EPIDEMIOLOGY AND MANAGEMENT OF *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* IN NEW YORK STATE

A Thesis

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Holly W. Lange

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ABSTRACT

Black rot caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) is a serious disease of crucifers which are a high value crop in New York. This pathogen can affect plants at any growth stage and cause widespread economic loss through reduction of yield and quality. The overall goal of this study was identifying the inoculum sources of Xcc in New York so that management strategies could be targeted to the appropriate location. To determine the diversity of strains of the pathogen more than 150 isolates of Xcc were collected over six years. Repetitive element polymerase chain reaction (rep-PCR) fingerprinting was the primary technique utilized to differentiate the isolates. The appearance of new fingerprint patterns in the state each year indicates that new infections do not primarily arise from the pathogen remaining in fields from one season to the next but are more likely introduced on seeds and/or transplants each year. Since the pathogen can overwinter in NY, rotation and control of debris are also important for eliminating inoculum sources.

Weeds have been found to harbor Xcc in other locations, but little is known of their role as an inoculum source in NY. 2004 was a year with a severe black rot epidemic. The following spring, five fields were targeted to determine if Xcc was present in weeds where inoculum levels had been high the previous year. Of the 77 weeds collected from May through July 2005, bacterial isolates from 21 grew on Xcc semi-selective media and eight were determined to be Xcc using an enzyme-linked immunosorbent assay test (ELISA). When inoculated into cabbage in the greenhouse, none of the isolates from weeds caused typical V-shaped lesions. The rep-PCR fingerprint patterns of isolates collected from the weeds were not the same as those

derived from cabbage isolates collected from fields or greenhouses the previous, or in following years. Bacterial isolates were also collected from cruciferous weeds in 2006 and 2007. Seven isolates out of 92 cruciferous weeds collected in these two growing seasons did cause V-shaped lesions in greenhouse inoculations. Four of these isolates were collected from a field showing black rot symptoms in the cabbage crop. With the exception of this field, the Xcc isolates from weeds had different rep-PCR fingerprint patterns compared to Xcc isolates from cabbage. This study provides evidence that while weeds can be infected with Xcc; the strains appear to be different than those responsible for field infections thus do not serve as a primary reservoir of Xcc inoculum.

The black rot pathogen can be seed-borne and even rigorous seed testing can not guarantee that every seed is free of bacteria. In greenhouse transplant facilities, conditions are ideal for the spread of Xcc with dense plant populations and overhead watering. Asymptomatic transplants can initiate field infections which are very difficult to control. The efficacy of copper hydroxide and benzothiadiazole to control the spread of black rot in inoculated flats was investigated. Leaf washes were used for dilution plate counts and real-time PCR to quantify pathogen numbers on cabbage seedlings. Both products did provide a significant reduction in the Xcc population over untreated control flats. Additionally, it was determined that younger seedlings could harbor larger numbers of Xcc bacteria than older seedlings, and that the age of the seedlings had an effect on the efficacy of copper hydroxide. Targeting cabbage transplants at the optimum age (the two-true leaf stage) for application of chemical controls is an effective management tool to decrease the population of Xcc in an environment favoring disease spread.

BIOGRAPHICAL SKETCH

Holly's interests in animals and science were fostered in her father's labs; playing with guinea pigs or donating blood for drug studies. She and her family spent the majority of their free time outside enjoying all that New England had to offer. She spent a summer at Syracuse University on an NSF scholarship studying biology, and the next two summers on Martha's Vineyard doing research on lobsters, learning to SCUBA dive, and riding her bicycle everywhere. A hands-on animal science education at the University of Connecticut included everything from grading eggs, milking cows, and welding, to livestock and dairy judging. A career as a dairy farmer and shepherd ensued, with four children, chickens, a horse, turkeys, pigs, and a vegetable garden and orchard joining the cows and sheep. The sale of the herd led her to a new start at the NY State Agricultural Experiment Station working with a variety of crops and pathogens (and people!).

To understand the new lab techniques and the plant systems and pathogens she was working with, she began to take classes at Fingerlakes Community College. To continue her education she began an M.S. program in plant pathology at Cornell University through the employee degree program.

Holly is married to Steve Brind'Amour, her cycling/kayak/ski/everything partner who never expected to have such an expansive knowledge of plant pathogens.

To Seth, Amanda, Eden and JohnWinthrop
Raising you was an adventure
and my finest achievement

To my parents who taught me to work hard
to love what you do
and to cherish your family

To my sister, Madelyn who is always there for me

and

To Steve
who makes it all worthwhile

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I wish to thank Cornell University for the opportunity to increase my knowledge and understanding of research and science through the study of plant pathology, and to obtain a degree while working full time. My major advisor, Dr. Christine Smart, is a gifted teacher with unfailing optimism that each problem and setback is an opportunity of some kind. My committee members Dr. Herb Aldwinckle and Dr. Steve Reiners were always supportive, enthusiastic, and ready to offer useful suggestions throughout my research, and I was able to interact with them as professors for some of my coursework. Silvia Restrepo and Maryann Borsick Herman taught me so much, were my good friends, and made our lab a fun place to be. Many other Smart lab members have assisted me in so many ways. The station community of faculty, technicians, students, post docs, and visiting scientists, has provided innumerable lessons, and skills; it is a stimulating and rewarding place to work. I appreciate that the entire staff at the Experiment station, whether greenhouse crew, field crew, or plumbers and electricians. They all work to make things run smoothly, aiding my research in so many ways.

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CHAPTER 1

TRACKING THE BLACK ROT OF CRUCIFERS PATHOGEN *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* IN NEW YORK STATE

Abstract

A severe black rot epidemic affecting New York crucifers in 2004 initiated a collection of *Xanthomonas campestris* pv. *campestris* (Xcc) isolates to characterize the population of this pathogen in the state. Over 150 isolates were collected over the next six years from ten NY counties. The goal was to understand the diversity and dynamics of the Xcc population for development of control strategies for this pathogen. Lesions of symptomatic plants were excised, ground and plated onto medium for Xcc identification. Enzyme-linked immunosorbent assays were utilized to differentiate the Xcc isolates, which causes a vascular reaction, from *Xanthomonas campestris* pv. *armoraciea* (Xca), which is a leaf spotting pathogen. The majority of isolates (120) were positive for Xcc with the balance being Xca or reacting with neither antigen. Each isolate was inoculated into greenhouse cabbage to identify whether the typical V-shaped lesions of black rot resulted. All isolates collected in 2004 were pathogenic on cabbage, in subsequent years some of the isolates caused a necrotic spot reaction. Repetitive element polymerase chain reaction (rep-PCR) fingerprinting was used to generate fingerprint patterns of each isolate to compare the isolates between fields, counties, cultivars, and years. New fingerprint patterns were identified in NY each year but the same fingerprint can remain on a farm when seedbed rotation is not employed. The isolates from 2004 were inoculated into a set of differential plants to determine which Xcc races were present, and if race affected pathogenicity. Race 1 was identified in 80% of the isolates tested with races 3 and 4 also represented. Since new strains of Xcc arrive in NY each growing season,

pathogen-free seed and transplants should be the management focus for control of black rot.

Introduction

Black rot disease of crucifers was first described by Louis Pammel on turnip in New York state in 1893 (Smith 1911). Black rot is now known to be caused by the Gram-negative bacterium *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (Xcc) and is a disease of economic significance for cruciferous crops in every part of the world (Williams, 1980). In tropical and sub-tropical areas, black rot epidemics can occur in each crop cycle. The disease is a threat every year in temperate climates when weather and inoculum levels become optimum for disease development.

In New York and other major crucifer growing areas in the Midwest and Northeast, black rot was a minor problem until several factors changed in the late 1960s and early 1970s (Williams 1980). There was an increase in the price of hybrid seed and many growers began using transplants. Seed was imported from regions known to have Xcc, and it is thought that the bacterium may have contaminated seed lots. Seed had been hypothesized to be the source of inoculum in many of the observed early epidemics (Walker and Tisdale 1920), and common practice at the time was to hot water treat seed at 50ºC for 25 min (Walker 1923). Unfortunately, much of the imported seed in the early 1970's had not been hot water treated. When the infested seeds were sown in seedbeds with clean seeds the infection often spread unnoticed until transplants were planted on-farm or shipped to be planted in locations where weather conditions were conducive for disease development (Williams 1980). This was the case in 1972, when the remnants of Hurricane Agnes struck central New York and the wind and flooding caused a major epidemic of black rot in the region (Hunter et al. 1975).

The bacteria enter the plant primarily through the hydathodes on the leaf margin (Hugouvieux et al. 1998). Hydathodes were discovered to be the openings utilized for entry into the plant during an early greenhouse experiment concerning slug transmission; plant-to-plant transmission via water splashing and hydathodes was clearly the most effective entry route for Xcc (Smith 1911). Guttation fluid that exudes from the hydathodes is easily contaminated with bacteria. The minerals, carbohydrates and amino acids and a pH of 7.0 make it an ideal medium for Xcc growth (Kohl and van der Wolf 2004) (Figure 1.1). Once inside the leaf, the bacteria multiply rapidly

Figure 1.1 Guttation droplets on the margin of a cabbage leaf. Arrow points to one droplet.

and move systemically, secreting an extracellular polysaccharide, xanthan, which plugs the xylem tissue (Sutton and Williams 1970). The typical yellow V-shaped lesions that originate at the leaf edge and extend inward are caused by blockage of water and nutrients which results in chlorosis and blackening of the veins (Sutton and Williams 1970) (Figure 1.2). Insect feeding sites, hail and wind damage, and field equipment can also wound the plant and allow entry of the pathogen. Temperatures between 22 and 28˚C are optimal for disease development. Wet conditions are conducive for epiphytic survival of the bacteria as well as movement between plants (Carisse et al. 1998).

Figure 1.2 Typical V-shaped lesions on cabbage leaves.

Black rot infections commonly arise from bacteria, on or in the crucifer seed, which are in a viable or revivable state (Cook et al. 1952; Schaad et al. 1980; Starr 1981). In addition to entering a plant through hydathodes, Xcc can enter the stomates of cotyledons, which are usually shed by the time transplanting occurs. The bacterium can survive in the xylem for months until conditions are favorable for multiplication. While contaminated seed is one source of inoculum, Xcc can survive on and in cruciferous weeds and these can serve as an inoculum source for field infections especially in areas where weeds survive year round (Kuan et al. 1986; Schaad and Dianese 1981). Cruciferous weeds do not always serve as a source of inoculum, as a recent study revealed that strains of *X. campestris* in weed populations in CA were unique to a site and distinct from strains in crop production areas (Ignatov et al. 2007). Crop debris and harvested plants as well as surface water can also harbor the pathogen (Kocks and Zadoks 1996; Kocks et al. 1998; Schaad and White 1974). Insects have also been shown to transmit the black rot pathogen. In one study, flea beetles (*Phyllotreta cruciferae*) sprayed with Xcc bacteria were shown to transmit the pathogen to all plants on which they fed, but they were not as efficient vectors after naturally feeding on diseased leaves (Shelton and Hunter 1985).

Although Xcc causes a vascular infection, two other *X. campestris* pathovars are not systemic, and enter the plant through stomates and invade the mesophyll tissue. These pathovars, *X. campestris* pv. *armoraciae* (Xca) initially isolated from horseradish (McCulloch 1929), and *X. campestris* pv. *raphani* (White 1930) (Xcr) which has a broader host range including tomato (White 1930), are also pathogenic to cruciferous crops. The symptoms are water soaked areas which form small lesions surrounded with a chlorotic region (White 1930) (Figure 1.3). Xca usually enters

Figure 1.3 Necrotic spots after pin inoculation with Xca on greenhouse cabbage.

through the stomates and is able to invade veins inducing a necrotic response (Hunter et al. 1987). The resulting dead tissue may fall out of the leaf giving a shot hole appearance and can cause collapse of the entire leaf. Both Xca and Xcr are thought to survive on seed and can cause serious field infections (Alvarez and Cho 1978). On young seedlings Xcc can cause lesions with a water soaked appearance before vascular symptoms develop (Alvarez et al. 1994), which can cause confusion with the other pathovars (Alvarez et al. 1994; Chen et al. 1994).

Repetitive element polymerase chain reaction fingerprinting, (rep-PCR), is a technique in which PCR primers designed from specific repetitive sequences amplify the regions between repetitive DNA elements on a bacterial chromosome. Rep-PCR has been used to determine phylogenetic differences in many bacterial species as well

as for the differentiation of strains within a species (de Bruijn et al. 1996; Versalovic et al. 1991; Versalovic et al. 1994). Unlike many other PCR protocols, no prior knowledge of the sample sequence is necessary to generate a fingerprint using rep-PCR. The technique has been used to generate fingerprint patterns that differentiate pathovars of *X. campestris* including Xcc and Xca (Massomo et al. 2003; Tsygankova et al. 2004). The rep-PCR technique has been used for microbiological studies in the environment, medicine, and plant pathology, as well as for diagnostics and epidemiology (Ishii and Sadowsky 2009). Rep-PCR was used for rapid diagnosis and differentiation to distinguish pathovars of *Xanthomonas* and *Pseudomonas* (Louws et al. 2005; Louws et al. 1994), to distinguish *X. campestris* pv. *vitians* strains in lettuce, tomatoes and peppers (Sahin et al. 2003), and assess the genetic diversity of Xcc (Valverde et al. 2007).

Warm temperatures and numerous rainstorms with high winds during the growing season of 2004 were ideal for the development of black rot in fields of cabbage and other crucifers in central New York. The severity of the losses led to the development of this project, with the overall goal of identifying the inoculum sources of Xcc in New York so that management strategies could be targeted to the appropriate location. The possible sources of inoculum included seed, the seedbeds where bare root transplants were grown, transplant plug production facilities, field debris, and weeds in and around the fields. The specific objective of this study was to determine if Xcc can over-winter so that the same strain is maintained from year to year or if new strains enter New York each year. The results of these studies will enable growers to utilize their resources more efficiently to manage black rot.

Materials and Methods

Isolate collection. Bacteria were isolated from cabbage, broccoli, Brussels sprouts, and cauliflower leaves that were collected from greenhouses, fields and winter storage facilities between 2004 and 2009. Tissue was excised from symptomatic leaves, surface sterilized with 0.6% NaOCl for three minutes, ground with sterile water using a mortar and pestle, plated onto King's B medium (King et al. 1954) and grown at 28ºC for 24-48 hours. Cultures that morphologically appeared to be *Xanthomonas* and did not fluoresce on King's B medium were cultured on YDC (Wilson et al. 1967) medium to check for yellow mucoid growth (typical of *Xanthomonas* species) and also cultured on *Xanthomonas* semi-selective SM medium (Chun and Alvarez 1983). When *Xanthomonads* are grown on SM the colonies are blue and are surrounded by a halo.

ELISA antigen test. Enzyme-linked immunosorbent assay (ELISA) analysis was performed using the Agdia (Elkhart, IN) bacterial reagent sets to differentiate between pathovars. Bacterial cultures were grown overnight in nutrient broth and following the manufacturer's protocol; reagents for Xcc and Xca were used. The two isolates with fully sequenced genomes; Xcc (strain 8004) and ATCC 33913 were used as positive controls for Xcc, and isolate Xca 071 (Sakata seeds) was used as the Xca positive control. *Pseudomonas syringae* pv. *tomato* and coating buffer alone were included as negative controls. Results were recorded for each isolate using visual assessment and confirmed with a microplate reader (Beckman Coulter Inc., Brea, CA). The test was performed at least twice for each isolate.

Inoculation test for pathogenicity. Two cultivars (cv) of cabbage susceptible to black rot were grown in the greenhouse with a 16 hour photoperiod at 24ºC. *Brassica oleraceae* cv 'Fresco' and 'Gonzales' were used and inoculated at the three true-leaf stage. Each Xcc field isolate was grown on YDC medium for one to two days at 28ºC.

A sterile pin dipped into the bacterial culture was pricked into the outer margin of one leaf eight times and into the petiole and midrib of a second true leaf on the same plant. The third leaf of each plant was left uninoculated. Control plants were pricked with a known Xcc isolate, a known Xca isolate, or a sterile pin. Two plants per isolate were inoculated and symptoms were scored visually at 14 days post inoculation. Plants were scored as having no reaction, a typical black rot symptomatic V-shaped yellow to brown lesion along the leaf margin, or a necrotic spot reaction.

Rep-PCR was carried out on each Xcc isolate collected following a previously described protocol (Louws et al. 1998) using a PTC-100 Peltier thermal cycler (MJ Research, Waltham, Ma)**.** Amplification was carried out in 0.2 ml thin-walled tubes (Fisher Scientific, Waltham, MA). The reaction mixture consisted of 5µl 5x Gitchster buffer, 0.2 µl BSA (20mg/ml), 2.5 µl 100% DMSO, 1.25 µl 25mM dNTP, 1 µl 100mM box A1R primer, 13.65 µl water, 0.4 µl Taq DNA polymerase in a total volume of 24µl (Louws et al, 1998). Box A1R primer (5'- CTA CGG CAA GGC GAC GCT GAC G-3') was purchased from Integrated DNA Technologies Inc (Coralville, IA). Bacterial cells were grown overnight in nutrient broth, the OD 600 adjusted to 0.1, and 12 µl was added to the 24 µl mix (Massomo et al. 2003). Extracted genomic DNA was alternatively used at 200ng per reaction, and the program run for 35 cycles of 95ºC for 2 min, 94 ºC for 3 s, 92 ºC for 30 s, 53 ºC for 1 min, 65 ºC for 8 min followed by 65 ºC for 8 minutes and held at 4 ºC (de Bruin et al., 1996). The PCR amplicons (8 µl) were loaded onto a 1.5% agarose gel using 0.5X TAE buffer and with ethidium bromide (10mg/ml) added, and run at 4ºC for 18 hours at 67V. A 100bp ladder (New England Biolabs, Ipswich, MA) was used as a size marker and banding patterns were observed under ultraviolet light using a Kodak Gel Logic C200 imaging system (Carestream Molecular Imaging, Rochester, NY).

Race determination. Isolates from 2004 (Table 1.2) were used to inoculate a set of differential host plants to determine the race based on the gene for gene relationship between *Brassica* cv and races of Xcc. Plants used to determine race in this study included; Seven Top turnip (resistant to race 2 and some race 4), Just Right turnip (resistant to race 4), Florida Broad Leaf mustard (resistant to 1, 3, and 4), Miracle cauliflower (resistant to race 3), Wirosa savoy cabbage (susceptible to all races) and PI 199947 (resistant to races 1, 3 and 4) (Kamoun et al. 1992; Vincente et al. 2001). Two NY isolates previously identified as race 1 and race 4 were used as positive controls. The Xca isolate 071 was used as a negative control. The differential plants were grown in the greenhouse as described above, and inoculated at the three true-leaf stage. Two plants of each differential were inoculated with each Xcc isolate tested, and the experiment was repeated three times. To inoculate the plants, a sterile pin was dipped into a one to two day old bacterial culture and pricked into the secondary veins around the outside edge of one true leaf. Control plants were pricked with a sterile pin with no bacterial culture. The plants were rated for disease symptoms 14 days after inoculation on a scale of zero to three. A rating of 0 = no symptoms; 1 = yellowing at the inoculation site; 2 = V-shaped lesions less than 4 cm^2; 3 = V-shaped lesions greater than 4 cm^2 as previously described (Vincente et al. 2001).

Results

Isolate collection. From 2004 to 2009, over 150 isolates were collected from Brassica plants displaying black rot symptoms across New York State (Table 1.2). In 2008 and 2009 several samples from neighboring states were also collected. The collection date, the crop type and cv if known, the source of the planting material, and the county of sample origin were recorded for each isolate collected (Table 1.2). The majority of samples came from symptomatic plants (V-shaped lesions) in grower's fields and from greenhouse transplant facilities which tended to have leaf spot or water

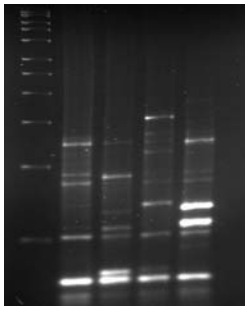
soaking symptoms. We also isolated from bare root transplants and cabbage heads in storage. Four isolates included in this study were already in culture and sent to our lab. Two were isolated from a black rot infected commercial seedbed which was the source of bare root transplants shipped to growers in New York in 2008. The other two isolates were isolated from cabbage seed at a seed testing facility.

ELISA Each isolate collected over the six year sampling period was tested with Xcc and Xca antigens. The majority of samples (120) tested Xcc positive with 16 showing a positive Xca reaction, five not reacting with either antigen, and eight reacting with both (Table 1.2). Of those that tested positive for Xcc, the majority were isolated from diseased plants with V-shaped lesions; however several were also isolated from transplants with necrotic leaf spots. Of the 16 that tested ELISA positive for Xca, one was isolated from plants collected at a greenhouse transplant production facility, two were from bare root transplants, and 13 were isolated from diseased plants growing in the field. Of the five isolates that did not react with either the Xcc or Xca antigen, two were isolated from a greenhouse production facility and three were from diseased plants in the field. Of the eight isolates that reacted with both antigens, three were isolated in 2005 from diseased plants in the field, two in 2006, and three were isolated in 2009.

Pathogenicity test. The inoculated leaves exhibited either the chlorotic V-shaped lesions with blackening of veins typical of black rot, or a localized necrotic spot at the inoculation site. Symptoms typically appeared between five and ten days post inoculation and plants were scored for symptom type at 14 days. The control plants inoculated with a sterile pin without bacteria showed no reaction at the inoculation site, while those inoculated with a known Xcc isolate produced V-shaped lesions. The known Xca isolate (071) left a necrotic spot with a chlorotic ring at the inoculation site. Typical V-shaped lesions were recorded in 125 of the tested isolates

which represented 87% of the isolates collected from cruciferous crops growing in production fields. Of the 33 isolates collected from greenhouse transplant facilities, ten of these, all from 2009, showed V-shaped lesions. The isolates that were collected from greenhouse transplant facilities in the other three years all showed necrotic spot symptoms as did seven of the sixteen 2009 isolates from transplant facilities. All of the isolates recovered from bare root transplants showed vascular infections typical of Xcc. Of the isolates derived from field samples, 11 tested positive for Xca yet caused V-shaped lesions in pathogenicity testing. Only two field samples failed to cause V-shaped lesions, one was Xcc positive with ELISA, the other reacted to both antigens. All of the isolates collected in 2004 caused V-shaped lesions in pathogenicity tests. This was not the case in subsequent years as some isolates each year caused necrotic spots rather than vascular infection. The days before symptom expression varied, as did the size and number of lesions.

Rep-PCR. Box A1R rep-PCR primers revealed distinct fingerprint patterns among the isolates collected. The fingerprint patterns were numbered sequentially beginning with the first four patterns identified during the 2004 growing season (Figure 1.4).



L 1 2 3 4

Figure 1.4 Rep-PCR fingerprint patterns collected in 2004. All 24 isolates fell into these four patterns. L represents the 1kb ladder.

There were a total of 21 different fingerprint patterns generated over the six years of the study. The majority of patterns were identified only in a single year; however

several patterns were repeated in more than one year. Pattern 2 was found in isolates collected from 2004 through 2007, however after 2004, the pattern was found only on a single farm (Figure 1.5).

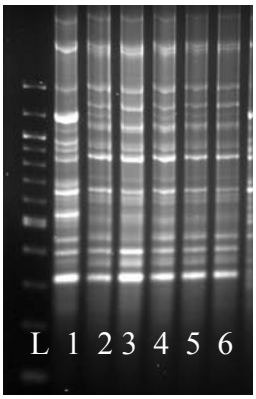


Figure 1.5 This rep-PCR fingerprint pattern shows isolates collected from one farm from 2004 through 2007. In 2004 (1, 2) there were two patterns, after that year the same pattern was seen each year. 2005 (3) 2006 (4, 5) 2007 (6).

Fingerprint patterns 5 and 6 were identified in isolates collected in 2005, 2006 and pattern five was found again in 2007. Fingerprint pattern 1 collected from four different counties in the state were all the same cabbage cv. Two Xcc isolates from seed samples of the same cultivar also matched this pattern (Figure 1.6).

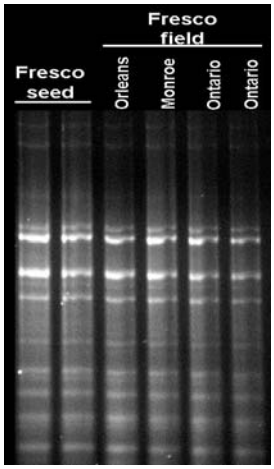


Figure 1.6 Rep-PCR fingerprints of Xcc collected from 'Fresco' cabbage seed and from field samples in three NY counties in 2004.

The patterns did not cluster according to geographic area except for pattern 2 which came from the same farm in four subsequent years, and patterns 5 and 6 which were only isolated from a greenhouse transplant facility. The two new patterns (9 and 10) seen in 2006 were very widespread and accounted for 79% of the isolates collected that year, with the remaining number being repeated from previous years. Not surprisingly, a storage cabbage with black rot symptoms collected in 2007 but grown in 2006 also had the pattern number ten. In 2008, the isolates from bare root transplants from a commercial seedbed in Georgia all had the same fingerprint (12). Two isolates collected from the seedbed the transplants were started in also had this fingerprint as did two field isolates collected at the end of the growing season (Figure 1.7).

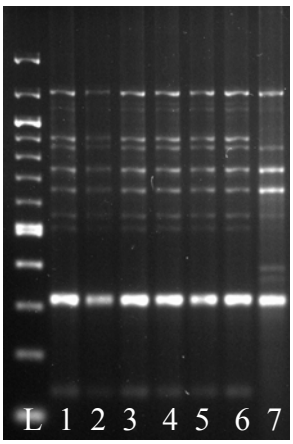


Figure 1.7 2008 rep-PCR fingerprint pattern 12. L is 100bp ladder as a marker, isolates from seedbed (1,2), isolates from bare root transplants from the seedbed (3-5), isolates from NY fields in Sept and Oct (6,7).

Fifteen of the sixteen isolates collected from three different greenhouse transplant facilities in 2009 fell into two fingerprint patterns. The isolates 0927 and 0928 were from transplants showing leaf spot symptoms, while 0927B and 0928B were isolated from V-shaped lesions. All four were rep-PCR pattern16. The 2009 isolates collected from four states had the most diverse Xcc population of our

sampling period. Overall, there was a great diversity in the population of Xcc in NY in our collection of isolates over this six year period.

Table 1.1 Summary of isolates ELISA, pathogenicity and rep-PCR pattern results

			ELISA		Pathogenicity		
Year	Xcc	Xca	Xcc/Xca	Neither	V-shaped[a]	Spot	rep-PCR patterns
2004	23	1	0	1	27	0	4
2005	12	3	3	0	8	12	6
2006	35	4	2	2	37	10	4
2007	8	0	0	2	7	3	3
2008	20	8	0	1	26	1	5
2009	20	0	3	0	21	6	6

[a] numbers may not agree as some isolates were not included in totals

Race differentiation. The isolates from 2004 were inoculated into a series of differential crucifer cvs in the greenhouse to identify the pathogenic variants or races, found in the NY samples based on the resulting interactions. Sixteen of the 2004 isolates tested caused vascular infections in all of the differentials except Florida Broad-leaf mustard indicating that they were race 1. Neither 0483 nor 04128 caused V-shaped lesions on Seven Top turnip, Just Right turnip or Florida Broad-leaf mustard, while each produced lesions on the Savoy cabbage and Miracle cauliflower. This indicates 0483 and 04128 belong to race 4. The three remaining isolates (0454, 04102, and 04105) appear to be race 3 as they were the only ones that did not cause V-shaped lesions on the Miracle cauliflower which is resistant to race 3 Xcc.

Table 1.2 Isolates collected over six years (2004-2009) from crucifer field samples with isolate number, cultivar, county collected from, greenhouse pathogenicity, ELISA results, source of plant material, and the collection date.

Isolate #	Variety	State or NY County	Greenhouse Infection	ELISA[a]	Box PCR	Source	Collection Date
0407	Fresco	Orleans	V-shaped	Xcc	1	transplant	5/28/2004
0419	red	Erie	V-shaped	Xcc	4	field	7/1/2004
0446	Amtrak	Ontario	V-shaped	Xcc	2	field	8/10/2004
0450	cauliflower	Orleans	V-shaped	Xcc	2	field	8/6/2004
0453	Fresco	Ontario	V-shaped	Xcc	1	field	8/16/2004
0454	Fresco	Ontario	V-shaped	Xcc	3	field	8/16/2004
0455	Fresco	Monroe	V-shaped	Xcc	1	field	8/12/2004
0458	red	Erie	V-shaped	Xcc	4	field	8/17/2004
0470	Kaitlin	Yates	V-shaped	Xcc	3	field	8/19/2004
0471	unknown	Yates	V-shaped	Xcc	3	field	8/19/2004
0473	Fresco	Yates	V-shaped	Xcc	1	field	8/25/2004
0474	cauliflower	Orleans	V-shaped	Xcc	3	field	8/23/2004
0483	unknown	Niagara	V-shaped	Xcc	3	field	9/1/2004
0484	Amtrak	Genesee	V-shaped	Xcc	2	field	9/1/2004
0489	unknown	Monroe	V-shaped	Xcc	2	field	9/1/2004
04102	unknown	Schoharie	V-shaped	Xcc	4	field	9/14/2004
04103	unknown	Schoharie	V-shaped	Xcc	3	field	9/14/2004
04105	unknown	Albany	V-shaped	Xcc	3	field	9/14/2004
04107	unknown	Schoharie	V-shaped	Xcc	3	field	9/14/2004
04109	F-118	Schoharie	V-shaped	Xcc	2	field	9/14/2004
04110	F-207	Schoharie	V-shaped	Xcc	2	field	9/14/2004
04117	Kaitlin	Ontario	V-shaped	Xcc	3	field	9/23/2004
04118	unknown	Yates	V-shaped	Xcc	2	field	9/23/2004
04125	unknown	Orleans	V-shaped	Xca	3	field	10/25/2004
04128	Amtrak	Orleans	V-shaped	nd	2	field	10-28-2004
1991E3	Fresco	Colorado	V-shaped	nd	1	STA labs	10-29-2004
1991E5	Fresco	Colorado	V-shaped	nd	1	STA labs	10-29-2004
0515	Brutus	Ontario	necrotic spot	Xcc	5	transplants[b]	6/24/2005
0516	Trans Am	Ontario	necrotic spot	Xca	6	transplants	6/24/2005
0518	cauliflower	Erie	V-shaped	Xca	7	field	7/1/2005
0522	Super Elite	Ontario	necrotic spot	Xcc	5	transplants	7/1/2005
0523	Trans Am	Ontario	necrotic spot	Xca	6	transplants	7/1/2005
0537	Rushmore	Ontario	necrotic spot	Xcc	6	greenhouse[c]	7/18/2005
0538	BL 10 broc	Ontario	necrotic spot	Xcc	6	greenhouse	7/18/2005
0539	Cheddar	Ontario	necrotic spot	Xcc	6	greenhouse	7/18/2005
0540	Graffiti	Ontario	necrotic spot	Xcc	6	greenhouse	7/18/2005
0541	Cassius	Ontario	necrotic spot	Xcc	6	greenhouse	7/18/2005
0557	Moreton	Ontario	V-shaped	Xcc	8	field	8/1/2005
0575	red	Ontario	V-shaped	Xcc	8	field	8/17/2005
0576	Brutus	Ontario	V-shaped	Xcc	7	field	8/19/2005
05108	red	Erie	V-shaped	both	7	field	9/22/2005
05109	Cheddar	Erie	V-shaped	both	7	field	9/22/2005

[a] nd: not determined [b] transplants: collected from field, [c] greenhouse: transplants in greenhouse

Table 1.2 (Continued).

Isolate #	Variety	State or NY County	Greenhouse Infection	ELISA	Box PCR	Source	Collection Date
05110	Super Elite	Erie	V-shaped	both	7	field	9/22/2005
05115	cauliflower	Orleans	V-shaped	Xcc	2	field	10/21/2005
0606	Kaitlin	Ontario	necrotic spot	Xcc	6	greenhouse	5/25/2006
0607	Kaitlin	Ontario	necrotic spot	Xcc	6	greenhouse	5/25/2006
0608	Amtrak	Ontario	necrotic spot	Xcc	6	greenhouse	5/25/2006
0609	Amtrak	Ontario	necrotic spot	Xcc	6	greenhouse	5/25/2006
0611	Amtrak	Ontario	necrotic spot	neither	5	greenhouse	6/1/2006
0656	unknown	Ontario	V-shaped	Xcc	9	field	8/8/2006
0657	unknown	Ontario	V-shaped	Xcc	9	field	8/8/2006
0659	Avalanche	Orleans	V-shaped	Xcc	2	field	8/9/2006
0660	Franklin bs	Orleans	V-shaped	Xcc	2	field	8/9/2006
0663	Megaton	Yates	V-shaped	Xcc	10	field	8/11/2006
0668	cauliflower	Ontario	V-shaped	Xca	9	field	8/14/2006
0671	2576	Ontario	V-shaped	Xca	10	field	8/15/2006
0682	Rotunda	Orleans	V-shaped	Xcc	9	field	8/23/2006
0683	Rotunda	Orleans	necrotic spot	Xcc	9	field	8/23/2006
0684	Rotunda	Orleans	necrotic spot	Xcc	9	field	8/23/2006
0685b	Paradox	Ontario	V-shaped	Xcc	10	field	8/28/2006
0685s	Fresco	Ontario	V-shaped	Xcc	10	field	8/28/2006
0695	red	Monroe	V-shaped	Xcc	10	field	8/30/2006
0696	unknown	Monroe	V-shaped	Xca	10	field	8/30/2006
0697	Amtrak	Monroe	V-shaped	Xcc	9	field	8/30/2006
06109	unknown	Ontario	V-shaped	neither	10	field	9/1/2006
06111	unknown	Ontario	V-shaped	Xcc	10	field	9/1/2006
06113	Bobcat	Ontario	V-shaped	Xcc	9	field	9/5/2006
06118	unknown	Orleans	V-shaped	Xcc	2	field	9/11/2006
06119	broccoli	Orleans	V-shaped	Xcc	2	field	9/11/2006
06129	cauliflower	Ontario	V-shaped	Xcc	10	field	9/18/2006
06130	cauliflower	Ontario	V-shaped	Xcc	9	field	9/18/2006
06131	Mentor	Monroe	necrotic spot	Xcc	10	field	9/21/2006
06132	Mentor	Monroe	V-shaped	Xcc	10	field	9/21/2006
06133	Mentor	Monroe	V-shaped	Xcc	10	field	9/21/2006
06143	ECBE	Wayne	V-shaped	Xca	10	field	9/25/2006
06148	unknown	Orleans	V-shaped	Xcc	10	field	9/28/2006
06149	unknown	Orleans	V-shaped	Xcc	10	field	9/28/2006
06150	unknown	Orleans	V-shaped	Xcc	10	field	9/28/2006
06151	Br sprouts	Yates	V-shaped	Xcc	10	field	10/3/2006
06152	unknown	Orleans	V-shaped	Xcc	10	field	10/9/2006
06153	unknown	Orleans	V-shaped	Xcc	10	field	10/9/2006
06154	unknown	Orleans	V-shaped	both	10	field	10/9/2006
06155	unknown	Orleans	V-shaped	Xcc	10	field	10/9/2006
06159	Ancoma	Wayne	necrotic spot	both	10	field	10/26/2006

Table 1.2 (Continued).

Isolate #	Variety	State or NY County	Greenhouse Infection	ELISA	Box PCR	Source	Collection Date
06164	Storage 4	Orleans	V-shaped	Xcc	9	field	10/31/2006
06166	Storage 4	Orleans	V-shaped	Xcc	9	field	10/31/2006
06167	Storage 4	Orleans	V-shaped	Xcc	9	field	10/31/2006
0701	Superstor	Ontario	V-shaped	Xcc	10	field	2-28-2007
0707	Mentor	Ontario	necrotic spot	neither	5	greenhouse	5-7-2007
0718	Huron	Ontario	necrotic spot	neither	5	greenhouse	7-7-2007
0719	Super Elite	Ontario	necrotic spot	Xcc	11	greenhouse	7-7-2007
0792	broccoli	Orleans	V-shaped	Xcc	2	field	10-10-2007
0793	cabbage	Orleans	V-shaped	Xcc	2	field	10-10-2007
0794	Trans Am	Orleans	V-shaped	Xcc	9	field	11-7-2007
0795	Trans Am	Orleans	V-shaped	Xcc	9	field	11-7-2007
0796	red storage	Orleans	V-shaped	Xcc	9	field	11-7-2007
0797	red storage	Orleans	V-shaped	Xcc	9	field	11-7-2007
Ga08159	unknown	Georgia	V-shaped	Xcc	12	seedbed	5-13-2008
Ga08137	unknown	Georgia	V-shaped	Xcc	12	seedbed	5-13-2008
08050	Cairo	Orleans	V-shaped	Xcc	12	bare root	5-10-2008
08054	Cairo	Orleans	V-shaped	Xcc	12	bare root	5-10-2008
08055	Cairo	Orleans	V-shaped	Xcc	12	bare root	5-10-2008
08056	Cairo	Orleans	V-shaped	Xcc	12	bare root	5-10-2008
08057	Cairo	Orleans	V-shaped	Xcc	12	bare root	5-10-2008
08092	Cheers	Orleans	V-shaped	Xcc	12	bare root	5-13-2008
08275	Fresco	Orleans	V-shaped	Xca	10	field	8-27-2008
08276	Amtrak	Orleans	V-shaped	Xcc	10	field	8-27-2008
08278	cabbage	Erie	V-shaped	Xca	10	field	9-10-2008
08279	cabbage	Genesee	V-shaped	Xcc	10	field	9-10-2008
08283	cabbage	unknown	V-shaped	Xcc	12	field	9-10-2008
08284	broccoli	Orleans	V-shaped	Xcc	2	field	9-10-2008
08288	cabbage	Ontario	V-shaped	Xcc	10	field	9-17-2008
08289	Kaitlin	Ontario	V-shaped	Xcc	13	field	9-17-2008
08290	Megaton	Yates	V-shaped	Xcc	10	field	9-19-2008
08292	Moreton	Yates	V-shaped	Xcc	10	field	9-19-2008
08294	organic	Ontario	V-shaped	Xcc	13	field	9-23-2008
08295	cabbage	Ontario	V-shaped	Xca	14	field	9-23-2008
08296	transplant	Florida	V-shaped	Xca	14	transplants	9-23-2008
08299	Trans Am	Monroe	V-shaped	Xcc	12	field	10-13-2008
08300	Kaitlin	Monroe	V-shaped	Xca	14	field	10-13-2008
08301	Amtrak	Monroe	V-shaped	Xca	15	field	10-13-2008
08302	Superstar	Monroe	V-shaped	Xcc	15	field	10-13-2008
08303	Fresco	Monroe	V-shaped	neither	15	field	10-13-2008
08304	Huron	Monroe	V-shaped	Xca	14	field	10-13-2008
08305	cabbage	Genesee	V-shaped	Xca	14	field	10-13-2008
08306	Bronco	Orleans	V-shaped	Xcc	2	field	10-15-2008

Table 1.2 (Continued).

Isolate #	Variety	State or NY County	Greenhouse Infection	ELISA[a]	Box[a] PCR	Source	Collection Date
09002	Megaton	Ontario	necrotic spot	both	18	greenhouse	5-8-2009
09003	Megaton	Ontario	necrotic spot	Xcc	17	greenhouse	5-8-2009
09008	Megaton	Michigan	V-shaped	Xcc	16	greenhouse	5-11-2009
09009	Megaton	Georgia	V-shaped	Xcc	16	greenhouse	5-19-2009
09010	Megaton	Georgia	necrotic spot	Xcc	17	greenhouse	5-19-2009
09011	Megaton	Georgia	V-shaped	Xcc	16	greenhouse	5-19-2009
09012	Megaton	Ontario	V-shaped	Xcc	nd	greenhouse	5-20-2009
09013	Megaton	Ontario	necrotic spot	both	17	greenhouse	5-20-2009
09015	Jubilee	Georgia	necrotic spot	Xcc	17	greenhouse	6-1-2009
09017	Megaton	Georgia	V-shaped	nd	16	greenhouse	6-1-2009
09018	Megaton	Michigan	V-shaped	Xcc	16	greenhouse	6-1-2009
09024	Bravo	Georgia	V-shaped	Xcc	19	08 crop	6-5-2009
09026	Megaton	Georgia	necrotic spot	Xcc	17	greenhouse	6-12-2009
09027	Megaton	Michigan	V-shaped	Xcc	16	greenhouse	6-12-2009
09027B	Megaton	Michigan	V-shaped	Xcc	16	greenhouse	6-12-2009
09028	Milestone	Michigan	V-shaped	Xcc	16	greenhouse	6-12-2009
09028B	Milestone	Michigan	V-shaped	Xcc	16	greenhouse	6-12-2009
09075	Moreton	Ontario	V-shaped	Xcc	20	field	7-16-2009
09114	Apex cauliflr	Onondaga	V-shaped	Xcc	20	field	8-12-2009
09121	Moreton	Ontario	V-shaped	Xcc	20	field	8-18-2009
09122	Moreton	Ontario	V-shaped	Xcc	20	field	8-18-2009
09139	cabbage	Ontario	V-shaped	Xcc	20	field	9-16-2009
09144	Diplomat broc	Pennsylvania	V-shaped	Xcc	20	field	9-17-2009
09146	Amazing caul	Pennsylvania	V-shaped	Xcc	16	field	9-22-2009
09147	cabbage	Monroe	V-shaped	Xcc	16	field	9-25-2009
09148	cabbage	Monroe	V-shaped	both	21	field	9-25-2009

[a] nd not determined

Discussion

A severe outbreak of black rot in central New York State in 2004 initiated the collection of isolates from infected fields. The 2004 growing season was wet and warm with wind driven rains, ideal conditions for Xcc to multiply and spread and cause black rot epidemics (Figure 1.8). In the month of July, over 98 mls of rain fell,

another 49 mls in August, and almost 82 additional mls by mid- September. Most of the samples were collected in August and September when fields of crucifers were

Figure 1.8 Black rot infected cabbage field August, 2004.

showing severe black rot symptoms. Four distinct fingerprint patterns were elucidated using rep-PCR, with no apparent grouping by region of the state. The isolates did appear to group by cv in one case; all but one of the isolates from the cabbage cv 'Fresco' had the fingerprint pattern 1 and two Xcc isolates from a seed lab testing Fresco seed also had this fingerprint (Figure 1.6). This would seem to implicate the seed as the source of inoculum in this instance.

The ELISA results were not always consistent with pathogenicity assays, an Xcc positive result causing a vascular infection, and conversely, an Xca result caused a leaf spotting reaction. A similar result was previously reported in a study using monoclonal antibodies to identify pathogens by pathovar, Xcc and Xca were not serologically distinguished in some cases (Alvarez et al. 1994). Some of the isolates reacted with both Xcc and Xca antigens, and several didn't react with either yet showed necrotic spots upon inoculation. For most of the NY isolates, an Xcc positive ELISA test correlated with V-shaped lesions when tested on cabbage in the greenhouse. The exceptions to this were the 2005 isolates 0518, 05108, and 05109

(Table 1.2), which were very virulent in pathogenicity tests yet were not clearly Xcc by ELISA after repeated testing. These isolates were all rep-PCR fingerprint pattern 7. Among the isolates collected from greenhouse transplant facilities, both Xcc and Xca positives resulted in a necrotic spot reaction in greenhouse pathogenicity tests for all years except 2009. These isolates fell into two rep-PCR fingerprints (Table 1.2, 5 and 6) and were found in 2005, 2006, and 2007, always associated with transplant facilities and never causing vascular infections. The seedlings in these facilities are scrutinized at all times by scouts and managers, and their vigilance and management tools controlled these outbreaks soon after they were discovered. This was not the case in 2009 when greenhouse isolates indicated Xcc with ELISA and ten of the sixteen isolates caused V-shaped lesions when inoculated back into cabbage. Initially, all of the isolates came from one cabbage cv. This was the only year that vascular infections resulted from inoculations of greenhouse isolates. With one exception, all of the isolates which caused a necrotic spot had the same rep-PCR fingerprint pattern 17 (Table 1.2).

Only one isolate from 2005 had a rep-PCR pattern identical to an isolate from 2004, and four new patterns were identified in 2005 following the 2004 black rot epidemic. This indicates that while Xcc can over winter in NY, new isolates enter the state each year. In 2006, twice as many isolates were collected as in the previous two years. Two new fingerprint patterns emerged, and three were repeats. The repeated patterns were over-wintering or being reintroduced the following year. In this year, for the first time, four isolates collected from fields showed only necrotic spots when put into greenhouse cabbage. In the previous two years all field isolates had caused vascular infection when inoculated into plants for pathogenicity testing.

The 2007 growing season saw very little black rot infection. Only one new rep-PCR pattern was identified in strains collected this year, all the others were

repeated from previous years. A few field isolates (Table 1.2 patterns 2, 9, and 10) were carried over from year to year implicating lapses in rotations, destruction of plant debris, sanitation of equipment, weed control or reintroduction of the same strain. Fingerprint 2 was identified from a farm in Orleans County, NY (Figure 1.6). The farmer had a convenient seedbed location and he did not rotate from this area. The fact that the samples from various types of crucifers started in this bed for five consecutive years all had the same fingerprint pattern incriminates the seedbed as the inoculum source. Once he abandoned this site, the farm was free of black rot the following growing season.

2008 began with bare root cabbage transplants of many different cvs with black rot symptoms coming into New York. A field infection in the seedbed area in Georgia where the transplants were grown was implicated as the inoculum source based on cultures isolated from this field (Table 1.2 Ga 159 and Ga 137) which had the same rep-PCR fingerprint (pattern 12). Isolates collected from symptomatic plants late in the season were also found to have pattern 12 indicating that the pathogen survives in the xylem of the plant until conditions are favorable for disease expression (Figure 1.7). One isolate with fingerprint pattern 12 was collected from a hail damaged field next to a field planted with Georgia transplants. Hot and dry weather after these cabbage seedlings were transplanted, and a growing season of even moisture probably kept the disease from becoming more widespread that year.

Sixteen of the 24 isolates tested with a set of *Brassica* differentials in 2004 were race 1. All four rep-PCR fingerprint patterns (1-4) were represented in this race. There were two isolates; 0483 and 04128, which were identified as race 4. These were both collected in western NY and had fingerprint patterns different from each other. The remaining three isolates had differential results of race 3, two of these had the same fingerprint pattern, and the other was different. Races 1 and 4 are important in

cabbage and cauliflower, and the predominant races found in the United States (Vincente et al. 2001). There did not appear to be differences in pathogenicity or virulence based on the race of the isolates as determined by the differentials.

In the six years of this population study, we found 21 rep-PCR fingerprint patterns in the Xcc isolates collected from across NY. There was a wide variation of strains across the state and more importantly, new strains were identified each year. While the rate at which new rep-PCR fingerprints develop is unknown, the fact that an isolate with the same pattern was identified in a field for five years provides important evidence that new fingerprints do not develop within a strain over the course of a single season (or even several seasons) in NY.

This signifies that while crop residue and weeds can harbor the pathogen, these inoculum sources do not appear to be a primary one in NY. The pathogen can over-winter and reside on-farm and it can also arrive on seeds or transplants. This offers implications for disease management, as growers now know that it is very important to reduce Xcc on seed by purchasing only hot water treated seed. Additionally, if Xcc is suspected in transplant facilities, removal of symptomatic plants and copper treatment is critical to reduce the chance of a black rot epidemic in the field.

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CHAPTER 2

CRUCIFEROUS WEEDS AS RESERVOIRS OF *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* IN NEW YORK STATE

Abstract

The relative role of weeds as a source of the pathogen *Xanthomonas campestris* pv. *campestris* (Xcc) in New York is unknown. In other locations cruciferous weeds have been shown to harbor Xcc, contributing to field infections of black rot. In 2004, there was a severe outbreak of black rot in western and central NY. To determine if weeds serve as a pathogen reservoir for subsequent seasons in NY, five fields that had a significant black rot problem in 2004 were chosen for the study in 2005. Weed species were collected every two weeks, a total of seven times, throughout the spring and summer from each site. Xcc-like bacteria were isolated from sampled cruciferous weeds from all five sites, but non-cruciferous weeds were not found to be hosts of Xcc. Most of the isolates were collected from two weed species; wild mustard (*Brassica kaber*) and Shepherd's purse (*Capsella bursa-pastoris*). DNA fingerprint analysis, using repetitive element (rep)-PCR, revealed that all bacteria isolated from weeds in 2005 were different from those isolated from cabbage in the same field in 2004. Greenhouse grown cabbage seedlings were inoculated with each of the bacterial isolates collected from weed species in 2005, and all isolates were non-pathogenic. Isolates were further analyzed using enzyme-linked immunosorbent assay (ELISA) and internal transcribed spacer region (rDNA) sequencing to determine the species and pathovar and multiple *Xanthomonads* were identified.

In 2006 and 2007, a weed collection from fields planted with crucifer crops was undertaken. Of the isolates collected from weeds in 2006 (37) and 2007 (55), six caused V-shaped lesions when inoculated into cabbage seedlings in the greenhouse. One of these came from a field which was infected with black rot. The other was

collected from the edge of a soybean field on a farm that also raised crucifer crops. This study indicates that while cruciferous weeds may be infected with Xcc, or Xcc-like bacteria, they do not appear to serve as a primary source of Xcc inoculum for black rot infections in subsequent years in NY.

Introduction

Black rot disease caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) is a serious and costly disease affecting crucifers grown world-wide (Williams 1980). This plant pathogen can infect a wide range of crucifers, both crop and weed species (Kuan et al. 1986; Schaad and Dianese 1981) at any stage of growth. The bacterium is seed borne (Cook et al. 1952; Schaad et al. 1980; Walker and Tisdale 1920), which enables the pathogen to spread long distances as infected transplants. Short distance dispersal is limited to approximately 12 meters (Schaad and Dianese 1981), and is facilitated by wind blown rain and bacteria blown from dead leaf tissue (Kuan et al. 1986). The Xcc bacterium enters the plant through hydathodes, gaining access into the leaf in the guttation fluid (Hugouvieux et al. 1998; Smith 1911) which creates an ideal environment for the bacteria to multiply and move through the vascular tissues of the plant. This mode of entry results in the typical yellow to tan V-shaped lesions originating on the edge of the leaf and extending into the veins which become blackened. The bacterial colonization leads to blockage of water and nutrient movement through the xylem (Sutton and Williams 1970). The pathogen can also gain entry through wounds caused by insect feeding, hail, or cultivation damage to the plant. The Xcc bacteria can survive epiphytically for long periods of time on plant surfaces protected from the environment by their pigmentation and mucilaginous extracellular polysaccharide (Leben 1981).

The *Brassicaceae* (Mustard) family has been cultivated for centuries. Most of the crop species belong to the genus *Brassica*. The species of crucifers that we

consider weeds in and around our cultivated fields have had a long association with the crop plants, most became naturalized weeds after escape from cultivation (Al-Shehbaz 2002). Cruciferous weeds are abundant and successful in NY (Fig. 2.1) as well as around the world. In Europe, cruciferous weed species were unintentionally spread as people traveled along trade routes, planting crop seed that

Figure 2.1 A field planted with small Christmas trees over-run with rape mustard across the road from cabbage fields on April 28, 2006.

was contaminated with weed seed, however cruciferous weeds were intentionally planted to mark the routes between missions in the 1800s in California (Ignatov et al. 2007). *Capsella bursa-pastoris* (shepherd's purse),the second most common weed on earth (Al-Shehbaz 2002), was cultivated as a medicinal herb (Defelice 2001), and *Brassica nigra* (black mustard) was a major mustard crop in the United States until the 1950s (Westman et al. 1999). Other mustard species are used as a cover crop for weed control in many organic systems (Kristiansen 2006), and cruciferous weeds have also been used as a trap crop to limit damage to crops from insect pests (Badenes-Perez et al. 2005). Most cruciferous weeds have small seeds which persist for long periods of time even when deeply buried in the soil. They can become established in both cultivated and non-cultivated areas, flower over many months, and have multiple generations per year. They often overwinter as rosettes, and in the case of the common

New York weed, *Barbarea vulgaris* (yellow rocket), can produce between 1000 and 10,000 seeds in a season (Uva et al. 1997). The weeds often emerge and flower before spring cultivation (Fig. 2.2).

Figure 2.2 Flowering wild mustard in cabbage debris in May.

It is a challenge to chemically control cruciferous weeds in fields of closely related crops, and there are very few new herbicides being developed for vegetables despite their high value. Vegetables are considered a specialty or minor crop due to their significantly lower acreage compared to agronomic crops (Fennimore and Doohan 2008). New herbicides must also be tested thoroughly as they can alter plant physiology, changing the susceptibility of crop plants to pathogens (Wisler and Norris 2005). Weeds are likely to be genetically more diverse than crop plants, and selection over time enables them to survive diseases and other crop pests (Schroeder et al. 2005). In a study by Westman et al., (1999), a large weed collection was challenged with Xcc by both mist and wound inoculation. The results of the disease reactions varied between species, within a species, and even between entries. Most weeds do not show black rot symptoms (Dane and Shaw 1996; Morris and Knox-Davies 1980; Schroeder et al. 2005; Wisler and Norris 2005), yet studies have shown that Xcc can survive both epiphytically and endophytically on many weed species (Dane and Shaw

1996; Kuan et al. 1986) and these bacteria are more successful colonizers on weed species closely related to their crop hosts (Wilson and Hirano 1999).

Weeds have the potential to serve as a source of Xcc inoculum for many crucifer crops. The role of Xcc in weeds initiating crop infections has been studied in several areas of the United States. Schaad and Dianese (1981) found that many cruciferous weed species were in, and near to, crop fields in Georgia which were infected with Xcc. They determined that an infected weed spread inoculum up to 12 m from a focus of infection, and that Xcc could be isolated from seeds collected from infected weeds. They did not find Xcc in samples of non-cruciferous weeds (Schaad and Dianese 1981). Expanding the study to California, Schaad and Dianese collected weeds from areas distant from crop production areas and were able to isolate Xcc from cruciferous weeds. In a study of Xcc isolates collected from cruciferous weed species in CA, (Ignatov et al. 2007), found that the strains isolated from weeds in crop producing areas were genetically distinct from those in non-producing sites. The dynamic interaction among Xcc, crucifer crops, and closely related weeds provides information to help identify sources of inoculum during the growing season and for subsequent years. This study was undertaken to provide information about the relative role of weeds as hosts and inoculum sources in the epidemiology of Xcc in NY.

Materials and Methods

Plant Materials and Isolate collection. Five sites which had severe black rot infections in cabbage during the 2004 growing season were chosen for bi-weekly sampling in 2005. A total of 77 weeds were collected over the seven sampling dates. During the first two collections, weeds were found and collected in the fields as well as in the border areas, but as the season progressed it was rare to find weeds within fields and collection was limited to the edges of the fields (Table 2.1). Leaf samples

from collected weeds were surface sterilized for 3 min with a 0.6 % NaOCl solution. The majority of weeds collected were healthy with no visible signs of disease, however if there was chlorotic or necrotic tissue these areas were used for isolation. Tissue was ground using a mortar and pestle with sterile water. An inoculation loop dipped into the resulting liquid was used to streak colonies onto King's B medium (King et al. 1954; Wilson et al. 1967). Yellow non-fluorescent colonies were plated on YDC medium (Wilson et al. 1967) or *X. campestris* semi-selective SM medium (Chun and Alvarez 1983).

In 2006 and 2007, cruciferous weeds were sampled from area fields rotating into or out of cabbage. Thirty seven samples from thirteen fields were collected in 2006 on six dates between April 25 and November 15. In 2007, two sites were repeated from 2006, and 16 more sites were sampled with 55 weeds sampled from April 19 until Aug 20. Weed species collected were predominantly shepherd's purse, wild mustard (*Brassica kaber*), yellow rocket, and pennycress (*Thaspi arvense*); additionally wormseed mustard (*Erysimum cheiranthoides*), wild radish (*Raphanus sativus*), and bittercress (*Cardamine hirsuta*) were occasionally found.

ELISA antigen test. Enzyme-linked immunosorbent assay (ELISA) analysis was performed using the Agdia (Elkhart, IN) bacterial reagent sets to differentiate between pathovars. Following the manufacturer's protocol, reagents for Xcc and *X. campestris* pv. *armoraciae* (Xca, a closely related pathovar) were used to identify isolates that could potentially cause disease on cabbage. Results were recorded for the isolates from weed samples as well as positive and negative controls.

Inoculation test for pathogenicity. *B. oleraceae* cultivar 'Gonzales' was grown in the greenhouse with 16 hours of light at 24ºC. When the seedlings had three true leaves, a pin dipped into a 24 hour old bacterial culture was pricked into the outer margin of one leaf eight times and also into the petiole and midrib of a second true leaf on the

same plant (Vincente et al. 2001). The third leaf was left uninoculated. Two plants per isolate were inoculated and symptoms were scored visually 14 days after inoculation with a V-shaped yellow to brown lesion along the leaf margin with vein blackening a positive pathogenicity result.

Rep-PCR. Repetitive element polymerase chain reaction (rep-PCR) fingerprinting was carried out following the previously described protocol (Louws et al. 1998) using a PTC-100 Peltier thermal cycler (MJ Research, Waltham, MA)**.** Amplification was carried out in 0.2 ml thin-walled tubes. The mixture consisted of 5µl 5x Gitchster buffer, 0.2 µl BSA 20mg/ml, 2.5 µl 100% DMSO, 1.25 µl 25mM dNTP, 1 µl 100mM box primer, 13.65 µl water, 0.4 µl *Taq* DNA polymerase in a total volume of 24µl (Louws et al, 1998).Box A1R primer (5'- CTA CGG CAA GGC GAC GCT GAC G-3') (Massomo et al. 2003) was purchased from Integrated DNA Technologies Inc. Bacterial cells were grown overnight in nutrient broth, the OD 600 adjusted to 0.1, and 12 µl was added to the 24 µl mix. Extracted genomic DNA was alternatively used at 100ng per reaction, and the program run for 35 cycles of 95ºC for 2minutes, 94 ºC for 3 s, 92 ºC for 30 s, 53 ºC for 1 min, 65 ºC for 8 min followed by 65 ºC for 8 min and held at 4 ºC (de Bruijn et al. 1996). The PCR amplicons (8 µl) were loaded onto a 1.5% agarose gel with ethidium bromide, using 0.5X TAE buffer and run at 4ºC for 18 hours at 67V. Banding patterns were viewed under ultraviolet light using a Kodak Gel Logic C200 imaging system (Carestream Molecular Imaging, Rochester, NY).

rRNA Internal Transcribed Spacer Region Sequencing. PCR products of the 21 isolates which grew on SM medium were sequenced using ITS primers G1 (5' GAAGTCGTAACAAGG 3') and L1 (5' CAAGGCATCCACCGT 3')(Jensen et al. 1993). A 50 µl mix consisted of: 5 µl 10X buffer, 1 µl 10 mM dNTPs, 1 µl 50µM G1, 1 µl 50µM L1, 41.8 µl water, 0.2 µl *Taq* DNA polymerase, and 4 µl of an overnight liquid bacterial culture that was boiled for 5 min. The PCR program ran for 25 cycles

after an initial 94 ºC for 3 min: 94 ºC for 1 min, 55 ºC for 2 min, 72 ºC for 2 min, followed by 72 ºC for 7 min. Bands were visualized on a 0.5% agarose gel with EtBr with a UV light after running for one hour at room temperature. Sequences of the amplicons were obtained from Cornell Life Sciences Core Laboratories.

Results

Weed collections. In 2005, bacterial isolations were attempted from each of the 77 weeds collected. All weed species were initially sampled but only cruciferous weeds were found to harbor potential *X. campestris* pathovars based on growth on YDC and SM media. Therefore the study focused on these 56 weeds (Table 2.1). Of the 56 cruciferous weeds collected from the five sites, putative Xcc bacteria were isolated from 21 of the weeds based on growth on SM Xcc semi-selective medium.

In 2006, 37 cruciferous weeds were collected between April and Oct from 13 sites in five counties in NY. Previous crops at the sites were corn and soybeans and all fields were being planted to crucifers. Of the 37 weeds, 12 were further identified as potential *X. campestris* (Table 2.2) based on growth on SM medium.

Table 2.1 weed isolates collected from 5 sites in Ontario County, NY in 2005.

Sample[a] site 1	2005 Date	Weed species	KB[b] medium	SM[c] medium	ITS[d] sequence	Greenhouse[e] inoculation
1051	5/5	cabbage regrowth	yellow	-	n/d	n/d
1052	5/5	cabbage regrowth	white	-	n/d	n/d
1053	5/5	cabbage regrowth	yellow	-	n/d	n/d
1054	5/5	mustard	yellow	-	n/d	n/d
1055	5/5	mustard	yellow	-	n/d	n/d
1056	5/5	purselane	yellow	-	n/d	n/d
1057	5/5	cabbage regrowth	white	-	n/d	n/d
1058	5/9	cabbage regrowth	white	-	n/d	n/d
1059	5/9	shepherd's purse	yellow	+	Xcc	-
10510	5/9	mustard	yellow	+	Xcc	-
10511	5/26	cabbage regrowth	ng	n/d	n/d	n/d
10512	5/26	cabbage regrowth	ng	n/d	n/d	n/d
10513	5/26	plantain	ng	n/d	n/d	n/d
10514	5/26	pepperweed	yellow	+	n/d	-
10516	6/6	buttercup	yellow	-	n/d	-
10517	6/20	plantain	ng	n/d	n/d	n/d
10518	7/5	mustard	yellow	+	n/d	-
10519	7/19	plantain	yellow	-	n/d	-
site 2						
2051	5/9	plantain	white	n/d	n/d	n/d
2052	5/9	mustard	white	-	n/d	-
2053	5/27	mustard	ng	n/d	n/d	n/d
2054	5/27	mustard	yellow	+	Xcc	-
2055	5/27	mustard	ng	n/d	n/d	n/d
2056	6/6	mustard	ng	n/d	n/d	n/d
2057	6/6	mustard	yellow	+	n/d	-
2058	6/20	groundsel	yellow	+	X	-
2059	6/20	yellow rocket	yellow	+	X	-
20510	7/15	plantain	white	-	n/d	-
20511	7/19	yellow rocket	yellow	-	n/d	n/d
site 3						
3051	5/9	shepherd's purse	yellow	-	n/d	-
3052	5/27	plantain	ng	n/d	n/d	n/d
3053	5/27	ragwort	ng	n/d	n/d	n/d
3054	6/6	yellow rocket	yellow	+	Xcc	-
3055	6/6	mustard	yellow	+	n/d	-
3056	6/6	plantain	ng	n/d	n/d	n/d
3057	7/5	yellow rocket	yellow	+	n/d	-
3058	7/5	yellow rocket	ng	n/d	n/d	n/d
3059	7/19	field pepperweed	yellow	-	n/d	-

[a]Samples in red had an rDNA sequence identical to Xcc [b] colony growth on KB, ng is no growth n/d not determined [c] + indicates blue colonies with clearing on SM medium [d] n/d not determined [e] V-shaped lesion in inoculated greenhouse cabbage = +, no reaction = -.

Table 2.1 (Continued).

Sample[a]	2005 Date	Weed species	KB[b] medium	SM[c] medium	ITS[d] sequence	Greenhouse[e] inoculation
site 4						
4051	5/5	shepherd's purse	yellow	+	X	-
4052	5/9	shepherd's purse	yellow	+	Xcc	-
4053	5/26	mustard	yellow	+	Xcc	-
4054	5/26	mustard	yellow	+	Xcc	-
4055	6/6	yellow rocket	yellow	-	n/d	-
4056	6/20	yellow rocket	yellow	-	n/d	-
4057	7/19	shepherd's purse	yellow	+	Xcc	-
4058	7/19	pennycress	yellow	+	n/d	-
4059	8/23	weed	yellow	-	n/d	-
40510	8/23	sow thistle	yellow	-	n/d	n/d
40511	8/23	weed	yellow	-	n/d	n/d
site 5						
5051	5/5	mustard	white	-	n/d	n/d
5052	5/5	shepherd's purse	tan	-	n/d	n/d
5053	5/11	mustard	yellow	+	Xcc	-
5054	5/11	mustard	yellow	-	not done	-
5055	5/11	shepherd's purse	yellow	+	Xcc	brown circle
5056	5/11	mustard	yellow	-	n/d	n/d
5057	5/11	shepherd's purse	yellow	+	Xcc	-
5058	5/11	mustard	white	n/d	n/d	n/d
5059	5/26	mustard	yellow	+	Xcc	-
50510	6/6	shepherd's purse	yellow	-	na	-
50511	6/6	plantain	ng	-	na	-
50512	6/20	yellow rocket	white	-	n/d	n/d
50513	7/5	yellow rocket	ng	-	n/d	n/d
50514	7/19	yellow rocket	yellow	no	n/d	-

[a]Samples in red had an rDNA sequence identical to Xcc [b] colony growth on KB, ng is no growth n/d not determined [c] + indicates blue colonies with clearing on SM medium [d] n/d not determined [e] V-shaped lesion in inoculated greenhouse cabbage = +, no reaction = -.

In 2007, crucifer weeds were collected from 18 sites in two NY counties three times in the month of May (Table 2.3). A total of 55 samples were collected from fields that had been planted in cabbage the previous season, or were going to be in cabbage in 2007. One of the fields was known to have had black rot in 2006. Potential *Xanthomonas campestris* isolates were obtained from 17 of the 55 samples, based on growth on SM semi-selective medium. These 17 isolates were used in further studies (Table 2.3).

Table 2.2 Isolates collected from cruciferous weed species in 2006.

Site[a]	NY county	2006 date	Sample[b] #	Weed species	KB[c]	SM[d]	Greenhouse[e] inoculation
1	Wayne	4/28	1	bittercress	ng	-	n/d
		4/28	2	wild mustard	ng	-	n/d
		4/28	3	shepherds purse	yellow	-	-
2	Wayne	4/28	4	wild mustard	ng	-	n/d
		4/28	5	wild mustard	yellow	-	-
3	Wayne		pine trees	rape			
		4/28	6		lt yellow	-	-
4	Wayne		cabbage debris				
		4/28	7	cress	white		n/d
		4/28	8	rape	ng	-	n/d
5	Ontario						
		4/28	9	wild mustard	yellow	-	n/d
		4/28	10	shepherds purse	yellow	+	+
		10/14	20	shepherds purse	yellow	+	-
6	Ontario						
		4/28	11	wild mustard	yellow	-	-
7	Monroe	5/4	12	wild radish	yellow	+	-
		5/4	13	wild mustard	white	-	n/d
8	Ontario		cauliflower				
		5/4	14	pennycress	yellow	+	-
9	Erie	5/10	15	yellow rocket	yellow	-	-
10	Erie	5/10	16	yellow rocket	yellow	+	-
		5/10	17	pepperweed	yellow	+	-
		5/10	18	shepherd's purse	yellow	-	-
11	Ontario		yard edge				
		5/25	19	wild mustard	yellow	+	-
12	Genesee	10/31	21	cabbage shepherd's purse	ng	n/d	n/d
		10/31	22	wormseed must	ng	n/d	n/d
		10/31	23	sh purse	yellow	Xcc	+

[a] Sites were not the same as in 2005 [b] red were positive in greenhouse pathogenicity test [c] color and growth on Kings B medium, ng is no growth, fluor is fluorescent growth, [d] + indicates blue colonies with halo on SM medium, n/d not determined, [e] V-shaped lesions on inoculated cabbage positive (+), no reaction or necrotic spot negative (-), n/d not determined

Table 2.2 (Continued**).** Isolates collected from cruciferous weed species in 2006.

Site [a]	NY county	2006 Date	Sample [b] #	Weed species	KB [c]	SM [d]	Greenhouse [e] inoculation
12	Genesee	10/31	24	wormseed must	yellow	+	+
		10/31	25	wild mustard	ng	n/d	n/d
		10/31	26	wormseed must	yellow	+	-
		10/31	27	wormseed must	yellow	+	+
		10/31	28	yellow rocket	ng	n/d	n/d
		10/31	29	wild mustard	yellow	+	+
		10/31	30	wild mustard	ng	n/d	n/d
		10/31	31	wild mustard	ng	n/d	n/d
		10/31	32	wild mustard	ng	n/d	n/d
13	Erie	11/15	33	yellow rocket	yellow	+	-
		11/15	34	yellow rocket	yellow	+	-
		11/15	35	mustard	ng	n/d	n/d
		11/15	36	mustard	ng	n/d	n/d
		11/15	37	mustard	ng	n/d	n/d

[a] Sites were not the same as in 2005 [b] red were positive in greenhouse pathogenicity test [c] color and growth on Kings B medium, ng is no growth, fluor is fluorescent growth, [d] + indicates blue colonies with halo on SM medium, n/d not determined, [e] V-shaped lesions on inoculated cabbage positive (+), no reaction or necrotic spot negative (-), n/d not determined

Table 2.3 cruciferous weeds collected in 2007.

Site [a]	Location (County)	2007 Date	Sample [b] #	2007 Crop	2006 [c] Crop	Weed [d] Species	KB [e] Medium	GH [f] Inoculation
12	Genesee	4/19	12-1	border	cabbage	sh purse	yellow	-
		5/20	12-30	border	cabbage	y rocket	Yellow	-
		5/31	12-34	peas	cabbage	wmustard	white	n/d
14	Ontario	5/4	14-2	residue	cabbage	sh purse	yellow	-
		5/4	14-3	residue	cabbage	y rocket	yellow	-
		5/4	14-4	residue	cabbage	cabbage	yellow	-
		5/30	14-32	cabbage	corn	y rocket	yellow	-
15	Ontario	5/4	15-5	stubble	corn	pennycress	yellow	-
6	Ontario	5/4	6-6	residue	cabbage	sh purse	yellow	-
		5/4	6-7	residue	cabbage	y rocket	fluor	n/d
16	Genesee	5/10	16-8	cabbage	n/d	sh purse	yellow	+
		5/10	16-9	cabbage	n/d	y rocket	yellow	-
		5/10	16-10	cabbage	n/d	pepperw	fluor	n/d
		5/10	16-11	cabbage	n/d	pennycress	yellow	-
		7/10	16-40	cabbage	n/d	sh purse	flour	n/d
		7/10	16-41	cabbage	n/d	w radish	yellow	n/d
		7/10	16-42	cabbage	n/d	sh purse	yellow	n/d
		7/10	16-43	cabbage	n/d	w mustard	yellow	n/d
		7/10	16-44	cabbage	n/d	w mustard	ng	n/d
17	Genesee	5/10	17-12	alfalfa	alfalfa	sh purse	yellow	-
		5/10	17-13	border	alfalfa	w mustard	yellow	n/d
		5/10	17-14	border	alfalfa	t mustard	yellow	-
		7/10	17-35	cabbage	alfalfa	w mustard	yellow	n/d
18	Genesee	5/10	18-15	border	alfalfa	sh purse	white	n/d
		5/10	18-16	border	alfalfa	y rocket	yellow	-
		5/10	18-17	alfalfa	alfalfa	pennycress	yellow	n/d
19	Genesee	5/10	19-18	cabbage	n/d	sh purse	pink	n/d
		5/10	19-19	cabbage	n/d	y rocket	yellow	-
		5/10	19-20	border	n/d	pennycress	fluor	n/d
20	Genesee	5/10	20-21	fallow	cabbage	y rocket	ng	n/d
		5/10	20-22	fallow	cabbage	cabbage	fluor	n/d
		5/10	20-23	fallow	cabbage	pepperw	yellow	-
21	Genesee	5/10	21-24	fallow	beans	sh purse	yellow	-
		5/10	21-25	fallow	beans	pennycress	yellow	-
		5/10	21-26	fallow	beans	yr mustard	yellow	-
22	Genesee	5/10	22-27	fallow	beans	pennycress	yellow	-
		5/10	22-28	fallow	beans	sh purse	yellow	+
		5/10	22-29	fallow	beans	pepperweed	yellow	-
23	Ontario	6/25	23-36	cover cr	cabbage	pennycress	yellow	-
		6/25	23-37	cover cr	cabbage	sh purse	yellow	-
24	Genesee	7/6	24-38	cabbage	n/d	wsmustard	yellow	-
		7/6	24-39	cabbage	n/d	w mustard	yellow	-
25	Ontario	10/27	25-47	fallow	soybean	w mustard	ng	n/d
26	Ontario	10/27	26-48	wheat	cabbage	w mustard	ng	n/d
27	Ontario	10/27	27-49	cabbage	n/d	y rocket	white	n/d

[a] two sites repeated from 2006 (12 and 6), [b] red numbers caused V-shaped lesions in greenhouse cabbage, [c] n/d not determined [d] sh purse shepherd's purse, w mustard wild mustard, ws mustard wormseed, t mustard tumble, yr mustard yellow rape, y rocket yellow rocket, cabbage regrowth from stems in field, [e] color and colony growth on King's B medium, ng is no growth, fluor fluorescent growth, [f] V-shaped lesion on greenhouse cabbage positive (+), no reaction or necrotic spot negative (-)

Table 2.3 (Continued).

Site [a]	Location (County)	2007 Date	Sample[b] #	2007 Crop	2006[c] Crop	Weed[d] Species	KB[e] Medium	GH[f] Inoculation
28	Ontario	8/20	28-50	cabbage	pumpkin	w mustard	ng	n/d
		8/20	28-51	cabbage	pumpkin	sh purse	ng	n/d
29	Ontario	5/20	29-52	border	squash	wmustard	ng	n/d
		5/20	29-53	border	squash	sh purse	ng	n/d
		5/20	29-54	border	squash	pennycress	ng	n/d
		5/20	29-55	border	squash	pennycress	ng	n/d

[a] two sites repeated from 2006 (12 and 6), [b] red numbers caused V-shaped lesions in greenhouse cabbage, [c] n/d not determined [d] sh purse shepherd's purse, w mustard wild mustard, ws mustard wormseed, t mustard tumble, yr mustard yellow rape, y rocket yellow rocket, cabbage regrowth from stems in field, [e] color and colony growth on King's B medium, ng is no growth, fluor fluorescent growth, [f] V-shaped lesion on greenhouse cabbage positive (+), no reaction or necrotic spot negative (-)

ELISA antigen test. Weed isolates which resembled Xcc morphology on YDC or SM media were tested with Xcc and Xca antigens. In 2005, of the 14 isolates tested with ELISA, 7 were positive for Xcc, 2 were Xca positive, 2 reacted with both, and 3 didn't react with either antigen. Of the 12 tested in 2006, there were 6 that were Xcc positive, 5 positive for Xca, and 1 didn't react to either antigen. The 2007 isolates had only 1 that was Xcc positive, 6 positive for Xca, and 10 that reacted with neither Xcc nor Xca. The ELISA tests were variable and often the results were confounding. Isolates that tested Xcc did not always cause a V-shaped lesion, isolates that tested Xca did not always cause a leaf spotting reaction and isolates that reacted with neither in one case caused a V-shaped lesion when inoculated back into cabbage.

Inoculation test for pathogenicity. Greenhouse inoculations of all weed isolates collected in 2005 which were positive for Xcc by morphology or rDNA sequencing did not result in any V-shaped lesions but caused a yellowing at the inoculation sites (Fig. 2.3A). A known pathogenic isolate of Xcc caused a V-shaped lesion within seven days of inoculation (Fig. 2.3B).

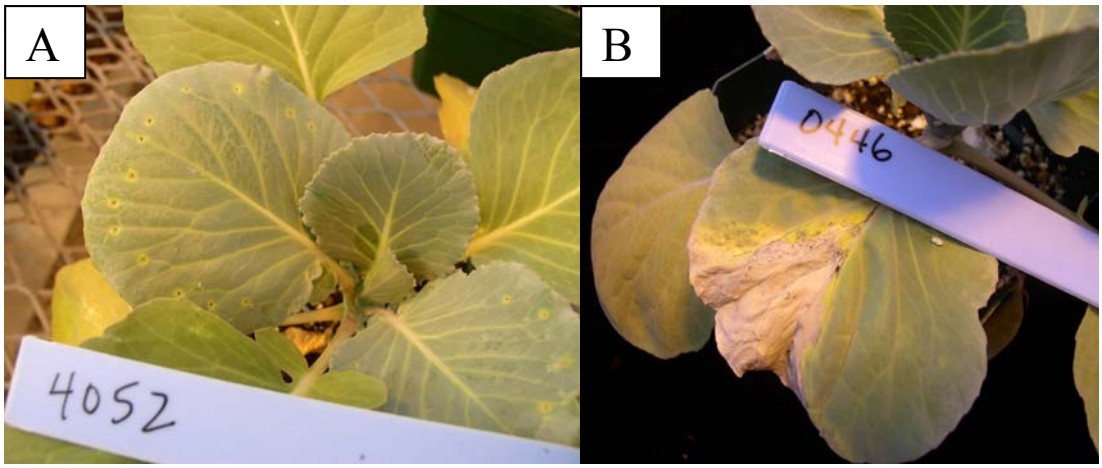

Figure 2.3 (A) weed #2 from site 4, 2005 (B) V-shaped lesion from a known Xcc isolate

In the 2006 inoculations, isolate 5-10 (Table 2.2) from shepherd's purse produced a V-shaped lesion on cabbage in the greenhouse. This isolate was collected April 28 from a field that had been in soybeans the previous year. Twelve weed samples were collected on Oct 31 from field 12, a cabbage field with multiple black rot symptomatic plants. Xcc-like bacteria were isolated from five of the 12 weeds collected; 1 shepherd's purse, 3 wormseed mustard, and 1 wild mustard. Four of those samples (12-23, 12-24, 12-27 and 12-29) produced V-shaped lesions in greenhouse inoculation assays (Table 2.2). When the 17 weed isolates from 2007 were inoculated into cabbage in the greenhouse, two isolates, both from shepherd's purse, caused V-shaped lesions (Table 2.3). One of these isolates was from a weed that was collected in a cabbage field and the other was growing in a fallow field which had been in snap beans the year before. It was not known if there had been black rot infection in the cabbage field.

Rep-PCR. The rep-PCR genomic fingerprints identified six patterns from the twelve 2005 weed isolates that had been identified as Xcc or Xca using ELISA (Fig. 2.4). None of the fingerprint patterns were the same as patterns identified from infected

fields in 2004 (data not shown). There were six fingerprint patterns in each of the subsequent year's weed collections. The fingerprint patterns of the 2006 and 2007 weed isolates did not match any field isolates from previous years or those collected from cabbage that season with the exception of the weed isolates from site 12 in 2006. Four of the weed isolates had the same fingerprint pattern as a cabbage isolate that was collected from the same field which was infected with black rot at the same time the weeds were collected.

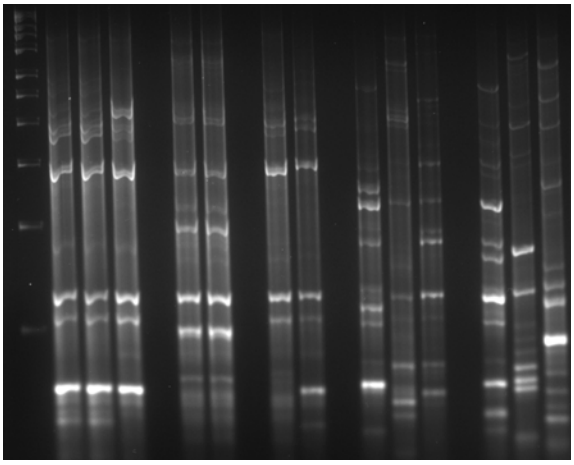

Figure 2.4 weed isolates from 5 sites collected in 2005. L 100bp ladder, Lanes 1,2 site 1, lane 3 site 3, lanes 4,5,6,8,11,12, site 4, lanes 7,9,10, site 5 lane 13 site 2 1)1059 2)10510 3)3054 4)4052 5)4053 6)4054 7)5059 8)4057 9)5053 10)5055 11)4059 12)4051 13)2054

Ribosomal (r-DNA) sequencing. Twelve of the 21 weed isolates collected in 2005 (Table 2.1 red) were identified by rDNA spacer region sequences to be identical or very similar to Xcc. A known Xca isolate was found to be identical to the sequence of Xcc. We did not repeat the sequencing with weed samples in the following years as this technique did not differentiate between Xcc and Xca or indicate isolates that were pathogenic on cabbage.

Discussion

A collection was undertaken in 2005 to determine if weeds found in and around fields that had severe black rot in 2004 would harbor Xcc for the next season. It was initially easy to find weeds in the surveyed fields but as the season progressed and herbicides were applied and crops planted, the collection moved more to the field borders. There were many types of crucifer weeds in all of the sites, however no species appeared to have disease symptoms, only yellowing of lower leaves was noted on a few specimens. This yellowing did not seem to correlate with the pathovar of *X. campestris* isolated from the weed, or the ability of the isolate to be pathogenic to cabbage. The absence of symptoms has been reported from other groups that have conducted surveys on bacterial isolates collected from weed species (Dane and Shaw 1996; Morris and Knox-Davies 1980; Schroeder et al. 2005; Wisler and Norris 2005), although Kuan (1986) found V-shaped lesions but no blackening of veins on *B. campestris* (field mustard) in California and in Oklahoma Xcc was recovered from shepherd's purse with chlorotic V-shaped lesions (Zhao et al. 2000). It appears that through selection pressure, the weeds have become tolerant or resistant to, pathogens such as Xcc (Schroeder et al. 2005). It has also been shown that Xcc can survive on weed hosts in the absence of a crop host. In a study by Dane and Shaw (1996), using a bioluminescent strain of Xcc, they found the inoculated strain frequently moved from cabbage onto both crucifer and non-cruciferous weeds during the winter months in Alabama. We did not find Xcc in non-cruciferous weeds in NY, which is similar to findings in Georgia, California and Oklahoma (Schaad and Dianese 1981; Zhao et al. 2002).

As has been reported with previous use of monoclonal antibodies to distinguish Xcc from Xca, the strains are not completely serologically distinguishable, but overlap with each other and may be more closely related than other *X. campestris*

pathovars (Alvarez et al. 1994). The ELISA results confirmed that of the 144 NY weed isolates collected over three years, 14 were Xcc positive and 17 were positive for Xca, or had enough similarity to those pathovars to react with the antigens provided with the test kit. The results were not reliable in predicting the pathogenicity of isolates as some isolates that were Xcc positive with ELISA caused necrotic spots, some that were Xca caused V-shaped lesions, and in one case an isolate (12-24) which didn't react with either antigen caused V-shaped lesions when inoculated into cabbage in the greenhouse.

The collection of bacterial isolates from weeds in 2005 following a year of severe black rot outbreaks did not yield any isolates which were pathogenic to cabbage when inoculated into plants in the greenhouse. One would expect that field epidemics would result in high levels of inoculum which could migrate to weed species, especially species that over-winter as rosettes. In site 1, cabbage regrowth and weeds that were collected in May were growing in cabbage debris remaining in the field from the previous season. A study in Washington examined bacteria isolated from weeds over a three year period. The isolates were indistinguishable from Xcc but avirulent on cabbage. Schultz and Gabrielson (1986) found cabbage debris in the same field and isolated Xcc from the debris that did cause disease on cabbage.

In 2006, a shepherd's purse was found to have an isolate which did cause a V-shaped lesion when inoculated into cabbage in the greenhouse. This isolate was collected on Apr. 28, and since no crucifer crop was in the field at the time, it most likely was infected the previous season. Since this species flowers from spring until fall, the Xcc could have been on the seed (Schaad and Dianese 1981), but this is unlikely as the field had been planted to soybeans. A more likely cause was movement of the pathogen on field equipment in soil or field debris from another site or being blown in on field debris. The rep-PCR pattern from this isolate (Table 2.2 (5-10) was

unlike any fingerprint from field infections in 2004 or 2005. Cabbage transplants were set into this field in May and the weeds were controlled after field preparation and planting. An isolate collected from another shepherd's purse at the site in Oct, which now had mature cabbage, yielded yellow bacteria which were Xca positive with ELISA, but this isolate did not cause V-shaped lesions in the greenhouse pathogenicity test. No black rot symptoms were observed in this field, if the Xcc found in the first weed was widespread; it seems that it would have spread to the cabbage which was not the case.

Four weed isolates out of 12 collected on Oct 31, 2006 from a cabbage field with black rot lesions also caused V-shaped lesions when inoculated into greenhouse cabbage. The rep-PCR fingerprint patterns of the weed isolates were identical to the isolates from cabbage isolates in the same field. While it is possible that the weeds were the source of inoculum, the widespread infection in the cabbage field without wet, windy weather present which would facilitate inoculum spread, as well as the low weed numbers, implicates the cabbage plants as the inoculum source which then moved to the weeds.

Again in 2007, two of the weed isolates out of the 55 collected caused V-shaped lesions when inoculated into cabbage. These were both collected on the same farm. This farm reported black rot infections in cabbage in 2006. One of these isolates was from shepherd's purse collected on May 10 on the border of a field of new cabbage transplants. There were no noticeable black rot symptoms in the field and while yellow bacteria were also isolated from three other weed samples, these were not pathogenic on cabbage. We returned to the same field in July and took another 5 weed samples which did not have isolates that were pathogenic on cabbage and the cabbage in the field still showed no symptoms of black rot. The season was dry and there was very little Xcc infection in the area until Oct and Nov of that year. The other

isolate causing a V-shaped lesion was also taken from shepherd's purse in May in an unworked field that had been in snap beans the previous year. This was one of 6 samples taken from the same area and the only one that was pathogenic on cabbage. The rep-PCR fingerprint patterns of these two weeds were not the same as each other or any of the isolates collected from cabbage. It seems that the Xcc found in the weeds in NY is rarely pathogenic to cabbage although the strains that infect cabbage readily infect cruciferous weeds. In no case did the rep-PCR fingerprint patterns from weed isolates match the cabbage isolates in the same location from the previous year. If the isolates in weeds originated from the field infection, the fingerprint pattern would be the same as was the case in 2006 when we did find a matching pattern between cabbage and weed isolates taken from the same field during a black rot infection. This was also true in a study of *X.campestris* pv.*vitians* in lettuce (Barak et al. 2001). The rep-PCR fingerprints taken from weeds during an epidemic were the same as the field infection patterns and were pathogenic on lettuce, but bacteria isolated from weeds two months after lettuce harvest had different fingerprint patterns and they were not pathogenic on lettuce. In our weed isolate collection, within a field the weed isolates had the same fingerprint pattern at several sites (Fig. 2.4, sites 1 and 4) which was also reported by (Zhao et al. 2002) where they found diversity of Xcc in different fields but the strains were similar within fields. Although Schaad and Dianese (1981) found Xcc in many weed isolates as well as weed seed, they had no data to prove that weed infections of Xcc arose from weeds. The sites where they found the most weeds with Xcc were abandoned crucifer fields. In a study in Thailand where 85% of fields were infected with black rot, the pathogen was not isolated from seed and it could not be traced to weeds as hand weeding had completely eliminated them as an inoculum source (Schaad and Thaveechai 1983).

Ignatov (2007) found that in CA, a state with the largest, most diverse population of cruciferous weeds, different strains of Xcc were unique to a site and the strains from crop producing areas were genetically distinct from those where no crucifers were grown. The rep-PCR fingerprint patterns of the weed isolates collected over three years also reflect this diversity in NY. The cruciferous weeds of NY harbor Xcc bacteria but they did not appear to be the same strains as found in crucifer field outbreaks of black rot. This was also the case in South Africa where wild radish was found to have populations of Xcc, but there was no indication that the weeds had a role in the black rot epidemics of *Brassica* crops there (Morris and Knox-Davies 1980). Shepherd's purse, in particular, was the weed we found in almost every location and this was the weed harboring Xcc that was pathogenic in greenhouse inoculations. The 2005 growing season did not see the wind driven rains of the previous year, but there was black rot found in crucifer fields throughout the state from July to Oct. If weeds serve as reservoirs of Xcc, the isolates collected in 2005 should have reflected at least some of the rep-PCR fingerprint patterns of the 2004 epidemic. If the cruciferous weeds serve as an inoculum source, black rot infections would be focused along field borders where most weeds are located during the growing season which was not the case. Our findings indicate that while Xcc can infect cruciferous weeds and some of these isolates can infect cabbage when inoculated into cabbage in a greenhouse, the weeds do not serve as a reservoir of the pathogen which readily infects crops.

Controlling weeds, especially those closely related to crop species is a sound disease management practice, but it does not appear that weeds can be implicated as a major source of inoculum for black rot epidemics in crucifer crops in NY.

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CHAPTER 3

REDUCING THE SPREAD OF *XANTHOMONAS CAMPESTRIS* PV.*CAMPESTRIS* DURING PRODUCTION OF CABBAGE TRANSPLANTS IN THE GREENHOUSE

Abstract

Black rot of cabbage and other Brassicas is caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc). This disease is primarily seed borne, and greenhouse conditions are conducive to spread of the pathogen. The objective of this study was to compare the efficacy of copper hydroxide and benzothiadiazole (BTH) to reduce the spread of Xcc in four week old cabbage plants. It is important to determine whether either of these registered products is effective in controlling the spread of Xcc in the greenhouse. The second objective was to determine if a control strategy was more effective on younger than on older cabbage seedlings in limiting the spread of Xcc on cabbage transplants. Copper hydroxide (Kocide 3000) and benzothiadiazole (Actigard 50WG) were applied at labeled rates on a seven day schedule beginning when the seedlings had two true leaves and the following day a strain of Xcc pathogenic to cabbage was sprayed onto the plants. Plant samples were taken weekly, washed with a saline plus tween solution and the resulting liquid was plated and added to real-time PCR reactions. The experiment was conducted three times.

There was a significant reduction in the number of Xcc bacteria recovered after the copper hydroxide and BTH treatments as compared to the untreated control and in the second objective, significant differences were found in the Xcc population in leaf washes depending on the age of the transplants. In this case, copper hydroxide

significantly reduced the Xcc population in the four and five week old plants as compared to the untreated control, which was not observed in the oldest group of transplants.

Introduction

Black rot caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) is the most important disease of *Brassica* crops in all regions where they are cultivated, and can cause major economic losses (Williams 1980). The growing season in New York State often has heavy rains accompanied by wind and warm temperatures which are conducive to the development of black rot. Periods of heavy morning dew also are ideal for spread of the disease. Black rot has caused major losses in NY, which is one of the leading U.S. states for cabbage production. In 2008, 9,900 acres of cabbage were grown with a farm gate value over $101 million (Boriss and Kreith 2009).

The pathogen can be spread through infected seed into the developing seedling and enter the vascular tissue where it becomes systemic in the plant (Cook et al. 1952; Kohl and van der Wolf 2004; Walker and Tisdale 1920). The seed industry standard for freedom of black rot is testing three samples of 10,000 seeds each (Franken et al. 1991) with detection of one seed in 10,000 possible (Schaad 1988) . This may not be an adequate sample size, for when conditions are favorable for disease a seed infection of 0.03% can cause an outbreak (Schaad et al. 1981). Seed treatments are a recommended practice, and while effective at reducing pathogen numbers, there is no seed sample that can be guaranteed to be pathogen free (Kuan 1988; Morrison 1999; Schultz et al. 1986).

The use of transplants decreases seed costs and produces plants that have been found to be higher yielding, have more uniform growth, and suffer less stress at emergence (Frantz et al. 1998). Greenhouse transplant production facilities generally

utilize high plant population density (Thomas 2003) coupled with overhead watering regimes. This creates a microclimate of low light, reduced air circulation, prolonged leaf wetness, high humidity, and close contact which are all conducive for pathogen multiplication and spread (Bains and Mirza 2002; Daughtrey 2004; Gitaitis 1990; Greathead 2003). In addition, symptoms on young plants often occur on the cotyledons which, following senescence, drop beneath the plant canopy where they can serve as an inoculum source. Unlike the transplants started in the relatively warm climate of the southern U.S., cabbage transplants are started for production in NY during a season of predominantly cool temperatures. These conditions are not conducive to Xcc symptom expression (Carisse et al. 1998; Schaad 1988) but allow the bacteria to survive under these conditions. In a previous study, bacteria could be detected several weeks after plants were inoculated with Xcc and placed in conditions unfavorable for symptom development (Timmer et al. 1987). Thus, the importance of inoculum control in greenhouse transplant facilities cannot be overstated.

Control of bacterial plant pathogens is difficult and often requires a combination of strategies. There are no treatments yet that can inhibit vascular pathogens like Xcc once they are inside the plant, sheltered from outside factors and in an environment suited to rapid multiplication. There has been some work using biocontrols such as bacteriophage (Jones et al. 2007), antagonistic bacteria (Monteiro et al. 2005), and plant derived toxins (Amusa and Odunbaku 2007; Luna et al. 2002) to control Xcc, but these are generally expensive to produce and have given variable results. In a study of antagonistic bacteria for black rot control, plants were pre-inoculated with *X. campestris* pv. *vesicatoria* or a mildly pathogenic strain of Xcc: neither reduced the incidence of black rot (Dane and Shaw 1996). Copper is the most widely used product for control of bacterial pathogens, but some bacteria including *Xanthomonads* have become tolerant or resistant to copper products (Marco and Stall

1983; Momol et al. 2002). Another control tactic which has been successful with other pathovars of *X. campestris* is the use of systemic acquired resistance (SAR) inducing products such as BTH which result in inhibition of pathogens (Louws et al. 2001). The plant is "primed" to resist attack, and because there is no direct effect on the pathogen, no bacterial resistance to SAR inducing products is expected to develop.

These experiments were designed to test the efficacy of copper hydroxide and BTH against high Xcc inoculum pressure using inoculated plants and greenhouse conditions favoring the multiplication and spread of the pathogen. In addition, we examined the effect of cabbage transplant age on the ability of copper hydroxide treatments to control Xcc. Combined; these studies have enabled the identification of strategies for greenhouse transplant facilities that would be the most effective in reducing Xcc in transplants.

Materials and Methods

Plant materials and disease greenhouse construction. Cabbage seeds, cultivar 'Surprise' (Bejo Seeds, Oceana, CA), were planted into Cornell mix (a soil-less peat) amended with vermiculite and perlite 4:1:1 in 200 cell flats. Each cell was 4 cm square and 6 cm deep. The seedlings were grown in a glass house at ca 24ºC day and 16ºC night with 16 hours of light and 6 hours of darkness initiated after germination. Water was applied with an Orbit water monitor (Orbit Irrigation Products, North Salt Lake, UT) system with Dan Sprinklers green spinner emitters (Netafim, Fresno, CA) delivering a fine mist three times a day for five minutes each watering. Ten nozzles (covering 7.8 m^2) were spaced every meter in two rows with 1m between rows (Fig. 3.1). The cabbage seedlings remained wet for much of the day to facilitate disease development and bacterial spread.

Figure 3.1 Greenhouse used for black rot control studies, with computerized watering unit in foreground.

Efficacy of copper hydroxide and BTH. Each replicate of the experiment consisted of 6 flats of cabbage seedlings. Two flats served as inoculated controls sprayed with water only, two flats were sprayed with copper hydroxide (Kocide 3000, DuPont, Wilmington, DE) at a rate of 2.8 kg/ha and two flats received sprays of BTH (Actigard 50WG, Syngenta, Greensboro, NC) at a rate of 52g/ha. Treatments were applied once per week for four consecutive weeks, and were initiated when the seedlings had two true leaves (approximately 4 wk). Sprays were applied with a hand held pump sprayer. The entire experiment was performed three times.

Effect of transplant age on pathogen population. To understand if transplant age plays a role in the ability of Xcc to survive and multiply on leaf surfaces leading to disease spread, copper hydroxide was sprayed at the labeled rate (as described above) on flats of cabbage seedlings of different ages. Treatments were initiated when the youngest plants were at the two true leaf stage (approximately 4 wk old) and the other flats were 1 and 2 wk older at the initial treatment. Sprays were applied on a seven day

schedule for four weeks. Two 200 cell flats of 'Surprise' *B. oleracea* were used for each age group as well a control flat for each age group which was sprayed with water.

Plant inoculation. Inoculum preparation was identical for both the efficacy and plant age experiments. The Xcc isolate 08304 used in this study was isolated from a diseased cabbage plant, cultivar 'Huron', collected in Monroe County, NY in 2008. The isolate was cultured on 100mm x 15mm Petri dishes with YDC (Wilson et al. 1967) agar for two days at 28ºC. Inoculum was prepared by scraping three Petri plates with a rubber policeman using sterile distilled water. Silwet L-77 was added as a surfactant (250 µl/L). A NanoDropND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) was used to determine the optical density of the inoculum. The OD 600 was approximately 0.2, approximately 10^9 Colony Forming Units (CFU). The inoculum was applied with a 500ml hand pumped atomizer bottle which applied fine droplets to run off, 24 h after the first treatment sprays were applied.

Sampling. Weekly, starting one day prior to the first spray treatment, five randomly chosen plantlets (2.5g) from each flat were cut below the cotyledons. For the plants of varying ages, 2.5g of leaves, rather than complete plants were utilized. Plant tissue was placed into 5ml of saline solution plus tween (.85g/L NaCl + 0.02% Tween 20) and shaken (170rpm) in a Labnet incubator shaker (Labnet, Woodbridge, NJ) for 30 minutes at 28ºC (Roberts, 1999). The resulting wash was serially diluted and 0.1ml from each dilution was placed onto each of two Petri plates containing YDC agar and spread with a sterile cell spreader on a turn table and incubated at 28ºC for 72 h until the colonies could be enumerated. The undiluted leaf wash solution and dilutions to 10^{-4} were plated. There were a total of two cell counts per dilution per flat, and two flats per treatment, for a total of 20 plates per treatment. To verify that Xcc bacteria were being counted, colonies that were yellow and mucoid on YDC agar from each

treatment were boiled for 5 minutes and spun down. A PCR mix containing 4µl of the resulting supernatant and Xcc specific primers zup 2309 5'-AAA TCA GGG GGA TGC GGT GG -3'and zup 2310 5'-TCC GGC CAG GGT CGA TAC AGT G -3' (Rijlaarsdam et al. 2004) was run at 95ºC for 5 min, then 30 cycles of 95ºC for 30sec, 63 ºC for 30 sec, 72 ºC for 30 sec, and a 72 ºC extension for 5 min.

The remainder of the wash was spun in an Eppendorf 5810R centrifuge (Hauppauge, NY) for 10 minutes at 4000 rpm at 28ºC. The supernatant was discarded and the pellet resuspended in 60µl sterile distilled water. Samples were collected every seven days, once pre-inoculation, and for four weeks post inoculation.

Real-time PCR. Bacterial pellets (from above) were resuspended in 60µl water and used in real-time PCR assays. A standard curve using Xcc genomic DNA from 0.01 to 100ng/ul with ten-fold dilutions were included on each PCR plate. Real-time PCR with SybrGreen was used in the following mix: 12.5µl SybrGreen Mix (BioRad, Inc., Hercules, CA), 2.5µl primer zup2309, 2.5µl zup 2310, 5µl water for each reaction plus 2.5µl of the resuspended pellet sample for a total of 25 µl per reaction. Real-time PCR was performed in a Bio-Rad iQ5 machine using the following program: 95ºC for 2 min, followed by 40 cycles of 95ºC for 10 sec, 58ºC for 30 sec, and 72ºC for 30 sec. Three real-time PCR reactions were performed on the resuspended pellet from each flat, for a total of six real-time PCR reactions per treatment.

Statistical analysis. Analyses of variance were determined using PROC GLM of the SAS software (SASXI, SAS Institute Inc, Cary, NC). The means were compared with Fisher's LSD test. Data included three replicates of the copper and BTH treatment results, and three replicates of the plant age trial. Each experiment (efficacy and plant age) was repeated three times which resulted in a total of 18 real-time PCR reactions for each treatment.

Results

Efficacy of copper hydroxide and BTH. The greenhouse design with computerized overhead irrigation and 200 cell flats for high plant population density was effective in establishing populations of Xcc with our spray inoculation method. Both the copper hydroxide and the BTH treatments reduced the number of Xcc bacteria recovered from the plant samples compared to the untreated control flats. The first collection, taken prior to inoculation, had no growth of Xcc (Fig. 3.2). Xcc bacteria were detected in the second set of samples taken (one week post inoculation) as yellow mucoid growth on YDC medium. The colonies were verified to be Xcc using the zup 2309 and zup 2310 primers in a PCR reaction with visualization of the amplicons on an agarose gel stained with ethidium bromide (data not shown). The Xcc populations from samples in the control flats peaked the fourth week after inoculation with an average of 24 x 10^6 CFU / ml. By the final collection on the fifth week, the average Xcc colony counts from dilution plates of the untreated flats had dropped to 17 x 10^6 / ml (Fig. 3.2)

The flats of seedlings with weekly copper hydroxide applications (Fig. 3.3) had colony counts which increased from an average of 7.2 x 10^3 one week post inoculation to 6 x 10^3 two weeks post inoculation, 2.2 x 10^5 three weeks post inoculation, and 150 at the final collection (Fig. 3.3). The BTH treated flats followed a similar pattern as the copper treatments (Fig. 3.3), with an average of 7 x 10^3 CFU at week 2, 8 x 10^3 at week 3, an increase to 2.2 X 10^4 CFU at week 4, and then a drop to 100 at week 5.

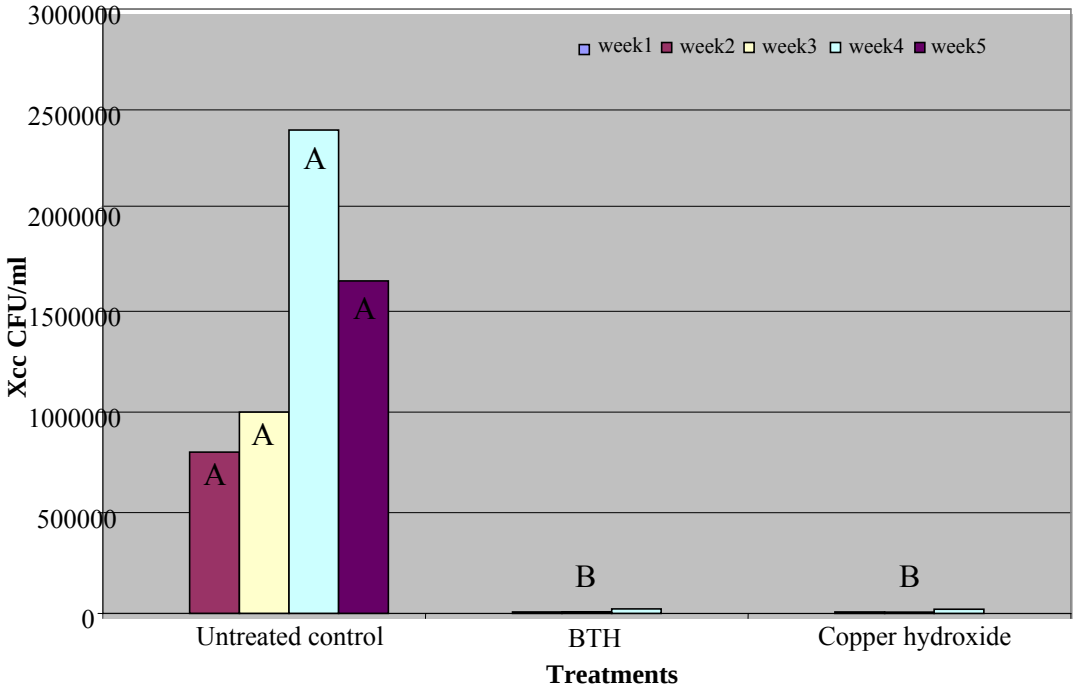

Figure 3.2 Xcc CFU detected by plating leaf wash from two flats of each treatment Values were 0 in week 1 and BTH and copper hydroxide data have values too low to be seen in this graph. Data were combined from three repetitions of the experiment. A: Untreated control had significantly more Xcc bacteria than either BTH or copper hydroxide (B).

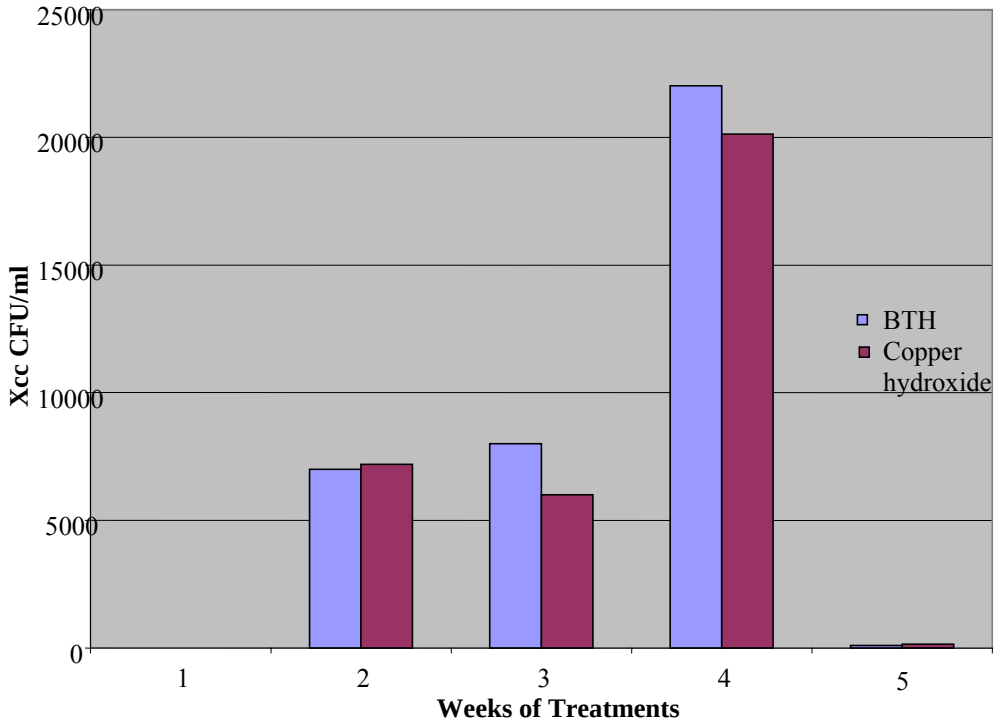

Figure 3.3 BTH (blue) and Copper hydroxide (purple) CFU counts over five weeks repeated three times. There were no significant differences between the two treatments. Data were combined from three repetitions of the experiment.

Real-time PCR. Results of real-time PCR assays were similar to those obtained with the dilution plate CFU count technique, utilizing the same leaf wash samples. Real-time results are presented as CT (cycle of threshold) values. A lower average CT (cycle of threshold) indicates that more Xcc DNA is present in a sample. In standard curve assays, 100ng/µl of Xcc DNA reached CT at approximately 12 cycles and each ten-fold dilution increased 3 cycles with the 0.01ng/ µl samples reaching CT at 25 cycles. The non-template control eventually reached CT at over 30 cycles. The first samples from leaf washes of the untreated control flats as well as the copper hydroxide and BTH treatments reached CT between 27-33 cycles, indicating that there were no Xcc bacteria in the sample (Fig. 3.4).

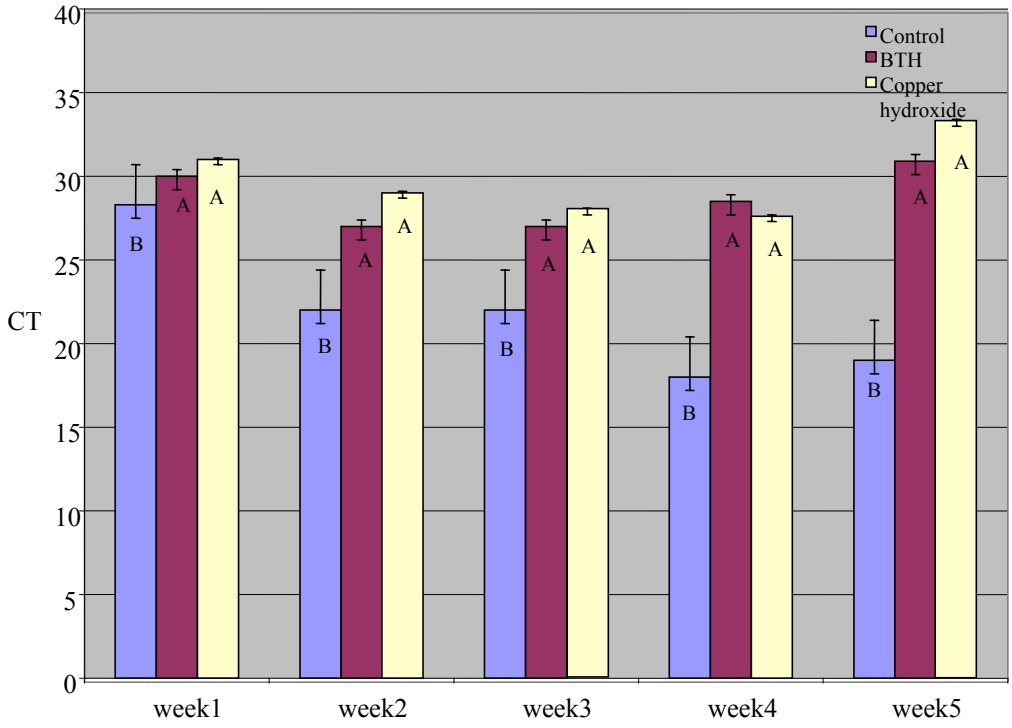

Figure 3.4 Cycle threshold (CT) means of the real-time PCR results detection of Xcc in leaf wash from different treatments. Different letters show significant differences between treatments. Data were combined from three replications of the experiment.

The flats of untreated control plants showed an increase of Xcc in the samples one week post inoculation with an average CT of 21.5 cycles. Samples collected two weeks post inoculation had an average CT mean of 22.1 cycles, followed by an increase in the Xcc population at three weeks post inoculation with an average CT of 18 cycles. There was a slight decrease in bacterial population at the final collection with an average CT of 19 cycles. Samples from the flats treated with BTH amplified at 27 cycles the second week (1 week after inoculation). The CT was 27.0, 28.5, and 30.9 at 3, 4, and 5 weeks after inoculation, respectively. The CT of the copper hydroxide treated flats was 29, 28.1, 27.6 and 33.3 at 2, 3, 4, and 5 weeks post inoculation. The data collected from three replications of the treatments confirms that there is a significant reduction in the number of Xcc bacteria present in leaf washes

from BTH and copper hydroxide treated flats compared to the flats with no treatment. The two treatments were not significantly different from each other in this experiment.

Plant Age Study. The three age groups of cabbage seedlings were sprayed with Kocide 3000 at the labeled rate and a day later the plants were inoculated by spraying Xcc over the flats. The copper hydroxide was applied every seven days for four weeks. Each age group had controls of the same age which were sprayed with water. There was no evidence of Xcc at the first collection in the flats prior to the first treatments and inoculation. The CT for both untreated controls and the treated flats were between 29.8 and 34.4 and the CT of the non-template control samples run at the same time was 32.9 (data not shown), indicating no amplification of Xcc DNA from the leaf wash solutions of any of the greenhouse samples prior to inoculation. The following week, Xcc was detected in all flats (Table 3.1). The four week old untreated

Table 3.1 Real-time PCR CT means of six samples of each treatment. Weeks are post inoculations of Xcc. Data were combined from three replications.

treatment	week 1	week 2	week 3	week 4
control 4 wk	18.5	19.1	18.3	19.7
treated 4 wk	21.9	21.6	21.2	19.2
control 5wk	20.2	21.3	23.0	18.8
treated 5 wk	25.6	25.5	24.9	22.9
control 6 wk	23.8	24.6	22.2	24.7
treated 6 wk	24.6	27.8	23.6	25.1

plants had the lowest CT (highest bacterial level) collected from the leaves of any of the groups. The four week old copper hydroxide treated group had the next lowest CT average followed by the other two control age groups. The six week old plants sprayed with copper hydroxide showed the lowest population of Xcc as seen in the highest CT means of all the treatments (Table 3.1, Fig. 3.5). These were followed closely by the five week old plants and these results were consistent for all three replicates of the experiment. The results show that the unsprayed four week old plants, the youngest

group in the experiment, had significantly higher populations of Xcc on the leaves than the other groups (Table 3.1). Although the treated six week old plants had the lowest CT means, the copper hydroxide treatment did not show a statistically significant reduction in Xcc population on the leaves. In the other two age groups (four and five weeks old); the copper hydroxide treatments did reduce the amount of Xcc collected in the leaf wash over the untreated controls of the same age plants.

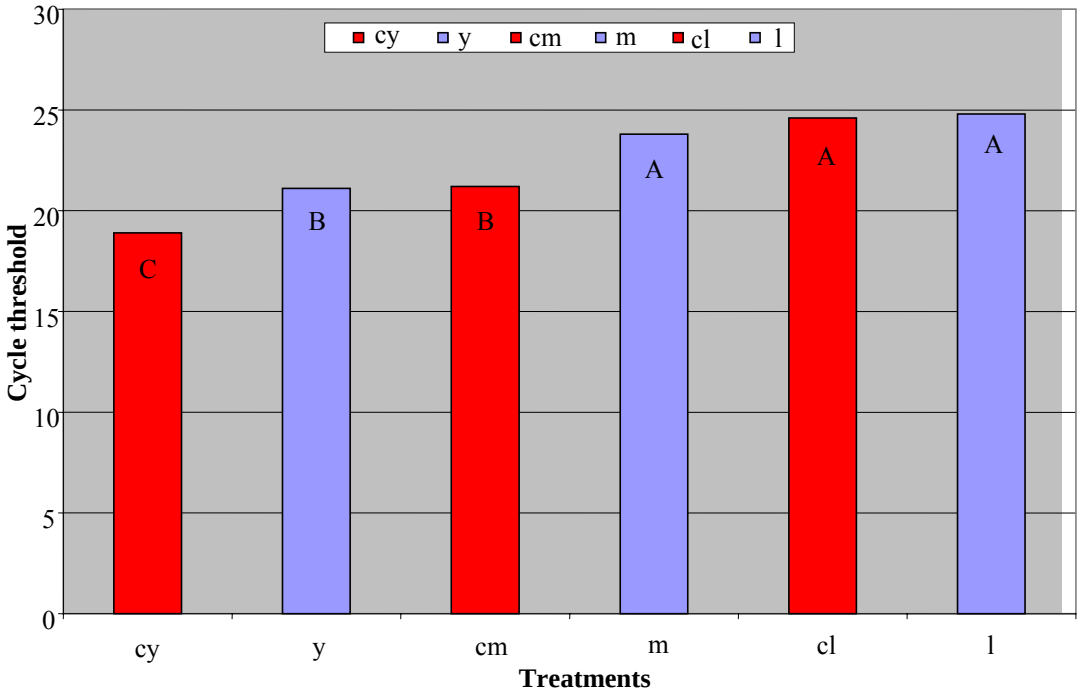

Figure 3.5 Two flats each of y (4), m (5), and l (6) week old cabbage plants were sprayed weekly with copper hydroxide after inoculation with Xcc. Untreated controls are in red for each age group (cy: 4 wk, cm: 5 wk, cl: 6 wk). The younger plants showed more disease and copper hydroxide was statistically less effective on this age group. Average CT means of four collections.
Letters which are the same are not statistically different from each other. Data were combined from three replications of the experiment.

Discussion

Because populations of Xcc can build to significant levels before symptom expression (Shigake et al. 2000; Timmer et al. 1987), it may be important to treat transplants during greenhouse production to control spread of Xcc prior to symptom development. To determine if it was possible to reduce the epiphytic population of Xcc on cabbage seedlings, two sets of experiments were performed. First, the efficacy of two commercially available products was tested, and one of these products was then used in a second set of experiments to study the effect of plant age in controlling Xcc. A leaf wash technique is an effective method to check for the presence and amount of Xcc on cabbage seedlings in the greenhouse, especially in the absence of symptoms. *X. campestris* is a plant pathogen with the capacity to grow epiphytically on leaves and there is evidence that this ability to adhere is correlated with its ability to cause disease (Jones et al. 1991; Romantshchuk 1992). Xcc has been shown to survive epiphytically for long periods (Kuan et al. 1986; Timmer et al. 1987) which can contribute to disease spread under high plant populations, high moisture conditions, and water splashing (Kocks et al. 1999; Shigake et al. 2000).

Real-time PCR is a fast, reliable technique without the added steps of DNA extraction and gel visualization with detection of very small amounts of pathogen an important feature although it does not distinguish dead from living cells (Schaad and Frederick 2002). It has been used to detect plant pathogens from seeds and plant materials when the equipment is available and pathogen sequences are known (Berg et al. 2006; Schaad and Frederick 2002; Walcott 2003). This technique was used to determine the presence of Xcc DNA in a leaf wash solution without extracting genomic DNA. The real-time PCR results were validated by the colony count data which were collected from the same samples. Contamination by other organisms was

not a factor with the real-time PCR as was sometimes the case when plating leaf washes onto medium.

Copper bactericides function as protectants and can only kill bacteria by contact when they are exposed on plant surfaces (Momol et al. 2002). They are also effective in reducing bacterial growth, even on resistant populations (Jones et al. 1991). Reducing the population of Xcc on leaf surfaces is key to reducing pathogen spread in a greenhouse environment. Regular applications of bactericides may help suppress pathogen spread (Greathead 2003). The weekly copper treatments we applied effectively reduced the numbers of bacteria on the cabbage leaves 200 fold compared to the untreated control flats.

The use of SAR-inducing products to control bacterial diseases has been documented for many vegetable pathogens. Some trials have shown the same efficacy using Actigard as found with copper products (Werner et al. 2002). Decreases in yield have been observed in some studies (Romero et al. 2001), while others have seen no yield difference with SAR treatments (Lang and Gent 2007). A study using the model plant *Arabidopsis thaliana* treated with salicylic acid to induce SAR, suppressed the growth of common bacteria including *X. campestris* pv. *campestris* without detriment to the plant mass or seed production (Traw et al. 2007). A plant defense system such as this would not be maintained over time if not beneficial to the plant. Treatment with the SAR product Actigard in this experiment was effective in reducing the amount of Xcc recovered in the leaf washes 100 fold compared to control flats treated with water. Some leaf curling was noted after treatment but this did not appear to be detrimental to the growth of the transplants. The BTH treatment was not statistically different in reducing the bacterial population on the leaves than the Kocide treatment.

The second objective of this study was to determine the role plant age might play in the efficacy of copper hydroxide. Well managed greenhouse facilities provide

efficient and economical production of large numbers of uniform, well grown transplants but this environment of higher than ambient temperatures and humidity, and vast numbers of a fast growing, succulent monoculture, is ideal for the spread of bacterial pathogens (Bains and Mirza 2002). The very nature of the process of growing plants makes exclusion of all pathogens virtually impossible, however the goal is to keep pathogens from flourishing (Powell 2007). Management to control pathogen spread includes control of the greenhouse environment, and maintenance of optimal plant growth, as well as timely applications of products known to effectively limit pathogen spread. The amount of disease is initially influenced by the amount of bacteria, but spread and secondary infections are primarily due to overhead watering causing splash to adjoining plants, not increased infection from seed to seedling (Roberts et al. 1999). Reduction in horizontal spread by controlling splash has been shown to be as effective as the use of copper products (Onsando 1988). Control measures implemented at the seedling stage may reduce both the rate of transmission and disease development which would in turn minimize the potential for epidemics once the plants are in the field (Mochizuki and Alvarez 1996).

The four week old cabbage seedlings were developing rapidly and had a thinner wax coating on the leaves than the older plants which may be why they supported a larger population of Xcc on their leaves throughout the experiment. The less waxy leaf surface may have provided a more favorable environment for bacterial attachment and multiplication. The application of copper hydroxide at this age significantly reduced the population of Xcc over the untreated control. While the five week old plants showed a lower population of Xcc over the four week old plants on both the untreated and treated flats, the CT mean of the untreated 5 week old plants were not statistically different from that of the treated four week group. The treated group of five week old plants had a statistically lower number of bacteria as seen with

the real-time data than either the treated or untreated youngest plants. Although the CT mean for both the untreated and treated six week old plants were higher than any of the other groups, they were not statistically different from that of the copper hydroxide treated 5 week old plants. The older plants in this experiment, with this cabbage cultivar, had lower populations of Xcc than the youngest (4 week old) age group, but only in the 4 and 5 week old plants did the repeated treatments of copper hydroxide reduce the leaf population of Xcc to a significant degree. The knowledge of how different age groups of cabbage transplants respond to treatment with copper can be a valuable tool for managing greenhouse spread of Xcc. It would likely be more economical to cull the four week old transplants with high leaf populations of Xcc to reduce inoculum and control pathogen spread than to use the space and resources to produce Xcc infested plants. This study also provides evidence that applying copper hydroxide at a younger plant age is more likely to decrease the population of Xcc that is present on the leaves. Asymptomatic plants may harbor significant levels of Xcc for future black rot infections when environmental conditions and inoculum levels are optimal for disease.

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