or levels of *E. coli* in the surface water irrigation sources surveyed.

Table 1.6. Average *Salmonella* and *E. coli* counts by site

| Site | *Salmonella* average | *E. coli* average | Difference |
|---|---|---|---|
| A | 159.5 | 0 | 159.5 |
| B | 120 | 20 | 100 |
| C | 132 | 24 | 108 |
| D | 46.67 | 11 | 55.67 |
| E | 206.47 | 14 | 192.47 |
| F | 154 | 10.5 | 143.5 |
| G | 153.2 | 6 | 147.2 |
| H | 146 | 37 | 109 |
| I | 203.67 | 77.25 | 126.42 |
| J | 93.6 | 17.4 | 76.2 |
| K | 104 | 5 | 99 |
| L | 300 | 5 | 295 |
| M | 109.33 | 1 | 108.33 |
| N | 126.2 | 6 | 120.6 |
| O | 161.75 | 171 | -10.25 |
| P | 107.8 | 6 | 101.8 |
| Q | 93 | 11 | 82 |

All numbers are in CFU/100 ml

Table 1.7. HIS oomycetes found in this study, source, isolation method and site

| Species | Frequency (n=210) | Isolation[a] | Source | Sites[b] |
|---|---|---|---|---|
| *Ph. lacustris* | 54 | W, P, C, L | Pond, Creek, Canal | A, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, S, U,V, X, Y, Z, AA, DD, FF, JJ, LL |
| *Ph. hydropathica* | 44 | W, P, C, L | Pond, Creek | A, C, D, E, F, G, H, J, L, M, N,O, P, Q, R, S, T, U, V, Z, AA, JJ |
| *Ph. irrigata* | 20 | W, P | Pond, Creek, Canal | B, C, D, I, K, L, M, N, R, Z, AA, T, CC, DD |
| *Ph.* spp | 15 | W, P, C | Pond, Creek | A, C, D, G, I, M, N, O, P, Q, R, T, W, AA, HH |
| *Ph. citricola* | 8 | W, P, C | Pond, Creek | C, D, E, I, J, K, L, JJ |
| *Ph. cryptogea* | 3 | W, C | Pond, Creek | C, G, Z |
| *Ph. gonapodyides* | 2 | W, P, C | Pond, Creek | I, R |
| *Ph. sansomeana* | 2 | P, C | Pond | F, G |
| *Ph. capsici* | 2 | P, C | Pond | M, U |
| *Ph. gallica* | 1 | P | Pond | K |
| *Ph. nicotianae* | 1 | P | Pond | J |
| *Py.* spp | 80 | W, P, C, L | Pond, Creek, Canal | A, B, C, D, E, F, G, H, I, J, K, L,M, N, O, P, Q, R, S, T, U, V,W, X, Y, Z, AA, BB, KK, EE |
| *Py. litorale* | 35 | W, P, C, L | Pond, Creek, Canal | A, C, D, E, G, H, I, Q, R, S, U, V, X, Y, AA, CC, HH, JJ |
| *Py. helicoides* | 17 | W, P, C, L | Pond, Creek | A, C, D, G, H, I, L, M, N, P, Q, S, HH |
| *Py. catenulatum* | 6 | W, C, L | Pond | A, G, H, K, L |
| *Py. marisipium* | 3 | W | Pond | A, F, K |
| *Py. myriotylum* | 2 | C, L | Pond | J, K |
| *Py. amasculinum* | 1 | W | Pond | L |
| *Py. mercuriale* | 1 | C | Pond | F |
| *Py. oedochilum* | 1 | W | Pond | L |
| *Py. irregulare* | 1 | W | Pond | G |
| *Py. vexans* | 1 | W | Creek | JJ |
| *Py. adhaerens* | 1 | W | Pond | M |

[a] Isolation method used: Water filter (W), or bait; pear (P), cucumber (C), lemon (L)

[b] Letters correspond to site locations in Table 1.1

Table 1.8. Frequency of HIS oomycetes by each source, isolation method, and site

| | Irrigation Source | | | | Isolation Method | | | | Site | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Phytophthora* species** | Total n=210 | Pond n=155 | Creek n=47 | Canal n=8 | Pear n=145 | Cucumber n=145 | Lemon n=145 | Filter n=210 | Total n=38 | Pond n=24 | Creek n=13 | Canal n=1 |
| *Ph. lacustris* | 54 | 36 | 18 | 0 | 35 | 7 | 3 | 30 | 26 | 17 | 8 | 1 |
| *Ph. hydropathica* | 48 | 44 | 4 | 0 | 24 | 13 | 6 | 16 | 22 | 18 | 4 | 0 |
| *Ph. irrigata* | 20 | 13 | 5 | 2 | 7 | 0 | 0 | 13 | 14 | 9 | 4 | 1 |
| *Ph.* spp. | 15 | 12 | 3 | 0 | 1 | 3 | 0 | 11 | 12 | 9 | 3 | 0 |
| *Ph. citricola* | 8 | 1 | 7 | 0 | 2 | 3 | 0 | 6 | 5 | 1 | 4 | 0 |
| *Ph. cryptogea* | 3 | 1 | 2 | 0 | 0 | 2 | 0 | 1 | 3 | 1 | 2 | 0 |
| *Ph. gonapodyides* | 2 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 1 | 0 |
| *Ph. sansomeana* | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 2 | 0 | 0 |
| *Ph. capsici* | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 2 | 0 | 0 |
| *Ph. gallica* | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Ph. nicotianae* | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Py.* spp | 71 | 49 | 20 | 2 | 18 | 21 | 9 | 42 | 30 | 19 | 10 | 1 |
| *Py. litorale* | 35 | 26 | 7 | 2 | 12 | 11 | 3 | 14 | 19 | 12 | 6 | 1 |
| *Py. helicoides* | 17 | 13 | 4 | 0 | 6 | 5 | 3 | 6 | 15 | 11 | 4 | 0 |
| *Py. catenulatum* | 6 | 6 | 0 | 0 | 0 | 2 | 3 | 2 | 5 | 5 | 0 | 0 |
| *Py. diclinum* | 6 | 5 | 1 | 0 | 0 | 3 | 1 | 2 | 6 | 6 | 0 | 0 |
| *Py. marisipium* | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 0 | 0 |
| *Py. lutarium* | 3 | 3 | 0 | 0 | 0 | 1 | 2 | 0 | 3 | 3 | 0 | 0 |
| *Py. myriotylum* | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 2 | 0 | 0 |
| *Py. amasculinum* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| *Py. mercuriale* | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Py. oedochilum* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| *Py. irregulare* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| *Py. vexans* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Py. adhaerens* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |

**Longitudinal study of *Salmonella* spp.** A regression tree for distinguishing *Salmonella* positive samples using variables for presence of *E. coli*, irrigation source, and all precipitation variables resulted in a single-node tree with precipitation ≥ 0-.64 cm (cumulative total 3 days prior to sampling) as the split. Fifty-four percent (n=88) of samples with less than 0.64 cm precipitation were positive, while 26% (n=35) of samples with greater than 0.64 cm precipitation were positive. A regression tree for *Salmonella* counts using the variables for precipitation (3 days prior to sampling) and *E. coli* counts showed samples with 0.38 - 0.64 cm precipitation associated with the highest *Salmonella* counts (146 CFU/100ml average). Samples receiving 0 – 0.38 cm had average counts of 57 CFU/100 ml, while samples receiving more than 0.64 cm of precipitation had average *Salmonella* counts of 11 CFU/100 ml. No splits occurred with *E. coli* counts as a predictor variable, suggesting that the levels of the two bacteria were not strongly correlated.

**Longitudinal study of HIS oomycetes.** A regression tree was computed for oomycete positive samples using irrigation source as the predictive variable. The tree resulted in a root node only suggesting that irrigation source did not have strong predictive power for distinguishing positive and negative samples. A regression tree was also computed for oomycete counts using irrigation source and turbidity as predictive variables. The tree for oomycete counts had one split for irrigation source showing that canal and creek samples (n=54) had, on average, higher oomycete counts, 19 CFU/L, than pond samples (n=156) with an average oomycete count of 12 CFU/L.

Cluster analyses were performed for oomycete counts and turbidity for creek and pond samples separately (Figures 1.4 and 1.5). A cluster analysis was not done for the canal site because of small sample size. For creek samples, the majority (18/25) belong to a cluster with average turbidities and oomycete counts of 2.4 NTU and 29 CFU/L. The remaining samples (7/25) were

in a cluster characterized by samples with higher average turbidities and oomycete counts of 4.0 NTU and 73 CFU/L. For pond samples, the majority (85/90) were in a cluster characterized by average turbidities and oomycetes counts of 5.7 NTU and 18 CFU/L. The remaining samples (5/85) were characterized by greater average oomycete counts and higher turbidity levels of 63.9 NTU and 78 CFU/L.

## DISCUSSION

Over the course of this study, *E. coli*, *Salmonella* spp. and HIS oomycetes were identified in surface water irrigation sources in New York. The presence and levels of *E. coli* in surface water irrigation sources was not closely associated with any of the parameters tested in this study. Contaminated runoff from precipitation events and proximity to livestock are considered likely sources for *E. coli* contamination of surface waters (USEPA 2012), but this study did not find a strong link between those or any other parameters tested and *E. coli* prevalence. Approximately 1/3 of all samples were positive for generic *E. coli*, showing that a risk exists for introducing *E. coli* into the produce preharvest environment by using surface water for irrigation. Fruit and vegetable growers are concerned with high levels of *E. coli* in their irrigation water because it is associated with fecal contamination and greater risk of introducing pathogenic bacteria, which could lead to foodborne illness associated with their produce. The new FSMA requires many growers to test their irrigation water for generic *E. coli*. If *E. coli* levels are > 235 CFU/100 ml for one sample, or a mean of >126 CFU/100 ml for five samples, the grower will have to discontinue use of the contaminated irrigation source until action is taken to rectify the water source and retesting shows *E. coli* levels below threshold (FDA 2013). Three samples from this study had *E. coli* levels above the regulatory threshold of >235 CFU/100 ml, but no sites had a mean greater than 126 CFU/100 ml over 5 samples. Further investigation is necessary to

understand why and when a surface water sample could exceed regulatory thresholds, as this information would be very valuable to any grower using surface water in the produce production environment.

*Salmonella* positive samples and high population levels were most correlated with periods of rainfall less than 0.64 cm (3 days before sampling), when growers are more likely to use water for irrigation. The lowest levels of *Salmonella* were associated with heavy rainfall amounts (>0.64 cm). Inconsistencies have been previously reported with the correlation of *Salmonella* levels and precipitation (McEgan et al. 2013; Strawn et al. 2013). Other factors, such as soil type, could influence the association of precipitation and Salmonella levels in irrigation sources (Strawn et al. 2013).

*Salmonella* was not strongly correlated with the presence of *E. coli,* suggesting that testing for *E. coli* would not provide information about *Salmonella* levels in irrigation sources. As a consequence, irrigation water can be a major source of *Salmonella* that is not routinely tested. In this study, *Salmonella* was more prevalent and had greater average levels for most sites than *E. coli* in surface irrigation water, and had more samples exceeding >300 CFU/100 ml. Other studies have also found *Salmonella* to be highly prevalent in surface water (Haley et al. 2009; Jokinen et al. 2011; Strawn et al. 2013). Major sources of water contamination by *Salmonella* and *E. coli* are similar; wildlife, livestock, and humans (Navarro et al. 2012; Hutchinson et al. 2005; Kinde et al. 1997). The greater prevalence and concentration of *Salmonella* compared to *E. coli* may be due to persistence of *Salmonella* in surface water sources compared with other potential pathogenic enteric bacteria as has been previously shown (Wright 1989; Chao et al. 1987).

All samples (n = 6) from one pond (W) in Tompkins County were found to be absent of *E. coli* and *Salmonella* spp. This result could be due to sampling frequency (once a month) where additional sampling could determine if this site is consistently free of *E. coli* and *Salmonella*. A unique feature of pond W is that it is the only embankment style pond in this study, and it is located at a higher elevation compared to the surrounding cultivated land. All of the other ponds sampled in this study were excavation style ponds. Embankment ponds are built on sloped terrain where a high embankment is constructed at the end of the pond with the lowest elevation to trap water. Excavation ponds are built on level ground, usually at the lowest elevation on the farm where groundwater is likely to be close to the surface. Pond style could play a role in the presence of organisms such as *E. coli* and *Salmonella* and should be further investigated.

Oomycetes were highly prevalent in all water sources indicating that using any surface water source for irrigation or other agricultural application runs a high risk for introducing plant pathogenic oomycetes into a growing environment. Several other studies have found high oomycete prevalence in surface waters (Ivey 2011; Gevens et al. 2007; Oudemans 1999, Steadman et al. 1975). We found higher levels of oomycetes in irrigation water sources from creeks compared to ponds. The canal site had similar levels of oomycetes to that observed with creeks but since only one canal site was sampled in this study, further investigation is necessary to describe oomycete prevalence in canals used for irrigation purposes. Irrigation ponds are primarily fed from groundwater and drainage tiles, while creeks are primarily fed from surface runoff, this could play a role in the difference in oomycete counts between ponds and creeks. Surface runoff is likely to contain oomycete infested water and debris that can enters creeks anywhere along the banks. Water entering a pond through ground water or a drainage tile passes through layers of soil where some of the oomycetes may be trapped. Turbidity levels were found

to be positively associated with oomycete levels in the surface waters sampled in this study. Greater turbidity may be associated with larger amounts of runoff water or debris containing oomycetes that enter a surface water source and could explain the positive correlation. In addition, turbidity could play a role in the persistence of oomycetes in surface water. Components of turbidity such as clay, silt, organic matter and microorganisms could play a role in survival for some oomycetes and protect them from ultraviolet light exposure as well.

The most prevalent (present ≥ 10% of samples) HIS oomycetes isolated in this survey were *Ph. lacustris, Ph. hyrdopathica, Ph. irrigata,* and *Py. litorale*, each of these plant pathogens have previously been found in surface water sources and are not considered major threats for fruit and vegetable growers (Venkatesan et al. 2013; Hong et al. 2008; Hong et al. 2010; Nechwatal et al. 2012). While *Ph. irrigata* and *Py. litorale* have been show to produce disease on some vegetable crops in greenhouse studies, no disease has been attributed to these species from plants grown in the field (Venkatesan et al. 2013; Hong et al. 2008). Other *Phytophthora* spp. species isolated during this survey include; *Ph. citricola, Ph. cryptogea, Ph. gonapodyides, Ph. sansomeana, Ph. capsici*, and *Ph. nicotianae*. These species have previously been isolated from surface water sources and are known to cause disease on some fruit and vegetables crops (Oudemans 1999; Hong et al. 2011; Yamak et al. 2002; Erwin & Ribeiro 1996; Hansen et al. 2009). *Phytophthora gallica* has not been previously reported from surface water and is pathogenic on some alder and beech trees, but is not known to infect cultivated fruit and vegetable crops (Jung et al. 2008). Many of the *Pythium* spp. isolated during this survey including; *Py. helicoides, Py. catenulatum, Py. marisipium, Py. myriotylum, Py. irregulare, Py. vexans,* and *Py. adhaerens,* have previously been reported from surface water sources (Zappia 2012; Sanchez et al. 2001; Abdelzaha et al. 1994; Gill 1970; Shokes 1979; Lodhi et al. 2004). Four *Pythium* spp.; *Py. amasculinum, Py.*

*mercuriale*, *Py. adhaerens* and *Py. oedochilum* isolated in this water survey have not been previously reported from surface water sources. Each *Py*. spp. found in this survey has been previously isolated from a diseased crop plant (Chellemi et al. 2000; Frezzi 1956; Abdekzaha et al. 1994; Wantanabe et al. 2006; Paulitz et al. 2004; Vawdry et al. 2005; Sparrow 1932, Japan Society 2013; Wheeler et al. 2013; Belbahir et al. 2008) .

The results from this study show that the prevalence of generic *E. coli*, *Salmonella* and HIS plant pathogenic oomycetes in surface water in New York State is high. Using surface water for irrigation puts a grower at risk for introducing potential human and plant pathogens into their growing environment. Water quality parameters, irrigation site properties, and precipitation data may be useful in helping predict the prevalence of potential pathogen contamination of surface water used for irrigation. Further studies are necessary to better understand specific factors that influence pathogen contamination and persistence in surface water, as this information could be used to reduce occurrences of foodborne illness and plant disease.

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# CHAPTER TWO

# ULTRAVIOLET LIGHT INACTIVATION OF HUMAN AND PLANT PATHOGENS IN UNFILTERED SURFACE IRRIGATION WATER

**ABSTRACT**

Fruit and vegetable growers continually battle plant diseases and food safety concerns. Surface water is commonly used in the production of fruits and vegetables and can harbor both human- and plant-pathogenic microorganisms that can contaminate crops when used for irrigation or other agricultural purposes. Treatment methods for surface water are currently limited and there is a need for suitable treatment options. A liquid processing unit that uses ultraviolet light for the decontamination of turbid juices was analyzed for its efficacy in the treatment of surface waters contaminated with bacterial or oomycete pathogens; *Escherichia coli, Salmonella enterica, Listeria monocytogenes, Clavibacter michiganensis* subsp. *michiganensis, Pseudomonas syringae* pv. *tomato,* and *Phytophthora capsici*. Five-strain cocktails of each pathogen containing approximately $10^8$ or $10^9$ CFU/L for bacteria, or $10^4$ or $10^5$ zoospores/L for *Ph. capsici,* were inoculated into aliquots of two turbid surface water irrigation sources and processed with the UV unit. Pathogens were enumerated before and after treatment. In general, as the turbidity of the water source increased the effectiveness of the UV treatment decreased, but in all cases, 99.9% or higher inactivation was achieved. Log reductions ranged from 10.0 to 6.1 and from 5.0 to 4.2 for bacterial pathogens and *Ph. capsici*, respectively.

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Jones LA, Worobo RW, Smart CD. 2014. UV light inactivation of human and plant pathogens in unfiltered surface irrigation water. Appl. Environ. Microbiol. 80(3): 849-854. doi: 10.1128/aem.02964-13.

## INTRODUCTION

For decades water has been a vector for human- and plant-pathogenic microorganisms (Centers for Disease Control and Prevention 2013; Hong & Moorman 2005). Illnesses caused by waterborne microorganisms can occur through the consumption of fruits and vegetables that have come in contact with contaminated water (Hillborn et al. 1999; U.S. Food and Drug Administration 2006). Plant pathogens are also spread through water and can lead to plant disease and yield losses. In the food production environment, agricultural water is defined as any water that is used in the growing, harvesting or packing of produce where water is likely to contact produce directly or surfaces that produce are likely to come in contact with (U.S. Food and Drug Administration 2013). Agricultural water, including that used for irrigation, pesticide or herbicide application, freeze protection, or produce wash water is under increasing scrutiny as a vehicle for foodborne human and plant disease-causing microorganisms. Irrigation water is one of the main avenues by which pathogenic microorganisms can reach produce, especially if the irrigation water is obtained from a surface water reservoir. Irrigation water is typically taken from groundwater, surface water, or municipal sources. Water from groundwater and municipal sources is generally free of pathogenic microorganisms but breaches can still occur. Surface water sources are considered high-risk for pathogen contamination because they are open to many routes by which both plant disease and human foodborne illness causing microorganisms can enter. Although surface water is a high risk source, many growers continue to use surface water because it remains the most feasible and economic choice.

Mitigation strategies may be necessary for growers to continue using surface water for agricultural applications, particularly for leafy greens or produce that is intended to be consumed raw. Human pathogenic bacteria can enter surface water sources through fecal material from

wildlife and human activities or contaminated runoff and debris. Three important bacterial species responsible for many foodborne illnesses are pathogenic *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes*, with illness occurring through consumption of contaminated fresh fruits and vegetables. These pathogenic bacteria have been recovered from the environment, including irrigation sources, where produce is grown (Strawn et al. 2013; Jay et al. 2007; Centers for Disease Control and Prevention 2008; Ijabadeniyi et al. 2011). Illnesses from these pathogens can lead to death and cause significant economic losses for growers if the bacteria are traced back to their farms.

The Food and Drug Administration (FDA) has recently drafted a set of food safety regulations as part of the Food Safety Modernization Act (FSMA), with a principal focus on fresh produce due to their high risk for contamination. Produce consumption accounts for about half of the foodborne illness outbreaks each year, and many of these cases are due to the bacterial pathogens *E. coli*, *S. enterica* and *L. monocytogenes* (Painter et al. 2013; U.S. Department of Health and Human Services 2013). Much of the produce focus of FSMA is on the prevention of contamination in the growing environment. These proposed regulations have brought considerable attention to agricultural water and its role in the spread of disease causing organisms. Testing of irrigation water for generic *E. coli*, an indicator of fecal contamination, will likely be required for some growers, especially if the water is from a surface water source. If generic *E. coli* levels are above regulatory thresholds the irrigation water would not be usable unless a mitigation strategy is applied and further testing reveals generic *E. coli* levels below threshold.

Much emphasis is being placed on human pathogen contamination of produce but growers are also concerned about plant-pathogenic organisms which can lead to large yield and economic

losses. A major mode of dispersal for many plant pathogens is through water which can become contaminated through infested debris, soil, or runoff. All major groups of plant pathogens including bacteria, viruses, fungi, nematodes, and oomycetes have been found in irrigation water (Hong & Moorman 2005). Several plant pathogenic isolates of the Gram-negative bacterium *Pseudomonas syringae* have been isolated from many different surface water sources (Morris et al. 2007; Morris et al. 2008). In New York surface water, we have recovered pathogens including the oomycete *Phytophthora capsici*, and the Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (see Chapter 1).

Given that irrigation water is a major carrier for human and plant pathogens, a mitigation strategy that could deal with both groups would be of great benefit to any grower. There are currently several methods available for the treatment of water such as chlorine, ozone, UV, and filtration but not all methods are suitable for surface water sources due to its complexity and variability. Water quality parameters such as pH, turbidity, color, dissolved solids, and microbial load can adversely affect treatment efficacies and can change seasonally or even hourly in surface water with weather events or human activities.

UV light has been used successfully for treating human bacterial and protist pathogens in drinking water (U.S Environmental Protection Agency 2006). It has also been used to successfully disinfect water contaminated with plant-pathogenic oomycetes and bacteria in nursery settings where recycling is a common method of water and nutrient conservation (Stanghellini et al. 1984; Lazarova & Bahri 2005). UV light treatment of drinking and nursery water can be effective, since they are high quality with low turbidity (<1.0 nephelometric turbidity units, NTU) and microbial loads. Previously, UV light was not considered suitable for the treatment of surface water due to high turbidity (>1.0 NTU) levels which can block or absorb

UV light shielding pathogens from treatment. One UV treatment system, UV CiderSure (FPE, Inc., Rochester, NY) however, has been designed to overcome this problem and is capable of consistently achieving a minimum 5-log reduction of *E. coli* O157:H7 in unfiltered apple cider. Apple cider is a liquid of varying high solids content and high turbidity in the range of 1000-2400 NTU (Koutchma 2009). The UV processing unit is designed to deliver the same UV dose to all pathogens using computational fluid dynamics and adjustable flow rates. In this study, the UV processing unit designed to treat turbid liquids with high solids contents was evaluated for efficacy in decontaminating surface waters contaminated with bacterial and oomycete pathogens including; *E. coli* O157:H7, *S. enterica*, *L. monocytogenes Pseudomonas syringae* pv. *tomato*, *Clavibacter michiganensis* subsp. *michiganensis*, and *Phytophthora capsici*.

## MATERIALS & METHODS

**Water Sources.** For this experiment, the UV inactivation of each pathogen was tested in three water sources. Two of the water sources were from actively used surface water irrigation sources, a creek (Tompkins Co., NY) and a pond (Ontario Co., NY). Phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$,) or reverse osmosis (RO) water were used as low turbidity water sources for all experiments with bacterial pathogens and *Ph. capsici*, respectively. Surface water was collected in the fall of 2012 and stored in 55 gallon drums (food grade plastic). Surface water was stirred thoroughly, then pH (Hanna HI 2211 pH/ORP meter, Woonsocket, RI) and turbidity (HACH 2100P portable turbidimeter, Loveland, CO) measurements were recorded before use. All turbidity values were recorded in nephelometric turbidity units. RO water was produced as needed (Barnstead Nanopure II, Thermo Fisher Scientific, Waltham, MA).

**Human and plant pathogen strains.** Six pathogen species; *E. coli*, *S. enterica*, *L. monocytogenes*, *Ps. syringae*, *C. michiganensis*, and *Ph. capsici* were used in this study. Five strains of each pathogen were used to prepare a five-strain cocktail for UV inactivation experiments (Table 2.1). *E. coli*, *S. enterica*, and *L. monocytogenes* strains are clinical or food isolates obtained from Dr. Weidman's food safety laboratory at Cornell University. After storage at -80˚C, human bacterial pathogens were passed once through tryptic soy broth (TSB, Hardy Diagnostics, Santa Maria, CA). All *Ps. syringae*, *C. michiganensis*, and *Ph. capsici* strains were obtained from the Smart lab and were isolated from field samples collected in New York. All *Ps. syringae*, *C. michiganensis* strains were isolated from tomato. *Ph. capsici* strains were isolated from pepper (0664-1), pumpkin (0759-8, MMZ-4A), zucchini (0752-15) and butternut squash (06180-4).

**Media and culture conditions.** *E. coli*, *S. enterica*, and *L. monocytogenes* were maintained on tryptic soy agar (TSA, Hardy Diagnostics, Santa Maria, CA) while *Ps. syringae*, *C. michiganensis*, and *Ph. capsici* were maintained on King's B (KB) agar (King et al, 1954), $D_2$ANX agar (Chun 1982), and PARP agar (Jeffers and Martin 1986), respectively. For preparation of the five-strain cocktails, *E. coli* and *S. enterica* were grown for 18 h at 37˚C; *L. monocytogenes* was grown for 24 h at 37˚C, in tryptic soy broth (TSB, Hardy Diagnostics, Santa Maria, CA), and *Ps. syringae* was grown at 28˚C for 18 h in KB broth. *Clavibacter michiganensis* was grown for 48 h at 28˚C in Luria-Bertani (LB) broth (Bertani 1951). All bacteria were incubated on a rotary platform shaker at 250 rpm. *Phytophthora capsici* was cultured on 15% V8 agar for 7 days at room temperature

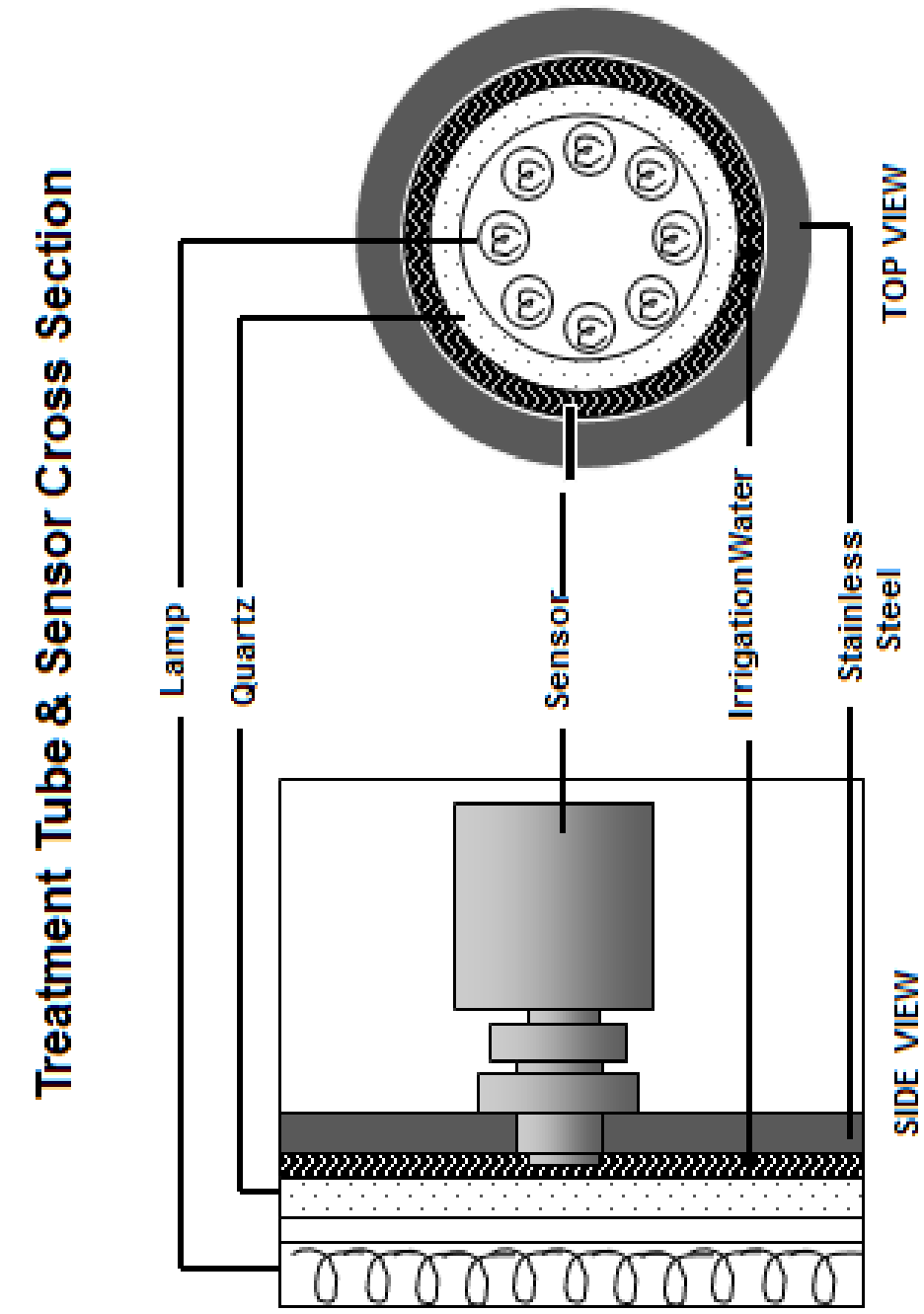
Table 2.1 Pathogens and strains used for UV inactivation studies

| PATHOGEN | STRAINS |
|---|---|
| Human Pathogens | |
| *Escherichia coli* O157:H7 | 933, 2722, ATCC 43895, ATCC 35150, ATCC 4389 |
| *Salmonella enterica* subsp. *enterica* | serovars; Hartford, Montevideo, Rubislaw, Gaminara, Cuban |
| *Listeria monocytogenes* | 2812, 2289, L99, 104025, F2586 – VI |
| Plant Pathogens | |
| *Pseudomonas syringae* pv. *tomato* | 09150, 09110, 09084, 0761, 0578 |
| *Clavibacter michiganensis* subsp. *michiganensis* | 11015, 10-4, 0767, 09085, 0690 |
| *Phytophthora capsici* | 0664-1, 06180-4, 0759-8, 0752-15, MMZ-4A |

(25-28˚C) under continuous fluorescent light for sufficient sporangia production. Zoospore suspensions were produced according to the protocol used by Dunn et al., 2013.

**UV radiation and inactivation.** UV radiation, 254 nm, was delivered to water samples using a thin film CiderSure unit (UV CiderSure 3500, FPE, Inc., Rochester, NY) for all experiments in this study (Figure 2.1). Water is initially drawn through 1 inch diameter tubing and then dispersed through a thin, 5mm thick cylinder surrounding 8 central UV lamps. The maximum penetration depth of UV irradiation is set at the thickness of the treatment cylinder at 5mm. The UV irradiance penetrating through the water sample is measured by sensors in the treatment cylinder, opposite the side of the UV lamps. The unit contains two UVX-25 sensors (UVP, Inc., Upland CA) that measure UV irradiance penetrating the liquid sample every 50 milliseconds. The flow rate automatically adjusts according to a preprogrammed algorithm based on information gathered from the sensors to overcome differences in water quality parameters, i.e., solids contents, turbidity, and color, of water being processed and delivers the same UV dose of 14.2mJ/cm$^2$ to all samples (Hanes et al. 2002). The efficacy of UV inactivation for each pathogen species was tested separately in three water sources. A five-strain cocktail of a pathogen species was added to 1liter water samples. Multiple-strain cocktails are commonly used in human and plant pathogen studies for more comprehensive assessments because it is common for more than one strain to be present in a foodborne illness or plant disease outbreak (Padley et al. 2008; Schlesser 2006). Bacterial cocktails were produced by adding 10 ml of stationary phase culture of each strain together and thoroughly mixing. One milliliter of the cocktail was added to 1liter of water for approximately $10^8$-$10^9$ CFU/L, 10ml were added to 1liter of water for approximately $10^9$-$10^{10}$ CFU/L. Zoospores of *Ph. capsici* were enumerated using a hemacytometer (Hausser Scientific, Horsham,

Figure 2.1. Longitudinal and cross section of CiderSure UV treatment tube. (Figure courtesy of Dr. Randy Worobo, Food Science, Cornell University)


Treatment Tube & Sensor Cross Section
Lamp
Quartz
Sensor
Irrigation Water
Stainless Steel
TOP VIEW
SIDE VIEW

PA). Equal numbers of zoospores from each strain were combined to produce a five-strain cocktail for inoculation of 1liter water samples at $10^4$ and $10^5$ zoospores/L. The higher inoculum levels were included to ensure the assessment of the upper inactivation rates. Three 1liter samples were prepared from individual cocktail preparations for each pathogen concentration and water source pairing. Subsamples, 1ml, were taken immediately from inoculated 1liter water samples for enumeration before UV inactivation. Samples were gently mixed prior to subsampling, UV inactivation, and filtering. The 1liter water samples were processed immediately through the UV irradiation unit and treated samples were collected in sterile 1liter bottles and filtered for enumeration. Non-inoculated samples from each water source were also processed for each pathogen. All UV inactivation experiments were repeated once for a total of six 1liter samples for each pathogen concentration and water source pairing.

**Enumeration.** Pathogen populations in both inoculated and non-inoculated samples were enumerated before and after UV inactivation on selective or semi-selective media. Before UV exposure, bacteria (CFU/L) were enumerated by serial dilution plating and *Ph. capsici* zoospores (zoospores/L) were enumerated with a hemacytometer. After UV inactivation, 1liter samples were filtered using sterile magnetic filter funnels (PALL, Port Washington, NY). For bacterial pathogens, 47 mm, 0.45 μm pore size filters (Thermo Fisher Scientific, Waltham, MA) were used. For *Ph. capsici*, 47 mm, 5.0 μm pore size filters (EMD Millipore, Billerica, MA) were used and placed onto the corresponding semi-selective medium for the enumeration of the pathogen of interest. One or more filters were used per 1liter sample to prevent clogging. Enumeration of *E. coli* was performed on Violet Red Bile Agar (VRBA, Hardy Diagnostics, Santa Maria, CA), samples were incubated at 37˚C for 18 h. *Salmonella enterica* CFU/L were enumerated on bismuth sulfite agar (Criterion, Santa Maria, CA), samples were incubated at 37˚C for 18 h.

*Listeria monocytogenes* CFU/L were enumerated on Oxford Medium Base amended with Modified Oxford Antimicrobic Supplement (Difco, Franklin Lakes, NJ); samples were incubated at 37˚C for 24 h. *Pseudomonas syringae* CFU/L were enumerated on *Pseudomonas* Agar Base containing a C-F-C supplement (Oxoid Limited Hampshire, UK). All *Ps. syringae* CFU/L were transferred to KB agar for confirmation (production of fluorescein). *Clavibacter michiganensis* CFU/L were enumerated on $D_2$ANX and incubated at 28˚C for 48 h. *Phytophthora capsici* zoospores were enumerated on PARP and were incubated at room temperature (25-28˚C) for 5 days, mycelial growth was transferred to 15% V8 agar, and incubated for 28˚C for up to 7 days for morphological conformation of *Ph. capsici.*

**Calculations and statistics.** Percent inactivation $[(N_o - N)/ N_o]$ was calculated for each pathogen concentration by water source pairing. Pathogen counts were also converted into logarithmic units and log reduction was calculated as log $(N/N_o)$ where N corresponds to the after treatment count and $N_o$ the initial count. Data were analyzed by analysis of variance using R statistical software (R Core Development Team 2008). Tukey's honestly significant difference (HSD) was used to determine significant differences ($\alpha = 0.05$) between log reduction means or % inactivation means of all pathogen concentration and water source pairings.

## RESULTS

**UV inactivation of pathogens by water source.** The efficacy of UV inactivation of each of the six pathogen species was analyzed in the three water sources that varied in pH and turbidity. The pH and turbidity of the water sources were monitored throughout the UV inactivation experiments, the average and range of both parameters are presented for each water source in Table 2.2. The RO water had an average neutral pH (6.96), while the PBS, creek, and pond water exhibited alkaline pH levels (8.01, 8.32 and 8.21, respectively). The pH did not vary more

than 0.19 pH units in any water source. The turbidity of the RO water and PBS were consistent at 0.1 NTU. The turbidity in the creek water was higher and more variable and ranged from 3.0-4.4 NTU. Turbidity was by far the highest and most variable in the pond water which ranged from 15.8-22-7 NTU.

The average % inactivation and log reduction values for all pathogens by water source can be found in Tables 2.3-2.8. Percent inactivation for all pathogens by water source were 99.9% or greater. The log reductions range from 10.0 to 6.1 and from 5.0 to 4.2 for bacterial pathogens and *Ph. capsici*, respectively (Tables 2.3-2.8). In all water sources *C. michiganensis* consistently had larger total numbers of CFU/L after UV treatment when compared to the other bacterial pathogens (Tables 2.3, 2.5, 2.7). *Escherchia coli* and *S. enterica* had similar numbers of CFU/L after UV treatment in all water sources and were consistently lowest of the bacterial pathogens (Tables 2.3, 2.5, 2.7). *Pseudomonas syringae* and *L. monocytogenes* had intermediate numbers of CFU/L after UV treatment among the bacterial pathogens, they performed similarly to each other in PBS, but in the creek and pond water *L. monocytogenes* had greater surviving CFU/L than *Ps. syringae* (Tables 2.3, 2.5, 2.7).

**UV inactivation of pathogens by species. *E. coli* O157:H7.** One hundred percent inactivation was achieved for the lower concentrations and 99.9% inactivation was achieved at higher concentrations in each water source (Tables 2.3, 2.5, 2.7). No significant difference in log reduction was found among water sources at the lower

Table 2.2 Average and range of pH and turbidity values for water sources used in UV inactivation experiments

| | **pH** | | **Turbidity (NTU)** | |
|---|---|---|---|---|
| **Water Source** | average | range | average | range |
| PBS | 8.01 | 7.88-8.10 | 0.1 | 0.1 |
| RO | 6.96 | 6.90-7.00 | 0.1 | 0.1 |
| Creek | 8.32 | 8.26-8.37 | 3.9 | 3.0-4.4 |
| Pond | 8.21 | 8.17-8.36 | 19.6 | 15.8-22.7 |

Table 2.3 CFU/L before and after UV processing, % inactivation, and average log reduction of bacterial pathogens in in PBS (all values represent averages (n=6))

| PATHOGEN | 5-strain cocktail / L | Before UV CFU / L | After UV CFU / L | % Inactivation | Log reduction (SD) |
|---|---|---|---|---|---|
| *E. coli* O157:H7 | 1ml | $2.12 \times 10^9$ | 0 | 100 | 9.5 (0.27) |
| | 10ml | $1.24 \times 10^{10}$ | 1 | 99.9 | 10.0 (0.26) |
| *S. enterica* | 1ml | $6.87 \times 10^8$ | 0 | 100 | 8.8 (0.16) |
| | 10ml | $5.47 \times 10^9$ | 3 | 99.9 | 9.4 (0.41) |
| *L. monocytogenes* | 1ml | $1.53 \times 10^9$ | 3 | 99.9 | 8.8 (0.44) |
| | 10ml | $9.47 \times 10^9$ | 10 | 99.9 | 9.0 (0.25) |
| *Ps. syringae* pv. *tomato* | 1ml | $2.67 \times 10^8$ | 2 | 99.9 | 8.1 (0.21) |
| | 10ml | $6.00 \times 10^9$ | 24 | 99.9 | 8.5 (0.70) |
| *C. michiganensis* subsp. *michiganensis* | 1ml | $6.43 \times 10^8$ | 20 | 99.9 | 7.5 (0.23) |
| | 10ml | $7.47 \times 10^9$ | 193 | 99.9 | 7.5 (0.28) |

Table 2.4. Zoospores/L before and after UV processing, % inactivation, and log reduction of *Ph. capsici* in RO water (all values represent averages (n=6))

| PATHOGEN | Before UV zoospores / L | After UV zoospores / L | % Inactivation | Log reduction (SD) |
|---|---|---|---|---|
| *Ph. capsici* | $5 \times 10^4$ | 0 | 100 | 4.7 (0.0) |
| | $5 \times 10^5$ | 5 | 99.9 | 5.0 (0.18) |

Table 2.5. CFU/L before and after UV processing, % inactivation, and average log reduction of bacterial pathogens in creek water (all values represent averages (n=6))

| PATHOGEN | 5 -strain cocktail / L | Before UV CFU / L | After UV CFU / L | % Inactivation | Log reduction (SD) |
|---|---|---|---|---|---|
| *E. coli* O157:H7 | 1ml | $1.73 \times 10^9$ | 0 | 100 | 9.5 (0.36) |
| | 10ml | $6.47 \times 10^9$ | 2 | 99.9 | 9.5 (0.72) |
| *S. enterica* | 1ml | $4.67 \times 10^8$ | 0 | 100 | 8.6 (0.23) |
| | 10ml | $8.00 \times 10^9$ | 44 | 99.9 | 8.6 (0.67) |
| *L. monocytogenes* | 1ml | $1.97 \times 10^9$ | 19 | 99.9 | 7.9 (0.42) |
| | 10ml | $1.17 \times 10^{10}$ | 809 | 99.9 | 7.1 (0.25) |
| *Ps. syringae* pv. *tomato* | 1ml | $1.15 \times 10^9$ | 26 | 99.9 | 7.7 (0.34) |
| | 10ml | $1.03 \times 10^{10}$ | 198 | 99.9 | 7.7 (0.22) |
| *C. michiganensis* subsp. *michiganensis* | 1ml | $4.65 \times 10^8$ | 62 | 99.9 | 6.9 (0.19) |
| | 10ml | $5.51 \times 10^9$ | 1,817 | 99.9 | 6.5 (0.16) |

Table 2.6. Zoospores/L before and after UV processing, % inactivation, and log reduction of *Ph. capsici* in creek water (all values represent averages (n=6))

| PATHOGEN | Before UV zoospores / L | After UV zoospores / L | % Inactivation | Log reduction (SD) |
|---|---|---|---|---|
| *Ph. capsici* | $5 \times 10^4$ | 0 | 100 | 4.7 (0.0) |
| | $5 \times 10^5$ | 8 | 99.9 | 4.8 (0.21) |

Table 2.7. CFU/L before and after UV processing, % inactivation, and average log reduction of bacterial pathogens in pond water (all values represent averages (n=6))

| PATHOGEN | 5-strain cocktail / L | Before UV CFU / L | After UV CFU / L | % Inactivation | Log reduction (SD) |
|---|---|---|---|---|---|
| *E. coli* O157:H7 | 1ml | $2.67 \times 10^9$ | 0 | 100 | 9.5 (0.12) |
| | 10ml | $8.00 \times 10^9$ | 460 | 99.9 | 7.3 (0.33) |
| *S. enterica* | 1ml | $6.47 \times 10^8$ | 4 | 99.9 | 8.3 (0.35) |
| | 10ml | $7.00 \times 10^9$ | 108 | 99.9 | 7.9 (0.21) |
| *L. monocytogenes* | 1ml | $2.10 \times 10^9$ | 123 | 99.9 | 7.2 (0.13) |
| | 10ml | $1.13 \times 10^{10}$ | 4,576 | 99.9 | 6.3 (0.21) |
| *Ps. syringae* pv. *tomato* | 1ml | $1.68 \times 10^9$ | 138 | 99.9 | 6.9 (0.42) |
| | 10ml | $1.11 \times 10^{10}$ | 1,986 | 99.9 | 6.8 (0.22) |
| *C. michiganensis* subsp. *michiganensis* | 1ml | $1.92 \times 10^9$ | 311 | 99.9 | 6.8 (0.18) |
| | 10ml | $1.84 \times 10^{10}$ | 16,033 | 99.9 | 6.1 (0.16) |

Table 2.8. Zoospores/L before and after UV processing, % inactivation, and log reduction of *Ph. capsici* in pond water (all values represent averages (n=6))

| PATHOGEN | Before UV zoospores / L | After UV zoospores / L | % Inactivation | Log reduction (SD) |
|---|---|---|---|---|
| *Ph. capsici* | $5 \times 10^4$ | 1 | 99.9 | 4.7 (0.0) |
| | $5 \times 10^5$ | 32 | 99.9 | 4.2 (0.15) |

concentrations (Figure 2.2). For the higher concentrations, log reduction was not different between PBS (10.0) and creek water (9.5) however, log reduction was significantly less (7.3) in pond water (Figure 2.3). No CFU/L were recovered in uninoculated controls before or after UV inactivation.

***S. enterica***. At the lower concentrations in PBS and creek water, 100 % inactivation was achieved, while 99.9% inactivation was achieved for the lower concentration in pond water and at the higher concentrations in each water source (Tables 2.3, 2.5, 2.7). At the lower concentrations there were no log reduction differences between PBS (8.8) and creek (8.6) water or between creek and pond (8.3) water. A significant difference was found between PBS and pond water (Figure 2.2). For the higher concentrations there were significant differences in log reduction between each pair of water sources (Figure 2.3). No *S. enterica* CFU/L were recovered in uninoculated controls before or after UV inactivation.

***L. monocytogenes***. For all concentrations and water source pairings, 99.9% inactivation was achieved (Tables 2.3, 2.5, 2.7). Significant differences in log reduction were observed among all water sources at both the lower and higher pathogen concentrations (Figures 2.2 and 2.3). No *L. monocytogenes* CFU/L were recovered in uninoculated controls before or after UV inactivation.

***Ps. syringae***. For all concentrations and water source pairings, 99.9% inactivation was achieved (Tables 2.3, 2.5, 2.7). Log reductions at the lower concentrations were different in pond water (6.9) compared to PBS (8.1) or creek water (7.7), but not between PBS and creek water (Figure 2.2). Log reductions at higher concentrations were different between each water sources (Figure 2.3). Background *Ps*. spp. were present in uninoculated pond water, 55 CFU/L, before UV

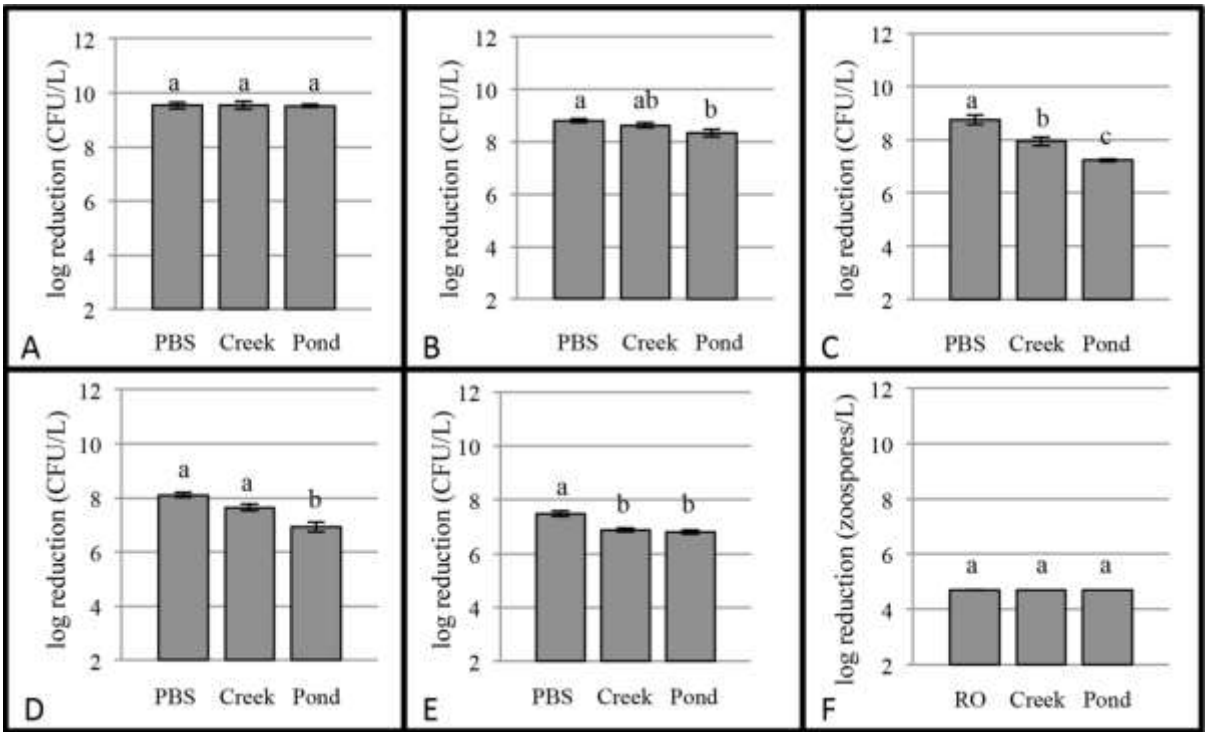



Figure 2.2. Average log reduction for the lower inoculum levels of each pathogen in each water source. A: *E. coli* O157:H7; B: *S. enterica*; C: *L. monocytogenes*; D: *Ps. syringae* pv. *tomato*; E: *C. michiganensis* subsp. *michiganensis*; F: *Ph. capsici* (no error bars are shown because all samples resulted in 100% inactivation). Different letters indicate significantly different groups based on a Tukey's HSD test ($\alpha$ =0.05).

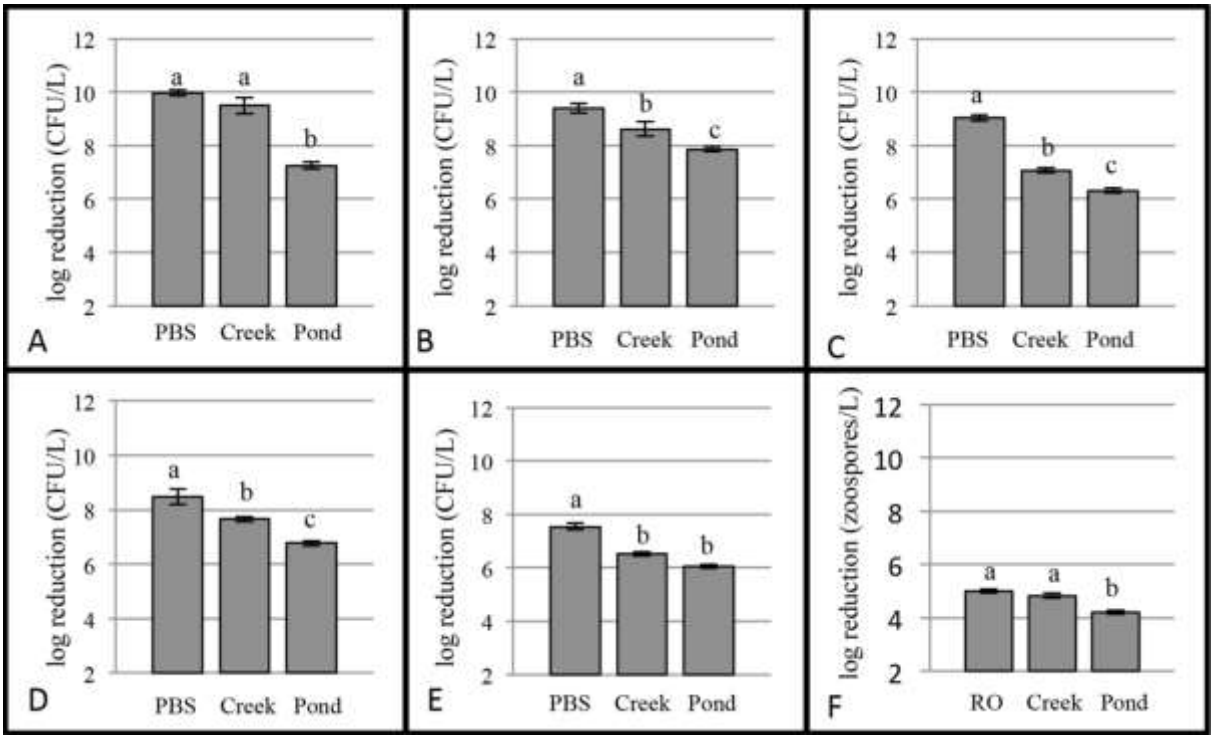



Figure 2.3. Average log reduction for the higher inoculum levels of each pathogen in each water source. A: *E. coli* O157:H7; B: *S. enterica*; C: *L. monocytogenes*; D: *Ps. syringae* pv. *tomato*; E: *C. michiganensis* subsp. *michiganensis*; F: *Ph. capsici.* Different letters indicate significantly different groups based on a Tukey's HSD test ($\alpha$ =0.05).

inactivation, and 0 CFU/L after UV inactivation. For inoculated samples background *Ps.* spp. were included in the before and after UV inactivation values.

***C. michiganensis***. For all concentrations and water source pairings, 99.9% inactivation was achieved (Tables 2.3, 2.5, 2.7). Differences in log reductions at the lower concentrations were found between PBS (7.5) and creek (6.9) or pond (6.8) water but not between creek and pond water (Figure 2.2). Differences in log reductions were found between each water source at the higher concentrations (Figure 2.3). No *C. michiganensis* CFU/L were recovered in uninoculated controls before or after UV inactivation.

***Ph. capsici***. One hundred percent inactivation was achieved at the $10^4$ zoospores/L level in RO and creek water, 99.9% inactivation was achieved in pond water (Tables 2.4, 2.6, 2.8). At $10^5$ zoospores/L 99.9 % inactivation was achieved in the three water sources (Tables 2.4, 2.6, 2.8). No significant difference in log reduction was found between water sources at $10^4$ zoospores/L (Figure 2.2). At $10^5$ zoospores/L differences in log reductions were found between each water source (Figure 2.3). No *Ph. capsici* isolates were recovered in uninoculated controls before or after UV inactivation.

## DISCUSSION

The surface water sources for this study were chosen because they were actively used irrigation sources that are representative of the pH and turbidity of surface waters in New York State as determined by an irrigation water survey conducted by Jones and Smart (see Chapter 1). For UV inactivation experiments, water sources were monitored for pH and turbidity to ensure uniformity of water sources during experimentation. Additionally, the values of these two water quality parameters are important when considering a water treatment option. Chlorination is one

of the most effective and economical water disinfection methods but is not recommended for water with a pH over 7.5 or water with high levels of particulates, due to low levels of hypochlorous acid formation and binding to organic matter, respectively. The pH of the surface waters in this study were on average 8.32 and 8.21 for the creek and the pond, respectively, making these sources poor choices for chlorine disinfection. Studies have found that pH is not a significant factor in UV treatment efficacy (Basaran et al. 2004; Quintero-Ramos et al. 2004).

Turbidity, on the other hand, has been found to adversely affect UV treatment, but the relationship between turbidity level and UV efficacy is not consistent. Water components that influence turbidity have variable UV blocking and absorbing qualities and therefore turbidity can only be used as a general guideline to determine UV transmittance. In general, as turbidity increases, UV transmittance and bactericidal efficacy decreases (Spellman 2004; Qian 2011). The average log reduction results from this study support this trend, as tests conducted in more turbid water were generally less effective at inactivating pertinent challenge pathogens when compared to a less turbid source. In some cases, for example with *E. coli* and *Ph. capsici*, there was no significant log reduction difference between the 0.1 NTU (PBS or RO) water source and the 3.9 NTU (creek) source. This may be due to the fact that complete or almost complete inactivation occurred in the less turbid water making log reduction equal to the initial pathogen concentrations and not wholly representative of the full measure of UV efficacy. In New York State, the turbidity of surface water used for irrigation is commonly between 1 NTU and 20 NTU, but can vary considerably throughout the year (see Chapter 1). The creek water had an average turbidity of 3.9 NTU and the pond at 19.6 NTU, these values are representative for the range of turbidities of the majority of surface water sources in New York.

Percent inactivation for all pathogens and water source pairings was found to be 99.9% or greater. These data show that UV light as a mitigation strategy can be effective against a broad spectrum of pathogens in complex surface water sources. When analyzing % inactivation data differences in UV efficacy among water sources is not apparent, but with examination of the average log reduction data we can begin to discern how UV efficacy is affected by the different water sources. For the bacterial pathogens, log reductions ranged from 10.0 (*E. coli* in PBS) to 6.1 (*C. michiganensis* in pond water). No validation standards have been developed for the treatment of bacterial or oomycete pathogens in surface water that is intended for irrigation. To be recognized as a valid treatment method of juices by the FDA the treatment must obtain a 5-log reduction of a pertinent pathogen in juice (U.S. Food and Drug Administration 2005). The log reduction results for bacterial pathogens from this experiment would meet the FDA's requirements for juice. No such standard exists for oomycetes. Log reduction values for *Ph. capsici* zoospores were less than those for all bacterial pathogens because the highest concentration of zoospores we could obtain was $5x10^5$. Thus, it would be impossible to have a 6 or greater log reduction as was observed with the bacterial pathogens. The zoospores were clearly highly susceptible to UV treatment as there was 100% inactivation in pond water at the lower pathogen concentration ($5x10^4$). The only other pathogen with 100% inactivation in pond water was the lower concentration of *E. coli*. *Clavibacter michiganensis*, in all water sources, was the least susceptible to UV treatment, followed by *L. monocytogenes* in creek and pond water, among the bacterial pathogens, with the highest CFU/L after treatment. Typically, Gram-positive bacteria are more recalcitrant to UV radiation than Gram-negative bacteria (Block 1983).

UV may not be applicable to all irrigation situations, particularly those applications requiring very high volumes. The path length through which UV can penetrate water is very small and limits the volume of water that can be treated at one time. Highly turbid waters may not be good candidates for UV treatment without pre-filtering to remove some of the UV blocking and absorbing components, however, even at relatively high turbidity levels (20 NTU) we saw 99.9% inactivation. Turbidity is not the only water quality parameter that can affect the efficacy of UV treatment, dissolved solids, such as iron can also absorb UV light and decrease the UV transmittance.

More than half of the irrigation water in the US is from surface water sources, and a recent survey of NY vegetable growers responding to a survey found that 57% use surface water (Bihn 2013). With recent FDA regulations for human pathogens in irrigation water, more options will be needed to treat these water sources. The data from this study suggest that UV light is a feasible treatment method to greatly reduce bacterial and oomycete pathogen populations in surface irrigation water without pretreatment. Additional research is needed to investigate the feasibility of this disinfection method utilizing additional water sources and on a larger scale that would be in line with irrigation water volumes currently used by a range of produce growers. UV light treatment systems have the potential to significantly lower the risk of both plant and human pathogen contamination of crops from surface water through irrigation or other agricultural applications and could be integrated into effective food safety and plant health programs.

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# CHAPTER THREE

## Development of a diagnostic protocol for distinguishing more virulent *Pto* strains from less virulent strains

**ABSTRACT**

A severe outbreak of bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato* (*Pto*) occurred in central New York in 2009. An isolate collected from this outbreak, *Pto* 09150, was found to be highly virulent on tomato. To gain a better understanding of the differences between this aggressive strain and other strains of *Pto*, and to develop a diagnostic assay for aggressive strains of this pathogen, the genome of *Pto* 09150 was sequenced. A genomic comparison found that *Pto* 09150 was very similar to a previously sequenced isolate, *Pto* T1, and that genetic determinants that might be accountable for the level of virulence seen in this strain could be identified. These determinants were used to develop a PCR-based diagnostic tool to distinguish more virulent *Pto* 09150 and similar strains from less virulent *Pto* strains such as DC3000 and the closely related *P. syringae* pv. *maculicola* strains. The major virulence determinants found in the draft genome of *Pto* 09150 were the genes for coronatine biosynthesis, a flgII-28 allele that differs from *Pto* T1, and a large repertoire of type III effectors (T3E). Primers for conventional PCR were developed based on the T3Es, *avrA*, *hopW*, *hopN*, and *hopR* and were tested with several strains of *Pto*, other pseudomonads, and several other bacterial pathogens of tomato. Primers developed for *avaA* and *hopW* were diagnostic for more virulent strains of *Pto* while primers for *hopN* and *hopR* were diagnostic for less virulent *Pto* strains and related *P. syrin*ge pv. *maculicola* strains.

## INTRODUCTION

*Pseudomonas syringae* pv. *tomato* (*Pto*) is one of the most studied plant pathogens and serves as a model for plant-pathogen interactions. The Gram- negative bacterium is the causal agent of bacterial speck disease of tomato (*Solanum lycopersicum*) and affects tomato production worldwide (Koike et al. 2007). Bacterial speck can be severe and lead to substantial economic losses (Strobel 2004). The pathogen is disseminated through infested seed and can survive in infected debris and epiphytically on weeds (McCarter et al. 1983). Virulence among *Pto* strains can vary widely and several other bacterial pathogens of tomato can cause symptoms similar to those caused by *Pto* making management of bacterial speck more challenging (Kozik et al. 2006). Diagnostics specific to the more virulent *Pto* strains would enhance disease management practices.

A highly virulent strain of *Pto* was isolated from an Ontario County, NY tomato field during the late summer of 2009. In preliminary greenhouse studies the field strain (09150) proved to be more aggressive than *Pto* DC3000 and many field isolated strains. DC3000 is the *Pto* strain most widely used in laboratory studies but is not representative of *Pto* strains that cause disease in the field (Cai et al. 2011). The following season mild speck-like symptoms observed on tomato plants at the same farm were found to be *P. viridiflava*. *Pseudomonas viridiflava* is the causal agent of bacterial leaf blight and stem pith necrosis of tomato and symptoms can sometimes be confused with those of bacterial speck (Jones & Jones 1984; Malathrakis 1987). Other bacterial pathogens of tomato that could potentially be mistaken for *Pto* are *P. syringae* pv. *maculicola*, *Xanthomomas* spp. (*X. vesicatoria*, *X. euvesicatora*, *X. gardneri*, and *X. perforans*) causing bacterial spot of tomato, and *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial wilt and canker of tomato (Hendson et al. 1992; Jones et al. 2004; EPPO 2005).

Previously, DNA based detection of *Pto* from infected tomato, was developed with sequences from many strains regardless of virulence level (Zaccardelli et al. 2005; Fanelli et al. 2007; Gropp & Guttman 2004). Some protocols are based on the genes for the production of the phytotoxin coronatine (Nabizadah et al. 1997; Cuppels et al. 2006). Coronatine is present in many *P. syringae* pathovars and a positive result could be the consequence of a strain other than *Pto* (Bereswill et al. 1994). Also, coronatine is not consistently found in all strains of *Pto,* such as *Pto* T1, and a false negative could result (Almeida et al. 2009). These detection approaches may not be optimal for all situations. A detection protocol that can distinguish more virulent strains from less virulent strains would better aid growers in their pest management decisions.

Several complete genomes and many draft genomes of *Pseudomonas* spp. including many *P. syringae* pathovars are currently available for genomic studies (http://www.pseudomonas.com/; http://www.pseudomonas-syringae.org/ ). *Pseudomonas syringe* isolates can be divided into three major phylogenetic groups with "Group I" including pathovars *tomato, maculicola,* and *actinidae* (Sarkar et al. 2004; Baltrus et al. 2011). Multilocus sequence typing of Group I strains indicates that more virulent isolates of *P. syringae* pv. *tomato,* exemplified by strain *Pto* T1, form a subgroup referred to alternatively as Ia or IV (Yan et al. 2008; Cai et al. 2011). In contrast, *Pto* DC3000 is more typical of the second subgroup, referred to as Ib, which includes strains that are more closely related to brassica pathogens in *P. syringae* pv. *maculicola* (*Pma*). Some of these, like *Pto* DC3000, cause comparatively mild symptoms on tomato. The two sub-groups are referred to here as "T1-like" and "DC3000/Pma-like". DC3000 is atypical of *Pto* strains with respect to its host range; besides tomato it can infect *Arabidopsis thaliana* and cauliflower (*Brassica oleracea* var. *botrytis*), while most other *Pto* strains only cause disease on tomato (Wiebe & Campbell 1993; Cupples & Ainsworth 1995).

In this study we attempted to better understand how the field isolate 09150 corresponds to the two *Pto* subgroups to identify sequence signatures for use in development of diagnostic tools. To accomplish this, the genome of *Pto* 09150 was sequenced using Illumina (Illumina, Inc., San Diego, CA) technology. Virulence factors, including phytotoxins, pathogen associated molecular patterns, and type III effector (T3E) repertoires were used to determine targets for distinguishing more virulent tomato pathovars from the less virulent pathovars and other bacterial pathogens of tomato. Here we report a PCR-based subspecific diagnostic assay developed from genomic comparison of *Pto* strains with variable levels of virulence.

## MATERIALS & METHODS

**Bacterial cultures and DNA extraction.** Bacterial isolates used in this study were obtained from frozen cultures stored at -80˚C from the culture collections from the laboratories of C. Smart and A. Collmer at Cornell University, and are listed in Table 3.1. Isolates of *Pseudomonas, X. perforans*, and *C. michiganensis* were maintained on King's B agar, yeast dextrose carbonate agar, and D2ANX agar, respectively, at 28˚C (King et al. 1954; Wilson et al. 1967; Chun 1982). For DNA extraction, overnight cultures were grown in Luria-Bertani (LB) broth at 28˚C on a rotary shaker at 200 rpm (Bertani 1951). DNA extraction was performed according to the protocol by Chen and Kuo (1993). For DNA extraction of *Pto* infected and healthy tomato tissue the protocol from Keb-Llanes et al. (2002) was used with modifications (polyvinylpyrrolidone and β- mercaptoethanol were omitted from extraction buffer A).

Table 3.1. Bacterial isolates used in this study

| Species | Isolate | Host of isolation | Origin | Year | Source | Reference |
|---|---|---|---|---|---|---|
| *Pseudomonas syringae* pv. *tomato* (*Pto*) | 0578[a] | *S. lycopersicum* | NY, USA | 2005 | C. Smart | This study |
| | 0761[a] | *S. lycopersicum* | NY, USA | 2007 | C. Smart | This study |
| | 09084[a] | *S. lycopersicum* | NY, USA | 2009 | C. Smart | This study |
| | 09150[a] | *S. lycopersicum* | NY, USA | 2009 | C. Smart | This study |
| | 12042[a] | *S. lycopersicum* | NY, USA | 2012 | C. Smart | This study |
| | 13093 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |
| | 13110 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |
| | K40[a] | *S. lycopersicum* | USA | 2005 | A. Collmer | Cai 2011 |
| | A9[a] | *S. lycopersicum* | USA | 1996 | C. Smart | Kunkeaw 2010 |
| | JL1065[a] | *S. lycopersicum* | CA, USA | | A. Collmer | Whalen 1991 |
| | NCPPB 1108 | *S. lycopersicum* | Jersey, UK | 1961 | A. Collmer | |
| | Max13 | *S. lycopersicum* | France | | A. Collmer | Zaccardelli 2005 |
| | T1 | *S. lycopersicum* | Canada | | A. Collmer | Ronald 1992 |
| | DC3000[a] | *S. lycopersicum* | Guernsey, UK | 1961 | A. Collmer | Collmer 2002 |
| *Pseudomonas syringae* pv. *antirrhini* (*Pan*) | Pan126[a] | *A. majus* | | 1965 | A. Collmer | |
| *Pseudomonas syringae* pv. *maculicola* (*Pma*) | M3[a] | *Brassica olerancea* var. *botrytis* | USA | 1937 | A. Collmer | Debener 1991 |
| | M6[a] | *Brassica olerancea* var. *botrytis* | UK | 1965 | A. Collmer | Debener 1991 |
| *Pseudomonas syringae* pv. *syringae* (*Psy*) | B728[a] | *Phaseolus vulgaris* | WI, USA | | C. Smart | Loper 1987 |

continued

| Species | Isolate | Host of isolation | Origin | Year | Source | Reference |
|---|---|---|---|---|---|---|
| *Pseudomonas viridiflava* | 10078[a] | *S. lycopersicum* | NY, USA | 2010 | C. Smart | This study |
| *Pseudomonas fulva* | 0430[a] | *S. lycopersicum* | NY, USA | 2004 | C. Smart | This study |
| *Pseudomonas fluorescens* | 09110[a] | *S. lycopersicum* | NY, USA | 2009 | C. Smart | This study |
| *Pseudomonas putida* | 09112[a] | *S. lycopersicum* | NY, USA | 2009 | C. Smart | This study |
| *Xanthomonas perforans* | 13091 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |
| *Clavibacter michiganensis* subsp. *michiganensis* | 13048 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |
| | 13085 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |
| | 13117 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |
| | 13129 | *S. lycopersicum* | NY, USA | 2013 | C. Smart | This study |

[a] Isolates used in virulence assay, all isolates were used in primer development

**Genomic sequencing of *Pto* 09150.** Genomic DNA from *Pto* 09150 was extracted according to the protocol from Chen and Kuo (1993) with modification (an extraction with an equal volume phenol:chloroform:isolamy alcohol (25:24:1) was added) and was sequenced using Illumina HiSeq (Illumina, Inc., San Diego, CA) at the Cornell University's Genomics Core Facility in Ithaca, NY. Initial coverage of 2434X was reduced to 2013X after quality trimming and error correction with Quake (version 0.2.2) (Kelley et al. 2010). De novo assembly was conducted using Velvet (version 1.1.04) (Zebrino & Birney 2008) and mapping assembly against the *Pto* T1 reference genome with MIRA (version 3.4.0 prod_linux-gnu_x86_64_static) (Chevreaux et al. 2004). The assemblies were manually analyzed using MAUVE (Darling et al. 2004). Overlapping contigs were merged using Minimus2 from the AMOS package (version 3.0.0., www.sourgeforce.net). A final round of scaffolding was performed using SSPACE (version 1.1) with default parameters (Boetzer et al. 2011). Genome annotation was conducted with RAST (Aziz et al. 2008) and HrpL binding site prediction with Pred_cutoff (Saha et al, 2013). To determine the phylogenetic placement of *Pto* 09150 sequences corresponding to the 9-gene MLST schema described by Almeida et al, (2010) were identified from the 09150 assembly. Sequences used for the MLST analyses were obtained from the Plant Associated and Environmental Database (www.PAMDB.org), for *Pto* isolates; Max13, kuzzen 100, T1, NCPPB 1108, JL1065, *Pan* 126, DC3000, ICMP3443; *Pma* isolates M3 and M6; and *P. syringae* pv. *syringae* B728a. Sequences for *P. viridiflava* UASWS0039, *P. fluorescens* NCIMB 11764, *P. syringae pv. glycinea* B076, and *P. syringae aesculi* 2250 were obtained from the National Center for Biotechnology (www.ncbi.nlm.nih.gov). A phylogenetic tree was produced using the neighbor-joining method in Mega6 (Saitou et al. 1987; Tamura et al. 2013). The evolutionary

distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004).

**Primer design.** Sequences for T3E genes, *avrA, hopW1, hopN1*, and *hopR1*, were aligned using MEGA6 (Table 3.2). Primer sequences were designed for each gene using a visual assessment of aligned sequences combined with Integrated DNA technologies (IDT, Coralville, IA) OligoAnalyzer tool (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

**Primer testing**. Each primer combination (Table 3.2) was tested using the Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). A temperature gradient, 56-65 ˚C, was used to determine the optimum annealing temperature for each primer pair. Twenty-five microliter reactions contained; 1X EmeraldAmp PCR MasterMix (Clonetch, Mountain View, CA), 10 pmol forward primer, 10 pmol reverse primer, and 10ng template DNA. Multiplex PCR was performed with primers for *avrA* (215f and 1321r) and *hopR1* (427f and 1129r) with the same reaction mixture as above except that 7.5 pmol of each primer was used. PCR conditions were as follows; initial denaturation of 94˚C for 4 min, followed by 35 cycles at 94˚C for 45 sec, 56-65 ˚C for 30 sec, 72˚C for 45 sec, and a final extension step of 72˚C for 5 min. PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide, and run for 60 min at 100 V. Primers were tested with DNA from; all isolates listed in Table 3.1, infected tomato leaf tissue (*Pto* 09150, *Pto* DC3000), infected tomato fruit (*Pto* 13093, *Pto* 13110), and healthy tomato leaves. Infected and healthy tomato leaf tissue was collected from cultivar 'Mountain Fresh Plus'. Infected tomato fruit tissue was obtained from field samples collected from unknown cultivars.

**Virulence assay.** Five-week old tomato plants ('Mountain Fresh Plus') were inoculated with

Table 3.2. Sequences used for primer design

| Gene | Strain | *P. syringae* pathovar | Accession |
|---|---|---|---|
| *avrA* | NCPPB1108 | *Pto* | ADGA01000238.1 |
| | T1 | *Pto* | ABSM01000053.1 |
| | K40 | *Pto* | ADFY01000462.1 |
| | Max13 | *Pto* | ADFZ01000171.1 |
| | 09150 | *Pto* | this study |
| *hopW1* | NCPPB1108 | *Pto* | ADGA01000243.1 |
| | T1 | *Pto* | ABSM01000048.1 |
| | K40 | *Pto* | ADFY01000403.1 |
| | Max13 | *Pto* | ADFZ01000107.1 |
| | 09150 | *Pto* | this study |
| *hopN1* | DC3000 | *Pto* | AE016853.1 |
| *HopR1* | NCPPB1108 | *Pto* | ADGA01000036.1 |
| | T1 | *Pto* | ABSM01000021.2 |
| | K40 | *Pto* | ADFY01000071.1 |
| | Max13 | *Pto* | ADFZ01000207.1 |
| | 09150 | *Pto* | this study |
| | DC30000 | *Pto* | AE016853.1 |

| Gene | Primer Name and Position | Sequence | Annealing Temp ˚C |
|---|---|---|---|
| *avrA* | 547f | 5’-CGATCTCTGTCGAACAATGC-3’ | 65 |
| | 215f | 5’-CGCATG TTCAGCATTGTCAT-3’ | 65 |
| | 1321r | 5’-GAAGACCTTGGTTCTTTCGG-3’ | 65 |
| | 1219r | 5’-TTGCCTGGTCGATTGTCAAC-3’ | 65 |
| *hopW1* | 173f | 5’-AGGACTTCACAAGCCTTCTG-3’ | 65 |
| | 313f | 5’-GAACAGCAGACACTCAAAGG-3’ | 56 |
| | 1099r | 5’-CCTGTGTCCAATTTGTCCTC -3’ | 65 |
| | 1327r | 5’-CGTCTACGACCTTACCATCG-3’ | 56 |
| *hopN1* | 91f | 5’-AATGGAAGCGAGTGTCTGC-3’ | 58 |
| | 142f | 5’-TCTTCACACAGATCGAAAGGC-3’ | 58 |
| | 771r | 5’-CGATAGAGACCATCAGATCCG-3’ | 58 |
| | 816r | 5’-GATTCTGGTCTTGATGTATTGCG-3’ | 58 |
| *hopR1* | 427f | 5’-GAGATGGAACATGGCATCAG-3’ | 65 |
| | 705f | 5’-GATGGTGGAGTCTATCTGC-3’ | 65 |
| | 1129r | 5’-AGGTGAACAGTGTCGTCTC-3’ | 65 |
| | 1299r | 5’-CATGACCATCAAGCTGAACG-3’ | 65 |

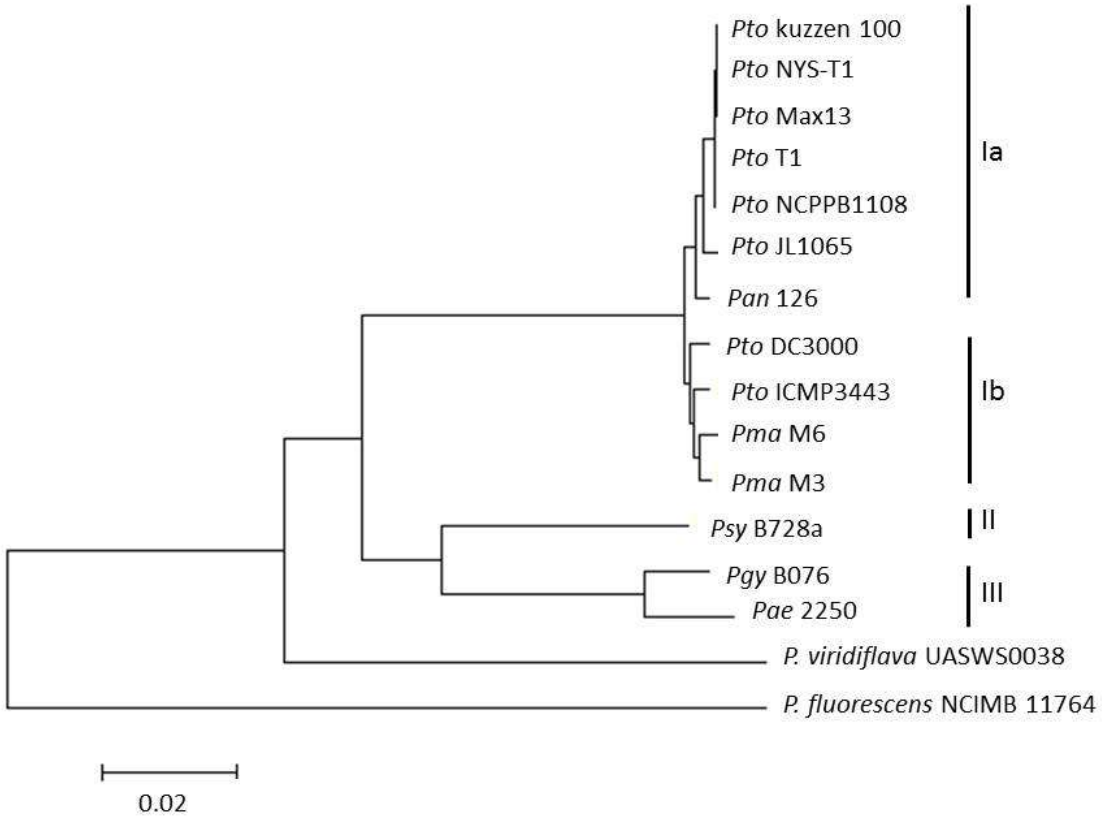
[a] Position corresponds to the 5’ end of the primer in reference to the adenine of the start codon of the corresponding gene, f designates forward primers and r designates reverse primers

pseudomonads (Table 3.1) from overnight bacterial cultures grown in LB broth at 28°C on rotary shaker (200 rpm). Inoculum was prepared by adjusting bacterial concentration to an OD of 0.05 in 50 ml of 1X phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$,) containing 0.25% Tween. Plants were dip-inoculated and kept in a greenhouse at 24°C (daytime) and 20°C (nighttime) with 14 h light per day. Two plants per isolate were inoculated for each repetition with a total of two repetitions performed. Plants were rated for disease by counting the number of specks per plant starting 6 days post inoculation when specks were first visible and continuing every 2 days for a total of 4 ratings. The area under the disease progress curve (AUDPC) was calculated for each isolate (Madden et al. 2007). Analysis of variance and Tukey's honestly significant difference was calculated using JMP Pro 10 (SAS Institute, Cary, NC) to determine significant differences ($\alpha = 0.05$) between means.

## RESULTS

**Genomic comparison data.** The 188 contigs in the *Pto* 09150 assembly were ordered using the *Pto* T1 draft genome (Almeida et al. 2009), and annotated using RAST (Aziz et al. 2008). Multilocus sequence typing assessment of *Pto* 09150 sequences phylogenetically placed it with ten Group I strains. Isolate *Pto* 09150 was found to cluster tightly with four strains in the T1-like subgroup and has been renamed *P. syringae* pv. *tomato* NYS-T1 (Figure 3.1). Known classes of virulence genes were manually reviewed with particular attention given to the Type III effector repertoire. Type III effectors play an important role in *P. syringae* pathogenesis and are regulated by the HrpL alternative sigma factor (Block & Alfano 2001; Lindeberg et al. 2012). Mapping HrpL binding sites in the genome has proven an efficient strategy for locating T3E genes as well as identification of other candidate members of the HrpL regulon. Predicted virulence

Figure 3.1. Evolutionary history of *Pseudomonas* spp. using the neighbor-joining method. Concatenated sequences and group designations are described by Yan et al. 2008. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



factors are listed in Table 3.4.

The T3E repertoires of *Pto* T1 and *Pto* DC3000 differ significantly, with only 14 T3E genes shared between them and 15 and 11 unique to *Pto* DC3000 and *Pto* T1, respectively (Almeida et al. 2009). In contrast, the effector repertoires of *Pto* T1 and *Pto* NYS-T1 appear to be identical, with only two differences observed in their overall virulence gene profile. First, *Pto* NYS-T1 encodes the structural genes for coronatine biosynthesis which are lacking in *Pto* T1. Secondly, the sequence of the *Pto* NYS-T1 flagellin structural protein (*fliC*) differs from that of *Pto* T1 in the flgII-28 region associated with induction of an immune response in solanaceous hosts (Clarke et al. 2013).

**Primer development.** Type III effector genes were selected as target loci for development of DNA-based diagnostic methods for use in distinguishing the two subgroups. To identify T3E loci conserved throughout the T1-like subgroup, effector repertoires were predicted for the draft genomes of three T1-like strains collected at different times and in different geographical regions (K40, NCPPB1106, and Max13) (Cai et al. 2011). Type III effector genes present in the five T1-like strains but absent in either full-length or truncated form from *Pto* DC3000 were *hopW1*, *avrA*, and *hopAE1*. The genes *hopW1* and *avrA* were selected for primer development, as *hopAE1* is present in two *Pma* strains closely related to *Pto* DC3000 (B. Vinatzer, personal communication). Four primer combinations derived from conserved regions of *avrA* and *hopW1* were evaluated (Tables 3.3 and 3.5). Products of the expected size were amplified from *Pto* T1, *Pto* NYS-T1, the T1-like strains K40, NCPPB1108, and Max13, as well as *Pto* JL1065 which also segregates with this subgroup. No products were amplified from either *Pto* DC3000 or the *Pma* strains M3 or M6 (Table 3.5, Figure 3.2). The absence of amplified products from members

Table 3.4. Predicted virulence factors for *Pto* DC3000, *Pto* T1, and *Pto* NYS-T1 from genomic data. Blue boxes indicate the presence of full length reading frames and an upstream *hrp*-binding sequence.

| | ***Pto* DC3000** | ***Pto* T1** | ***Pto* NYS-T1** |
|---|---|---|---|
| **Type III effectors** | | | |
| avrE1 | ■ | ■ | ■ |
| HopC1 | ■ | ■ | ■ |
| HopH1 | ■ | ■ | ■ |
| HopI1 | ■ | ■ | ■ |
| HopR1 | ■ | ■ | ■ |
| HopO1-2 | ■ | ■ | ■ |
| HopS2 | ■ | ■ | ■ |
| HopT1-2 | ■ | ■ | ■ |
| HopY1 | ■ | ■ | ■ |
| HopAA1-1 | ■ | ■ | ■ |
| HopF2 | ■ | ■ | ■ |
| HopD1 | ■ | ■ | ■ |
| HopQ1 | ■ | ■ | ■ |
| HopO1-1 | ■ | ■ | ■ |
| HopT1-1 | ■ | ■ | ■ |
| HopAF1 | ■ | ■ | ■ |
| HopW1 | | ■ | ■ |
| AvrA | | ■ | ■ |
| HopAS1 | | ■ | ■ |
| HopS1 | | ■ | ■ |
| HopAE1 | | ■ | ■ |
| HopAG1 | | ■ | ■ |
| HopAH1 | | ■ | ■ |
| HopAI1 | | ■ | ■ |
| AvrRpt2 | | ■ | ■ |
| avrD1 | | ■ | ■ |

| | ***Pto* DC3000** | ***Pto* T1** | ***Pto* NYS-T1** |
|---|---|---|---|
| HopAB2(avrPtoB) | ■ | | |
| HopA1 | ■ | | |
| HopB1 | ■ | | |
| HopM1 | ■ | | |
| HopN1 | ■ | | |
| HopE1 | ■ | | |
| HopG1 | ■ | | |
| HopV1 | ■ | | |
| HopAA1-2 | ■ | | |
| HopAD1 | ■ | | |
| HopAM1 (2 copies) | ■ | | |
| HopAO1 | ■ | | |
| HopAQ1 | ■ | | |
| AvrPto1 | ■ | | |
| **Hrp regulon (non-T3E)** | | | |
| PSPTO_0834-0836 | ■ | | |
| tyrosine phosphatase | | | ■ |
| **toxins** | | | |
| coronatine | ■ | | ■ |
| **NRPS** | | | |
| PSPTO_4699 | ■ | | |
| **PAMP elicitation** | | | |
| flg28 elicitor in FliC | ■ | ■ | |
| **other** | | | |
| YeeABC | | | ■ |

Table 3.5. PCR production amplification from primers developed in this study.

| | | *avrA* | | | | *hopW1* | | | | *hopN1* | | | | *hopR1* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Primers | 547f 1321r | 547f 1219r | 215f 1321r | 215f 1219r | 173f 1099r | 173f 1327r | 313f 1099r | 313f 1327r | 91f 771r | 91f 816r | 142f 771r | 142f 816r | 427f 1129r | 427f 1299r | 705f 1129r | 705f 1299r |
| Species | Isolate | | | | | | | | | | | | | | | | |
| *Pseudomonas syringae* | 0578 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| pv. *tomato* | 0761 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | 09084 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | 09150 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | 12042 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | 13093 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | 13110 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | K40 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | A9 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | JL1065 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | T1 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | Max13 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | NCPPB 1108 | + | + | + | + | + | + | + | + | - | - | - | - | + | + | + | + |
| | DC3000 | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + |
| | | | | | | | | | | | | | | | | | |
| *Pseudomonas syringae* pv. *antirrhini* | Pan126 | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + |
| *Pseudomonas syringae* | M3 | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + |
| pv. *maculicola* | M6 | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + |
| | | | | | | | | | | | | | | | | | |
| *Pseudomonas syringae* pv. *syringae* | B728a | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |

| | | *avrA* | | | | *hopW1* | | | | *hopN1* | | | | *hopR1* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Primers | 547f 1321r | 547f 1219r | 215f 1321r | 215f 1219r | 173f 1099r | 173f 1327r | 313f 1099r | 313f 1327r | 91f 771r | 91f 816r | 142f 771r | 142f 816r | 427f 1129r | 427f 1299r | 705f 1129r | 705f 1299r |
| | | | | | | | | | | | | | | | | | |
| *Pseudomonas viridiflava* | 10078 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | | | |
| *Pseudomonas fulva* | 0430 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | | | |
| *Pseudomonas fluorescens* | 09110 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | | | |
| *Pseudomonas putida* | 09112 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | | | |
| *Xanthomonas perforans* | 13091 | +[a] | +[a] | +[a] | +[a] | +[b] | - | - | - | - | - | - | - | - | - | +[b] | +[b] |
| | | | | | | | | | | | | | | | | | |
| *Clavibacter* | 13048 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *michiganensis* | 13085 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| subsp. *michiganensis* | 13117 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 13129 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | | | | |

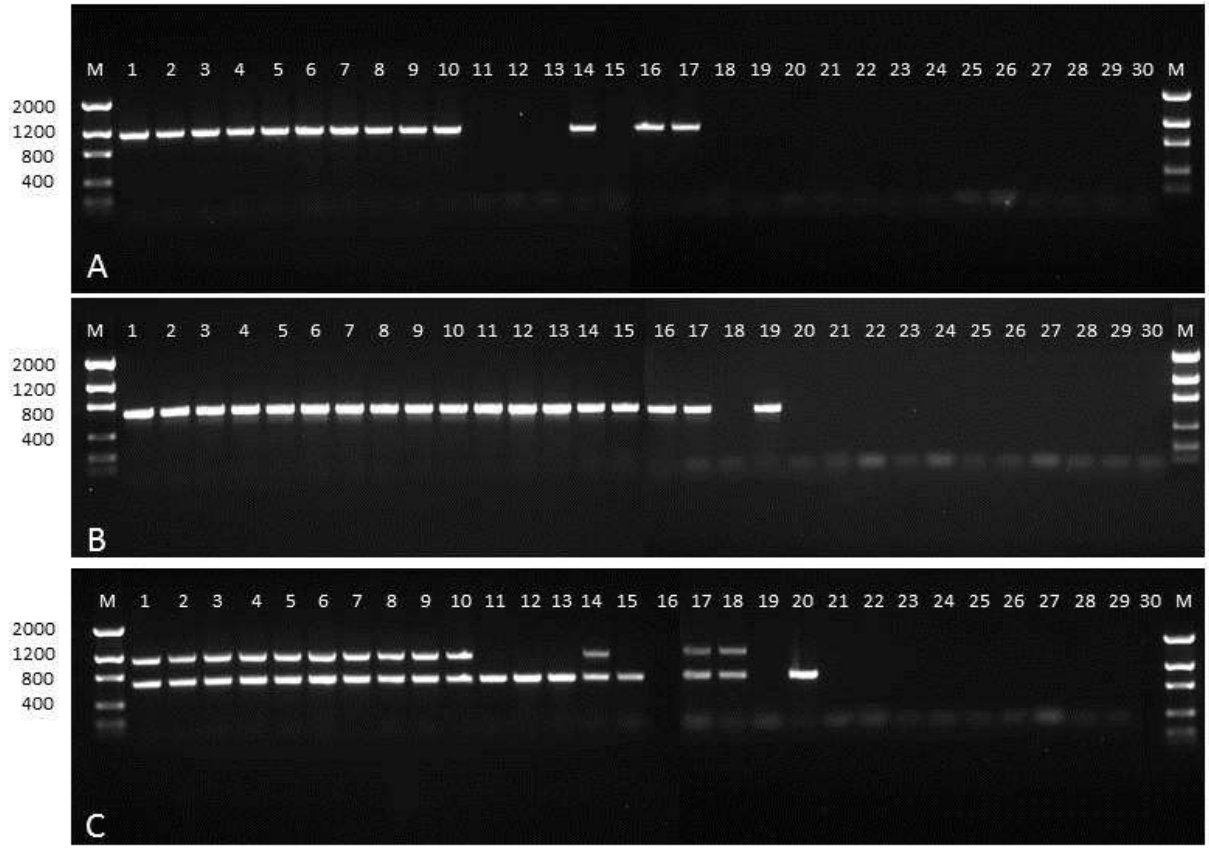
+ Target amplification with corresponding primer pair and isolate.

- No amplification with corresponding primer pair and isolate.

[a] Weak target amplification at lower annealing temperatures.

[b] Weak target amplification at all annealing temperatures.

Figure 3.2. Results of PCR amplification for T3Es; A: *avrA* primers 215f and 1321r; B: *hopR1* primers 427f and 1129r; and C: multiplex with *avrA* primers 215f and 1321r and *hopR1* primers 427f and 1129r. M: DNA ladder (Invitrogen, Low DNA Mass Ladder); 1: 0578; 2:0908; 3: NYST1; 4:12042; 5: K40; 6: A9; 7: JL1065; 8: T1; 9: Max13; 10: NCPPB1108; 11: DC3000; 12: M3; 13: M4; 14: leaf tissue infected with NYST1; 15: leaf tissue infected with DC3000; 16: fruit tissue infected with 13093; 17: fruit tissue infected with 13110; 18: healthy tomato leaf tissue; 19: 126; 20: B728a; 21: 10078; 22: 0430; 23: 09110; 24: 09112; 25: 13091; 26: 13048; 27: 13085; 28: 13177; 29: 13129; 30: negative water control.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 M
2000
1200
800
400
A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 M
2000
1200
800
400
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 M
2000
1200
800
400
C

of the DC3000/*Pma* subgroup confirms the utility of *avrA* and *hopW1* as loci distinguishing T1-like strains. The primers 215f and 1321r for *avrA* were selected for diagnostics to distinguish T1-like strains from the DC3000/Pma-like strains using an annealing temperature of 65°C.

The strain *Pto* DC3000 has nine effector genes lacking homologs among the sequenced T1-like strains. Of these, *hopN1* was selected as a candidate diagnostic locus for the *Pto* DC3000/*Pma* subgroup. The *hopN1* sequences in the Hop Database (www.pseudomonas-syringae.org) were aligned to identify conserved regions and two primer combinations evaluated on the members of the T1-like and DC3000/*Pma* subgroups. Amplified products of the expected size were apparent for *Pto* DC3000, *Pma* M3, and *Pma* M6 but not for any of the T1-like strains (Table 3.5).

The T3E *hopR1* is highly conserved throughout Group I *P. syringae* strains and was used for development of primers that can generally distinguish *P. syringae* strains from other bacteria. As shown in Table 3.5, all Group I *P. syringae* isolates tested yielded an amplified product. Neither *Psy* B728a in *P. syringae* Group II nor any of the non-*P. syringae* bacterial pathogens evaluated yielded a *hop1R* amplification product (Table 3.5, Figure 3.2).

A multiplex PCR was performed with *avrA* primers 215f and 1321r and *hopR1* primers 427f and 1129f. Results show target-sized amplification products for the *avrA* primer set for the T1-like strains. Target amplification was seen for *hopR1* primers in both the T1-like and DC3000/Pma like strains. No amplification was seen for the remaining pseudomonads or other bacterial pathogens of tomato (Table 3.5., Figure 3.2.).

Weak amplification for some of the primer combinations for *avrA*, *hopW1*, and *hopR1* was seen with *X. perforans* (Table 3.5). Many of the amplifications products for *X. perforans* were only visible at low annealing temperatures. No amplification was seen for any primer combinations

for DNA from healthy tomato tissue or water controls. Target amplification was seen for all samples from *Pto* infected tomato leaf and fruit tissue.

**Virulence assay.** The more virulent (AUDPC > 212.1) *Pto* strains include JL1065, A9, K40, and the NY field isolated strains; 0578, 0761, 09084, 09150, and 12042. The less virulent (AUDPC < 212.1) tomato pathogens include *Pto* DC3000, *Pma* M3, *Pma* M6, and *P. viridiflava* 10078. The negative controls included *Psy* B728a, *Pan* 126, *P. fluorescens, P. putida, P. fulva* and water, had an AUDPC value of less than 1 (Table 3.6).

## DISCUSSION

Our results have elucidated some genetic clues about the virulence of *Pto* NYS-T1. First of all, Pto NYS-T1 has a large effector repertoire. These T3Es are injected into host cells via the type III secretion system to manipulate the host's cellular machinery to enhance growth and survival within the plant. Type III effectors are the key virulence factors and host range determinants of Gram-negative plant pathogens (Jin et al, 2003; Alfano & Collmer 2004). Many T3Es individually have only a modest quantitative effect on virulence but collectively they can greatly promote pathogen growth and survival both in host plant and on non-host plants (White et al. 2000; Vinatzer et al. 2006). Other single or specific combinations of T3Es may be principally responsible for virulence on a given host (Cunnac et al. 2011).

Phytotoxins, such as coronatine, are virulence factors that can increase the aggressiveness of a pathogen (Bender et al. 1999). Many *P. syringae* pathovars produce coronatine but the presence of coronatine biosynthetic genes is not closely correlated with strain phylogeny or host range

Table 3.6. Mean area under the disease progress curve (AUDPC) of tomatoes inoculated with pseudomonads.

| Isolate | Species | *P. syringae* MLST group[a] | AUDPC[b] |
|---|---|---|---|
| water | negative control | | 0 a |
| B728a | *Psy* | III | 0 a |
| 09110 | *P. fluorescens* | | 0 a |
| 09112 | *P. putida* | | 0 a |
| 0430 | *P. fulva* | | 0 a |
| Pan126 | *Pan* | Ia | 0.8 a |
| 10078 | *P. viridiflava* | | 5.3 a |
| M6 | *Psm* | Ib | 11.8 a |
| M3 | *Psm* | Ib | 33.3 ab |
| DC3000 | *Pto* | Ib | 90.6 abc |
| JL1065 | *Pto* | Ia | 212.1 abc |
| 09084 | *Pto* | | 326.8 abc |
| A9 | *Pto* | | 394.8 abc |
| 12042 | *Pto* | | 425.3 abc |
| 0761 | *Pto* | | 453.6 bc |
| 09150 (NYS-T1) | *Pto* | Ia | 469.9 c |
| K40 | *Pto* | Ia | 481.8 c |
| 0578 | *Pto* | | 483.9 c |

[a] MLST group according to Yan et al. 2008

[b] different letters represent significantly different groups

among and within pathovars (Bereswill et al. 1994). The genes necessary for coronatine biosynthesis are present in the *Pto* NYS-T1 genome but not in the *Pto* T1 genome. Coronatine has been shown to enhance fitness of *P. syringae* pathovars by facilitating entry into plants through stomata and promoting growth in planta (Uppalapati et al. 2007). Even though the *Pto* NYS-T1genome contains the genes for coronatine biosynthesis production of coronatine stills needs to be experimentally confirmed. Another possible virulence factor is the flgII-28 epitope of the bacterial flagellin gene (*fliC*) of *Pto* NYS-T1 that differs from *Pto* T1 and *Pto* DC3000. The flgII-28 region is associated with a microbe associated molecular pattern which triggers an immune response in tomato and other solanaceous crops, and changes in the flgII-28 region could facilitate leaf invasion and thus enhanced virulence (Cai et al. 2011). Allelic variation of flgII-28 has been reported and some of these strains have elicited a reduced immune response from tomato (Clarke et al. 2011).

Comparative genomics was used to select targets for diagnostic PCR protocol development to distinguish the more virulent T1-like strains from less virulent DC3000/Pma-like strains and other bacterial pathogens of tomato. Primers for *avrA* and *hopW1* were able to distinguish T1-like strains from DC3000/Pma-like strains equally well among the isolates used in this study. Primers developed for *avrA* (215f and 1321r) were chosen over those for *hopW1* because this effector is as not widespread among the *P. syringae* pathovars (Lindeberg et al. 2012). The T3E *avrA* has only been found in the T1-like strains of *Pto*, *P. syringae* pv. *aesculi (Pae)*, a pathogen of horse chestnut *(Aesculus hipposcastinum*), and *P. syringae* pv. *glycinea (Pgy),* a pathogen of soy bean (*Glycine max*) (Baltrus et al. 2011). The pathovars *Pae* and *Pgy* are not pathogens of tomato and are in *P. syringae* MLST Group III; these pathovars could be differentiated molecularly from the Group I *Pto* strains using primers developed for *hopR1* (Figure 3.2).

Multiplex PCR was performed with primers for *avrA* and *hopR* and amplification was only seen for Group I *P. syringae* pathovars.

Several other common bacterial tomato pathogens (*C. michiganensis, P. viridiflava,* and *X. perforans*), pseudomonads (*P. syringae antirrhini, P. syringae* pv. *syringae, P. fluorescens. P. putida,* and *P. fulva*) were included as controls for primer development. No amplification products were seen from these control strains except for *X. perforans*. Weak amplification of *X. perforans* was seen with some primer combinations from *avrA*, *hopW1*, and *hopR1* and is attributed to the presence of virulence genes with limited similarity to these T3Es (Table 3.5). Primers for *avrA* produced weak target amplification only at low annealing temperatures, PCR with the temperatures listed in Table 3.3 did not amplify DNA from *X. perforans*. For some primer combinations for *hopW1* and *hopR1* weak amplification resulted for *X. perforans* and these primers were not considered for further development. The primers developed in this study can clearly distinguish T1-like strains from the DC3000/Pma-like strains tested in this study. To confirm the utility of these primers *Pto* strains from additional geographic regions could be tested.

The virulence assay showed T1-like strains to have larger AUDPC values than both the DC3000/Pma-like strains. Significant differences were not seen between any of the *Pto* strains used in the virulence study due to the large variation in symptoms among plants inoculated with the same strain and are likely attributed to plant size at time of inoculation. All tomato plants were sown at the same time but germination was variable leading to large variations in plant size at five weeks post seeding when plants were used for inoculations.

From this study we were able to discover several factors from the draft genome of *Pto* NYS-T1 that could be responsible for the level of virulence seen in the field. We were able to use these virulence determinants to develop a targeted DNA-based diagnostic protocol to distinguish the more virulent T1-like strains from less virulent DC3000/Pma-like strains. With the availability of multiple closed and draft genomes for many bacterial pathogens and the decreasing cost and time to sequence bacterial genomes this approach could be applied for subspecies level targeted diagnostics.

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## CONCLUSIONS

Irrigation is necessary for the production of fruits and vegetables, and many growers will choose to irrigate from a surface water source. This research gives us a better understanding of the risks of contaminating produce grown in New York State with pathogenic microorganisms by using surface water for irrigation. Human and plant pathogenic microorganisms have been found in surface irrigation water sources in many areas of the United States but parameters linked to the presence and concentrations of a particular organism can vary from region to region (Strawn et al. 2013; McEgan et al. 2013; Johnson et al. 2003). For New York growers the threat of introducing *E. coli*, *Salmonella* spp., or plant pathogenic oomycetes into their produce growing environments exists. Unfortunately, the presence or levels of *E. coli* cannot be strongly correlated to any of the variables considered in this study, such as presence of livestock near irrigation source, or amount of recent precipitation, which have been correlated to *E. coli* in studies elsewhere (Johnson et al. 2003; Cooley et al. 2007). It is possible that no strong correlation exists and *E. coli* presence in surface water is commonplace. Regulations for irrigation water quality are based on generic *E. coli* levels. Presence of generic *E. coli* was found to be common New York surface water reservoirs with 44/123 samples having 1 or more CFU/100 ml and 17/18 sites had at least one positive sample. Three of the 123 (2.4%) samples exceeded regulatory threshold levels and the corresponding irrigation sources would not be unusable until measures were taken to treat the water and retesting shows levels below threshold. No sites exceeded the 5-sample mean average of >126 CFU/100 ml suggesting that spikes in *E. coli* >235 CFU/100 ml levels are more likely to occur than rolling averages >126 CFU/100 ml that could lead to water quality testing failures. More research will be necessary to understand

the circumstances that lead to spikes of *E. coli* in surface water sources to help growers meet irrigation water quality standards.

*Salmonella* spp. were found more frequently in the irrigation sites than *E. coli.* The presence of *E. coli* was not strongly correlated with that of *Salmonella* suggesting that it may not be a suitable indicator for *Salmonella* spp. in surface water sources. In 17/18 sites the average levels of *Salmonella* were greater than *E. coli* levels suggesting that produce is at greater risk of being exposed to *Salmonella* than *E. coli* through surface irrigation water at the sites tested in this study. A significant portion (19%) of these samples had excessive levels (>300 CFU/100 ml) of *Salmonella*. According to the Centers for Disease Control and Prevention (http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html) *Salmonella* is the 2nd leading cause, only behind the norovirus, of foodborne illnesses in the US and is the leader in foodborne illnesses that end in death. About half of the *Salmonella* related outbreaks have been attributed to consumption of contaminated produce such as tomatoes, peppers, sprouts, and melons (Painter et al. 2013).

This research revealed that the presence and levels of *Salmonella* spp. in surface water irrigation sources in New York is associated with cumulative precipitation levels 3 days prior to sampling. More samples were positive with higher CFU/100 ml counts if they were collected when precipitation three days prior to sampling was less than 0.64 cm. This is a time when growers may be likely to irrigate. The relationship between *Salmonella* prevalence in surface water and recent precipitation has been studied in many regions, but results are not consistent suggesting that the situation is more complex and may rely on other factors. The results of the survey performed in this thesis show that *Salmonella* spp. are likely to be in surface waters in New York

and further research to understand how these bacteria enter an irrigation source, how long they persist, what preventive measures can be taken, and which water treatment options are effective against *Salmonella* would greatly benefit both growers and consumers.

Hymexazol-insensitive oomycetes were found to be very prevalent in all New York surface water sources. Fortunately, the most frequently found species are currently not of great concern for produce growers (see Chapter 1). On the other hand, many oomycetes that are capable of infecting fruit and vegetable crops were found. For example, *Ph. citricola* is responsible for leather rot of strawberries and root rot of raspberries, while *Py. catenulatum* can attack seedlings of corn, common bean, tomato, pepper, and eggplant (Erwin & Ribeiro 1996; Hendrix & Campbell 1969). Creeks were found to have more HIS oomycetes than ponds but the number of oomycetes found per liter may not directly correlate to the level of plant health risk. Specific detection of pertinent oomycetes for crops being irrigated may be necessary to assess the risk of using a particular irrigation source. Many uncharacterized *Phytophthora* and *Pythium* spp. were found in surface water sources and these could present challenges for detection (see Chapter 1). Detection methods include culturing, baiting, serological and DNA-based methods. Culturing and baiting methods take time, possibly several days to weeks, and morphological or DNA analyses are still needed for an identification. Serological methods for *Phytophthora* and *Pythium* may not provide resolution down to the species level. Many DNA-based detection protocols have been developed for specific oomycete pathogens but these protocols are rarely optimized for water samples. Also, the volumes used for detection methods are usually 1 liter or less because larger volumes can be difficult and expensive to process in the laboratory. Additional research for processing and identifying oomycetes from water sources is necessary to better understand the plant health risks of using surface water for irrigation.

The results from this research show that UV treatment can eliminate or greatly reduce the levels of some bacterial and oomycete pathogens in two diverse surface water sources. Ultraviolet light shows promise in the treatment of surface water and should be further developed as a water treatment option for growers. Many other pathogens can threaten fruit and vegetable production and UV treatment should be further assessed with additional pathogens. Also, other surface water sources should be examined to determine the full range of effectiveness of the UV treatment system. Current UV treatment systems may not be able to handle the volumes of water needed for irrigation, and more work will need to be done to develop UV technologies that can treat high throughput volumes of irrigation water. As we look to the near future, the need for irrigation water treatment options will grow as stricter and additional food safety regulations are put in place.

The *Pto* diagnostic protocol created from this study was enabled by comparison of the genome of a virulent New York field isolate to many other *Pto* and related *Pseudomonas* genomes. Sequencing of genomes is becoming more economical and the number of genomes available for research purposes is increasing exponentially (Lagesen et al. 2010). Bioinformatic tools are constantly improving and becoming more accessible, which enables exciting new research areas using whole genome comparisons. These comparisons can be used for the development of very targeted detection protocols that can provide more information than just species identification. Detection protocols that can offer more knowledge about target microorganisms can greatly improve and fine tune food safety and plant health programs for growers. The *Pto* diagnostic protocol developed in this study distinguishes virulent *Pto* strains from less virulent *Pto* strains and other pathogens that can cause similar symptoms. Growers can use this information to help them decide if control measures are necessary.

With more demands being placed on agricultural production, due to increasing populations, keeping our food supply safe and our plants healthy will only become more challenging. This work will add to the current body of agricultural research to support growers in their quest to keep produce safe and plants healthy.

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