DETERMINING THE CHARACTERISTICS OF *XANTHOMONAS CUCURBITAE* BACTERIUM FROM THE NORTH CENTRAL REGION AND DEVELOPING EFFECTIVE SEED TREATMENT FOR ERADICATION OF THE BACTERIUM IN PUMPKIN SEEDS

BY

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THESIS

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ABSTRACT

Bacterial spot of cucurbits, caused by *Xanthomonas cucurbitae*, has become a serious threat to pumpkin production in Illinois and other states in the North Central Region (NCR) of the United States. This study was conducted to investigate the characteristics of *X. cucurbitae* isolates from the NCR and to develop an effective seed treatment to eradicate the pathogen in pumpkin seeds. Characteristics of the pathogen included the cell multiplication of *X. cucurbitae* at different temperatures and pH levels and pathogenic variation among isolates of the pathogen. Fourteen *X. cucurbitae* isolates from seven states (Illinois, Indiana, Iowa, Kansas, Michigan, Ohio, and Wisconsin) were tested. The American Type Culture Collection (ATCC) strain 23378 was included in this study as positive control. The result showed that the range of minimum, maximum, and optimum temperatures for cell multiplication of *X. cucurbitae* were 4-6, 34-36, and 24-30°C, respectively. Cell multiplication and colony development of *X. cucurbitae* was optimum at pH 6.5-8. For determining pathogenic variation among the isolates, *X. cucurbitae* was inoculated onto leaves of three-week-old 'Howden' and 'Dickinson' pumpkins in a greenhouse. Development of the bacterial lesions was recorded from 12 to 168 h post inoculation. The post-inoculation period for the first appearance of the visible lesions was significantly affected by the isolates. However, diameters of the lesions 168 h post inoculation were not significantly different among the isolates. Survival of *X. cucurbitae* in pumpkin seed was studied using naturally-infected and artificially-inoculated pumpkin seed stored at 4 and 22°C. *X. cucurbitae* survived longer than 18 months in the seeds at both temperatures and remained viable. The storage temperature significantly affected survival of *X. cucurbitae* in the seed. The effectiveness of hot-water, hydrochloric acid (HCl), and sodium hypochlorite (NaClO) for eradication of *X. cucurbitae* in pumpkin seed was tested. The

results showed that a hot-water treatment at 55°C for either 10 or 15 min and HCl treatment at 0.5% concentration for 40 min eradicated *X. cucurbitae* in both naturally-infected and artificially-inoculated pumpkin seeds without any significant adverse effect on either seed germination or seedling vigor. None of the NaClO treatments eradicated *X. cucurbitae* in the seed.

To my family

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CHAPTER 1

INTRODUCTION

Pumpkin

Pumpkin belongs to the Cucurbitaceae family. This family contains more than 700 species, including some important crops, such as *Cucumis sativus* (cucumber), *Lagenaria siceraria* (gourd), *Citrullus lanatus* (watermelon), and *Cucumis melo* (melon). In general, pumpkin refers to cultivars of the species *Cucurbita pepo, C. maxima,* and *C. moschata* (Berg, 2008). The name of pumpkin was originally derived from the Greek word "pepon" which refers to "large melon." Then French adopted it as "pepon" and eventually British changed it to "pumpkin" (Michael et al., 2012). Typically, pumpkins are orange, round-shaped fruit with many creases running from the stem-end to the blossom end, and a thick-shell covering seed and pulp. However, there are variations existing in shape, size, weight, and color, even within one species. In most cases, a pumpkin is oblate in shape, with the equatorial diameter greater than the polar diameter, but the shape can also be round to pear-shaped (Whitaker and Bohn, 1950). Furthermore, the size of pumpkins ranges from 5 cm to more than 50 cm in diameter. The weight of pumpkins ranges from less than 0.5 kg to more than 500 kg. Commonly, pumpkins are orange, but some cultivars are also yellow or white (Babadoost and Zitter, 2009).

Pumpkin production

Pumpkin is a widely grown crop around the world for food, feed, and entertainment. As an edible fruit, pumpkin is a valuable source of vitamin C, dietary fiber, niacin, folic acid, and iron (Whitaker and Bohn, 1950). Pumpkin is also regarded as a Halloween staple, and carving pumpkins has become a Halloween tradition in October since mid-19th century.

China, India, the Russian Federation, Iran, and the United State (US) are the world's leading producers of pumpkins (FAOSTAT, 2014). Each year, approximately 5×10^8 kg of pumpkins are produced in the US, which is valued at about $150 million. Top pumpkin-producing states in US are Illinois, California, Ohio, Michigan, and New York (USDA, 2014). Approximately 90% of processing pumpkins in the US are produced and processed in Illinois (Babadoost and Islam, 2003). Production of processing pumpkins in Illinois increased from less than 1,000 ha in the 1930s to more than 6,000 ha in 2012 (Babadoost and Zitter, 2009). Illinois also produces about 5,000 ha of jack-o-lantern (Halloween pumpkins) every year (Babadoost and Islam, 2003).

Pumpkin diseases

Pumpkin plant and fruit can be infected by several plant pathogens during the growing season (Babadoost and Ravanlou, 2012; Jossey and Babadoost, 2008; Williams and Zitter, 1996). Some important fungal and oomycete diseases of pumpkins include downy mildew, caused by *Pseudoperonospora cubensis*; Fusarium crown and fruit rot, caused by *Fusarium* spp.; gummy stem blight and black rot of fruit, caused by *Didymella bryoniae*; Phytophthora blight, caused by *Phytophthora capsici*; powdery mildew, caused by *Podosphaeria xanthii*; Plectosporium blight, caused by *Plectosporium tabacinum;* and Sclerotinia blight, caused by *Sclerotinia sclerotiorum*. Three bacterial diseases are known to affect pumpkin plants and fruit in Illinois. These diseases are angular leaf spot, caused by *Pseudomonas syringae* pv. *lachrymans*; bacterial spot, caused by *Xanthomonas cucurbitae*; and bacterial wilt, caused by *Erwinia tracheiphila*. Cucumber mosaic, caused by *Cucumber mosaic virus*; papaya ringspot, caused by *Papaya ringspot virus*; squash mosaic, caused by *Squash mosaic virus*; watermelon mosaic, caused by *Watermelon mosaic virus*; and zucchini yellow mosaic, caused by *Zucchini yellow mosaic virus* are major viral diseases of

pumpkins in Illinois and other states in the US (Jossey and Babadoost, 2008; Williams and Zitter, 1996).

Bacteria spot of pumpkin

Bacterial spot, caused by *Xanthomonas cucurbitae*, was first reported in 1926 on 'Hubbard' squash in New York (William and Zitter, 1996). Subsequently, the disease was reported in other cucurbit growing areas in Asia, Australia, Europe, and North America on cucumber, pumpkin, squash, and watermelon (Bineeta et al, 1999. Pruvost et al, 2009). Surveys during 2009-2011 showed that pumpkin bacteria spot has become a prevalent disease in Illinois, causing up to 100% yield losses in commercial fields (Babadoost and Ravanlou, 2012).

The causal pathogen of bacterial spot was originally named *as Xanthomonas campestris* pv. *cucurbitae*. Later, the name was changed to *X. cucurbitae* (Kado, 2010). This pathogen can infect both leaves and fruit. Infected leaves develop small, round to angular, and yellow to beige lesions surrounded by a yellow halo. On some pumpkin cultivars, the lesions may appear more angular, similar to the lesions caused by angular leaf spot, caused by *Pseudomonas lachrymens* (Babadoost et al., 2012; Babadoost and Zitter, 2009; Babadoost and Ravanlou, 2012). Lesions may coalesce to cover large parts of the leaves. The lesions on fruit begin as small (1-3 mm in diameter), circular, and slightly sunken with a beige center and brown halo. The lesion may reach 10-15 mm in diameter. Infected fruit are colonized by secondary fungi and bacteria causing rapid decay of fruits, resulting in up to 100% yield losses (Babadoost and Zitter, 2009).

Taxonomy of *Xanthomonas cucurbitae*

X. cucurbitae belongs to the kingdom of Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Xanthomonadales, and family Xanthomonadaceae (Saddler and Bradbury, 2005). *X. cucurbitae* was classified as a pathovar of the species *X. campestris* until 1995, when a comprehensive DNA-DNA hybridization study was carried out on 183 strains of the genus *Xanthomonas*. Previously described species, *X. campestris* was divided into 16 DNA homology groups. In group 8, two strains of *X. campestris* pv. *cucurbitae* with G+C content of DNA around 66~68% were considered significantly distinct from all other groups and reclassified as a new species, *Xanthomonas cucurbitae* (Vauterin et al., 1995).

Characteristics of *X. cucurbitae*

Xanthomonas bacteria are straight rods and Gram negative. On sugar containing media, *Xanthomonas* spp. form shiny, yellow, mucoid colonies and produce copious exopolysaccharides, which is known as "xanthan gum." Colonies of bacteria of the genus *Xanthomonas* are smooth, round and butyous when young, but when older, colonies may show surface markings such as striations and become lobed (Kado, 2010).

X. cucurbitae is aerobic and has non-spore forming rod-shaped cells, measuring 0.5-1.3 × 0.4-0.6 µm with only one polar flagellum. The colonies of *X. cucurbitae* are yellow, convex shiny and mucoid on yeast extract-dextrose-CaCO3 agar medium (Rademaker et al., 2005). The optimum temperature for multiplication of *X. cucurbitae* ranges from 25 to 30°C. The bacterium is reported not to be able to multiply above 35°C (Bineeta et al., 1999).

Epidemiology of bacterial spot disease

Very little has been published on the biology of *X. cucurbitae* and epidemiology of bacterial spot. Babadoost and Ravanlou (2012) reported that *X. cucurbitae* can develop typical symptoms of bacterial spot on all plants belonging to the family Cucurbitaceae. Artificial inoculation of bean, broccoli, pepper, mustard, radish, snow pea, turnip, tomato, and wild mustard resulted in hypersensitive reactions (Babadoost and Ravanlou, 2012). *X. cucurbitae* has been reported to be a seedborne pathogen (William and Zitter, 1996). Infected seed give rise to seedlings with bacterial lesions on cotyledon leaves and on true leaves. In addition, *X. cucurbitae* can survive in association with crop residue. Bacteria spot usually appears during the summer season during periods of high temperature and moisture. Infected seed plays an essential role in long distance dispersal of the pathogen. *X. cucurbitae* is splash-dispersed within the fields (Babadoost and Ravanlou, 2012). Efforts to isolates *X. cucurbitae* from soil have not been successful (Babadoost and Zitter, 2009).

Pathogenic variation in *Xanthomonas* spp.

Phytopathogenic diversity and host specificity of *Xanthomonas* spp. and pathovars have been studied. The "new host-new species" concept, for the genus *Xanthomonas* has shown different host ranges or different disease symptoms for different host species or cultivar (Vauterin and Swing, 1997). Pathogenic variation of 51 isolates of *X. oryzae*, showed a spectrum of virulence and these differences resulted in the separation of the isolates into four distinct groups (Reddy, 1976). For *X. campestris* pv. *phaseoli* (pathogen of common bacterial blight of bean), 30 isolates were tested for pathogenic variation, and the results showed distinct variation among the isolates, which resulted in the designation of eight physiological races of *X. campestris* pv. *phaseoli* (Opio, 1996). In addition, a study on the causal agent of bacterial black spot of mango (*X. campestris* pv.

mangiferaeindicae) also has reviewd distinct variation among isolates, and the isolates were separated into three different groups, *X. axonopodis* pv. *mangiferaeindicae, X. axonopodis* pv. *anacardii*, and *X. axonopodis* pv. *spondiae* (Ah-you et al., 2007).

Survival of *Xanthomonas cucurbitae*

Limited information is available on the survival of *X. cucurbitae*. Thapa (2014) reported that *X. cucurbitae* survived for more than 24 months in plant tissue (fruit and leaves) in the field. Although *X. cucurbitae* is reported as a seedborne pathogen, no published information on the survival of the bacterium in the seed is available.

Management of bacterial spot

Due to a lack of sufficient information on the etiology and epidemiology of the bacterial spot, no effective strategies for management of this disease have been developed. In general, for seedborne diseases, using pathogen-free seeds always plays a critical role in disease management (Moffet and Wood, 1979; William and Zitter, 1996). A previous study indicated that treating seed in hot water (54 or 56°C) for 30 min, and then soaking the seeds in 1% sodium hypochlorite plus a 1% spreader-sticker solution for another 40 min significantly reduced the incidence of the bacterial spot in the field, but these treatments were still not able to eradicate the pathogen from the seed (Moffet and Wood, 1979). Ozdemir and Zitter also reported that seed treatments with copper plus mancozeb, 1% peroxyacetic acid, and 1% sodium hypochlorite for 15 min eradiated the pathogen from the seeds (Ozdemir and Zitter, 2006). Another report showed that hot-water seed treatments at 55°C for 25 to 30 min was not effective in reducing seedborne inoculum of *X. cucurbitae* (Vincent-Sealy and Brathwaite, 1982). In 1989, Sinha reported that cucumber cultivars ‘Japanese

Long Green' and 'Collection 72-10' were moderately resistant to bacterial spot. Babadoost and Zitter (2009) suggested that crop rotation for at least 2 years and minimizing moisture in plant canopy by avoiding overhead irrigation or other methods may reduce the incidence and severity of bacterial spot of cucurbits. Thapa (2014) reported that application of copper oxychloride plus copper hydroxide (Badge), copper sulfate (Cuprofix), oxytetracycline (Mycoshield), copper sulfate pentahydrate (Phyton), copper hydroxide (Kocide-3000) plus acibenzolar-s-methyl (ActiGard), and copper hydroxide (Kocide-3000) plus famoxadone plus cymoxanil (Tanos) reduced the incidence and severity of bacterial spot on both leaves and fruit of pumpkins in the field (Thapa, 2014).

RESEARCH OBJECTIVE

Bacterial spot has become a serious threat to pumpkin production in Illinois and other states in the US. Adequate information on the etiology of the disease is not available for developing effective management strategies for the disease. The overall goal of this project was to determine characteristics of *X. cucurbitae* and to develop effective seed treatments to eradicate seedborne inoculum of *X. cucurbitae*. Specific objectives of this research were to:

i) determine characteristics of *X. cucurbitae* strains isolated from the NCR;

ii) determine the survival of *X. cucurbitae* in pumpkin seed; and

iii) develop an effective seed-treatment to eradicate *X. cucurbitae* in pumpkin seed.

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CHAPTER 2

CHARACTERISTICS OF *XANTHOMONAS CUCURBITAE* ISOLATES FROM THE NORTH CENTRAL REGION OF THE UNITED STATES

ABSTRACT

This study was conducted to investigate the effects of temperature and pH on cell multiplication of *Xanthomonas cucurbitae* isolates from the North Central Region (NCR) of the United States. Also, pathogenic variation among the isolates was investigated. Fourteen *X. cucurbitae* isolates from seven states (Illinois, Indiana, Iowa, Kansas, Michigan, Ohio, and Wisconsin) were tested. The American Type Culture Collection (ATCC) strain 23378 was included in this study as positive control. The effects of temperature on cell multiplication of *X. cucurbitae* on yeast-extract-dextrose-calcium-carbonate agar (YDC), Luria Bertani agar (LB), and nutrient agar (NA) were assessed. The range of minimum, maximum, and optimum temperatures for cell multiplication in the three culture media were 4-6, 34-36, and 24-30°C, respectively. The effects of pH on cell multiplication of *X. cucurbitae* was studied on LB and NA media, which showed that cell multiplication and colony development was optimal at pH 6.5-8 on both of the culture media. For determining pathogenic variation among the isolates, a suspension of *X. cucurbitae* (10^8 CFU/ml) from 48-h cultures was used to inoculate the leaves of three-week-old 'Howden' and 'Dickinson' pumpkins in the greenhouse with temperatures ranging from 24 to 28°C. The development of the lesions was recorded from 12 h to 168 h post inoculation. There were significant differences in the post-inoculation period for appearance of visible lesions among the isolates. Diameters of the lesions caused by the isolates, however, were not significantly different from each other 168 h post

inoculation. Four isolates of *X. cucurbitae* caused significantly larger lesions on 'Dickinson' pumpkin leaves than they did on 'Howden' pumpkin leaves.

MATERIALS AND METHODS

Characteristics of *Xanthomonas cucurbitae* isolates

Morphology of bacterial colony

Fifteen isolates, including 14 isolates from the North Central Region (NCR) and the ATCC strain 23378 (Table 2.1), were characterized for their color, shape, and size of colonies on yeast-extract-dextrose-calcium-carbonate agar (YDC), Luria Bertani agar (LB), and nutrient agar (NA) media. The isolates were cultured on the media in Petri dishes and the cultures were incubated at 24±1°C for 48 h in the dark, after which colony characteristics were recorded. The study was repeated once.

Effect of temperature on cell multiplication

All 14 isolates of *X. cucurbitae* from the NCR were included in this study. The ATCC strain 23378 was included as a positive control. Each of the isolates was cultured on YDC, LB, and NA media in 100 mm Petri dishes and the cultures were incubated at temperatures from 4 to 38°C, for 10 days. Colony development of the isolates was recorded daily. Minimum, maximum, and optimum temperatures for each isolate were determined. The experiment was repeated once.

Effect of pH on cell multiplication

All 14 isolates from the NCR and the ATCC strain 23378 of *X. cucurbitae,* used for studying colony characteristics, were included for investigating the effect of pH on cell multiplication. LB and NA media with pH adjusted to levels of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 were used. Bacterial suspensions with 10^8 CFU/ml of the isolates were prepared. A loopful bacterial suspension of each isolate was streaked onto each of the culture media. The cultures were incubated at 24±1°C in dark for 48 h, and colony development was recorded. A scale of 0-4 was used to rate the intensity of colony development, in which 0 = no colony developed, 1 = a quarter of the steaked lines was covered with the bacterial colonies, 2 = half of the streaked lines in the plate was covered with the bacterial colonies, 3 = three quarters of the streaked lines in the plate and 4 = the streaked lines in the plate was covered with the bacterial colonies. The experiment was repeated once.

Pathogenicity of X. cucurbitae

Fourteen *X. cucurbitae* isolates from the NCR (Table 2.3) were tested for their pathogenicity. The ATCC strain 23378 of *X. cucurbitae* and sterilized distilled water (SDW) were used as positive control and a negative controls, respectively. Isolates were cultured on LB medium in Petri dishes and incubated at 24±1°C for 48 h. Cell suspensions of each isolate were prepared separately by washing bacterial colonies with SDW into a 15 ml conical tube. The cell density of each isolate was adjusted to 10^8 CFU/ml with the use of a spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA) at DO_{600} (OD=0.5 at 600 nm).

‘Howden’ and ‘Dickenson’ pumpkins were used for assessing variation among the *X. cucurbitae* isolates. Pumpkin seeds were sown in 32-cell plastic trays filled with a mixture of soil:peat:perlite (1:2:1), and grown in a greenhouse with 24-28°C for 21 days. Plants were then inoculated with *X. cucurbitae*.

A modified syringe infiltration method developed by previous investigators (Barak et al., 2001; Katagiri et al., 2002) was used to inoculate plants with the *X. cucurbitae* isolates. For each isolate, four plants from each cultivar, and the first two true leaves of each plant, were inoculated. On each leaf, three infiltrations of inoculum, each with 300 µl of inoculum per infiltration, were made using a 10 ml syringe. Hours of post inoculation (hpi) for the appearance of the lesions and dimeters of the lesions were recorded. Appearance of lesions was recorded every 12 h beginning from the time of inoculation. Diameters of the lesions were measured 168 h (seven days) after inoculation.

Seven days after inoculation, all symptomatic leaves were collected in order to re-isolate *X. cucurbitae* from the lesions. Leaf samples were soaked in 75% ethanol for 3 min and then rinsed three times with SDW. Lesions were cut in pieces of approximately 5 mm in diameter. One piece of each leaf with a lesion was placed onto KC medium in a Petri dish to have the bacterium grow out onto the medium. Also, two pieces of leaves with lesion were transferred into a microcentrifuge tube with 1 ml of SDW. The tube was vortexed for 30 s to release bacterial cells from plant tissue into water. For each sample, a loopful of bacterial suspension was streaked onto KC medium in 100 mm Petri dishes. All of the cultures were incubated at 24±1°C for 48 h in darkness. *Xanthomonas*-like colonies were subcultured on YDC and LB for identification. Convex, mucoid, and yellow colonies on YDC were identified as *X. cucurbitae* and considered for further evaluation

in a polymerase chain reaction (PCR) test. The ATCC strain 23378 was used as a positive control. For the PCR test of *X. cucurbitae,* a bacterial suspension with a cell density of 10^8 CFU/ml in SDW was prepared from the cultures grown on YDC. A total of 25 µl reaction volume was prepared, which contained 12.5 µl 2x Gotaq Green Master Mix (Promega Corporation, Madison, WI), 1 µl forward primer (RST 2, 5′-AGG CCC TGG AAG GTG CCC TGG A-3′), 1 µl reverse primer (RST 3, 5′-ATC GCA CTG CGT ACC GCG CGC GA-3′), 10 µl of nuclease free water, and 0.5 µl of bacterial suspension (Meng et al., 2004; Zhao et al., 2002). PCR amplification was performed with initial denaturation at 95°C for 10 min; followed by 32 cycles of denaturing at 95°C for 40 s, annealing at 59°C for 40 s and extension at 72°C for 1 min; then a final extension at 72°C for 10 min. Gel with 1% agarose and SYBE Safe DNA Gel Stain was prepared. PCR amplicons and one kb DNA ladder were loaded into the gel and ran at 100 volts for 30 min. All results were visualized under Blue-light Transilluminator (Thermo Fisher Scientific, Inc. Carlsbad, CA).

Data analysis

For the effect of pH on cell multiplication, data from two trials were combined and analyzed by a one-way ANOVA, using the GLM procedure of SAS 9.4 (SAS Institute Inc. Cary, NC). Normality of data was examined using the UNIVARITAE procedure, and a homogeneity test was run using the BERLETT test. Tucky's Honest Significant Difference ($\alpha = 0.05$) was used for mean separation analysis.

For the pathogenicity tests, hours of post inoculation and lesion size from each pumpkin cultivar were tested using a one-way ANOVA, using the GLM procedure of SAS 9.4 (SAS Institute Inc. Cary, NC). Normality of the data was examined using the UNIVARITAE procedure, and a

homogeneity test was run using the BERLETT test. Means of hours of post inoculation and lesion size were compared according to the Tucky's Honest Significant Difference ($\alpha = 0.05$).

RESULTS

Morphology of *X. cucurbitae* colony

No differences were noted on the colony morphology of *X. cucurbitae* isolates collected from various locations in the NCR and the ATTC strain 23378. All isolates tested developed yellow, convex colonies on LB and NA media, and colonies were more mucoid on YDC medium (Figure 2.1).

The range of minimum, maximum, and optimum temperatures for cell multiplication of *X. cucurbitae* isolates were 4-6, 34-36, and 24-30, respectively (Table 2.1). There were no significant differences on either minimum, maximum, or optimum temperatures among the isolates. Culture medium did not significantly affect cell multiplication of the bacterium.

Tested isolates of *X. cucurbitae* multiplied fastest on NA with pH 7.5. But cell multiplication of the bacterium did not significantly differ on NA with pH 6.5, 7.0, 7.5, and 8.0 (Table 2.2). None of the isolates multiplied on the medium with pH 4.5 (Figure 2.2). Cell multiplication of the bacterium was significantly slower on the medium with pH 5.0 compared those with pH 5.5 or greater. Similarly, cell multiplication of *X. cucurbitae* was significantly slower on the medium with pH 5.5 than higher pH values tested. Moreover, cell multiplication of the bacterium was significantly slower on the medium with pH 6 than the cultures with pH 6.5-8.5. Cell multiplication

of the bacterium on the medium with pH 8.5 was significantly slower than that of the medium with pH 8.0 (Table 2.2).

Pathogenic variation of *X. cucurbitae* isolates

Symptoms of bacterial spot appeared on inoculated leaves within 7 days after inoculated with *X. cucurbitae*. *X. cucurbitae* was successfully isolated from leave lesions (Figures 2.3 and 2.4). Bacterial lesions developed significantly faster on leaves of both 'Howden' and 'Dickinson' pumpkins inoculated with the isolated KS455 compared to the other isolates (Table 2.3). There were significant differences in the time periods for development of leaf lesions on "Howden' and 'Dickinson' plants inoculated with the isolates KS455, KS456, OH256, and WI206 (Table 2.3). Inoculation with the 15 different isolates of *X. cucurbitae* resulted in no significant differences in lesion diameters of lesions that developed on either 'Howden" or 'Dickinson' (Table 2.3). However, there were significant differences in lesion diameters between 'Howden' and 'Dickinson' on leaves inoculated with the isolates MI358 (P = 0.0026), OH256 (P = 0.0001), OH261 (P = 0.0008), and WI206 (P = 0.0003) (Table 2.3).

DISSUSSSION

All of the 15 isolates of *X. cucurbitae* that were tested, including ATCC stain 23378, produced colonies with similar colors and morphology on all three culture media (YDC, LB, and NC). Yellow mucoid and convex colonies developed on YDC, as reported by other investigators (Kado, 2010; Rademaker et al., 2005). Thus, preliminary identification of *X. cucurbitae* can be done by culturing bacteria on YDC medium. Reliable, identification of *X. cucurbitae* isolates, however, is

achieved by PCR assays using primers RST 2 (5′-AGG CCC TGG AAG GTG CCC TGG A-3′), and RST 3 (5′-ATC GCA CTG CGT ACC GCG CGC GA-3′).

Previous investigators reported that *X. cucurbitae* cells did not multiply at above 35°C, and optimal temperatures for colony development were 25-30°C (Bineeta et al., 1999). The results of my studies agree with previous investigators, as the 4 isolates of *X. cucurbitae* from the NCR did not develope colonies on either YDC, LB, or NA agar at temperatures above 36°C. *X. cucurbitae* isolates from the NCR did multiply at temperatures as low as 4°C. All these findings indicate that *X. cucurbitae* could multiply in a wide-range of temperature in nature, and that this organism may cause infection in pumpkin and other cucurbits plants (Pruvost et al., 2009; Pruvost et al., 2010; Lamichhane et at., 2010; Babadoost and Ravanlou, 2012).

Although we did not find any published report on the effects of pH on colony development of *X. cucurbitae*, Esgalhado et al. (1995) reported that *X. campestris* developed colonies on a culture medium with pH levels between 6.0-7.5 at temperatures of 25-27°C. Our findings show that cell multiplication of *X. cucurbitae* was the fastest on NA (likely on other culture media too) at pH 6.5-8.0. We may conclude that *X. cucurbitae* could multiply rapidly on and in plant tissues with neutral to slightly alkaline pH and may cause rapid infection.

Limited information is available on the pathogenicity/virulence of *X. cucurbitae* on cucurbits. Ravanlou (2014) reported significant differences in the time period needed for the appearance of visible lesions in inoculated leaves among *X. cucurbitae* isolates from Illinois. Our studies also showed that the time periods for appearance of the visible lesions in inoculated leaves were

significantly different among the isolates from the NCR. However, one week after inoculations, all 14 isolates from the NCR produced typical lesions, and there were no significant differences in diameter of the lesions among the isolates. But diameters of the lesions caused by some isolates were significantly different on cultivars ‘Howden’ and ‘Dickinson’. Further studies are needed to determine interactions between isolates of *X. cucurbitae* and cultivars of pumpkins.

TABLES AND FIGURES

Table 2.1. Minimum, maximum, and optimum temperatures for cell multiplication of *Xanthomonas cucurbitae* on yeast-extract-dextrose-calcium-carbonate agar medium (YDC), Luria Bertani agar medium (LB), and nutrient agar medium (NA).

	Minimum temperature (°C)				Maximum temperature (°C)				Optimum temperatures (°C)			
Isolate	**YDC**	**LB**	**NA**	**LSD**	**YDC**	**LB**	**NA**	**LSD**	**YDC**	**LB**	**NA**	**LSD**
ATCC strain 23378	6	6	6	NS[z]	34	35	34	NS	24-29	24-29	24-29	NS
IA382	5	5	5	NS	35	35	34	NS	24-29	24-29	24-29	NS
IA389	5	5	5	NS	34	35	34	NS	24-29	24-30	24-29	NS
IL232	5	5	5	NS	34	35	35	NS	24-29	24-30	24-29	NS
IL234	5	5	5	NS	35	35	35	NS	24-30	24-30	24-30	NS
IN332	6	4	5	NS	35	36	35	NS	24-30	24-29	24-30	NS
IN334	5	4	5	NS	35	36	35	NS	24-29	24-29	24-29	NS
KS455	6	5	5	NS	35	35	34	NS	24-29	24-30	24-29	NS
KS456	6	5	5	NS	34	35	35	NS	24-29	24-29	24-30	NS
MI358	5	4	6	NS	35	36	35	NS	24-29	24-29	24-29	NS
MI359	6	6	5	NS	34	35	34	NS	24-29	24-29	24-29	NS
OH256	6	6	6	NS	35	35	35	NS	24-30	24-30	24-30	NS
OH261	6	6	6	NS	35	35	35	NS	24-30	24-30	24-29	NS
WI205	5	5	5	NS	35	34	35	NS	24-30	24-30	24-30	NS
WI206	6	5	5	NS	35	34	34	NS	24-29	24-29	24-29	NS
LSD	NS	NS	NS	___	NS	NS	NS	___	NS	NS	NS	___

[z] NS = not significant at $\alpha = 0.05$.

Table 2.2. Colony development of *Xanthomonas cucurbitae* isolates from the North Central Region on nutrient agar medium with different pH[y] levels.

pH[x]	Colony development[y]
4.5	0.00 f[z]
5.0	1.05 e
5.5	2.00 d
6.0	3.52 c
6.5	3.90 a
7.0	3.94 a
7.5	3.97 a
8.0	3.95 a
8.5	3.77 b

[x] pH of the culture medium was adjusted by NaOH or HCl. Data of 14 isolates from North Central Region were combined together before analysis.

[y] Intensity of colony development was rated using a 0-4 scale, where 0 = no colony developed, 1 = a quarter of the steaked lines was covered with the bacterial colonies, 2 = half of the streaked lines in the plate was covered with the bacterial colonies, 3 = three quarters of the streaked lines in the plate and 4 = the streaked lines in the plate was covered with the bacterial colonies.

[z] Values with a letter in common are not significantly different from each other according to the Tukey's Honest Significant Difference ($\alpha = 0.05$).

Table 2.3. Development of lesions on 'Howden' and 'Dickinson' pumpkins leaves inoculated with isolates of *Xanthomonas cucurbitae* collected from pumpkin fields in the North Central Region[v]

Isolate	Origin of the isolate	Appearance of visible lesion on leaves (hpi)[w]			Diameter of lesion 7 days post-inoculation		
		Howden	Dickinson	*P* value	Howden	Dickinson	*P* value
ATCC strain 23378	-	66 ab	66 ab[y]	NS	5.08 a	5.00 a	NS
IA382	Iowa	72 a	72 a	NS	5.79 a	6.46 a	NS
IA389	Iowa	69 a	66 ab	NS	5.66 a	5.96 a	NS
IL232	Illinois	69 a	72 a	NS	5.91 a	5.92 a	NS
IL234	Illinois	72 a	72 a	NS	5.58 a	6.04 a	NS
IN332	Indiana	72 a	75 a	NS	6.41 a	5.83 a	NS
IN334	Indiana	72 a	75 a	NS	5.79 a	5.40 a	NS
KS455	Kansas	57 b	51 b	0.0128	5.56 a	5.00 a	NS
KS456	Kansas	75 a	71 a	0.0426	5.91 a	5.92 a	NS
MI358	Michigan	72 a	69 a	NS	5.33 a	6.46 a	0.0026
MI359	Michigan	69 a	66 ab	NS	5.38 a	5.54 a	NS
OH256	Ohio	75 a	81 a	0.0095	5.21 a	6.67 a	<0.0001
OH261	Ohio	74 a	78 a	NS	5.29 a	6.33 a	0.0008
WI205	Wisconsin	72 a	75 a	NS	5.95 a	5.62 a	NS
WI206	Wisconsin	68 ab	75 a	0.0063	6.00 a	7.46 a	0.0003
SDW[z]	-	-	-		-	-	-

[v] Plant leaves were inoculated at six areas, each area was infiltrated with 300 µl of bacterial suspensions with 10^8 CFU/ml.
[w] Hours after inoculation.
[y] In each column, values with a letter in common are not significantly different from each other according to the Tukey's Honest Significant Difference test ($\alpha = 0.05$).
[z] SDW = sterilized distilled water.

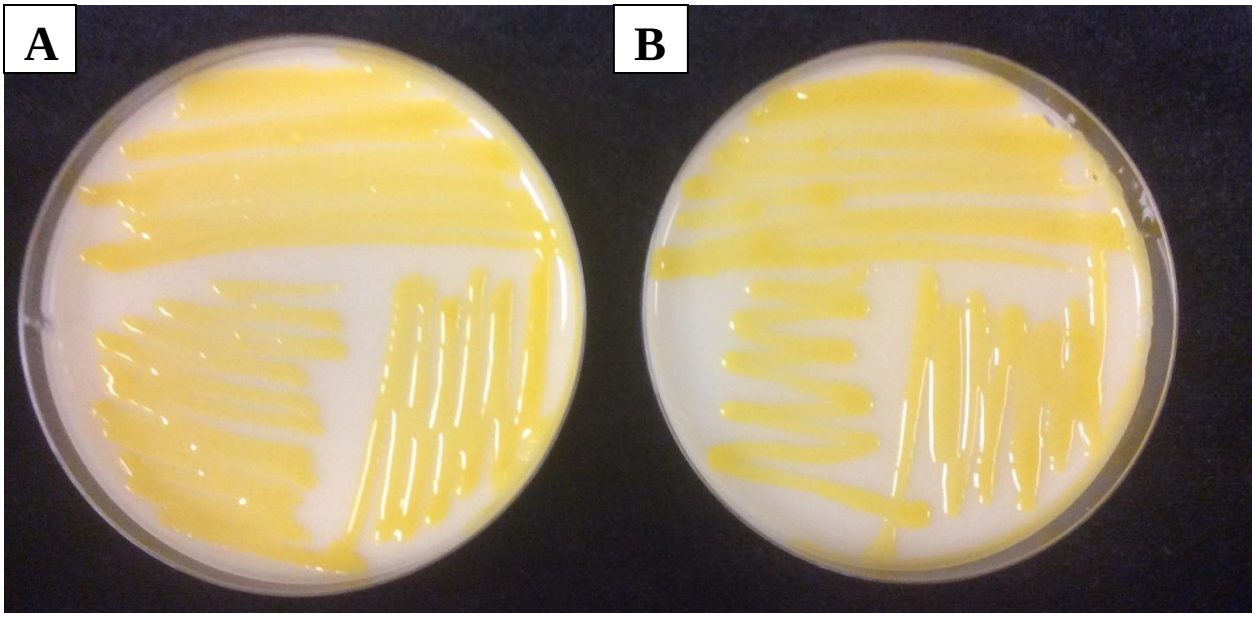

Figure 2.1. Yellow convex and mucoid colonies of *Xanthomonas cucurbitae* on yeast-extract-dextrose-calcium-carbonate agar medium (YDC). A, ATCC strain 23778; B, an isolate from the North Central Region.

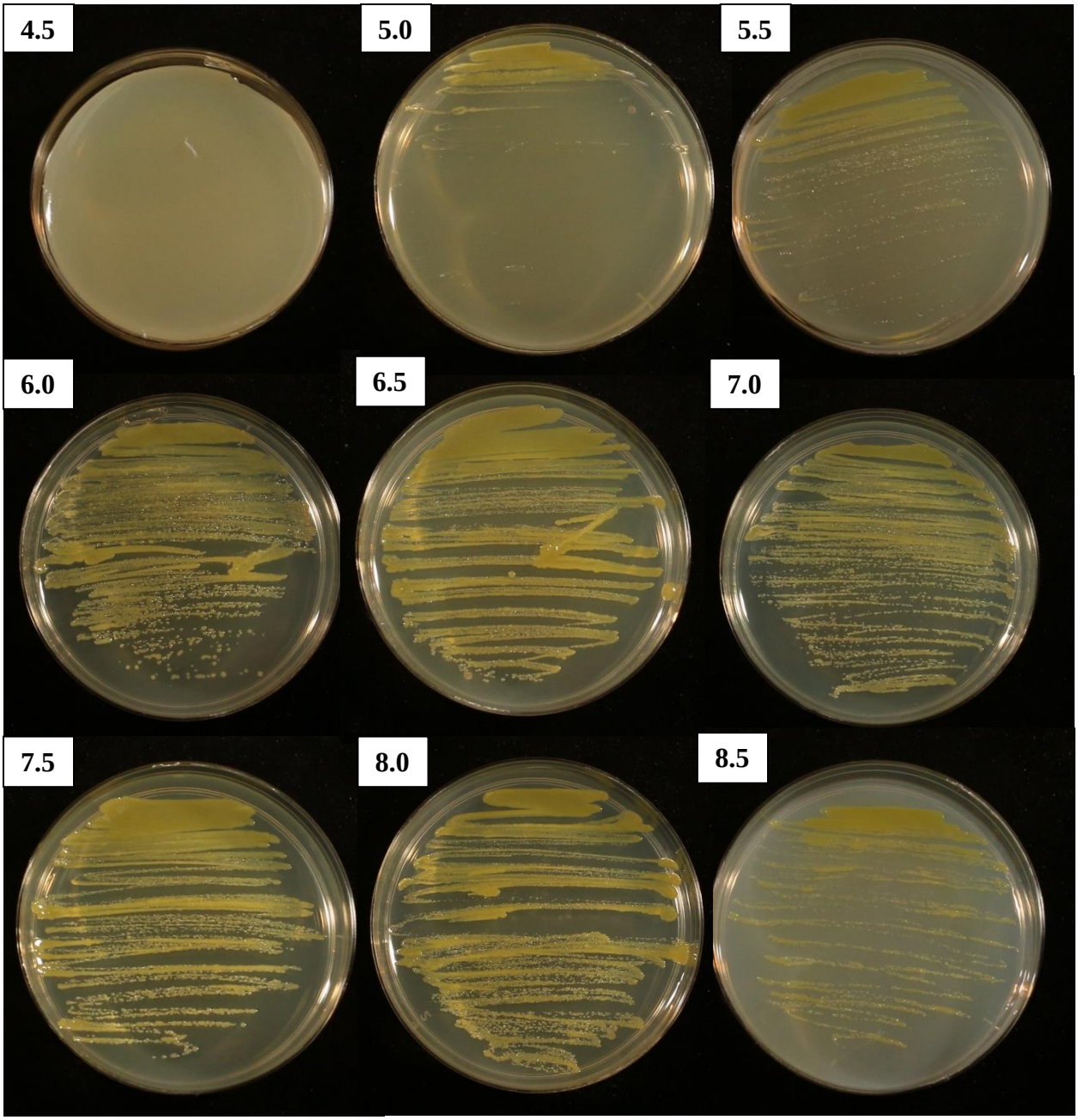

Figure 2.2. Colony development of *Xanthomonas cucurbitae* ATCC Strain 23378 on Luria Bertani agar mediums with different pH at 24±1°C in dark for 48 h.

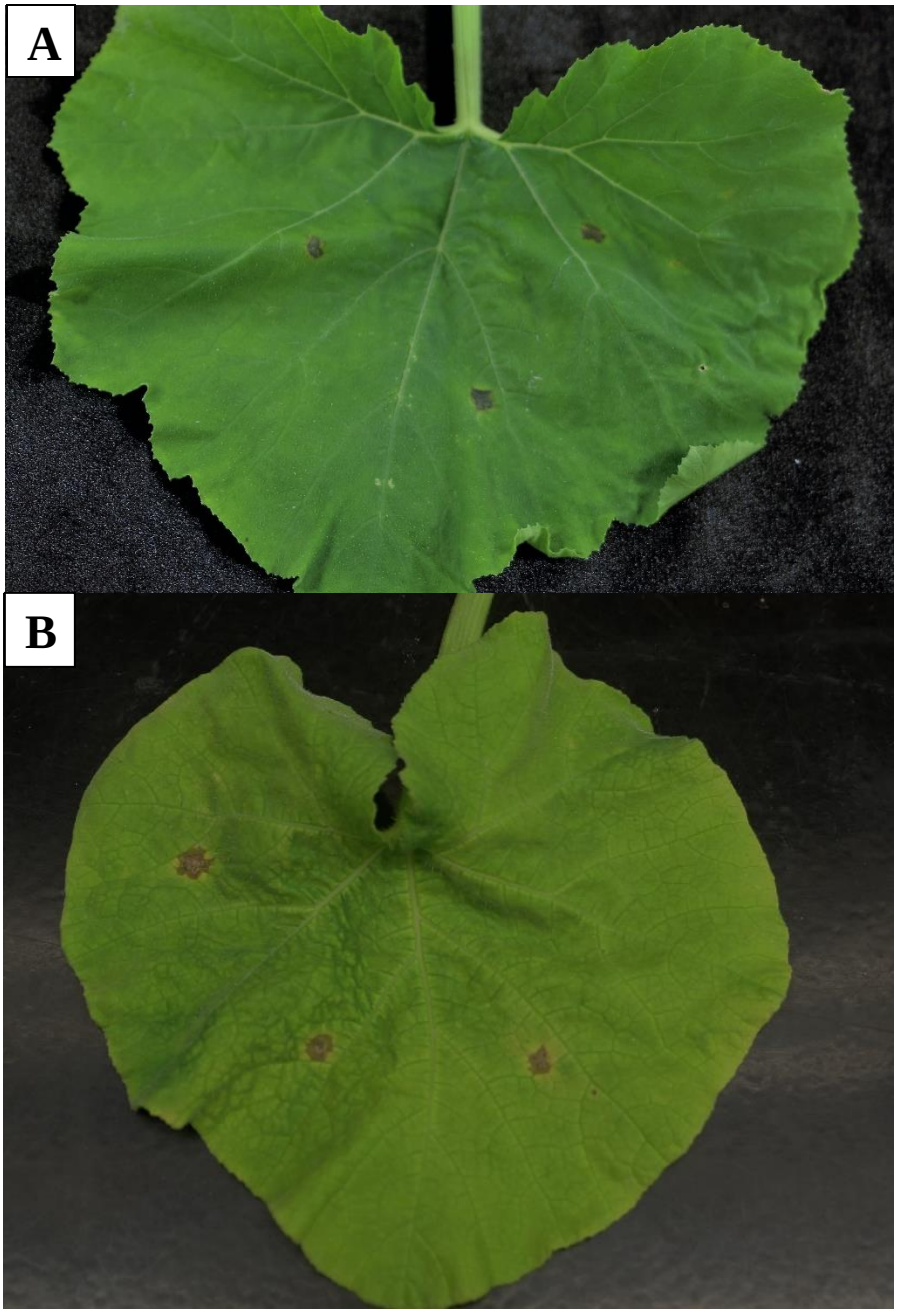

Figure 2.3. Lesions on pumpkin leaves 7 days after inoculation with *Xanthomonas cucurbitae*. A, 'Howden' pumpkin; B, 'Dickinson' pumpkin.

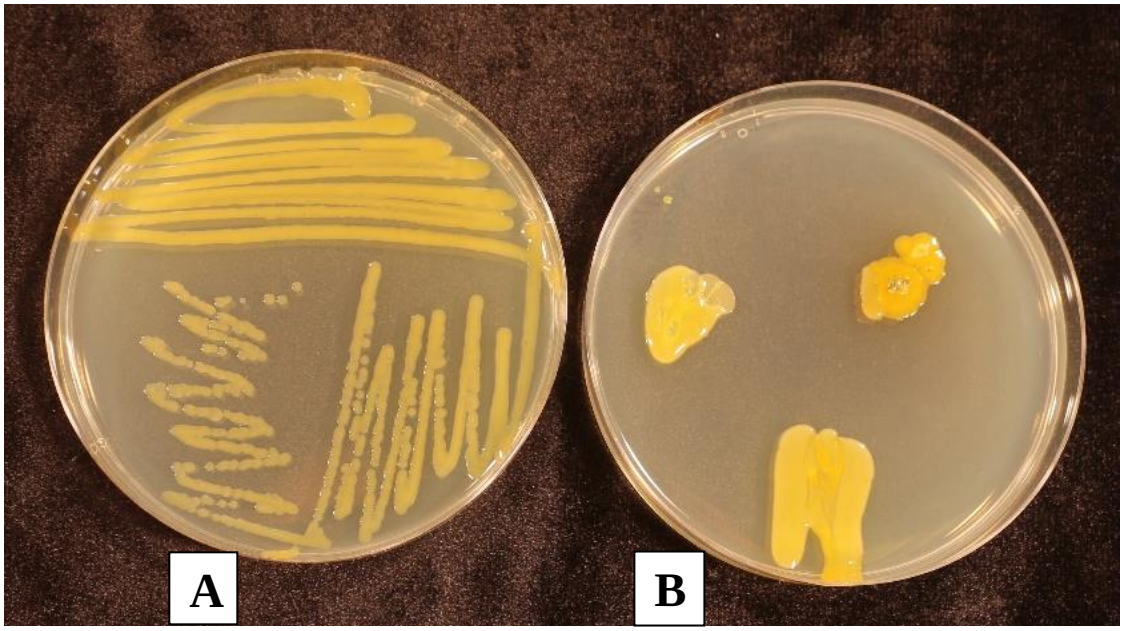

Figure 2.4. Isolating *Xanthomonas cucurbitae* from infected pumpkin leaves. A, leaf tissues with bacterial lesions were surface-disinfested and washed with sterile distilled ware (SDW), the tissues were cut in 5-mm pieces, the pieces were inserted into a test tube with SDW and shaken, and the water with suspected bacterial cells was streaked onto kasugamycin-cephalexin agar (KC)plates. B, surface-disinfested leaf tissues were washed with SDW and placed directly onto KC plates.

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CHAPTER 3

SURVIVAL OF *XANTHOMONAS CUCURBITAE* IN PUMPKIN SEED

ABSTRACT

Pumpkin bacterial spot, caused by *Xanthomonas cucurbitae*, is considered to be a seedborne disease. This study was conducted to assess the survival of *X. cucurbitae* in pumpkin seeds. Naturally-infected 'Howden' pumpkin seeds were collected in November 2013 and stored at 4°C. In July 2014, naturally-infected 'Howden' pumpkin seeds, along with artificially-inoculated 'Dickinson' pumpkin seeds, were placed in a laboratory at 22°C and in a room at 4°C for 18 months. Every 3 months, 150 seeds from each of three replications stored at 22 and 4°C were tested for the presence of *X. cucurbitae*. Each 150-seed sample was soaked in a saline solution and shaken overnight. Dilutions of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were prepared from the seed wash and 100 µl of each dilution was spread onto kasugamycin-cephalexin agar (KC). *Xanthomonas*-like colonies were sub-cultured on Luria Bertani agar (LB) for further morphological and molecular identification. Survival of *X. cucurbitae* was significantly ($P = 0.0039$) affected by the storage temperature, and the number of *X. cucurbitae* colonies recovered from stored seeds at both temperatures declined over time. For naturally infected seeds placed at room temperature, the number of recovered colonies after 3 months was significantly lower than the number of colonies recovered from the seed prior to placing them in storage. The number of recovered colonies after 6 months was significantly lower than those after 3 months in storage. Similarly, the number of recovered colonies after 9 months was significantly lower than the number of recovered colonies after 6 months. There were no significant differences in the number of recovered colonies after 9 months and 12 months of storage. After 15 months of storage, the number of recovered colonies

was significantly lower than numbers of recovered colonies after 9 and 12 months, but there were no significant differences between the recovered colonies after 15 and 18 months of storage. For artificially-inoculated seeds placed at room temperature, the number of recovered colonies after 3 months of storage was significantly lower than the number of recovered colonies prior to storage of seeds. Number of colonies recovered after 6 months was significantly lower than that of 3 months after storage. However, there was not significant difference between numbers of recovered colonies after 6 months and 9 months in storage. Number of recovered colonies after 12 months was significantly lower than the numbers of recovered colonies after 6 months and 9 months. Number of recovered colonies after 15 months was significantly lower than the number of recovered colonies after 9 months, but numbers of recovered colonies after 15 months and 18 months were not significantly different from each other. For naturally infected seeds stored at 4°C, the number of recovered colonies declined significantly over the time. However, the numbers of recovered colonies after 14 months and 17 months were not significantly different from each other. Numbers of recovered colonies after 20, 23, and 26 months were not significantly different from each other.

MATERIALS AND METHODS

Seed sources

Seeds of pumpkin cultivars ‘Howden’ and ‘Dickinson’ were used in this study. ‘Howden’ seeds were collected from either symptomatic or asymptomatic fruit at the University of Illinois Vegetable Research Farm, Champaign IL. Pumpkin fruits with 2% or greater of the surface covered with bacterial spots were cut and seeds inside the fruits were collected. These seeds were

considered to be naturally-infected. Seeds were also collected from asymptomatic pumpkin fruit and considered as non-infected (control) seeds. Samples of seeds collected from both symptomatic and asymptomatic fruit were tested for the presence of *Xanthomonas cucurbitae.* Seeds from the cultivar 'Dickinson' were obtained from Libby's Pumpkin Company and tested for the presence of *X. cucurbitae.* Non-infected seeds of 'Dickinson' were inoculated with three isolates of *X. cucurbitae*, including IL232, OH261, and ATCC strain 23378, and considered as artificially-inoculated seeds. Thus, naturally-infected 'Howden' seeds, artificially-inoculated 'Dickinson' seeds, and *X. cucurbitae*-free 'Howden' and 'Dickinson' seeds were used in this study.

For inoculation of seeds with *X. cucurbitae,* a modified method developed by Carisse et al. (1999) was used. *X. cucurbitae* was grown on Luria Bertani agar (LB) medium at 24°C in the dark for 48 h. Bacterial colonies from each isolate were washed from the agar surface using sterile distilled water (SDW), and suspensions adjusted to 10^8 CFU/ml were prepared using spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA) readings at OD_{600} (OD=0.5 at 600 nm). Seeds were either inoculated without surface-disinfestation or after surface-disinfestation. Seeds were surface-disinfested by soaking them in 1% NaOCl (2 ml NaOCl/seed for 3 min,) followed by three rinses with SDW (4 ml SDW/seed), each time for 1 min. After the surface-disinfestation, seeds were dried on sterile blotter paper in a laminar flow hood (The Baker Inc., Sanford, ME). Seeds were inoculated for either 6 or 20 min by soaking them in *X. cucurbitae* suspension (10^8 CFU/ml). Inoculated seeds were dried in the laminar flow hood and stored at room with 4 °C until they were used in the study.

Seed storage

Two storage condtions were used to conduct this study. These included storage in the laboratory at 22°C and in a cold room at 4°C. In both locations the experiment was arranged using using a randomized complete block design with the following treatments. Treatment in each block are listed in table 3.1.

Every 3 months, 150 seeds from each replication of each treatment from each location were tested for the presence of *X. cucurbitae*. Each sample of 150 seeds was placed in a sterile flask with 100 ml of washing saline solution (WSS). WSS was prepared by adding 7.5 g of NaCl and 200 µl of Tween-20 to 1,000 ml distilled water and then sterilized in an autoclave at 121°C for 20 min. Flasks with seeds in WSS were shaken on a rotary shaker (Fisher Scientific Co., Clinical Rotator 14-251-200) at 120 rpm overnight in the dark at 4°C. The seed-wash was filtered through four-layer of sterile cheesecloth and rinsed with 30 ml of sterile distilled water (SDW). Collected seed-wash was centrifuged at 14,000 g for 7 min. Supernatant was discarded and the pellet was re-suspended in the PBS buffer. The PBS buffer was prepared by dissolving 3.9 g KH_2PO_4, 2 g KCl, 80 g NaClO, and 17.9 g Na_2HP_4 in 1,000 ml distilled water with pH 7.4. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were prepared from the seed-wash in PBS buffer and 100 µl of each dilution was spread onto kasugamycin-cephalexin agar (KC) medium in each of 100 mm Petri dishes (Thapa, 2014). Petri dishes were incubated at 24±1°C for 4 days. *Xanthomonas*-like colonies were counted and sub-cultured on Luria Bertani agar (LB) medium for further identification.

Identification of *X. cucurbitae*

Colony morphology

Xanthomonas-like colonies on LB medium were transferred onto yeast-extract-dextrose-calcium-carbonate agar (YDC) medium. YDC medium was prepared by adding 10 g yeast extract, 20 g dextrose, 20 g calcium carbonate, and 15 g agar to 1,000 ml distilled water and autoclaving at 121°C for 20 min. ATCC strain 23378 of *X. cucurbitae* was used as positive control. Culture plates were incubated at 24±1°C for 48 h and then were examined for mucoid yellow colonies of *X. cucurbitae* (Rademaker et al. 2005).

Polymerase chain reaction (PCR) test

Suspension of *X. cucurbitae* with cell densities of 10^8 CFU/mltaken were prepared by adding mucoid, yellow colonies from LB plates into SDW. A total of 25 µl reaction volume was prepared, which contained 12.5 µl 2x Gotaq Green Master Mix (Promega Corporation, Madison, WI), 1 µl forward primer (RST 2, 5′-AGG CCC TGG AAG GTG CCC TGG A-3′), 1 µl reverse primer (RST 3, 5′-ATC GCA CTG CGT ACC GCG CGC GA-3′), 10 µl of nuclease-free water, and 0.5 µl of the bacterial suspension (Meng et al., 2004; Zhao et al., 2002; Leite, 1994). PCR amplification was performed with initial denaturation at 95°C for 10 min; followed by 32 cycles of denaturing at 95°C for 40 s, annealing at 59°C for 40 s, and extension at 72°C for 1 min; then, a final extension at 72°C for 10 min. Gel with 1% agarose containing SYBE Safe DNA Gel Stain was prepared. PCR products and one kb DNA ladder were loaded into the gel and then ran at 100 volts for 30 min. The results were visualized under Blue-light Transilluminator (Thermo Fisher Scientific, Inc., Carlsbad, CA).

Pathogenicity test

Isolates identified as *X. cucurbitae*, based on the colony morphology on YDC medium and PCR results, were tested for their pathogenicity. The isolates were grown on the LB medium at 24±1°C for 48 h. Then, bacterial suspensions with 10^8 CFU/ml in SDW were prepared and used to inoculate leaves of 3-week-old 'Howden' plants , using the syringe method (Barak et al., 2001; Katagiri et al., 2002). Approximatly 300 µl of inoculum was infiltrated into each inoculation spot on the abaxial side of the leaves. Inoculated plants were place in a greenhouse at 24-28°C and examined for the development of bacterial lesions 7 days after inoculation.

Data analysis

The data were analyzed using SAS 9.4 (SAS Institute Inc. Cary, NC). The data of colony forming units were natural-log transformed to meet the assumption of normality and homogeneity in order to conduct analysis of variance (ANOVA). The PROC GLM procedure was used to determine the effect of three fixed factors (storage temperature, seed treatments, and seed-inoculation time period) at $\alpha = 0.05$. The Tukey Honest Significant Difference test was used to compare differences among the number of colonies of different samples over time. Type I error was controlled at $\alpha = 0.05$. A regression analysis was conducted in R studio (R Studio. Boston, MA) to predict *X. cucurbitae* survival in seed at different temperatures. For each survival condition the mathematical equation $\hat{Y} = a + bx$ was used. In this equation, $\hat{Y}$ = predicted $\log_e$ number of recovered colonies, a = number of recovered colonies of the original samples prior to placing in the storage, b = rate of decline of number of recovered colonies over the time, and x = time (month).

RESULTS

The survival of *X. cucurbitae* was significantly ($P = 0.0039$) affected by the storage temperature. *X. cucurbitae* was recovered from both naturally-infected and artificially-inoculated seeds 18 months after placing the seed samples in the laboratory (Figure 3.1, and 3.2). But, the number of *X. cucurbitae* colonies recovered declined significantly ($P = 0.05$) over the 8 months period (Figure 3.3 and 3.4). The relationship between the numbers of recovered colonies of *X. cucurbitae* from naturally-infected seeds over the duration of the studywas $\hat{Y} = 10.4393 - 0.5876x$ ($R^2 = 0.8715$) (Figure 3.1). Similarly, the relationship between the number of *X. cucurbitae* colonies recovered from artificially-inoculated seeds over the duration of the study was $\hat{Y} = 17.7579 – 1.0065x$ ($R^2 = 0.9145$) (Figure 3.2). In naturally-infected seeds, the number of recovered colonies after 3 months was significantly lower than that of the original sample. The number of the recovered colonies after 6 months was significantly lower than the number of recovered colonies after 3 months in storage. Number of recovered colonies after 9 months was significantly lower than number of recovered colonies after 6 months. Number of the recovered colonies after 15 months was significantly lower than number of recovered colonies after 12 months. There were not significant differences between numbers of recovered colonies after 9 and 12 months and between the numbers of recovered colonies after 15 and 18 months of storage. Similar colony recovery was recorded for seed samples that had been artificially inoculated and stored. The only difference was that in artificially-inoculated seeds, the numbers of recovered colonies after 6 and 9 months were not significantly different from each other (Table 3.2).

X. cucurbitae was recovered from the naturally-infected and artificially-inoculated seeds after 26 and 18 months, respectively, in the room with 4°C (Figures 3.3 and 3.4). The number of *X. cucurbitae* colonies recovered from both naturally-infected and artificially-inoculated seeds at 4°C declined over the time. The relationship between the number of *X. cucurbitae* colonies recovered from naturally infected seeds with the time period of months was $\hat{Y} = 14.8577 - 0.5236x$ ($R^2 = 0.9071$) (Figure 3.3). The relationship between the number of the colonies recovered from artificially-inoculated seeds with the time periods of months was $\hat{Y} = 20.1656 – 0.9060x$ ($R^2 = 0.9401$) (Figure 3.4). In the naturally-infected seeds, the number of recovered colonies after 8 months was significantly lower than that of the original sample. Number of the recovered colonies after 11 months was significantly lower than the number recovered after 8 months. Number of recovered colonies after 14 months was significantly lower than the number of recovered colonies after 11 months. Number of recovered colonies after 20 months was significantly lower than the number recovered colonies after 17 months. There were not significant differences between the numbers of recovered colonies after 14 and 17 months and among the numbers of recovered colonies after 20, 23, and 26 months. Over the period of 18 months, the number of recovered *X. cucurbitae* colonies from artificially-inoculated seeds processed at any date was significantly lower than number of recovered colonies number 3 months prior to that date (Table 3.2).

DISCUSSION

Survival of some of *Xanthomonas* species in seeds has been studied, but there is no published report on the survival of *X. cucurbitae* in seeds. Bashan and Okon (1982) reported that *X. campestris*. pv. *vesicatoria* survived at least 100 days on tomato seeds. Persley (1979) reported that survival of *X. manihotis* on cassava seed ranged from 2 to 15 months in conditions of 5°C and

60% relative humidity. Thri, Murty, and Devadath (1984) found that *X. campestris* pv. *oryzae* survived for 170 - 180 days in kharif-harvested seeds and 120-130 days in rabi-harvested seeds. In their study, temperatures of rooms for stored seeds of kharif-harvested and rabi-harvested were different.

The result of our study showed that *X. cucurbitae* can survive in pumpkin seeds for longer than 26 months at 4°C and longer than 18 months at 22°C. Thapa (2014) reported that *X. cucurbitae* survived in pumpkin leaf and fruit tissues in the field for longer than 24 months. Our findings agree with Thapa's report that *X. cucurbitae* survives in pumpkin tissues for more than 18 months. Additional studies are needed to determine the exact period of survival of *X. cucurbitae* in seeds of pumpkin and other cucurbits under different storage conditions.

Ravanlou (2013) isolated *X. cucurbitae* from both seed surface and kernels inside the shell of pumpkin seeds. In our study, *X. cucurbitae* survived in naturally infected seeds for at least 26 months and the isolated bacteria were still viable and pathogenic, as plants that were inoculated with the isolated bacteria developed typical *X. cucurbitae* lesions. Similarly, *X. cucurbitae* was isolated from artificially-inoculated seed after 18 months, and these recovered bacterial were also still pathogenic. These results showed that *X. cucurbitae* survive on and in pumpkin seed for longer than 18 months and remained viable and pathogenic during that time.

TABLES AND FIGURES

Table 3.1. Inoculation of pumpkin seeds with *Xanthomonas cucurbitae*

Treatments[x]	Seed inoculation		Seed surface disinfestation[z]	Pumpkin cultivar
	Bacterial isolate[x]	Inoculation time (min)[y]		
Uninoculated-1	----	----	-	Howden
Uninoculated-2	----	----	+	Dickinson
Uninoculated-3	----	----	-	Dickinson
Naturally infected-1	----	----	-	Howden
Artificially Inoc-1	ATCC-23378	6	+	Dickinson
Artificially Inoc-2	ATCC-23378	20	+	Dickinson
Artificially Inoc-3	ATCC-23378	6	-	Dickinson
Artificially Inoc-4	ATCC-23378	20	-	Dickinson
Artificially Inoc-5	IL232	6	+	Dickinson
Artificially Inoc-6	IL232	20	+	Dickinson
Artificially Inoc-7	IL232	6	-	Dickinson
Artificially Inoc-8	IL232	20	-	Dickinson
Artificially Inoc-9	OH261	6	+	Dickinson
Artificially Inoc-10	OH261	20	+	Dickinson
Artificially Inoc-11	OH261	6	-	Dickinson
Artificially Inoc-12	OH261	20	-	Dickinson

[w] Naturally infected seed were collected from fruit of the pumpkin cultivar 'Howden' that were showing symptoms of bacterial spot. Artificially Inoc = artificially inoculated. ATCC-23378, IL232, and OH261 *X. cucurbitae* isolates were from the American Type Culture Collection, Illinois, and Ohio, respectively.

[y] Seeds were inoculated by soaking them in a *X. cucurbitae* suspension (10^8 CFU/ml) for either 6 or 20 min.

[z] + = seeded were surface-disinfested by soaking them in 0.6% NaOCl solution for 3 min, followed by three rinses with sterile distilled water; - = seeds were not surface-disinfested.

Table 3.2. Survival of *Xanthomonas cucurbitae* in pumpkin seeds

Storage Temperatures					
22°C			4°C		
Seeds[x]	**Time (month)[y]**	**Log_e (number of colony)[z]**	**Seeds**	**Time**	**log_e (number of colony)**
Natural	0	12.21 A	Natural	0	15.87 A
Natural	3	9.23 B	Natural	8	12.21 B
Natural	6	6.19 C	Natural	11	8.53 C
Natural	9	3.48 D	Natural	14	5.86 D
Natural	12	3.12 D	Natural	17	5.55 D
Natural	15	1.78 E	Natural	20	3.74 E
Natural	18	1.62 E	Natural	23	3.23 E
			Natural	26	2.13 E
Artificial	0	18.97 a	Artificial	0	18.95 a
Artificial	3	14.59 b	Artificial	3	17.75 b
Artificial	6	9.81c	Artificial	6	15.55 c
Artificial	9	9.29 c	Artificial	9	12.76 d
Artificial	12	5.84 d	Artificial	12	10.36 e
Artificial	15	1.64 e	Artificial	15	5.14 f
Artificial	18	1.09 e	Artificial	18	3.66 g

[x] Natural = naturally infected seeds; Artificial = artificially infected seeds.

[y] Time = recovered period after placing seeds samples in the laboratory (22°C) and cold-room (4°C).

[z] In each column, colonies collected from naturally infected seeds samples or artificially infected seed samples, at either 22°C or 4°C, with the letter were not significantly different from each other.

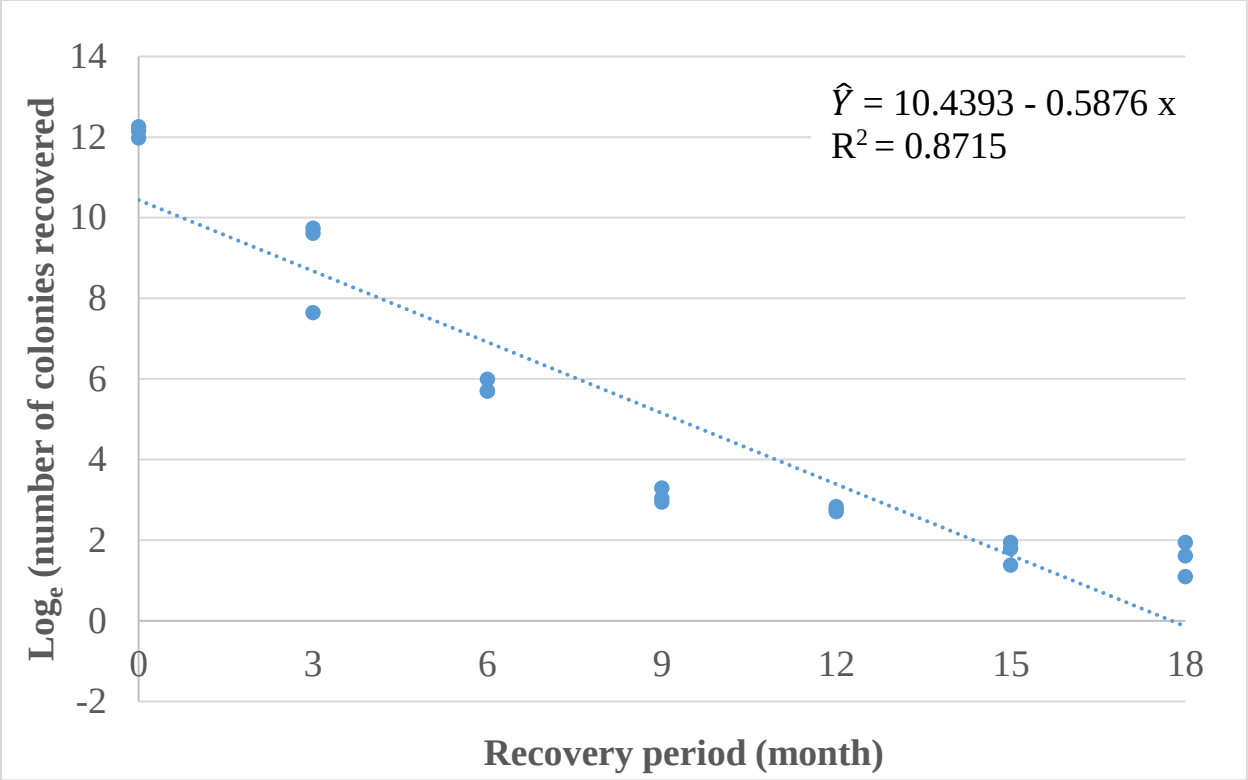

Figure 3.1. Recovery of *Xanthomonas cucurbitae* colonies from naturally infected seeds of 'Howden' pumpkin stored in the laboratory at 22°C for up to 18 months.

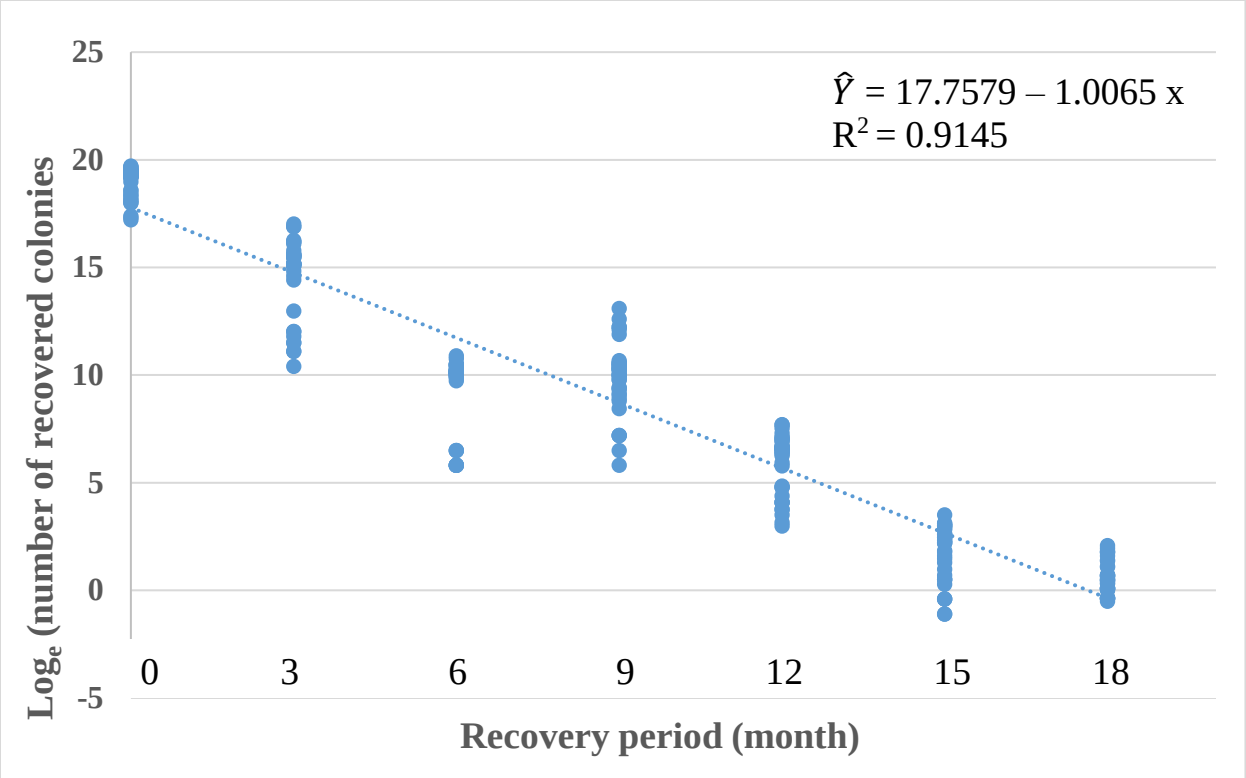

Figure 3.2. Recovery of *Xanthomonas cucurbitae* colonies from artificially-inoculated seeds of 'Dickinson' pumpkin stored in the laboratory at 22°C for up to18 months.

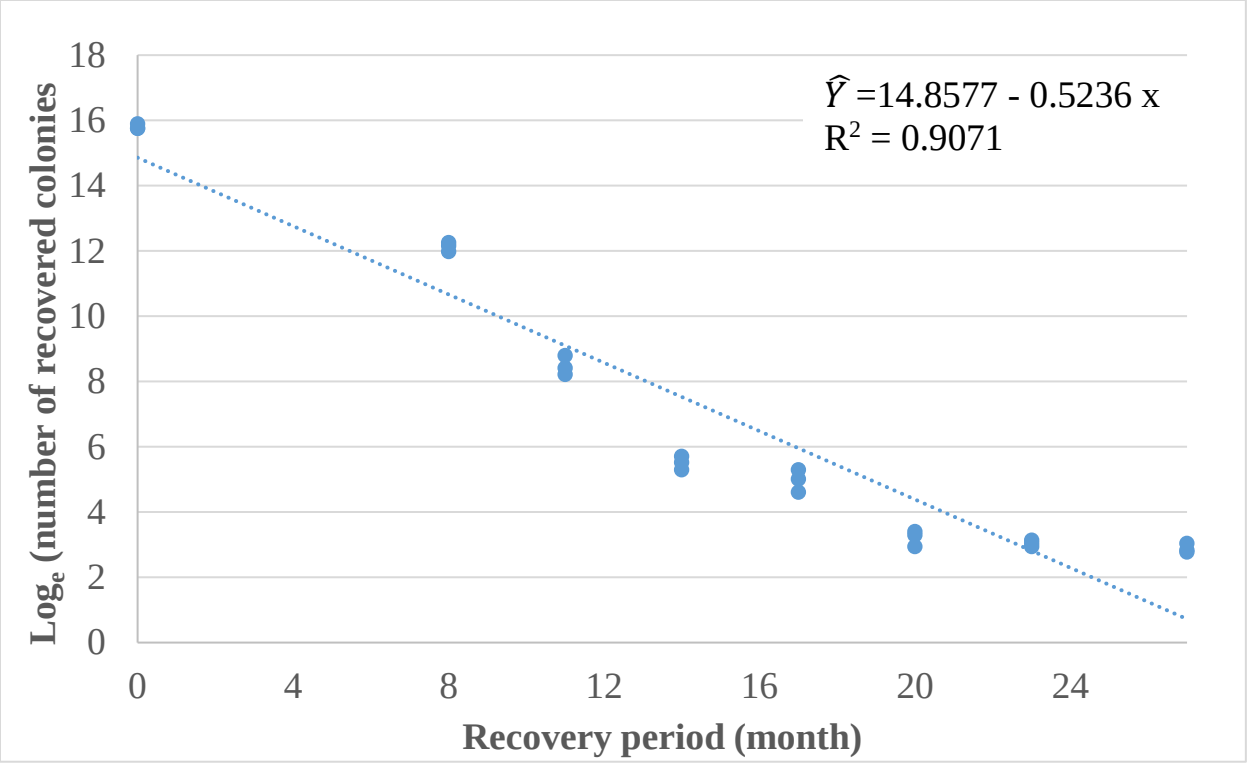

Figure 3.3. Recovery of *Xanthomonas cucurbitae* colonies from naturally infected seeds of 'Howden' pumpkin stored in the cold-room at 4°C for up to 26 months.

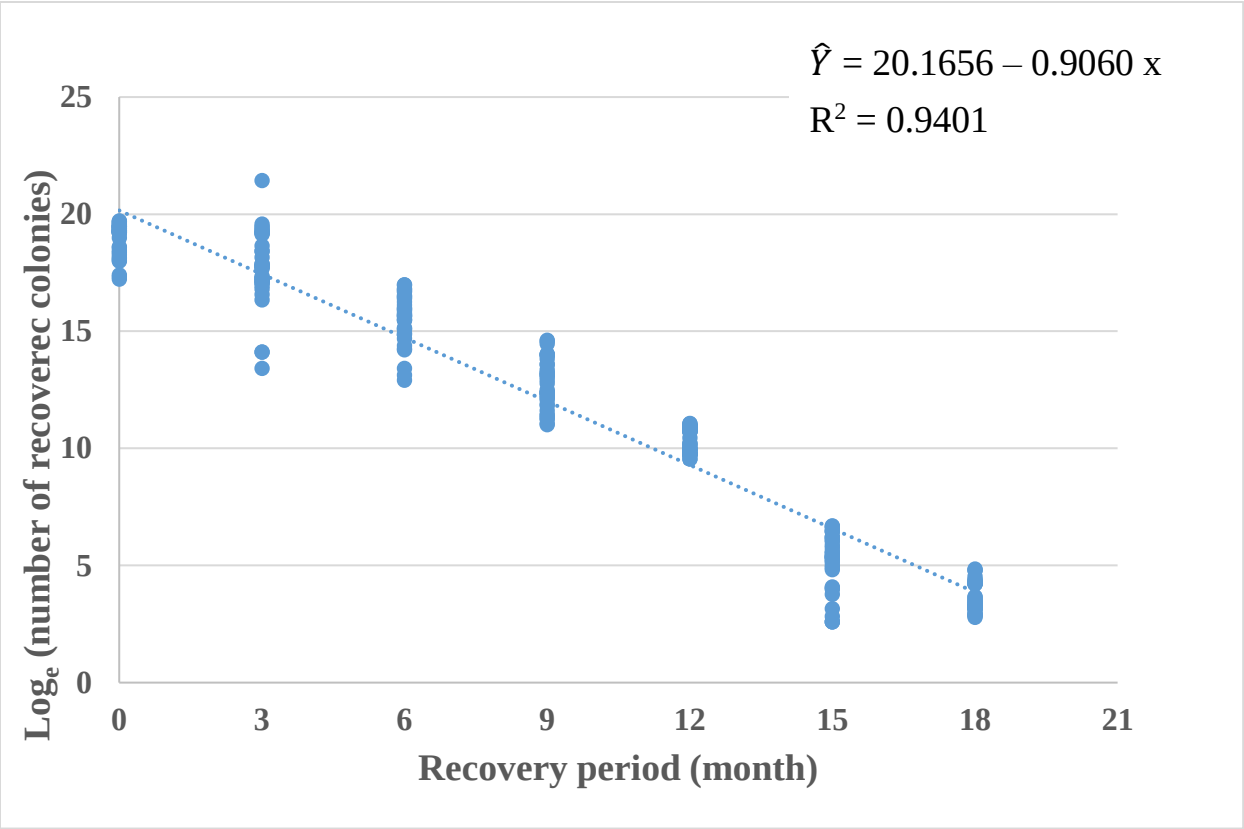

Figure 3.4. Recovery of *Xanthomonas cucurbitae* colonies from all artificially infected 'Dickinson' pumpkin seeds stored in the cold-room at 4°C for 18 up to months.

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CHAPTER 4

DEVELOPING EFFECTIVE SEED TREATMENT TO ERADICATE *XANTHOMONAS CUCURBITAE* IN PUMPKIN SEED

ABSTRACT

This study was conducted to develop effective treatments for pumpkin seed to eradicate *Xanthomonas cucurbitae*, the causal agent of cucurbit bacterial spot. Naturally-infected and artificially-inoculated seeds were used in this research. Three seed treatments, including hot-water, hydrochloric acid (HCl), and sodium hypochlorite (NaClO) treatments, were evaluated. A total of 48 hot-water treatments were conducted, which included 5, 10, 15, 20, 25, and 30 min durations of treatment at temperatures of 49, 50, 51, 52, 53, 54, 55, and 56°C. Hydrochloric acid (HCl) treatments included 0.5, 1.0, and 2.0% concentration of HCl, each for 10, 20, 30, 40, 50, and 60 min. Sodium hypochlorite treatments were 0.5, 1.0, 1.5, and 2.0 % of NaClO in water, each for 1, 2, 3, and 4 min. Hot-water treatment at 55°C for 10 or 15 min and hydrochloric acid treatment at 0.5% concentration for 40 min eradicated *X. cucurbitae* in both naturally-infected and artificially-inoculated pumpkin seeds without any significant adverse effect on either seed germination or seedling vigor. None of the NaClO treatments completely eradicated the pathogen in seeds.

MATERIALS AND METHODS

Seeds sources

Naturally-infected seeds

Xanthomonas cucurbitae-infected seeds of 'Howden' pumpkin were collected at the University of Illinois Vegetable Research Farm in Champaign, IL. Pumpkin fruit with typical lesions of bacterial spot, caused by *X. cucurbitae*, were collected, and seeds from the fruit were harvested. Collected seeds were washed under running tap water in a 5 mm-diameter steel sieve to remove extra fruit tissues associated with seeds. Washed seeds were spread onto two-layers of cheesecloth for 2 days to air dry them. Dried seeds were stored in Ziploc® plastic bags at 4°C until they were tested (Ravanlou, 2013).

Artificially-inoculated seeds

X. cucurbitae isolates, IL232 and OH261, and American type culture collection (ATCC) strain 23378, were used to inoculate seeds of the pumpkin cultivar 'Dickinson'. Inoculum of *X. cucurbitae* was prepared by culturing the bacterium on Luria Bertani agar (LB) at 24±1°C in the dark for 48 h. Then, bacterial colonies were washed off of the agar surface with sterile distilled water (SDW), and the concentration of the inoculum was adjusted to 10^8 CFU/ml, with the use of a spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA) at OD_{600} (OD=0.5 at 600 nm). Two hundred seeds of 'Dickinson' pumpkin, obtained from Libby's Pumpkin Co. (Libby's Pumpkin, Morton, IL), were placed in a sterile 500-ml flask containing 300 ml of *X. cucurbitae* inoculum and subjected to vacuum for 3 min. Treated seeds were placed onto sterilized blotter

paper in a laminar flow hood (The Baker INC, Sanford, ME) to dry. Dried, inoculated-seeds were stored in Ziploc® plastic bags at 4°C for future studies (Carisse et al., 2010).

Non-infected pumpkin seeds

Non-infected seeds of the pumpkin cultivar 'Howden' were collected from asymptomatic pumpkin fruit at the University of Illinois Vegetable Research Farm in Champaign, IL. Non-infected seeds of the pumpkin cultivar 'Dickinson' were provided by the Libby's Pumpkin Co. Both seed lots were tested for presence of *X. cucurbitae* prior to use.

Seed treatment

Hot-water treatment

Seeds were treated in water at temperatures of 49, 50, 51, 52, 53, 54, 55, and 56°C for the time periods of 5, 10, 15, 20, 25, and 30 min. An electrical water-bath (Precision Reciprocal Shaking Bath, Model 50, Thermo Fisher Scientific, Inc., Waltham, MA) was used for hot-water treatment of the seeds. The bath was filled to its 50% capacity and heated to obtain the target treatment temperature. Seed samples were loosely wrapped in two-layers of cheesecloth and immersed in the heated water. After immersing seeds in the water, the treatment temperature was re-established. The seed sample was then kept in the water for the prescribed amount of time. Then, the sample was immersed into SDW at room temperature to reduce the temperature to 20-22°C. Treated seeds were spread onto sterile paper towel in the laminar flow hood to dry. Seeds of both Howden' and 'Dickinson' cultivars were used in this study.

Hydrochloric acid treatment

All hydrochloric acid (HCl) treatments were conducted in the laboratory at a temperature of 22-23°C. Diluted HCl solutions were prepared by adding a concentrated HCl solution into SDW by gently stirring the solution. Solution of 0.5, 1.0, and 2.0% (v/v) HCl were used in this study, and seeds were treated for 10, 20, 30, 40, 50, and 60 min at each HCl concentration. Seeds were wrapped in two-layer of cheesecloth and immersed into the HCl solutions. HCl treated seeds were rinsed with SDW three times, each time for 3 min. Treated seeds were then spread onto sterile paper towel in the laminar flow hood and allowed to dry overnight. Seeds of both 'Howden' and 'Dickinson' cultivars were used in this study.

Sodium hypochlorite treatments

Commercial bleach (Clorox Co., Oakland, CA) with a NaClO concentration of 8.25% was used in this study. Diluted NaClO solutions with concentrations of 0.5, 1.0, 1.5, and 2.0% were prepared by adding the commercial bleach solution to SDW. Seeds were treated for 1, 2, 3, and 4 min in each of the prepared NaClO solutions. Seeds were wrapped loosely in two-layer cheese cloth and immersed into the NaClO solution for the prescribed amount of time. NaOCl treated seeds were rinsed with SDW three times, each time for 3 min. Treated seeds were spread onto sterile paper towel in the laminar flow hood and allowed to dry overnight. Seeds of both 'Howden' and 'Dickinson' cultivars were used in this study.

Seed germination and seedling vigor

Laboratory tests

For each germination test, 200 seeds were used. The germination test was repeated three times (a total of 600 seeds for each treatment). Untreated 'Howden' and 'Dickinson' seeds were used as controls. Seed germination paper (Seedburo Equipment Company. Des Plaines. IL) was used for the laboratory tests. Seeds were placed 0.5 cm apart on the moistened germination paper and the paper was rolled and placed on poly flat lay bags with 50 ml water. Bags were placed in an incubator with 24±1°C for 4 days. All tested seeds were examined, and the percentage of germinated seeds was calculated.

Seedling vigor was assessed using 10 seeds, with three replications (a total of 30 seeds for each treatment). Treated and untreated seeds were used in this study. Ten seeds were placed 2 cm apart on moistened germination paper, the paper was rolled, and incubated at 24±1°C for 4 days. Lengths of shoots and roots of the seedlings were measured at the end of incubation period.

Greenhouse tests

Greenhouse tests were conducted using 32-cell trays filed with a mixture of soil:peat:perlite (1:2:1). Seed germination was assessed by planting 20 seeds from each treatment and untreated seeds, with three replications (a total of 60 seeds for each treatment and control). Trays were placed in a greenhouse at 24-28°C. Three weeks after sowing, germinated seed were counted, and the percent of seed germination was calculated. Seedling vigor was evaluated 3 weeks after sowing seed, using a 0-4 scale, 0 for non-germinated seed and 4 for vigorously seedling.

Testing seeds and seedling for the presence of *X. cucurbitae*

Laboratory tests

Artificially-inoculated seeds of the pumpkin cultivar 'Dickinson', which were treated with hot-water, HCl, and NaClO, were tested for the presence of *X. cucurbitae* by sampling 200 seeds from each treatment with three replications (a total of 600 seeds for each treatment). Non-treated seeds were tested as a control. Each sample of 200 seeds was placed in a flask with 100 ml of washing saline solution (WSS). WSS was prepared by adding 7.5 g of NaCl and 200 µl of Tween-20 to 1,000 ml of distilled water and then sterilized in an autoclave at 121°C for 20 min. Flasks with seeds and WSS were shaken on a rotary shaker (Fisher Scientific Co., Clinical Rotator 14-251-200) at 120 rpm for 1 h in the dark at with a temperature of 4°C. The seed-wash liquid was filtered through four-layers of sterile cheesecloth and rinsed with 30 ml of SDW. The collected seed-wash was centrifuged at 14,000 g for 7 min. The supernatant was discarded, and the pellet was re-suspended in a phosphate buffer (PBS). The PBS buffer was prepared by adding 3.9 g KH_2PO_4, 2 g KCl, 80 g NaCl, and 17.9 Na_2HP_4 to 1,000 ml distilled water with pH 7.1. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were prepared from the seed-wash suspension, and then 100 µl of each diluted suspension was spread onto the semi-selective kasugamycin-cephalexin agar (KC) in 100 mm Petri dishes (Schaad et al., 2001). The Petri dishes were incubated at 24±1°C for 4 days. *Xanthomonas*-like colonies were counted and sub-cultured on YDC and LB media for identification of *X. cucurbitae*.

Greenhouse tests

Naturally-infected 'Howden' pumpkin seeds, which were collected from infected pumpkin fruit, were used in this study. The seeds were treated with hot-water, HCl, and NaOCl. Infected seeds

without treatment were used as a control. For each treatment, 20 seeds, with three replications (a total of 60 seeds for each treatment), were evaluated. Seeds were sown in 32-cell plastic trays (32 seeds per tray) containing a mixture of soil:peat:perlite (1:2:1). Trays were placed in a greenhouse at a temperature of 24-28°C in dark. After three weeks, seed germination and seedling vigor were assessed. During this time, leaves were checked daily for bacterial lesions. Leaves with suspected lesions were collected, and bacteria were isolated using two different methods. In the first method, leaves were surface-disinfested by soaking them in 75% ethanol for 1 min, followed by rinsing with SDW for three times, each time for 1min. Leaf tissues with lesions were cut (5 mm-diameter) and the leaf pieces were placed onto KC medium. In the second method, two 5-mm-diameter pieces of surface-disinfested leaf from each plant were transferred into a microcentrifuge tube with 1 ml SDW. The tube was vortexed for 30 s to release bacterial cells from plant tissue into the SDW. Then, a loopful of bacterial suspension was streaked onto KC medium in each of 100 mm Petri dishes. The cultures were incubated at 24±1°C for 48 h in darkness. *Xanthomonas*-like colonies from all culture plates were sub-cultured onto LB and YDC for identification of *X. cucurbitae*.

Identification of X. cucurbitae

Colony morphology. *Xanthomonas*-like colonies on LB medium were also sub-cultured on YDC medium. YDC medium was prepared by adding 10 g yeast extract, 20 g dextrose, 20 g calcium carbonate, and 15 g agar to 1,000 ml distilled water (Schaad et al., 2001). ATCC strain 23378 of *X. cucurbitae* was used as positive control. Culture plates were incubated at 24±1°C in darkness and examined for yellow mucoid colonies after 48 h.

Polymerase chain reaction (PCR) test. Bacterial suspensions with cell densities of 10^8 CFU/ml, from cultures with yellow mucoid colonies on YDC, were prepared by adding colonies to SDW. A total of 25 µl reaction volume was prepared, which contained 12.5 µl 2x Gotaq Green Master Mix (Promega Corporation, Madison, WI), 1 µl forward primer (RST 2, 5′-AGG CCC TGG AAG GTG CCC TGG A-3′), 1 µl reverse primer (RST 3, 5′-ATC GCA CTG CGT ACC GCG CGC GA-3′), 10 µl of nuclease free water, and 0.5 µl of bacterial suspension (Leite, 1994; Meng et al., 2004; Zhao et al., 2002). PCR amplification was performed with initial denaturation at 95°C for 10 min; followed by 32 cycles of denaturing at 95°C for 40 s, annealing at 59°C for 40 s, and extension at 72°C for 1 min; and then a final extension at 72°C for 10 min. Gel with 1% agarose containing SYBE Safe DNA Gel Stain was prepared. PCR products and one kb DNA ladder were loaded into prepared gel and ran at 100 volts for 30 min. The results were visualized under Blue-light Transilluminator (Thermo Fisher Scientific, Inc., Carlsbad, CA).

Pathogenicity test. Isolates identified as *X. cucurbitae*, based on the colony morphology on YDC medium and PCR results, were tested for their pathogenicity. The isolates were cultured on LB medium at 24±1°C for 48 h. Bacterial suspension of 10^8 CFU/ml in SDW was prepared and used to inoculate leaves of 3-week-old ‘Howden’ pumpkin plants in a greenhouse with the temperature at 24-28°C, using a syringe method (Barak et al., 2001; Katagiri et al., 2002). Approximately 300 µl of inoculum was infiltrated into each inoculation spot on the abaxial side of the leaves. Inoculated plants were examined for development of bacterial lesions over a 7 day period after inoculation. Lesions that developed on the leaves were collected and processed for isolation and identification of *X. cucurbitae*.

Experiment design and data analysis

The experiments for this study were conducted using split-plot designs, with completely randomized block, with three replications. In the hot-water treatment, temperature was the main plot, and time periods were considered as split-plots. In the HCl and NaClO treatments, concentration of the chemical was the main plot, and time periods were the split-plots. Every experiment was repeated once. Data from hot-water, HCl, and NaClO treatments were analyzed separately. For each seed treatment, data were analyzed using PROC MIXED of SAS 9.4 (SAS Institute Inc. Cary, NC). Normality of the data was determined, and homogeneity of the data was at $\alpha = 0.05$. If the raw data met the assumption of ANOVA, then the original data were presented. If transformation of the data was needed, the original data were either log–transformed or square root-transformed before analysis. Treatment means were compared using the Tukey's Honest Significant Difference. Type I error was controlled at $\alpha = 0.05$.

RESULTS

Hot-water seed treatment

Seed germination and seedling vigor tests in laboratory

Analysis of variance of the laboratory studies showed that percent germination of non-infected 'Howden' seeds was not significantly ($P = 0.0618$) affected by temperature of water-treatment, but water temperature significantly affected shoot length ($P = 0.0113$) and root length ($P = 0.0027$) (Table 4.1, Figure 4.1). Similarly, seed germination was not significantly affected ($P = 0.3349$) by time period of treatment in hot water. However, shoot length ($P = 0.0148$) and root length ($P = 0.0409$) were significantly affected by time period of treatment (Table 4.1). Interaction of water

temperature x time period of treatment did not significantly affect percent seed germination, but the interaction significantly affected shoot length and root length (Tables 4.1 and 4.4).

Water temperature of seed treatment significantly affected seed germination (P = 0.0034), shoot length (P = 0.0001), and root length (P = 0.0014) of the seedlings of non-infected 'Dickinson' pumpkin (Tables 4.1 and 4.5, Figure 4.2). Treatment time period significantly affected seed percent germination (P = 0.0001) and root length (P = 0.0128), but not shoot length (P = 0.11121) of the seedlings of 'Dickinson' pumpkin (Table 4.1).

Seed germination and seedling vigor tests in greenhouse

In the greenhouse studies, seed germination of non-infected 'Howden' pumpkin was not significantly (P = 0.0962) affected by water temperature, but seedling vigor was significantly (P = 0.0014) affected by water temperature (Table 4.2). Time period of treatment and the interaction of water temperature x treatment period did not significantly affect either percent seed germination or seedling vigor of 'Howden' pumpkin (Tables 4.2 and 4.6).

Seed germination of non-infected 'Dickinson' pumpkin was significantly affected by water temperature (P = 0.0001), time period of treatment (P = 0.0408), and the interaction of water temperature x treatment period (P = 0.0004) (Tables 4.1 and 4.7). Seedling vigor was not significantly affected by either water temperature, time period of treatment, or the interaction of water temperature x time period of treatment (Table 4.2). Treatment at 53°C for 30 min and 56°C for 30 min significantly reduced percentage of seed germination compared to the control seeds (Table 4.7). Also, treatment at 56°C for 30 min significantly reduced seedling vigor.

Seed germination and seedling vigor of infected 'Howden' pumpkin were significantly affected by water temperature, time period of treatment, and the interaction of water temperature x treatment period ($P \leq 0.0049$) (Table 4.3). Treatment at 55°C for 25 and 30 min and all treatments at 56°C significantly reduced seed germination and seedling vigor compared to untreated and some treated seeds (Table 4.8).

Presence of X. cucurbitae in pumpkin seeds

Treatment of either naturally-infected or artificially-inoculated pumpkin seeds in water with temperature 55°C for 10 min, 55°C for 15 min, 55°C for 20 min, 55°C for 25 min, 55°C for 30 min, 56°C for 5 min, 56°C for 20 min, 56°C for 25 min, and 56°C for 30 min eradicated *X. cucurbitae* from seeds. Other hot-water treatments did not completely eradicate *X. cucurbitae* in seeds.

HCl seed treatment

Seed germination and seedling vigor in laboratory

Seed germination of non-infected 'Dickinson' pumpkin was significantly affected by HCl concentration ($P = 0.0006$) and duration of treatment ($P = 0.0394$), but not by the interaction of HCl concentration x duration of treatment ($P = 0.3654$) (Table 4.1). Shoot length was significantly affected by HCl concentration ($P = 0.0001$), duration of treatment ($P = 0.0005$), and the interaction of HCl concentration x duration of treatment ($P = 0.0001$) (Table 4.1). Similarly, root length was significantly affected by HCl concentration ($P = 0.0001$), duration of treatment ($P = 0.0001$), and the interaction of HCl concentration x duration of treatment ($P = 0.0001$) (Table 4.1). Some HCl treatments significantly reduced both percent seed germination and seedling vigor (Table 4.9).

Seed germination and seedling vigor in the greenhouse

Percent seed germination of non-infected 'Dickinson' pumpkin in the greenhouse study was not significantly affected by either concentration of HCl, duration period of treatment, or the interaction of HCl concentration x duration of the treatment (Table 4.2). In contrast, seedling vigor was significantly affected by HCl concentration (P = 0.0001), duration of treatment (P = 0.0001), and the interaction of HCl concentration x duration of the treatment (P = 0.0063) (Tables 4.2 and 4.10). Treatment with 1.0 and 2.0 % HCl significantly reduced seedling vigor in most of the treatments (Table 4.10).

Percent seed germination of infected 'Howden' pumpkin was significantly affected by the interaction of HCl concentration x period of treatment (P = 0.0014) (Tables 4.3 and 4.11). Seedling vigor was also significantly affected by treatment period (P = 0.0001) and the interaction of HCl concentration x treatment period (P = 0.0001) (Table 4.3). All treated seeds had statistically the same or higher seedling vigor compared to untreated seeds (Table 4.11).

Presence of X. cucurbitae in pumpkin seeds

All HCl treatments eradicated *X. cucurbitae* in both naturally-infected and artificially-inoculated pumpkin seeds.

NaClO seed treatment

Seed germination and seedling vigor in laboratory

In naturally-infected 'Howden' pumpkin, percent seed germination was significantly affected by NaClO concentration (P = 0.0004), duration of treatment (P = 0.0062), and the interaction of

NaClO concentration x duration of treatment (P = 0.0001) (Table 4.1). Shoot length was also significantly affected by NaClO concentration (P = 0.0278), duration of treatment (P = 0.0019), and the interaction of NaClO concentration x duration of treatment (P = 0.0005) (Table 4.1). Similarly, root length was significantly affected by NaClO concentration (P = 0.0001), duration of treatment (P = 0.0002), and the interaction of NaClO concentration x duration of treatment (P = 0.0029) (Table 4.1). Percent seed germination, shoot length, and root length were significantly affected by NaClO treatments (Table 4.12).

Seed germination of non-infected 'Dickinson' pumpkin was significantly affected by NaClO concentration (P = 0.0004), duration of treatment time (P = 0.0062), and the interaction of NaClO concentration x time period of treatment (P = 0001) (Table 4.1). Seedling vigor was significantly affected by NaClO concentration (P = 0.0001), duration of treatment (P = 0.0002), and the interaction of NaClO concentration x duration of treatment (P = 0.0029) (Table 4.1). Some of the NaClO treatments significantly reduced seed germination and seedling vigor (Table 4.13).

Seed germination and seedling vigor in greenhouse

Percent seed germination and seedling vigor of infected 'Howden' pumpkin was significantly affected by NaClO concentration ($P < 0.0003$), duration of treatment ($P < 0.0049$), and the interaction of NaClO concentration x duration of treatment ($P < 0.0011$) (Table 4.3). Similarly, seedling vigor was significantly affected by NaClO concentration ($P < 0.0001$), duration of treatment (P = 0039), and the interaction of NaClO concentration x duration of treatment (P = 0.0060) (Table 4.3). Some of the NaClO treatments significantly reduced seed germination and seedling vigor (Table 4.12).

In the greenhouse, percent seed germination of 'Dickinson' pumpkin was significantly affected by concentration of NaClO (P = 0.0001) and the interaction of NaClO concentration x duration of treatment (P = 0.0356) (Table 4.2). However, duration of treatment did not significantly affect (P = 0.1903) seed germination. Seedling vigor was significantly affected by concentration of NaClO (P = 0.0001), duration of treatment (P = 0.0002), and the interaction of NaClO concentration x duration of treatment (P < 0.0001) (Table 4.2). Some of the NaClO treatments significantly reduced seed germination and seedling vigor (Table 4.14).

Presence of X. cucurbitae in pumpkin seeds

None of NaClO treatments completely eliminated *X. cucurbitae* from pumpkin seeds. Lesions that developed on pumpkin leaves were identified as *X. cucurbitae* based on their colony morphology on YDC and 1,400 bp amplicon in PCR tests (Figure 4.3).

DISCUSSION

An outbreak of pumpkin bacteria spot, caused by *X. cucurbitae*, was reported in Illinois in 2012 (Babadoost and Ravanlou, 2012). Field surveys in subsequent years indicated that bacterial spot of pumpkin is a serious threat to pumpkin production in Illinois and other states in the NCR. Due to the lack of sufficient information on the etiology and epidemiology of the bacterial spot, no effective strategies have been developed for management of the disease.

Since *X. cucurbitae* is considered to be a seedborne pathogen, eradication of seedborne inoculum is an effective approach to reducing losses caused by bacterial spot (Sahin and Miller, 1997; Akinolar et al., 2000). No effective seed treatment for eradication of *X. cucurbitae* in cucurbit seeds has been available. Thus, this study was conducted to develop effective methods for eradication of *X. cucurbitae* in pumpkin seed. Hot-water seed treatment has been successfully used for control of several seedborne pathogens (Nega et al., 2002; Moffett and Wood, 1979; Bennett, 2010). Also, HCl and NaClO have been used to eradicate or reduce seedborne inocula of several pathogens (Sahin and Miller, 1997; Özdemir and Zitter, 2006; Taylor and Harman, 1990; Pradhanang and Collier, 2007). The results of our study showed that both hot-water and HCl treatments successfully eradicate *X. cucurbitae* in pumpkin seeds, which agrees with the reports by Moffett and Wood that hot-water can be used to eradicate seedborne inoculum of *Xanthomonas* spp. This is the first report of successful seed treatment for control of seedborne *X. cucurbitae* in pumpkin. Further research will be needed to validate the effectiveness of the hot-water and HCl treatments for eradication of *X. cucurbitae* in other cucurbit seeds.

Seed treatment alone is not expected to effectively control bacterial spot, in pumpkin fields. Rather, seed treatment should be considered as one component and integraded approach for managing this disease. Other effective strategies for managing bacterial spot of pumpkin should include resistant cultivars identified through screening of the existing pumpkin cultivars/lines or produced through breeding; crop rotation for three years or longer with non-cucurbits, as *X. cucurbitae* survives in the fields for more than two years (Thapa, 2014); and timely applications of effective chemicals or biocontrol agents as reported by Thapa and Babadoost (2016).

TABLES AND FIGURES

Table 4.1. Analysis of variance for seed germination and seedling vigor of non-infected 'Howden' and Dickinson' pumpkins in the laboratory-based experiment.

Seed treatment (cultivar)	Factor	Value	df	Seed germination	Seedling vigor[t]	
					Shoot length	Root length
Hot-water (Howden)	Temperature[u]	P	7	0.0618	0.0113	0.0027
		F	7	2.59	1.96	5.37
	Time[v]	P	5	0.3349	0.0148	0.0409
		F	5	1.16	1.74	2.16
	Temperature * time	P	35	0.4762	0.0246	0.0019
		F	35	1.01	1.13	7.26
Hot-water (Dickinson)	Temperature	P	7	0.0034	<0.0001	0.0014
		F	7	5.48	8.27	4.48
	Time	P	5	<0.0001	0.1112	0.0128
		F	5	8.01	2.33	2.25
	Temperature * time	P	35	0.0016	<0.0001	0.0148
		F	35	2.23	3.72	1.4
Hydrochloric acid (Dickinson)	Concentration[w]	P	2	0.0006	<.0001	<0.0001
		F	2	10.28	36.5	112.38
	Time[x]	P	5	0.0394	0.0005	<0.0001
		F	5	2.17	39.6	410.02
	Concentration * time	P	10	0.3654	<.0001	<0.0001
		F	10	1.16	7.96	11.79
Sodium hypochlorite (Dickinson)	Concentration[y]	P	3	0.0004	0.0278	<0.0001
		F	3	8.73	3.16	12.02
	Time[z]	P	3	0.0062	0.0019	0.0002
		F	3	11.9	60.6	167.61
	Concentration * time	P	9	<0.0001	0.0005	0.0029
		F	9	14.59	3.67	3.03

[t] Data of shoot and root lengths were square root-transformed before analyses.

[u] Temperatures of water treatments were 49, 50, 51, 52, 53, 54, 55, and 56°C.

[v] Hot-water seed treatment was achieved by soaking seeds in hot water for 5, 10, 15, 20, 25, or 30 min.

[w] Concentrations of hydrochloric acid was 0.5, 1.0 and 2.0%.

[x] Hydrochloric acid seed treatment was conducted by soaking seeds in a hydrochloric

Table 4.1. (cont.)

acid solution for 10, 20, 30, 40, 50, or 60 min.

[y] Concentration of sodium hypochlorite was 0.5, 1.0 1.5, and 2.0%.

[z] Sodium hypochlorite seed treatments were conducted by soaking seeds in sodium hypochlorite solutions for 1, 2, 3, or 4 min.

Table 4.2. Analysis of variance for seed germination and seedling vigor of non-infected 'Howden' and 'Dickinson' pumpkins in the greenhouse based experiment.

Seed Treatment	Factor	Value	df	Seed germination[s]	Seedling vigor[t]
Hot-water (Howden)	Temperature[u]	P	7	0.0962	0.0014
		F	7	2.2	5.79
	Time[v]	P	5	0.1904	0.9537
		F	5	1.53	0.91
	Temperature * time	P	35	0.4811	0.8675
		F	35	1.00	1.04
Hot-water (Dickinson)	Temperature	P	7	<0.0001	0.0847
		F	7	4.41	1.78
	Time	P	5	0.0408	0.1889
		F	5	2.57	1.03
	Temperature * time	P	35	0.0004	0.8769
		F	35	2.51	0.97
Hydrochloric acid (Dickinson)	Concentration[w]	P	2	0.1501	<0.0001
		F	2	1.97	74.75
	Time[x]	P	5	0.2644	0.0001
		F	5	1.82	68.15
	Concentration * time	P	10	0.2947	0.0063
		F	10	1.23	2.63
Sodium hypochlorite (Dickinson)	Concentration[y]	P	3	<0.0001	<0.0001
		F	3	49.58	94.09
	Time[x]	P	3	0.1903	0.0002
		F	3	3.08	6.65
	Concentration * time	P	9	0.0356	<0.0001
		F	9	2.26	16.98

[s] Data of seed germination following hot-water treatments of both cultivar 'Howden' and 'Dickinson' and hydrochloric acid treatment were square root-transformed before analyses.

[t] Seedling vigor was assessed using a scale of 0 to 4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[u] Temperatures of water treatments were 49, 50, 51, 52, 53, 54, 55, and 56°C.

[v] Hot-water seed treatment was achieved by soaking seeds in hot water for 5, 10, 15, 20, 25, or 30 min.

[w] Concentration of hydrochloric acid was 0.5, 1.0, and 2.0%.

[x] Hydrochloric acid seed treatment was conducted by soaking seeds in hydrochloric

Table 4.2. (cont.)

acid solution for 10, 20, 30, 40, 50, or 60 min.

[w] Concentration of sodium hypochlorite was 0.5, 1.0 1.5, and 2.0%.

[y] Sodium hypochlorite seed treatment was conducted by soaking seeds in sodium hypochlorite solution for 1, 2, 3, or 4 min.

[z] Data of seed germination following hot-water treatments of both cultivar 'Howden' and 'Dickinson' and hydrochloric acid treatment were square root-transformed before analyses.

Table 4.3. Analysis of variance for seed germination and seedling vigor of naturally-infected 'Howden' pumpkin in the greenhouse based experiment.

Seed Treatment	Factor	Value	df	Seed germination[s]	Seedling vigor[t]
Hot-water	Temperature[u]	P	7	<0.0001	<0.0001
		F	7	15.73	14.89
	Time[v]	P	5	0.0017	<0.0001
		F	5	5.26	15.94
	Temperature * time	P	35	0.0049	0.0028
		F	35	2.76	3.64
Hydrochloric acid	Concentration[w]	P	2	0.1613	0.383
		F	2	1.9	0.97
	Time[x]	P	5	0.0698	<0.0001
		F	5	4.23	81.2
	Concentration * time	P	10	0.0014	<0.0001
		F	10	3.57	5.92
Sodium hypochlorite	Concentration[y]	P	3	0.0003	<0.0001
		F	3	5.67	17.83
	Time[z]	P	3	0.0049	0.0039
		F	3	3.12	3.59
	Concentration * time	P	9	0.0011	0.0060
		F	9	3.89	2.58

[s] Data of seed germination following hydrochloric acid seed treatment were natural log-transformed before analyses.

[t] Seedling vigor was assessed using a scale of 0 to 4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[u] Temperatures of water treatments were 49, 50, 51, 52, 53, 54, 55, and 56°C.

[v] Hot-water seed treatment was achieved by soaking seeds in hot water for 5, 10, 15, 20, 25, or 30 min.

[w] Concentration of hydrochloric acid was 0.5, 1.0, and 2.0%.

[x] Hydrochloric acid seed treatment was conducted by soaking seeds in hydrochloric acid solution for 10, 20, 30, 40, 50, or 60 min.

[w] Concentration of sodium hypochlorite was 0.5, 1.0 1.5, and 2.0%.

[y] Sodium hypochlorite seed treatment was conducted by soaking seeds in sodium hypochlorite solution for 1, 2, 3, or 4 min.

[z] Data of seed germination following hot-water treatments of both cultivar 'Howden' and 'Dickinson' and hydrochloric acid treatment were square root-transformed before analyses.

Table 4.4. Seed germination and seedling vigor of non-infected 'Howden' pumpkin in the laboratory based experiment following hot-water seed treatment.

Treatment		Seed Germination (%)	Seedling vigor	
Temperature (°C)	Time (min)		Shoot length[y]	Root length (cm)
Control	Control	98.67 a[z]	0.61 opq	6.99 p-s
49	5	99.33 a	1.39 a	14.88 a-d
49	10	99.00 a	1.38 ab	15.72 a
49	15	99.00 a	1.18 b-g	11.54 f-k
49	20	98.83 a	1.14 b-i	11.36 g-k
49	25	98.67 a	1.22 a-f	12.27 b-j
49	30	99.00 a	1.20 a-f	15.23 ab
50	5	99.33 a	1.34 abc	13.96 a-g
50	10	98.83 a	1.31 abc	14.98 abc
50	15	98.50 a	1.20 a-f	12.43 b-j
50	20	98.17 a	0.99 d-m	13.58 a-h
50	25	98.67 a	0.99 d-m	11.75 e-k
50	30	98.17 a	1.13 a-i	14.96 abc
51	5	98.67 a	1.17 a-h	12.50 b-j
51	10	99.50 a	1.25 a-e	14.69 a-e
51	15	98.33 a	1.10 a-k	12.67 b-j
51	20	98.17 a	0.80 j-p	12.33 b-j
51	25	99.67 a	0.86 i-n	11.51 f-k
51	30	98.17 a	1.04 c-k	14.46 a-f
52	5	98.33 a	1.10 a-j	10.76 h-n
52	10	96.83 a	1.22 a-f	13.06 a-i
52	15	98.93 a	1.09 a-k	11.50 f-k
52	20	98.33 a	0.94 e-n	11.99 c-k
52	25	98.67 a	1.07 b-k	11.26 h-l
52	30	97.67 a	0.86 i-n	10.43 i-o
53	5	98.67 a	1.26 a-d	10.78 h-n
53	10	98.50 a	1.25 a-e	9.83 j-q

Table 4.4. (cont.)

Treatment		Seed Germination (%)	Seedling vigor	
Temperature (°C)	Time (min)		Shoot length[y]	Root length (cm)
53	15	98.17 a[z]	0.92 g-n	10.49 i-n
53	20	99.00 a	1.28 a-d	11.28 g-l
53	25	98.00 a	1.10 a-j	11.34 g-l
53	30	98.17 a	0.73 l-q	9.09 k-r
54	5	99.00 a	1.09 a-k	11.88 d-k
54	10	98.83 a	1.09 a-k	11.10 g-m
54	15	98.67 a	1.09 a-k	11.39 g-k
54	20	98.17 a	1.11a-j	11.21 g-l
54	25	98.67 a	1.11a-j	9.86 j-p
54	30	98.67 a	0.78 k-q	8.00 n-r
55	5	97.33 a	0.85 h-n	8.32 l-r
55	10	97.50 a	1.03 c-k	11.73 e-k
55	15	98.33 a	0.92 h-n	9.18 k-r
55	20	98.17 a	0.89 h-n	10.56 i-n
55	25	98.33 a	0.66 n-q	8.02 n-r
55	30	98.00 a	0.54 pqr	6.31 rs
56	5	99.00 a	0.47qr	7.25 p-s
56	10	99.00 a	0.66 n-q	6.79 qrs
56	15	98.17 a	0.28 rs	7.45 o-s
56	20	98.17 a	0.68 m-q	8.24 m-r
56	25	97.33 a	0.15 s	4.96 s
56	30	96.83 a	0.12 s	6.64 p-s

[y] Length of shoot was measured in centimeter and then the data were natural log-transformed before analyses.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.5. Seed germination and seedling vigor of non-infected 'Dickinson' pumpkin in the laboratory based experiment following hot-water seed treatment.

Treatment		Seed Germination (%)	Seedling vigor	
Temperature (°C)	Time (min)		Shoot length (cm)	Root length (cm)
Control	Control	90.67 bcd[z]	2.11 b-g	8.75 a-f
49	5	94.17 abc	2.40 b-e	7.23 a-h
49	10	95.17 abc	4.15 a	9.65 abc
49	15	93.17 ab	1.70 b-g	6.15 d-i
49	20	92.50 a-d	1.15 efg	4.02 hij
49	25	91.17 a-d	2.59 b-e	9.42 a-d
49	30	91.17 a-d	2.87 abc	10.70a
50	5	92.67 a-d	2.09 b-g	8.57 a-f
50	10	93.17 a-d	3.13 ab	9.67 abc
50	15	93.50 a-d	1.52 c-g	6.48 c-h
50	20	91.50 a-d	1.33 d-g	7.17 b-h
50	25	92.17 a-d	2.50 b-e	9.67 abc
50	30	91.00 a-d	1.55 c-g	10.08ab
51	5	92.17 a-d	1.97 b-g	7.32 a-h
51	10	92.67 a-d	2.90 abc	8.75 a-f
51	15	94.83 abc	1.88 b-g	7.27 a-h
51	20	94.83 abc	1.87 b-g	7.33 a-h
51	25	91.33 a-d	2.27 b-f	8.43 a-f
51	30	92.00 a-d	1.97 b-g	8.50 a-f
52	5	93.83 a-d	2.00 b-g	7.62 a-g
52	10	93.50 a-d	1.93 b-g	7.28 a-h
52	15	93.17 a-d	2.21 b-f	8.70 a-f
52	20	92.67 a-d	1.87 b-g	6.43 c-h
52	25	91.17 a-d	2.37 b-f	9.13 a-d
52	30	91.83 a-d	2.08 b-g	8.60 a-f
53	5	95.50 a	1.68 b-g	7.10 b-h
53	10	92.00 a-d	2.03 b-g	6.72 b-h
53	15	91.67 a-d	2.18 b-g	7.33 a-h

Table 4.5. (cont.)

Treatment		Seed germination (%)	Seedling vigor	
Temperature (°C)	Time (min)		Shoot length (cm)	Root length (cm)
53	20	91.00 a-d[z]	1.55 c-g	4.85 g-j
53	25	90.50 cd	2.78 a-d	9.22 a-d
53	30	93.17 a-d	2.20 b-g	9.08 a-e
54	5	91.67 a-d	1.90 b-g	7.57 a-g
54	10	92.00 a-d	1.90 b-g	6.45 c-h
54	15	90.67 bcd	1.93 b-g	7.60 a-f
54	20	93.00 a-d	1.73 b-g	8.50 a-f
54	25	91.17 a-d	2.07 b-g	8.47 a-f
54	30	90.50 cd	2.05 b-g	5.65 e-j
55	5	91.33 a-d	2.38 b-f	7.53 a-g
55	10	90.67 bcd	2.40 b-f	6.33 c-h
55	15	90.67 bcd	1.92 b-g	6.51 c-h
55	20	91.99 a-d	1.55 c-g	5.50 g-j
55	25	92.00 a-d	1.68 b-g	4.70 g-j
55	30	90.67 bcd	1.68 b-g	4.90 g-j
56	5	94.33 abc	1.62 b-g	7.23 a-h
56	10	93.67 a-d	1.73 b-g	6.63 b-h
56	15	92.33 a-d	1.77 b-g	6.51 c-h
56	20	92.67 a-d	1.18 efg	4.53 g-j
56	25	91.33 a-d	0.68 g	2.88 ij
56	30	89.99 d	0.95 fg	2.57 j

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.6. Seed germination and seedling vigor of non-infected 'Howden' pumpkin in the greenhouse based experiment following hot-water seed treatment

Treatment		Seed germination[x]	Seedling vigor[y]
Temperature (°C)	Time (min)		
Control	Control	4.54 a[z]	3.67 a
49	5	4.54 a	3.61 a
49	10	4.51 a	3.65 a
49	15	4.56 a	3.60 a
49	20	4.55 a	3.61 a
49	25	4.53 a	3.67 a
49	30	4.51 a	3.70 a
50	5	4.54 a	3.83 a
50	10	4.55 a	3.68 a
50	15	4.53 a	3.67 a
50	20	4.53 a	3.67 a
50	25	4.51 a	3.70 a
50	30	4.54 a	3.63 a
51	5	4.51 a	3.57 a
51	10	4.51 a	3.57 a
51	15	4.52 a	3.78 a
51	20	4.51 a	3.60 a
51	25	4.52 a	3.58 a
51	30	4.52 a	3.55 a
52	5	4.53 a	3.50 a
52	10	4.53 a	3.57 a
52	15	4.51 a	3.60 a
52	20	4.55 a	3.53 a
52	25	4.51 a	3.52 a
52	30	4.53 a	3.52 a
53	5	4.53 a	3.72 a
53	10	4.54 a	3.80 a
53	15	4.53 a	3.77 a

Table 4.6. (cont.)

Treatment		Seed germination[x]	Seedling vigor[y]
Temperature (°C)	**Time (min)**		
53	20	4.54 a[z]	3.77 a
53	25	4.51 a	3.70 a
53	30	4.54 a	3.77 a
54	5	4.52 a	3.75 a
54	10	4.54 a	3.73 a
54	15	4.55 a	3.77 a
54	20	4.53 a	3.75 a
54	25	4.51 a	3.87 a
54	30	4.52 a	3.77 a
55	5	4.51 a	3.83 a
55	10	4.51 a	3.75 a
55	15	4.54 a	3.77 a
55	20	4.54 a	3.77 a
55	25	4.54 a	3.78 a
55	30	4.51 a	3.78 a
56	5	4.55 a	3.78 a
56	10	4.54 a	3.23 a
56	15	4.54 a	3.78 a
56	20	4.55 a	3.82 a
56	25	4.54 a	3.82 a
56	30	4.55 a	3.88 a

[x] Data of germination were natural log-transformed before analysis.

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.7. Seed germination and seedling vigor of non-infected ‘Dickinson’ pumpkin in the greenhouse based experiment following hot-water seed treatment

Treatment		Seed germination[x]	Seedling vigor[y]
Temperature (°C)	**Time (min)**		
Control	Control	4.51 ab[z]	3.63 abc
49	5	4.44 a-d	3.73 a
49	10	4.41 bcd	3.42 a-d
49	15	4.42 a-d	3.53 a-d
49	20	4.42 a-d	3.50 a-d
49	25	4.41 a-d	3.50 a-d
49	30	4.41 a-d	3.45 a-d
50	5	4.43 a-d	3.42 a-d
50	10	4.40 bcd	3.55 a-d
50	15	4.46 a-d	3.55 a-d
50	20	4.45 a-d	3.53 a-d
50	25	4.42 a-d	3.50 a-d
50	30	4.41 bcd	3.57 a-d
51	5	4.40 bcd	3.53 a-d
51	10	4.44 a-d	3.53 a-d
51	15	4.40 bcd	3.51 a-d
51	20	4.45 a-d	3.57 a-d
51	25	4.43 a-d	3.47 a-d
51	30	4.41 bcd	3.45 a-d
52	5	4.41 bcd	3.43 a-d
52	10	4.40 bcd	3.57 a-d
52	15	4.40 bcd	3.63 abc
52	20	4.41 bcd	3.65 bcd
52	25	4.45 a-d	3.38 a-d
52	30	4.45 a-d	3.53 a-d
53	5	4.42 a-d	3.50 a-d
53	10	4.41 bcd	3.52 a-d
53	15	4.41 bcd	3.52 a-d

Table 4.7. (cont.)

Treatment		Seed germination[x]	Seedling vigor[y]
Temperature (°C)	Time (min)		
53	20	4.45 a-d[z]	3.47 a-d
53	25	4.43 a-d	3.57 a-d
53	30	4.38 d	3.68 ab
54	5	4.43 a-d	3.53 a-d
54	10	4.47 a-d	3.62 a-d
54	15	4.50 ab	3.67 abc
54	20	4.42 a-d	3.52 a-d
54	25	4.44 a-d	3.60 a-d
54	30	4.45 a-d	3.52 a-d
55	5	4.45 a-d	3.55 a-d
55	10	4.39 bcd	3.60 a-d
55	15	4.45 a-d	3.58 a-d
55	20	4.46 a-d	3.60 a-d
55	25	4.45 a-d	3.63 abc
55	30	4.48 a-d	3.62 a-d
56	5	4.53 a	3.52 a-d
56	10	4.45 a-d	3.43 a-d
56	15	4.47 a-d	3.48 a-d
56	20	4.45 a-d	3.37 bcd
56	25	4.47 abc	3.33 cd
56	30	4.39 cd	3.28 d

[x] Data of germination were natural log-transformed before analysis.

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.8. Seed germination and seedling vigor of infected 'Howden' pumpkin in the greenhouse based experiment following hot-water seed treatment

Treatment		Seed germination	Seedling vigor[y]
Temperature (°C)	**Time (min)**		
Control	Control	65.00 a[z]	1.95 c-i
49	5	62.50 a	1.88 c-i
49	10	63.75 a	2.55 abc
49	15	63.75 a	2.55 abc
49	20	61.25 ab	1.84 c-i
49	25	63.75 a	1.91 c-i
49	30	58.75 ab	1.76 d-i
50	5	63.75 a	1.91 c-i
50	10	58.75 ab	2.25 a-g
50	15	56.25 ab	2.35 a-e
50	20	53.75 abc	1.61 g-k
50	25	63.75 a	1.91 c-i
50	30	53.75 abc	1.61 g-k
51	5	58.75 ab	1.76 d-i
51	10	60.00 ab	2.40 a-d
51	15	56.25 ab	2.25 a-g
51	20	61.25 ab	1.84 c-i
51	25	58.75 ab	1.76 d-i
51	30	51.25 a-d	1.54 g-k
52	5	62.50 a	1.88 c-i
52	10	68.75 a	2.75 a
52	15	61.25 ab	2.45 a-d
52	20	53.75 abc	1.61 f-j
52	25	61.25 ab	1.84 c-i
52	30	53.75 abc	1.61 f-j
53	5	61.25 a	1.88 c-i
53	10	56.25 ab	2.19 a-g
53	15	60.00 ab	2.34 a-f

Table 4.8. (cont.)

Treatment		Seed germination	Seedling vigor[y]
Temperature (°C)	Time (min)		
53	20	63.75 a[z]	1.91 c-i
53	25	60.00 ab	1.80 d-i
53	30	57.50 ab	1.73 d-j
54	5	47.50 a-d	1.43 i-l
54	10	60.00 ab	2.40 a-d
54	15	67.50 a	2.70 ab
54	20	55.00 abc	1.65 e-k
54	25	57.50 ab	1.73 d-g
54	30	50.00 a-d	1.50 h-k
55	5	55.00 abc	1.65 e-i
55	10	50.00 a-d	2.00 b-i
55	15	47.50 a-d	1.90 c-i
55	20	50.00 a-d	1.50 h-k
55	25	33.75 c-f	1.01 klm
55	30	31.25 def	0.94 klm
56	5	48.75 a-d	1.46 h-l
56	10	50.00 a-d	2.00 b-g
56	15	40.00 c-e	1.60 h-k
56	20	25.00 df	0.75 lm
56	25	16.25 f	0.49 m
56	30	13.75 f	0.41 m

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.9. Seed germination and seedling vigor of non-infected 'Dickinson' pumpkin in the laboratory based experiment following hydrochloric acid seed treatment

Treatment		Seed germination (%)	Seedling vigor	
Concentration (%)	Time (min)		Shoot length[x]	Root length[y]
Control	control	90.67 a-d[z]	1.28 e-g	2.08 b-f
0.5	10	94.67 a-b	1.41 d-g	2.24 c-e
0.5	20	93.00 a-c	1.64 c-e	2.57 b
0.5	30	94.00 a-b	1.54 b-f	2.58 b
0.5	40	94.67 ab	2.30 a	3.37 a
0.5	50	88.67 a-e	1.76 bcd	2.37 b-d
0.5	60	97.00 a	1.91 b	3.24 a
1.0	10	82.00 b-g	1.27 efg	1.84 d-g
1.0	20	79.99 d-g	1.16 hg	1.72 e-i
1.0	30	66.00 h	1.31 efg	1.44 g-i
1.0	40	76.00 e-g	1.16 hg	1.61 g-i
1.0	50	78.67 d-g	1.46 c-g	1.70 e-i
1.0	60	72.67 f-g	1.16 fgh	1.32 hi
2.0	10	70.00 hg	1.60 b-e	1.90 d-g
2.0	20	84.00 a-f	1.58 b-e	1.99 c-g
2.0	30	80.00 c-g	1.79 bc	2.56 bc
2.0	40	75.67 e-g	1.70 bcd	2.54 bc
2.0	50	77.33 e-g	1.50 c-g	2.15b-e
2.0	60	74.67 e-g	0.79 h	1.19 i

[x] The length of shoot was measured in centimeter and then the data were square root- transformed before analyses.

[y] The length of root was measured in centimeter and then the data were square root-transformed before analyses.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.10. Seed germination and seedling vigor of non-infected 'Dickinson' pumpkin in the greenhouse following hydrochloric acid seed treatment

Treatment		Seed germination[x]	Seedling vigor[y]
Concentration (%)	**Time (min)**		
Control	control	4.44 a[z]	3.01 c-f
0.5	10	4.44 a	3.36 a
0.5	20	4.44 a	3.30 abc
0.5	30	4.46 a	3.25 a-d
0.5	40	4.40 a	3.19 a-e
0.5	50	4.50 a	3.43 a
0.5	60	4.44 a	3.32 ab
1.0	10	4.50 a	2.88 fg
1.0	20	4.48 a	2.88 fg
1.0	30	4.43 a	2.83 g
1.0	40	4.41 a	2.87 fg
1.0	50	4.47 a	3.15 a-f
1.0	60	4.50 a	2.98 d-g
2.0	10	4.47 a	2.99 d-g
2.0	20	4.50 a	3.00 c-g
2.0	30	4.44 a	2.84 fg
2.0	40	4.47 a	2.99 d-g
2.0	50	4.46 a	2.79 g
2.0	60	4.51 a	2.90 efg

[x] Data of seed germination were natural log-transformed before analysis.

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.11. Seed germination and seedling vigor of infected 'Howden' pumpkin in the greenhouse following hydrochloric acid seed treatment

Treatment		Seed germination[x]	Seedling vigor[y]
Concentration (%)	**Time (min)**		
Control	control	3.11 b[z]	0.93 cde
0.5	10	3.48 ab	1.14 b-e
0.5	20	3.48 ab	1.01 bde
0.5	30	3.34 ab	1.00 cde
0.5	40	3.81 a	1.55 a
0.5	50	3.48 ab	1.56 a
0.5	60	3.11 b	0.89 cde
1.0	10	3.39 ab	0.83 de
1.0	20	3.48 ab	1.16 a-d
1.0	30	3.48 ab	1.00 cde
1.0	40	3.11 b	0.90 cde
1.0	50	3.81 a	1.01 cde
1.0	60	3.39 ab	1.22 a-d
2.0	10	3.75 ab	1.00 cde
2.0	20	3.48 ab	0.76 e
2.0	30	3.55 ab	0.92 cde
2.0	40	3.48 ab	0.94 cde
2.0	50	3.91 ab	1.50 ab
2.0	60	3.80 a	1.29 abc

[x] Data of seed germination were natural log-transformed before analysis.

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.12. Seed germination and seedling vigor of infected 'Howden' pumpkin in the greenhouse following sodium hypochlorite seed treatment

Treatment		Seed germination	Seedling vigor[z]
Concentration (%)	**Time (min)**		
Control	Control	83.75 a[z]	1.62 a
0.5	1	55.00 cde	1.38 c-f
0.5	2	51.25 de	1.27 fg
0.5	3	50.00 de	1.12 gh
0.5	4	55.00 cde	1.05 h
1.0	1	58.75 cde	1.34 def
1.0	2	63.75 b-e	1.33 ef
1.0	3	48.75 e	0.98 h
1.0	4	70.00 abc	1.43 b-e
1.5	1	80.00 ab	1.44 b-e
1.5	2	80.00 ab	1.55 ab
1.5	3	82.50 a	1.49 a-d
1.5	4	77.50 ab	1.48 a-d
2.0	1	67.50 a-d	1.32 ef
2.0	2	77.50 ab	1.51 abc
2.0	3	67.50 a-d	1.52 abc
2.0	4	72.50 abc	1.60 a

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.13. Seed germination and seedling vigor of non-infected 'Dickinson' pumpkin in the laboratory following sodium hypochlorite seed treatment

Treatment		Seed germination (%)	Seedling vigor	
Concentration (%)	Time (min)		Shoot length[x]	Root length[y]
Control	control	89.33 a[z]	1.59 ab	2.05 a-d
0.5	1	73.33 c-f	1.46 bcd	1.80 d-g
0.5	2	82.00 a-d	1.56 abc	1.83 d-g
0.5	3	80.67 a-e	1.40 bcd	1.65 fg
0.5	4	73.33 c-f	1.45 bcd	1.63 g
1.0	1	82.67 abc	1.44 bcd	1.88 c-g
1.0	2	62.67 g	1.27 d	1.69 e-g
1.0	3	66.00 fg	1.28 cd	1.99 a-d
1.0	4	80.67 a-e	1.80 a	2.19 ab
1.5	1	71.33 egf	1.44 bcd	1.94 b-f
1.5	2	72.67 c-g	1.48 bcd	1.95 b-e
1.5	3	76.67 b-e	1.52 abc	1.97 a-e
1.5	4	86.67 ab	1.54 abc	2.24 a
2.0	1	72.00 d-g	1.62 ab	2.00 a-d
2.0	2	84.67 ab	1.44 bcd	2.02 a-d
2.0	3	81.33 a-e	1.35 bcd	2.16 abc
2.0	4	82.67 abc	1.45 bcd	2.17 ab

[x] Length of shoot was measure in centimeter and then the data were square root-transformed before analyses.

[y] Length of root was measure in centimeter and then the data were natural log-transformed before analyses.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

Table 4.14. Seed germination and seedling vigor of non-infected 'Dickinson' pumpkin in the greenhouse following sodium hypochlorite seed treatment

Treatment		Seed germination	Seedling vigor[y]
Concentration (%)	**Time (min)**		
Control	Control	83.75 a[z]	1.62 a
0.5	1	55.00 cde	1.38 c-f
0.5	2	51.25 de	1.27 fg
0.5	3	50.00 de	1.12 gh
0.5	4	55.00 cde	1.05 h
1.0	1	58.75 cde	1.34 def
1.0	2	63.75 b-e	1.33 ef
1.0	3	48.75 e	0.98 h
1.0	4	70.00 abc	1.43 b-e
1.5	1	80.00 ab	1.44 b-e
1.5	2	80.00 ab	1.55 ab
1.5	3	82.50 a	1.49 a-d
1.5	4	77.50 ab	1.48 a-d
2.0	1	67.50 a-d	1.32 ef
2.0	2	77.50 ab	1.51 abc
2.0	3	67.50 a-d	1.52 abc
2.0	4	72.50 abc	1.60 a

[y] Seedling vigor was assessed using a scale of 0-4, as 0 = seed did not germinate; 4 = seedling was vigorously growing; and 1, 2, and 3 were used to describe seedling vigor between 0 and 4.

[z] In each column, values with a letter in common are not significantly different from each other according to Tukey's HSD test ($\alpha = 0.05$).

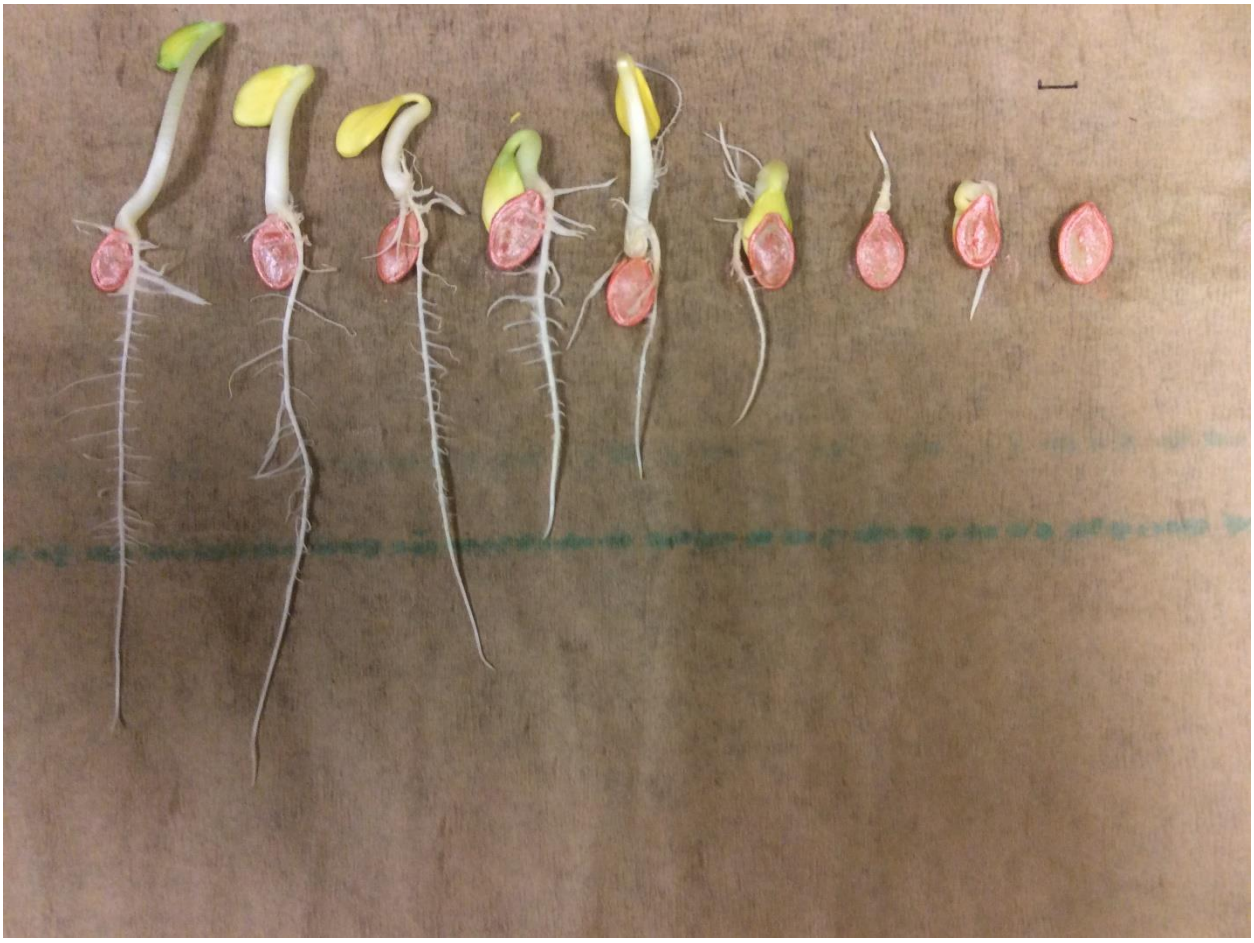

Figure 4.1 Seedling vigor of non-infected 'Howden' pumpkin following hot-water treatments. Right, ungerminated seed; left, germinated seed and vigorously growing seedling.

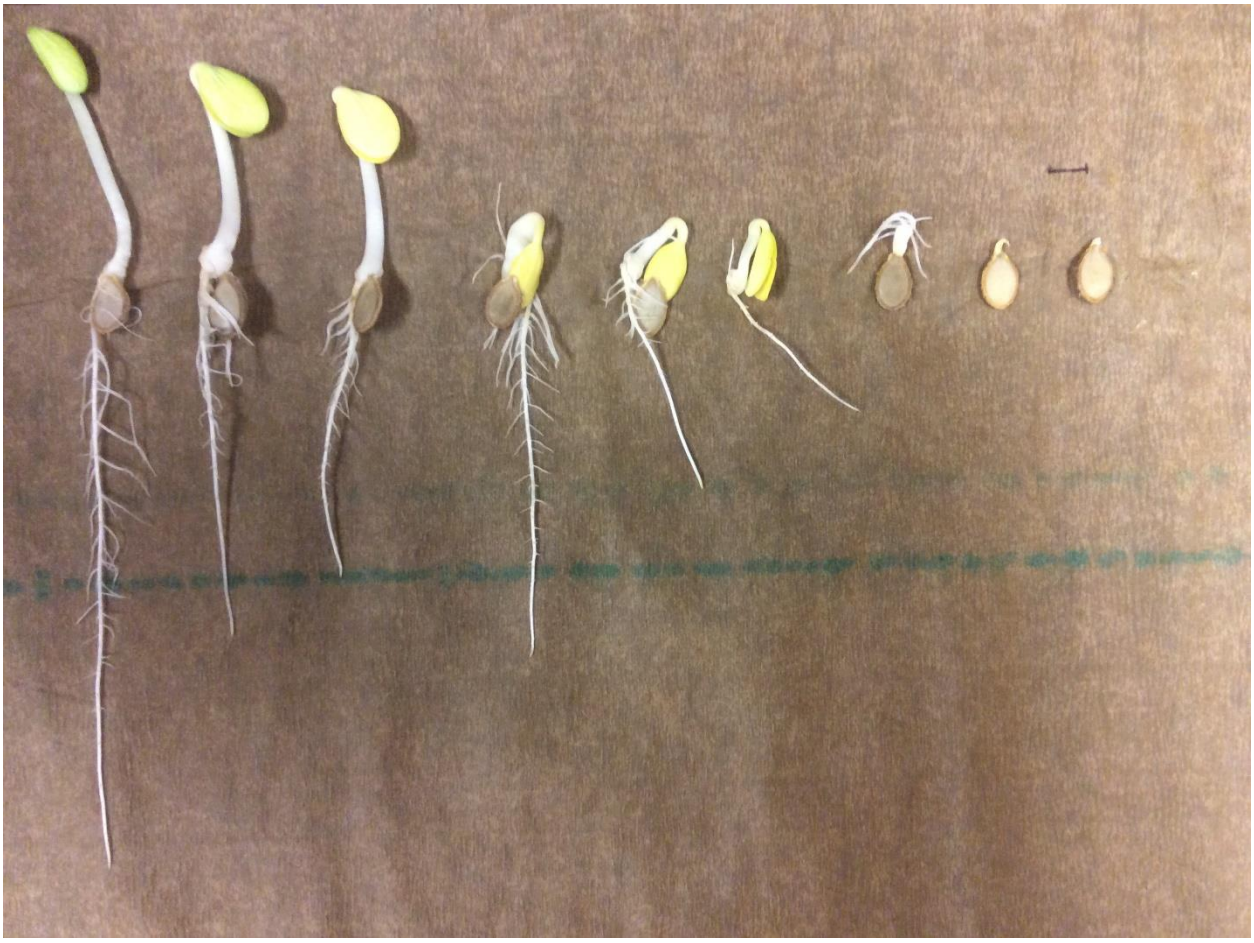

Figure 4.2 Seedling vigor of non-infected ‘Dickinson’ pumpkin following hot-water treatments. Right, ungerminated seed; left, germinated seed and vigorously growing seedling.

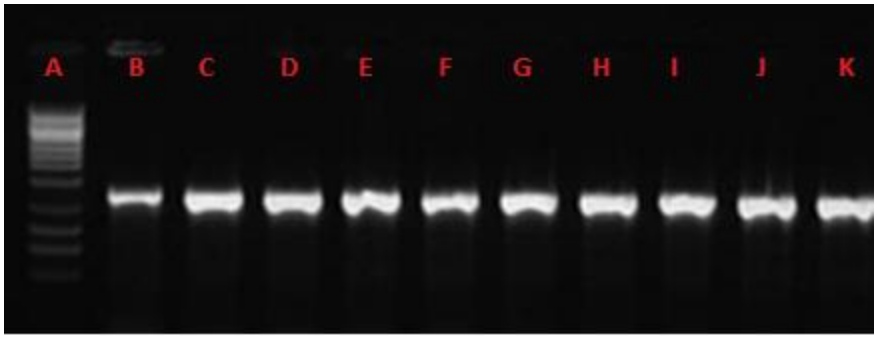

Figure 4.3 PCR product of *Xanthomonas cucurbitae* detected from seed samples with primers RST2/RST3. Produced amplicon was 1,400 bp. A,1 kb ladder; B, ATCC strain 23378; C-K, *X. cucurbitae* isolates from the North Central Region.

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