

Improved seed health tests for *Xanthomonas axonopodis* pv. *phaseoli*

in common bean

by

Yiqing He

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Microbiology

Program of Study Committee:
Gary Munkvold, Major Professor
Adam Bogdanove
Byron Brehm-Stecher
Anania Fessehaie

Iowa State University

Ames, Iowa

2010

Copyright © Yiqing He, 2010. All rights reserved.

TABLE OF CONTENTS

ABSTRACT	iv
CHAPTER 1. GENERAL INTRODUCTION	1
Introduction	1
Thesis Organization	2
Literature Review	3
Literature Cited	17
CHAPTER 2. IMPROVED EXTRACTION PROCEDURES FOR DETECTING <i>XANTHOMONAS AXONOPODIS</i> PV. <i>PHASEOLI</i> IN COMMON BEAN SEED	23
Introduction	24
Materials and Method	27
Results	31
Discussion	32
Literature Cited	33
Tables and Figures	36
CHAPTER 3. A DIAGNOSTIC REAL-TIME PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF <i>XANTHOMONAS</i> <i>AXONOPODIS</i> PV. <i>PHASEOLI</i> AND <i>XANTHOMONAS</i> <i>AXONOPODIS</i> PV. <i>PHASEOLI</i> VAR. <i>FUSCANS</i> IN SEEDS	41
Introduction	42
Materials and Method	46
Results	51
Discussion	54
Literature Cited	56
Tables and Figures	63

CHAPTER 4. EVALUATION OF SURVIVAL AND SEED TRANSMISSION	
OF <i>XANTHOMONAS AXONOPODIS</i> PV. <i>PHASEOLI</i> AND	
<i>XANTHOMONAS AXONOPODIS</i> PV. <i>PHASEOLI</i> VAR.	
<i>FUSCANS</i> BY QUANTITATIVE REAL-TIME PCR	76
Introduction	77
Materials and Method	82
Results	85
Discussion	87
Literature Cited	90
Tables and Figures	93
CHAPTER 5. GENERAL CONCLUSIONS	104
References	105
ACKNOWLEDGMENTS	107

ABSTRACT

Xanthomonas axonopodis pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* are important seedborne pathogens of *Phaseolus vulgaris*. In order to maintain seed quality and meet phytosanitary requirements, accurate seed health testing methods are critical. Currently employed selective-media-based methods for these pathogens include several variations in extraction procedures. In order to optimize pathogen extraction from *P. vulgaris* seeds, we assessed the influence of different extraction steps on the sensitivity of *X. axonopodis* pv. *phaseoli* detection, including incubation time/temperature, vacuum extraction and centrifugation of seed extract. The results showed that vacuum extraction and centrifugation of seed extracts increased sensitivity, and the highest sensitivity was obtained with the 3-hour vacuum extraction at room temperature followed by centrifugation. These results were confirmed on seventy 1000-seed subsamples from 14 different naturally infested seedlots. Our results suggest that a 3-hour vacuum extraction followed by centrifugation would be a valuable modification of the current method approved by the International Seed Testing Association (ISTA).

Based on DNA sequence information from RAPD fragments generated from *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*, real-time PCR methods were developed for detection and quantification of the pathogens in or on seeds. Assay specificity was tested against DNA of several *Xanthomonas* species and pathovars of *X. axonopodis*. None of the closely related *Xanthomonas* strains were amplified using this PCR assay. The detection limit of the TaqMan assay for purified DNA and cells was 20 fg and 20 CFU per 25 µl PCR reaction mixture, respectively. A linear model was developed for seed contamination level in relation to amplification

cycles based on sensitivity tests on seed samples spiked with inoculated seeds. seedlots On naturally infested *Phaseolus vulgaris* seedlots, real-time PCR detection was more sensitive than the selective medium assay. Real-time PCR should be useful for rapid, highly sensitive and specific detection of these seedborne pathogens to ensure seed quality control and meet phytosanitary regulations. To my knowledge this is the first published real-time PCR assay developed for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*.

Seed transmission frequency and survival in storage were compared between both common blight pathogens from different geographic origins. *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* isolates had a higher percentage of seed-seedling transmission than *X. axonopodis* pv. *phaseoli* isolates ($P<0.001$). Both variants reduced seedling emergence compared to the noninoculated control, and *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates reduced seedling emergence more than *X. axonopodis* pv. *phaseoli* ($P<0.001$). The incidence of bacterial blight symptoms was higher in seedlings from *X. axonopodis* pv. *phaseoli* var. *fuscans* inoculated seeds than in seedlings from seeds inoculated with *X. axonopodis* pv. *phaseoli* ($P<0.001$). Real-time PCR showed that a higher percentage of seedlings were infected with *X. axonopodis* pv. *phaseoli* var. *fuscans* than *X. axonopodis* pv. *phaseoli*. PCR results also revealed symptomless infection of seedlings. Survival (population size) of both bacteria on stored seeds was monitored over time using real-time PCR. Survival did not differ significantly between the two variants and real-time PCR gave higher population size estimates than a culture plating test after three months in storage ($P<0.05$).

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Xanthomonas axonopodis pv. *phaseoli* (Smith) Vauterin and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder (Vauterin, 1995) cause common bacterial blight of bean (*Phaseolus vulgaris* L.), a destructive disease worldwide. They are gram-negative rod shaped bacteria with a polar flagellum, using aerobic metabolism. *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were first named as *X. campestris* pv. *phaseoli* (Smith) Dye and *X. campestris* pv. *phaseoli* var. *fuscans* by the phenotypic approach, but later DNA-DNA hybridization information demonstrated genomic homology with *X. axonopodis* (Vauterin et al. 1995), resulting in reclassification to *X. axonopodis* pv. *phaseoli*. The two variants can be differentiated using isoenzyme profiling (El-Sharkawy and Huisingh 1971), plasmid profiling (Lazo and Gabriel 1987), or DNA-DNA hybridization (Hildebrand et al. 1990). The average genome sizes for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were 3850.6 ± 48.9 and 3584.3 ± 68.1 kb respectively (Chan and Goodwin 1999b). *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* also can be distinguished by the production of a diffusible brown pigment, due to secretion and subsequent oxidation of homogentisic acid which is involved in tyrosine catabolism for a number of bacteria. *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* appears to be disrupted in tyrosine catabolism and does not use tyrosine as a nutrient (Goodwin and Sopher 1994).

Common bacterial blight is one of the major diseases in bean production and infected/contaminated seeds are major source. Therefore, seed health test with high sensitivity and specificity is important in meeting phytosanitary requirements. Several

different approaches have been used for detecting *X. axonopodis* pv. *phaseoli* in seeds, including a dome test (Venette et al. 1987), phage test (Kahveci and Maden 1994), selective media (Mabagala 1992), PCR (Audy et al. 1994), and serology such as indirect immunofluorescence microscopy and ELISA (Wong 1991).

The research presented in this thesis was initiated in response to the NSHS review of seed health tests for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. The objectives of this study were to 1) optimize seed extraction methods to improve detection sensitivity of the ISTA-approved detection method (temperature, incubation time, vacuum and centrifugation) and evaluate the modified method on naturally infested seeds, 2) develop quantitative real-time PCR for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* detection in *P. vulgaris* seeds, and 3) compare pathogenic seed transmission frequency and survival on stored seed between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* with help of the newly developed real-time PCR assay.

Thesis Organization

Chapter 1 is the general introduction of the background of the research project and literature review.

Chapter 2 evaluates the extraction procedures for pathogen detection from common bean seeds. Yiqing He is a graduate student and primary researcher and author. Gary Munkvold is an associate professor and correspondence author. Both authors are from Department of Plant Pathology of Iowa State University.

Chapter 3 describes the development of diagnostic real-time PCR assay for detection and quantification of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* in seeds. Yiqing He is a graduate student and primary researcher and author. Anania Fessehaie is a postdoctoral

researcher. Lisa Shepherd is a seed health testing coordinator from Seed Science Center of Iowa State University. Gary Munkvold is an associate professor and correspondence author. Yiqing He, Anania Fessehaie and Gary Munkvold are from Department of Plant Pathology of Iowa State University.

Chapter 4 examines the survival and seed transmission of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* by quantitative real-time PCR method. Yiqing He is a graduate student and primary researcher and author. Gary Munkvold is an associate professor and correspondence author. Both authors are from Department of Plant Pathology of Iowa State University.

Chapter 5 gives general conclusions and recommendations for future research.

Literature Review

Xanthomonas axonopodis pv. *phaseoli* (Smith) Vauterin and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder (Vauterin, 1995) cause common bacterial blight of bean (*Phaseolus vulgaris* L.), a destructive disease worldwide. They are gram-negative rod shaped bacteria with a polar flagellum, using aerobic metabolism. *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were first named as *X. campestris* pv. *phaseoli* (Smith) Dye and *X. campestris* pv. *phaseoli* var. *fuscans* by the phenotypic approach, but later DNA-DNA hybridization information demonstrated genomic homology with *X. axonopodis* (Vauterin et al. 1995), resulting in reclassification to *X. axonopodis* pv. *phaseoli*. The two variants can be differentiated using isoenzyme profiling (El-Sharkawy and Huisinigh 1971), plasmid profiling (Lazo and Gabriel 1987), or DNA-DNA hybridization (Hildebrand et al. 1990). A physical map of the *X. axonopodis* pv. *phaseoli* var. *fuscans* (BXP65) chromosome has been constructed by PFGE and Southern hybridization (Chan and Goodwin 1999a).

Xanthomonas axonopodis pv. *phaseoli* var. *fuscans* isolates showed greater pathogenicity in the field than *X. axonopodis* pv. *phaseoli* isolates, generally causing greater stem collapse (Rudolph, 1990). In another report, *X. axonopodis* pv. *phaseoli* var. *fuscans* strains showed greater pathogenicity than *X. axonopodis* pv. *phaseoli* strains having the same origin. African strains were most pathogenic. The largest variation in pathogenicity came from *X. axonopodis* pv. *phaseoli* strains that originated in Caribbean and South American countries (Mutlu et al. 2008). Pathogenic variation was greater within *X. axonopodis* pv. *phaseoli* than within *X. axonopodis* pv. *phaseoli* var. *fuscans* strains (Mutlu et al. 2004).

Disease impact and distribution:

The principal host of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* is *Phaseolus vulgaris*, but other legume species are naturally infected, including *P. lunatus*, *Vigna aconitifolia* and *V. radiata*. *Lablab purpureus* and *Mucuna deeringiana* are possibly natural hosts. *Phaseolus coccineus*, *P. acutifolius* and *Lupinus polyphyllus* are hosts only by artificial inoculation (Bradbury, 1986). Common bacterial blight is one of the most destructive diseases in bean producing areas worldwide (Saettler, 1989). Heavy and early infection, high humidity, temperatures fluctuating between 20 and 30°C and alternate dry and wet weather can lead to more than 40% yield losses in susceptible cultivars (Saettler, 1989). It was reported in Ethiopia that for every percentage of common bacterial blight severity increase there was a loss of approximately 3.9 to 14.5 kg/ha seed (Tadele 2006). Disease surveys conducted in South Africa indicated common bacterial blight occurred in 83 and 85% of localities in seed and commercial production areas, respectively, which showed high incidences and wide spread distribution (Fourie 2002). No geographical differentiation was observed for genetic diversity among *X. axonopodis* pv. *phaseoli* or *X. axonopodis*

pv. phaseoli var. *fuscans* strains. The lack of geographical differentiation could be a result of continuous introduction and movement of new genotypes among regions (Mahuku et al. 2006).

Disease symptoms and epidemiology:

Common bacterial blight symptoms first appear as flaccid, small water-soaked spots on the underside of leaflets. These spots enlarge and merge, becoming dried and brown. A narrow, bright lemon-yellow border of tissue encircles the lesion. The flaccid leaf areas resulting from infection of *X. axonopodis* *pv. phaseoli* had significantly higher stomatal resistances compared to nearby turgid areas on the same leaf and the pathogen-induced water stress was highly localized (Goodwin and Sopher 1994). On either a chlorophyll or leaf area basis, photosynthesis and export declined over time after inoculation. By 14 days after inoculation, photosynthesis and export were 25 and 50% lower, respectively, in infected leaflets, even though only 7-10% of the total area measured was necrotic (Jiao et al. 1996). The pathogen attacks all aerial plant parts, but symptoms are more severe and conspicuous on leaves and pods (Mahuku et al. 2006).

Seed-borne bacteria are the primary inoculum (Saettler, 1989), but the pathogens can survive for months in leaves and other plant debris on the soil (Saettler, 1989; Fourie, 2002). Infection of approximately 1 in 10,000 seeds was capable of causing an outbreak of blight (Sutton and Wallen et al. 1970). The minimal population of *X. axonopodis* *pv. phaseoli* required to initiate infection in the field was 10 CFU/seed and there was a positive correlation between seed symptoms and the population of *X. axonopodis* *pv. phaseoli* per seed (Opio et al. 1993). A range of tolerances, ranging from 0 seeds infected in samples of 1,000-45,000, are used in seed quality programs by US seed companies to ensure that the pathogen is not transmitted in commercial seed lots (Maddox 1997).

Although seeds usually are the most important source of primary inoculum, the development of *X. axonopodis* pv. *phaseoli* epidemics depended more on the level of horizontal resistance and climatic conditions than on the size of *X. axonopodis* pv. *phaseoli* populations present in bean seeds (Maringoni et al. 1995). A diagrammatic scale was developed and validated to assess disease severity in the common bacterial blight/*Phaseolus* bean pathosystem (Diaz et al. 2001).

Seed Infection:

Seedborne inoculum is important in the survival and dissemination of these pathogens, and they can be transmitted from seed to growing plants, initiating damaging epidemics (Zaumeyer and Thomas 1957). Seed contamination can occur through the pods or through systemic infection of the mother plant. The disease can reduce seed size and quality. Pathogen incidence on seeds is correlated with disease incidence levels in the field. On white-seeded varieties, yellow or brown spots may appear on the seed coat, particularly near the hilum. On dark-seeded varieties, this discoloration is not visible (Zaumeyer and Thomas 1958). Discolored seeds were on average 5.3-26.4% lighter than normal seeds (Fininsa 2003). Severely infected seed may be shriveled and show poor germination or produce weakened plants.

Seeds can be externally contaminated or internally infected. Infection of flower buds and young pods can result in the transmission of *X. axonopodis* pv. *phaseoli* through the vascular system to the seed, leading to internal infection (Aggour et al. 1989). *Xanthomonas axonopodis* pv. *phaseoli* is a systemic pathogen that penetrates the ovule through the funiculus. It enters the sutures of the pod from the vascular system of the pedicel and passes into the raphe leading into the seed coat. The bacterial inoculum recovered from plant tissue with visible symptoms of blight infection was always much lower in resistant and moderately resistant cultivars than susceptible

cultivars (Cafati and Saettler 1980), but resistance to common blight foliar symptoms does not influence bacterial translocation to seeds of inoculated plants (Torres and Maringoni 1997).

Examining the efficiency of inoculation, Valarini et al. (1991), found that 100% incidence of superficial seed contamination was achieved immediately after seed-bacterium contact while 100% seed infection, which would give a significant reduction in seed germination, was obtained only after 36 hours,. For inoculation under field conditions, sand blast injury was shown to be highly effective (Faria and Melo 1989).

Plants grown from infected seeds frequently bear lesions on the cotyledons or primary nodes. In highly susceptible varieties, the lesions on leaves continue expanding until the leaves appear scorched or sun scalded. Such leaves soon become ragged and torn by wind and rain. Later, they wither and drop off. The tops may break over during a rain or strong wind.

Pathogen survival:

Seed is an important mechanism for survival and dissemination of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. However, it remains unclear whether there are consistent differences in survival characteristics between the two variants. Bacteria in/on the seed can survive longer than the seed itself (Dreo et al. 2003), although seeds from fields with 100% of infected plants presented low pathogen incidences when stored for 3-4 years. Sun drying and different harvesting systems did not affect pathogen incidence on seeds (Valarini et al. 1992). Inoculum of *X. axonopodis* pv. *phaseoli* can be localized superficially or internally in the seed (Valarini et al. 1996). *Xanthomonas axonopodis* pv. *phaseoli* was capable of long-term survival having been recovered from 15 year-old bean seeds (Schuster and Sayre 1967). In seeds, the survival of seedborne *X. axonopodis* pv. *phaseoli* was reported to be

reduced from 64 to 36-37% incidence during the first 6 months but the contamination rate was maintained after 30 and 60 months at both -18 and 5°C. The optimal temperature for bacterial survival and maintenance was 5°C and optimal conditions for seed conservation were the same as those for the maintenance of *X. axonopodis* pv. *phaseoli* (Marques et al. 2005).

In crop residue, under rainy conditions *X. axonopodis* pv. *phaseoli* survived up to 3 weeks above and 1 week below ground in infected plant tissue. Under dry conditions it survived 8 weeks above and 5 weeks below ground in infected plant tissue (Chavez-L and Granada 1988). Crop residue also can provide a source for epiphytic colonization of other crop plants. In a dry bean - onion crop rotation scheme, epiphytic *X. axonopodis* pv. *phaseoli* was recovered from symptomless onion plants in fields cropped to dry beans the prior year, but not from fields cropped to plants other than dry bean. Therefore, close rotation of onion and dry bean may allow *X. axonopodis* pv. *phaseoli* to persist epiphytically (Gent et al. 2005b). Some studies have shown that survival rate differed between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. *Xanthomonas axonopodis* pv. *phaseoli* survival was higher in sterile than non-sterile soil, and was higher compared to that of *X. axonopodis* pv. *phaseoli* var. *fuscans* (Torres et al. 2009). Under mild temperatures and low rainfall, *X. axonopodis* pv. *phaseoli* var. *fuscans* survived for 2 to 6 months in leaflets on the soil surface, and for 1 to 4 months in those incorporated in the soil regardless of the depth. Under higher rainfall and temperatures, the survival was from 1.5 to 2 months in leaflets on the surface and from 1 to 1.5 months in those buried 10 or 15 cm deep (Torres et al. 2009).

Survival of closely related bacterial plant pathogens on seed and crop residue also has been studied. *Xanthomonas vesicatoria* populations on inoculated seeds

dropped substantially within 30 days after inoculation in one study. Low seed moisture content also interfered with *X. vesicatoria* survival and transmission (Correa et al. 2008). In contrast, *Xanthomonas campestris* var. *vitians* survived at least 23 months after inoculation on lettuce seeds and the survival rates were higher in seeds stored at 10°C compared to room temperature (Ohata et al. 1982). In seeds of wheat and triticale, *X. translucens* pv. *cerealis* survival was only slightly reduced during the first two years after the harvest, but after 42 months the pathogen was not recoverable. In another study, survival of *X. translucens* pv. *cerealis* in crop debris was longer than 30 months under laboratory conditions and shorter than 8 months under field conditions (Malavolta et al. 2000). Also on infected tissue, the population of another closely related pathogen *X. axonopodis* pv. *glycines* was found to remain viable for 110 days in infected leaves on the soil surface and for 29 days in infected leaves buried at 15 cm depth but did not survive at all at a depth of 30 cm (Khare and Khare 1995). *Xanthomonas axonopodis* pv. *allii* could be recovered from infested onion leaves 9 months after they were placed on the soil surface or buried to a depth of 25 cm, but culturable populations of the pathogen declined more rapidly in buried leaves (Gent et al. 2005a). *Xanthomonas axonopodis* pv. *manihotis* survived for 48 h in manihoeira incubated at 15°C, and less than one day at 20, 25, 30 and 35°C (Theodoro and Maringoni 2002).

Disease management:

Disease management practices include planting pathogen-free seeds, rotation of beans with non-host plants with at least 2 years, use of resistant cultivars, treatment of seed with antibiotics such as streptomycin, foliar applications of copper-based bactericides before symptoms appear, and elimination of weed hosts. Seeds are typically produced in arid areas where common blight is rare; plants are inspected

during production and seeds of both resistant and susceptible genotypes should be certified pathogen-free.

Seed treatments such as tolylfluanid have been reported to reduce seed-to-plant transmission compared to untreated seeds in laboratory and glasshouse experiments (Lopes et al. 2008). From the aspect of introduction of antibiotics into seeds, immersion in either 25% PEG or 60% glycerol solutions did not diminish germination, although seedling vigor was slightly reduced. PEG solutions were more effective than glycerol solutions for introduction of antibiotics into seeds. However, concentrations of tetracycline and chlorotetracycline in PEG solutions that effectively reduced *X. axonopodis* pv. *phaseoli* were phytotoxic. PEG solutions with streptomycin reduced but did not eradicate internal populations of the bacterium from naturally contaminated seeds and caused few phytotoxic effects (Liang et al. 1992). Both hot water and dry heat have been successful in treating bean seeds for *X. axonopodis* pv. *phaseoli*. Also treatment with Streptocycline (100 g/ml) + captan (0.2%), or hot water (52°C for 10 min) followed by streptocycline (100 g/ml) were reported as very effective, eradicating the bacterium from naturally infected seed and reducing the number of infected seedlings from 80% to 5% in inoculated seed lots (Jindal 1991). External seed contamination could also be reduced by application of streptomycin (Taylor and Dudley 1977). It was translocated within the plant but not into the developing seeds (Mitchell et al 1952). However, natural resistance of bacteria from bean phyllosphere to streptomycin was reported to be quite common (Weller and Saettler 1977).

Several saprophytic bacteria (*Pseudomonas* spp., *Bacillus* sp. and *Erwinia herbicola* [*Pantoea agglomerans*]) were tested for biological control activity against *X. axonopodis* pv. *phaseoli* and they showed antagonistic effects, but no significant

antagonistic effect was observed on snap bean seeds 7 days after inoculation (Arsenijevic et al. 1998).

Elimination of susceptible weeds from the surrounding areas is recommended to control spread of the disease in *Phaseolus vulgaris*, because reciprocal infection between the weed and crop could occur with the crop becoming infected at the pre-flowering and pod-formation stages (Ovies and Larrinaga 1988).

Seed Health Testing:

Several different approaches have been used for detecting *X. axonopodis* pv. *phaseoli* in seeds, including a dome test (Venette et al. 1987), phage test (Kahveci and Maden 1994), selective media (Mabagala 1992), PCR (Audy et al. 1994), and serology such as indirect immunofluorescence microscopy and ELISA (Wong 1991).

Monoclonal antibodies were produced specifically against *X. axonopodis* pv. *phaseoli* but could react somewhat with other *Xanthomonas* strains and certain other bacteria strains (Yucel, Bermek and Cirakoglu 1997). Commercial ELISA kits are available. Only a few of the published methods have been accepted as routine diagnostic tools for seed health testing.

The determination of seed contamination was difficult due to the small number and uneven distribution of the bacterium. Chemical seed treatment and other microorganisms present in or on seeds could also hinder isolation.

A number of variations on seed extraction methods have been tested. Seed crushing was found to be more effective as an extraction method for *X. axonopodis* pv. *phaseoli* than soaking whole seeds in sterilized saline phosphate buffer (Maringoni, Kimati and Kurozawa 1998). Nevertheless, typical routine testing still involves whole-seed soaking because of the labor requirement for crushing large numbers of seed samples in commercial testing laboratories. Soaking whole seeds in sterilized distilled

water for 18-24 h at 5°C was more effective than soaking ground seeds 2 hours, at room temperature (Valarini et al. 1992, Valarini and Menten 1992). A method approved by ISTA (International Seed Testing Association) involves extraction by soaking at 5°C ($\pm 1^\circ\text{C}$) overnight. Recently, a National Seed Health System (NSHS) review panel evaluated eight published or industry-adopted methods. Four were designated as class B and needed further improvement and evaluation. The main variations among the methods were in extraction procedures: either 5°C ($\pm 1^\circ\text{C}$) overnight incubation or room temperature 2-3h. Vacuum and centrifugation were used in some extraction procedures.

For routine seed testing of bean, both complementary semi-selective media, MT and XCP1, are recommended (Remeeus and Sheppard 2006). It has been reported that MT is less selective but can recover more target colonies, whereas MT can also detect other seed-borne pathogenic *Pseudomonads* (Remeeus 2006). However, another report found XCP1 more efficient than MT in both quantification and detection of *X. axonopodis* pv. *phaseoli* in whole bean seed extracts (Tebaldi et al. 2007). It was noted that recovery of *fuscans* type *X. axonopodis* pv. *phaseoli* is in general lower on MT than on XCP1 (Sheppard 2007). Moreover, the recovery of *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *phaseoli* var. *fuscans*, *P.s. syringae* and *P.s. phaseolicola* were all higher on MT media than on tryptone glucose extract agar (Goszczyńska and Serfontein 1998).

PCR used for detection of plant pathogens was first reported in 1989 (Puchta and Sanger 1989) and now is commonly used for plant tissue, although not widely adopted for seed health testing. Primers to detect and identify phytopathogenic *Xanthomonas* strains by specific amplification of the *Hrp* gene cluster in *X. axonopodis* pv. *vesicatoria* were reported and were believed to be sensitive and specific to

phytopathogenic *Xanthomonads* (Leite et al. 1994). These primers generated a constant PCR product in several *X. axonopodis* pv. *phaseoli* isolates (Nunes et al. 2008), but these could not be applied as commercial health test in that they were not specific or uniform enough for all *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*.

PCR primers can be designed according to unique DNA sequences in the target bacteria. However, these genes are normally present as a single copy per cell, so 16S rRNA genes, present as multiple copies, are often used (Pastrik and Maiss 2000). The disadvantage of PCR primers from the 16S rRNA is that they have not been found to be highly specific (Seal et al. 1993, Walcott and Gitaitis 2000). Therefore, internal transcribed spacer (ITS) regions between the 16S and 23S ribosomal genes seem to be more promising as a target to design primers (Gurtler and Barrie 1995). Some phytopathogen detection methods are designed based on the ITS region sequence, thus it provided a way other than RAPD PCR analysis to design pathovar specific detection for *X. axonopodis* pv. *phaseoli*.

Several PCR methods have been reported to detect *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Audy et al. (Audy et al. 1994) designed primers based on a 3.4-kb plasmid(p7) DNA fragment, which specifically directed the amplification of a 730-bp fragment from DNA of 27 pathogenic *X. axonopodis* pv. *phaseoli*. An additional fragment of 550 bp was occasionally amplified from the DNA of *X. axonopodis* pv. *phaseoli* var. *fuscans* strains. Primers Xf1 and Xf2, based on a sequence conserved amplified region (SCAR) derived from RAPD PCR analysis of *X. axonopodis* pv. *phaseoli* var. *fuscans*, amplified a DNA fragment of 450 bp from all *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates and did not amplify any product from *X. axonopodis* pv. *phaseoli* isolates, or from any other DNAs tested (Toth et al. 1998). *X.*

axonopodis pv. *phaseoli*-targeted primers were then designed, but missed two virulent isolates out of 42 and amplified a fragment from the *X. axonopodis* pv. *vitians* genome (Halfeld-Vieira et al. 2001).

To differentiate between isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*, RAPD PCR was used as a rapid approach. A RAPD PCR primer OP-G11 generated a 820bp PCR product from *X. axonopodis* pv. *phaseoli* var. *fuscans* and a 900-bp product from *X. axonopodis* pv. *phaseoli* (Birch et al. 1997). However, it was also reported that some *X. axonopodis* pv. *phaseoli* isolates did not always generate the 900-bp fragment.

Rep-PCR was used to generate DNA fingerprinting to classify other seed pathogens such as *Acidovorax avenae* subsp. *citrulli* on cucurbit (Walcott and Gitaitis 2000). Rep-PCR and PCR-RFLP of the ribosomal genes have been used to genotypically distinguish the two *X. axonopodis* pv. *phaseoli* variants and to calculate genetic diversity among populations (Mahuku et al. 2006). Pulsed-field gel electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP) using *Xba*I confirmed that the two bacteria were genetically different (Chan and Goodwin 1999b).

In *X. axonopodis* pv. *phaseoli*, ISXax1 was identified as a novel insertion sequence which was restricted to *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *phaseoli* var. *fuscans* and *X. axonopodis* pv. *vesicatoria* which could be used as part of an identification procedure (Alavi et al. 2007). Suppression subtractive hybridization was performed to isolate DNA fragments present in these bean pathogens and absent from closely related *Xanthomonads*. Some virulence gene candidates were identified, such as homologs of hemagglutinins, TonB-dependent receptors, zinc-dependent metalloproteases, type III effectors, type IV secretion system components and

unexpectedly, homologs of the type III secretion apparatus components (SPI-1 family) (Alavi et al. 2008). These could contribute to further research on pathogenic mechanisms, resistance cultivar breeding and also pathovar specific detection design.

Real-time PCR has been used to quantify population size of bacterial pathogens such as *Listeria monocytogenes* (Reichert-Schwillinsky et al. 2009), *E.coli* (Ibekwe et al. 2004), and others. To solve quantitative analysis problems, real-time PCR has been applied successfully to identify many phytopathogens (Schena et al. 2004). For bacteria, Schaad first used it for *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers and it has become widely used as developed diagnostic tool for many bacteria pathogens (Schaad et al. 1999). Real-time PCR methods have not previously been applied for detection of *X. axonopodis* pv. *phaseoli* from seed. In selective detection of *Pseudomonas syringae* pv. *tomato*, primers and probes designed according to the sequence of RAPD PCR product were used to make dot blot hybridization and real-time PCR (Fanelli et al. 2007). This demonstrates an approach for designing specific TaqMan primers and probes to detect the existence of target pathogens.

Seed transmission:

Seeds are the most important source of primary inoculum for common blight outbreaks and the bacterium can be transmitted from seeds to seedlings at a high frequency. Incidence of seed infection is closely correlated with disease incidence in the seed production field (Valarini et al. 1992). There is usually a positive correlation between seed symptoms and the population of bacteria per seed. In Uganda, the population of *X. axonopodis* pv. *phaseoli* in farmers', commercial and research seeds averaged 10^5 to 10^9 CFU/100 seeds and the incidence of seed infection was 0.3 to 16.1%. Several studies have reported thresholds for seedborne infection. Infection incidence as low as 1 in 10,000 seeds was capable of causing an outbreak of blight

(Sutton and Wallen et al.1970). The minimum population required to initiate disease in the field in another study was 10 CFU/seed while a 0.2% seed infection level resulted in a serious disease outbreak (Opio et al. 1993). In Canada, 0.5% seed infection level has been shown to lead to disease epidemics (Zaumeyer and Thomas 1957). The fact that symptomless and slightly diseased seeds gave rise to severely infected seedlings demonstrated that the normal cleaning by removing colored and shriveled seeds would have limited effect on reducing the common bacterial blight infection of a seed stock if it was obtained from an infected field. Weller and Saettler (1980) showed that the minimum population to initiate infection depended on different bean genotypes and locations. Seed weight and germination can be significantly affected by incidence of *X. axonopodis* pv. *phaseoli* in the field and on the seeds (Valarini and Menten 1991). Similar studies of other *Xanthomonas* spp. have been conducted and some results can be related to *X. axonopodis* pv. *phaseoli*. Cotton seeds inoculated with *X. axonopodis* pv. *malvacearum* transmitted disease in up to 68% of seedlings and 16.66% of the emerged seedlings wilted and died (Gholve and Kurundkar 2007). Epiphytic populations of *X. axonopodis* pv. *glycines* developed on both resistant and susceptible seedlings that grew from seeds with either external or internal populations. In the field the pathogen dispersed from a diseased source plant equally in all directions and at the same rate on either resistant or susceptible cultivars (Groth and Braun 1989).

The effects of inoculum load and watering regime on the transmission of *Xanthomonas campestris* pv. *campestris* from seed to seedlings of cauliflower were investigated (Roberts et al. 1999). Effects of watering regime treatments on symptoms and on the proportion of contaminated but symptomless plants were similar. Initially, they were influenced only by the dose of bacteria with little difference between the

watering regimes, but later the proportion of plants with symptoms was greater for plants subjected to overhead watering, due to spread and secondary infection. For *X. campestris* pv. *cajani* in pigeonpea seed samples, heavy infection of seed caused failure of seed germination. Moderate infection caused hypocotyl splitting, browning of the radicle and necrosis of cotyledonary tissues (Sharma, Agrawal and Singh 2002). *Xanthomonas campestris* pv. *campestris* in broccoli seeds did not affect either seed germination or vigor and there were no significant differences among genotypes regarding seed transmission (Tebaldi et al. 2007).

The research presented in this thesis was initiated in response to the NSHS review of seed health tests for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. The objectives of this study were to 1) optimize seed extraction methods to improve detection sensitivity of the ISTA-approved detection method (temperature, incubation time, vacuum and centrifugation) and evaluate the modified method on naturally infested seeds, 2) develop quantitative real-time PCR for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* detection in *P. vulgaris* seeds, and 3) compare pathogenic seed transmission frequency and survival on stored seed between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* with help of the newly developed real-time PCR assay.

Literature cited

- Aggour, A. R., D. P. Coyne, A. K. Vidaver & K. M. Eskridge (1989) Transmission of the common blight pathogen in bean seed. *Journal of the American Society for Horticultural Science*, 114, 1002-1008.
- Alavi, S. M., S. Poussier & C. Manceau (2007) Characterization of ISXax1, a novel insertion sequence restricted to *Xanthomonas axonopodis* pv. *phaseoli* (Variants *fuscans* and non-*fuscans*) and *Xanthomonas axonopodis* pv. *vesicatoria*. *Applied and Environmental Microbiology*, 73, 1678-1682.
- Alavi, S. M., S. Sanjari, F. Durand, C. Brin, C. Manceau & S. Poussier (2008) Assessment of the genetic diversity of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp. *fuscans* as a basis to identify putative

- pathogenicity genes and a type III secretion system of the SPI-1 family by multiple suppression subtractive hybridizations. *Applied and Environmental Microbiology*, 74, 3295-3301.
- Arnaudsantana, E., E. Penamatos, D. P. Coyne & A. K. Vidaver (1991) Longevity of *Xanthomonas axonopodis* pv. *phaseoli* in naturally infested dry bean (*Phaseolus-vulgaris*) debris. *Plant Disease*, 75, 952-953.
- Arsenijevic, M., A. Obradovic, D. Stevanovic & M. Ivanovic (1998) Antagonistic effect of some saprophytic bacteria to *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli*. *Bulletin OILB/SROP*, 21, 297-300.
- Audy, P., A. Laroche, G. Saindon, H. C. Huang & R. L. Gilbertson (1994) Detection of the bean common blight bacteria, *Xanthomonas-campestris* pv *phaseoli* and x-c *phaseoli* var *fuscans*, using the polymerase chain-reaction. *Phytopathology*, 84, 1185-1192.
- Bradbury, J. F.(1986) *Guide to plant pathogenic bacteria*. xviii + 332pp.
- Birch, P. R. J., L. J. Hyman, R. Taylor, A. F. Opio, C. Bragard & I. K. Toth (1997) RAPD PCR-based differentiation of *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. *European Journal of Plant Pathology*, 103, 809-814.
- Chan, J. & P. H. Goodwin (1999a) A physical map of the chromosome of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* BXPf65. *Fems Microbiology Letters*, 180, 85-90.
- (1999b) Differentiation of *Xanthomonas campestris* pv. *phaseoli* from *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* by PFGE and RFLP. *European Journal of Plant Pathology*, 105, 867-878.
- Diaz, C. G., R. B. Bassanezi & A. Bergamin Filho (2001) Development and validation of a diagrammatic scale for *Xanthomonas axonopodis* pv. *phaseoli* in phaseolus bean. *Summa Phytopathologica*, 27, 35-39.
- Dreo, T., T. Demsar & M. Ravnikar (2003) Quarantine of *Xanthomonas campestris* pv. *phaseoli* in beans. *Zbornik predavanj in referatov 6. Slovenskega Posvetovanja o Varstvu Rastlin, Zrece, Slovenije, 4-6 marec 2003*, 123-127.
- El-Sharkawy, T. A. & D. Huisinigh, (1971) Differentiation among *Xanthomonas* species by polyacrylamide gel electrophoresis of soluble proteins. *Journal of General Microbiology*, 68, 155-165
- Fanelli, V., C. Cariddi & M. Finetti-Sialer (2007) Selective detection of *Pseudomonas syringae* pv. *tomato* using dot blot hybridization and real-time PCR. *Plant Pathology*, 56, 683-691.
- Faria, J. C. D. & P. E. D. Melo (1989) Inoculation of common beans with *Xanthomonas-campestris* pathovar *phaseoli* under field condition. *Pesquisa Agropecuaria Brasileira*, 24, 987-990.
- Fininsa, C. (2003) Relationship between common bacterial blight severity and bean yield loss in pure stand and bean-maize intercropping systems. *International Journal of Pest Management*, 49, 177-185.
- Fourie, D. (2002) Distribution and severity of bacterial diseases on dry beans (*Phaseolus vulgaris* L.) in South Africa. *Journal of Phytopathology-Phytopathologische Zeitschrift*, 150, 220-226.
- Goodwin, P. H. & C. R. Sopher (1994) Brown pigmentation of *Xanthomonas-campestris* pv *phaseoli* associated with homogentisic acid. *Canadian Journal of Microbiology*, 40, 28-34.

- Goszczynska, T. & J. J. Serfontein (1998) Milk-Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *Journal of Microbiological Methods*, 32, 65-72.
- Gurtler, V. & H. D. Barrie (1995) Typing of *Staphylococcus-aureus* strains by PCR-amplification of variable-length 16s-23s rDNA spacer regions - characterization of spacer sequences. *Microbiology-Uk*, 141, 1255-1265.
- Halfeld-Veira, B. A., R. M. Souza, A. R. Figueira & A. De Jesus Boari (2001) Identification of *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* through the PCR technique. *Fitopatologia Brasileira*, 26, 737-740.
- Hildebrand, D. C., N. J. Palleroni & M. N. Schroth (1990) Deoxyribonucleic-acid relatedness of 24 *Xanthomonas* strains representing 23 *Xanthomonas-campestris* pathovars and *xanthomonas-fragariae*. *Journal of Applied Bacteriology*, 68, 263-269.
- Jiao, J. R., P. Goodwin & B. Grodzinski (1996) Photosynthesis and export during steady-state photosynthesis in bean leaves infested with the bacterium *Xanthomonas campestris* pv. *phaseoli*. *Canadian Journal of Botany-Revue Canadienne De Botanique*, 74, 1-9.
- Jindal, K. K. (1991) Physical and chemical agents for the eradication of *Xanthomonas campestris* pv. *phaseoli* from bean seeds. *Plant Disease Research*, 6, 68-71.
- Kahveci, E. & S. Maden (1994) Detection of *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola* by bacteriophages. *Journal of Turkish Phytopathology*, 23, 79-85.
- Lazo, G. R. & D. W. Gabriel (1987) Conservation of plasmid DNA-sequences and pathovar identification of strains of *Xanthomonas-campestris*. *Phytopathology*, 77, 448-453.
- Leite, R. P., G. V. Minsavage, U. Bonas & R. E. Stall (1994) Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA-sequences related to the *hrp* genes of *Xanthomonas-campestris* pv. *vesicatoria*. *Applied and Environmental Microbiology*, 60, 1068-1077.
- Liang, L. Z., J. M. Halloin & A. W. Saettler (1992) Use of polyethylene-glycol and glycerol as carriers of antibiotics for reduction of *Xanthomonas-campestris* pv. *phaseoli* in navy bean-seeds. *Plant Disease*, 76, 875-879.
- Lopes, L. P., P. F. R. Alves, C. Zandona, M. P. Nunes & Y. R. Mehta (2008) A semi-selective medium to detect *Xanthomonas axonopodis* pv. *phaseoli* in bean seeds and its eradication through seed treatment with tolylfluanid (Meio semi-seletivo para detectar *Xanthomonas axonopodis* pv. *phaseoli* em sementes de feijoeiro e sua erradicacao atraves do tratamento de sementes com o fungicida tolylfluanid.). *Summa Phytopathologica*, 34, 287-288.
- Mabagala, R. B. (1992) An improved semiselective medium for recovery of *Xanthomonas-campestris* pv. *Phaseoli*. *Plant Disease*, 76, 443-446.
- Maddox, D. A. (1997) Regulatory needs for standardized seed health tests. *Plant pathogens and the worldwide movement of seeds*, 81-92.
- Mahuku, G. S., C. Jara, M. A. Henriquez, G. Castellanos & J. Cuasquer (2006) Genotypic characterization of the common bean bacterial blight pathogens, *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* by rep-PCR and PCR-RFLP of the ribosomal genes. *Journal of Phytopathology*, 154, 35-44.

- Maringoni, A. C., H. Kimati & C. Kurozawa (1995) Presence of the *Xanthomonas campestris* pv. *phaseoli* in dry bean seeds and epidemiological consequences. *Fitopatologia Brasileira*, 20, 449-457.
- (1998) Comparison between two extraction methods of *Xanthomonas campestris* pv. *phaseoli* from bean seeds. *Pesquisa Agropecuaria Brasileira*, 33, 659-664.
- Marques, A. S. d. A., P. M. Guimaraes, J. P. d. Santos & T. M. Vieira (2005) Survival and viability of *Xanthomonas axonopodis* pv. *phaseoli* associated with bean seeds stored under controlled conditions. *Fitopatologia Brasileira*, 30, 527-531.
- Mumford, R., N. Boonham, J. Tomlinson & I. Barker (2006) Advances in molecular phytodiagnostics - new solutions for old problems. *European Journal of Plant Pathology*, 116, 1-19.
- Nicolaisen, M., H. N. Rasmussen, K. Husted & S. L. Nielsen (2001) Reverse transcription-detection of immobilized, amplified product in a one-phase system (RT-DIAPOPS) for the detection of potato virus Y. *Plant Pathology*, 50, 124-129.
- Nunes, W. M. d. C., M. J. Corazza, S. A. C. D. d. Souza, S. Tsai & E. E. Kuramae (2008) Characterization of *Xanthomonas axonopodis* pv. *phaseoli* isolates. *Summa Phytopathologica*, 34, 228-231.
- Opio, A. F., J. M. Teri & D. J. Allen (1993) Studies on seed transmission of *Xanthomonas campestris* pv. *phaseoli* in common beans in Uganda. *African Crop Science Journal*, 1, 59-67.
- Ovies, J. & L. Larrinaga (1988) Transmission of *Xanthomonas campestris* pv. *phaseoli* by a wild host. *Ciencia y Tecnica en la Agricultura, Proteccion de Plantas*, 11, 23-30.
- Pastrik, K. H. & E. Maiss (2000) Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. *Journal of Phytopathology-Phytopathologische Zeitschrift*, 148, 619-626.
- Puchta, H. & H. L. Sanger (1989) Sequence-analysis of minute amounts of viroid RNA using the polymerase chain-reaction (PCR). *Archives of Virology*, 106, 335-340.
- Roberts, S.J., L.H. Hiltunen, P.J. Hunter & J. Brough, (1999) Transmission from seed to seedling and secondary spread of *Xanthomonas campestris* pv. *campestris* in Brassica transplants: effects of dose and watering regime. *European Journal of Plant Pathology* 105: 879-889.
- Rudolph K. (1990) Toxins as taxonomic features. In: Klement Z, Rudolph K and Sands DC (eds) *Methods in Phytobacteriology*, 251-267.
- Remeus, P.M. and Sheppard, J.W. (2006) *ISTA Method Validation Reports* 3, 1-11
- Saettler, A.W. (1989) Common bacterial blight. *Bean Production Problems in the Tropics*. 261-184
- Schaad, N. W., Y. Berthier-Schaad, A. Sechler & D. Knorr (1999) Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. *Plant Disease*, 83, 1095-1100.
- Schena, L., F. Nigro, A. Ippolito & D. Gallitelli (2004) Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology*, 110, 893-908.
- Schuster, M. L. & R. M. Sayre (1967) A coryneform bacterium induces purple-colored seed and leaf hypertrophy of *Phaseolus vulgaris* and other leguminosae. *Phytopathology*, 57, 1064-&.

- Seal, S. E., L. A. Jackson, J. P. W. Young & M. J. Daniels (1993) Differentiation of *Pseudomonas-solanacearum*, *Pseudomonas-syzygii*, *Pseudomonas-pickettii* and the blood-disease bacterium by partial 16s-ribosomal-RNA sequencing - construction of oligonucleotide primers for sensitive detection by polymerase chain-reaction. *Journal of General Microbiology*, 139, 1587-1594.
- Severin, V. (2004) Survival of *Xanthomonas axonopodis* pv. *phaseoli* in soil. *Analele Institutului de Cercetare-Dezvoltare pentru Protectia Plantelor*, 33, 43-49.
- Sheppard, J., Kurowski, C. and Remeus, P.M. (2007) Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris*. *International Rules for Seed Testing* 7-021
- Sutton MD and Wallen VR (1970) Epidemiological and ecological relationship of *Xanthomonas phaseoli* and *X. c.* var *fuscans* on bean in Southern Ontario, 1961–1968. *Canadian Journal of Botany* 48: 1329–1334
- Tadele, T. (2006) Effect of common bacterial blight severity on common bean yield. *Tropical Science*, 46, 41-44.
- Tebaldi, N. D., R. d. C. Panizzi & R. Sader (2007) Detection, transmission and effect of *Xanthomonas campestris* pv. *campestris* in the physiological quality of broccoli seeds. *Summa Phytopathologica*, 33, 290-293.
- Torres, J. P. & A. C. Maringoni (1997) Reaction of dry bean of *Xanthomonas campestris* pv. *phaseoli* and seed transmission of the bacterium. *Fitopatologia Brasileira*, 22, 546-549.
- Toth, I. K., L. J. Hyman, R. Taylor & P. R. J. Birch (1998) PCR-based detection of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* in plant material and its differentiation from *X. c.* pv. *phaseoli*. *Journal of Applied Microbiology*, 85, 327-336.
- Valarini, P. J., J. A. H. Galvao & D. D. A. Oliveira (1996) *Xanthomonas campestris* pv. *phaseoli*: Importance of seed inoculum in the epidemiology of the common bacterial blight of beans. *Fitopatologia Brasileira*, 21, 261-267.
- Valarini, P. J. & J. O. M. Menten (1992) Methodology for detection of *Xanthomonas campestris* pv. *phaseoli* in bean seeds. *Fitopatologia Brasileira*, 17, 373-383.
- Valarini, P. J., J. O. M. Menten & M. A. Lollato (1992) Incidence of the common bacterial blight in the field and transport of *Xanthomonas campestris* pv. *phaseoli* through bean seeds, obtained by different methods. *Summa Phytopathologica*, 18, 160-166.
- Vauterin, L., B. Hoste, K. Kersters & J. Swings (1995) Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, 45, 472-489.
- Venette, J. R., R. S. Lamppa, D. A. Albaugh & J. B. Naves (1987) Presumptive procedure (dome test) for detection of seed-borne bacterial pathogens in dry beans. *Plant Disease*, 71, 984-990.
- Vieira, B. d. A. H. & R. M. d. Souza (2000) Virulence of isolates of *Xanthomonas axonopodis* pv. *phaseoli* and its variant *fuscans*. *Ciencia e Agrotecnologia*, 24, 94-102.
- Vieira, R. F., J. E. S. Carneiro & J. P. Lynch (2008) Root traits of common bean genotypes used in breeding programs for disease resistance. *Pesquisa Agropecuaria Brasileira*, 43, 707-712.
- Walcott, R. R. & R. D. Gitaitis (2000) Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. *Plant Disease*, 84, 470-474.

- Weller, S. A., J. G. Elphinstone, N. C. Smith & D. E. Stead (2000) Detection of *Ralstonia solanacearum* from potato tissue by post-enrichment TaqMan PCR. *Bulletin OEPP*, 30, 381-383.
- Wong, W. C. (1991) Methods for recovery and immunodetection of *Xanthomonas campestris* pv *phaseoli* in navy bean seed. *Journal of Applied Bacteriology*, 71, 124-129.
- Yucel, F., E. Bermek & B. Cirakoglu (1997) Production of monoclonal antibodies specific for *Xanthomonas campestris* pv. *phaseoli*. *Turkish Journal of Biology*, 21, 125-131.
- Zaumeyer, W. J. & H. R. Thomas (1958) New snap and Pinto beans resistant to several diseases. *Phytopathology*, 47, 454 p.

CHAPTER 2. IMPROVED EXTRACTION PROCEDURES FOR DETECTING *XANTHOMONAS AXONOPODIS* PV. *PHASEOLI* IN COMMON BEAN SEED

A paper to be submitted to *European Journal of Plant Pathology*

Yiqing He, Gary Munkvold

Abstract

Xanthomonas axonopodis pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* are important seedborne pathogens of *Phaseolus vulgaris*. In order to maintain seed quality and meet phytosanitary requirements, accurate seed health testing methods are critical. Currently employed selective-media-based methods for these pathogens include several variations in extraction procedures. In order to optimize pathogen extraction from *P. vulgaris* seeds, we assessed the influence of different extraction steps on the sensitivity of *X. axonopodis* pv. *phaseoli* detection. Seeds were inoculated with *X. axonopodis* pv. *phaseoli* to reach inoculum levels from 10^1 CFU/seed to 10^5 CFU/seed; one contaminated seed was mixed into 1000-seed subsamples of clean and healthy *P. vulgaris* seeds. Thirty 1000-seed subsamples were tested for each different extraction condition. Extraction procedures included soaking whole seeds in sterilized saline phosphate buffer overnight at 4°C or at room temperature for 3 h, with or without vacuum extraction, and concentrating the seed extract by centrifuging. The seed extract dilutions were cultured on semi selective agar media MT and XCP1. The percentages of positive subsamples were recorded and compared to measure the effects of each extraction step on detection sensitivity. The results showed that vacuum extraction and centrifugation of seed extracts increased sensitivity, and the highest sensitivity was obtained with the 3h vacuum extraction followed by centrifugation. These results were confirmed on naturally infected seeds; *Xanthomonas axonopodis* pv. *phaseoli* was detected in 35 and 48 of 70 naturally infested samples using overnight soaking and 3-

hour vacuum extraction, respectively (a significant difference). Our results suggest that a 3-hour vacuum extraction followed by centrifugation would be a valuable modification to the current method approved by the International Seed Testing Association (ISTA).

Introduction

Xanthomonas axonopodis pv. *phaseoli* (Smith) Vauterin and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder (Vauterin, 1995) cause common bacterial blight of bean (*Phaseolus vulgaris* L.), one of the most destructive diseases in bean production worldwide (Saettler, 1989). *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* can be distinguished by a diffusible brown pigment produced in culture, due to secretion and subsequent oxidation of homogentisic acid which is an intermediate in tyrosine catabolism for a number of bacteria. *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* appears to have a disrupted tyrosine catabolism pathway and cannot use tyrosine as a nutrient (Goodwin and Sopher 1994). The two variants of *Xanthomonas axonopodis* pv. *phaseoli* also can be distinguished by isoenzyme profiling (El-Sharkawy and Huisingh 1971), plasmid profiling (Lazo and Gabriel 1987), or DNA-DNA hybridization (Hildebrand et al. 1990). *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* are equally important as seed quality factors and phytosanitary concerns. Some studies have indicated that *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates are more aggressive than *Xanthomonas axonopodis* pv. *phaseoli* (Mutlu et al. 2004) but others have found no relationship between virulence on beans and the capacity to produce dark pigment in culture or the geographical origin of the isolates (Vieira et al. 2008, Vieira and Souza 2000).

Common blight symptoms first appear as flaccid, small water-soaked spots on the underside of leaflets. These spots enlarge and merge, becoming dried and brown. A narrow, bright lemon-yellow border of tissue encircles the lesion. Infected pods exhibit circular, water-soaked areas that often produce yellow masses of bacterial ooze. Later, spots dry and appear as reddish-brown lesions. Pod infection often causes discoloration, shriveling and bacterial contamination of seeds; however, some seed may appear healthy. Cells of *X. axonopodis* pv. *phaseoli* may invade and travel within the vascular (water-conducting) tissue of the bean plant. Yield is affected most if blight develops before the pod-fill stage.

Although *X. axonopodis* pv. *phaseoli* can survive for months in leaves and other plant debris on the soil (Saettler, 1989; Fourie, 2002), seed-borne bacteria are the primary inoculum sources (Saettler, 1989). The main route of spread is by planting infected seeds. Bacteria in/on the seed can survive longer than the seed itself (Dreo et al. 2003). Infection of approximately 1 in 10,000 seeds was capable of causing an outbreak of blight (Sutton et al. 1970). The minimal population of *X. axonopodis* pv. *phaseoli* required to initiate infection in the field was 10 CFU/seed and there was a positive correlation between seed symptoms and the population of *X. axonopodis* pv. *phaseoli* per seed (Opio et al. 1993). A range of tolerances, ranging from 0 seeds infected in samples of 4,000-45,000, is used in seed quality programs in US seed companies to ensure that the pathogen is not transmitted by commercial seed lots (Maddox 1997).

Several different approaches have been used for detecting *X. axonopodis* pv. *phaseoli* in seeds, including a dome test (Venette et al. 1987), phage test (Kahveci and Maden 1994), selective media (Mabagala 1992, Reemeus and Sheppard 2006, Sheppard et al. 2007), PCR (Audy et al. 1994), and serology such as indirect

immunofluorescence microscopy and ELISA (Wong 1991). Only a few of the published methods have been accepted as routine diagnostic tools. For routine seed testing of bean, two complementary semi-selective media, MT and XCP1, are recommended. It has been reported that MT is less selective but more sensitive, and can also be used to detect another seed-borne bacterial pathogen *Pseudomonads* (Remeeus and Sheppard, 2006). However, another report found XCP1 more efficient than MT in both quantification and detection of *X. axonopodis* pv. *phaseoli* in whole bean seed extracts (Tebaldi et al. 2007). It was noted that recovery of fuscans type *X. axonopodis* pv. *phaseoli* is in general lower on MT than on XCP1 (Remeeus and Sheppard, 2006). Moreover, the ratio of recovery of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* on MT media in comparison to tryptone glucose extract agar ranged from 95 to 115%, *P.s. syringae* from 103 to 140% and *P.s. phaseolicola* from 91 to 132% (Goszczyńska and Serfontein 1998).

Published seed health testing methods for these pathogens and other seedborne *Xanthomonads* include several variations in the extraction procedure. The seed crushing method was found to be more effective as an extraction method for *X. axonopodis* pv. *phaseoli* than soaking whole seeds in sterilized saline phosphate buffer (Maringoni et al. 1998). However, soaking whole seeds in sterilized distilled water for 18-24 h at 5° C was better than soaking ground seeds for 2 hours at room temperature (Valarini et al. 1992, Valarini and Menten 1992). The optimal extraction method for *Xanthomonas hortorum* pv. *carotae* was suggested as overnight (16-18 h) incubation at 4-7° C (Asma, 2005). The same method was used for *Xanthomonas* spp. detection from onion seeds (Roumagnac et al. 2000, Sakthivel et al. 2001). The current seed health test method approved by the International Seed Testing Association (ISTA) includes soaking whole seeds overnight in saline buffer at 5° C (Sheppard et al., 2007).

The U.S. National Seed Health System (NSHS) is authorized by the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) to provide resources to assist seed companies in meeting phytosanitary regulations. Its main objectives include: to conduct development research on and facilitate the implementation of standardized seed health tests and phytosanitary inspection procedures; to provide accreditation to private entities to carry out testing activities; to promote international phytosanitary reform and foster trade. One component of NSHS activities is to convene review panels for the evaluation of seed health testing methods. A NSHS review panel evaluated eight published or industry-adopted seed health tests for *X. axonopodis* pv. *phaseoli*. Four were designated as class B, “temporary standard”, including three selective media assays. Temporary standard methods have NSHS approval but are in need of additional supporting data or further improvement before they can be fully approved. The review panel recommended further research on optimization of seed extraction methods for selective media assays. Most variations among selective media assays were seed soaking time and temperature, the use of vacuum extraction, or concentration of the seed extract by centrifugation. The objective of this study was to follow NSHS review panel recommendations by evaluating the effects of incubation time/temperature, vacuum extraction and centrifugation on improving *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* detection sensitivity in bean seeds.

Materials and Methods

Bacterial strains and growth conditions

Bacterial isolates used in this study to inoculate *Phaseolus vulgaris* beans were *X. axonopodis* pv. *phaseoli* B1, originally from Idaho, US. Bacterial cultures were grown routinely on nutrient agar (Becton, Dickinson and Company, MD) or YDC

medium. Isolation of *X. axonopodis* pv. *phaseoli* or *X. axonopodis* pv. *phaseoli* var. *fuscans* from naturally infected seed or inoculated plants was conducted on semiselective agar media MT (Goszczyńska and Serfontein, 1998; Sheppard, 2007) and XCP1 (McGuire *et al.*, 1986; Sheppard, 2007). Bacterial cultures were enriched on nutrient agar and YDC agar. Long-term, bacteria were stored on silica beads (Microbank, Austin, TX) by shaking beads in a cell suspension, removing excess fluid, and freezing at -80°C.

Pathogenicity Test

Seeds of Charlevoix dark red kidney bean (*Phaseolus vulgaris*), a bean genotype susceptible to common bacterial blight, were planted under greenhouse condition in pasteurized soil in 20 cm pots. Plants were allowed to grow until the first true leaf was fully established. Plants were watered 2 h before inoculation to ensure sufficient moisture. Seedlings were inoculated by contaminating a sterile needle with bacteria from 2-day-old cultures grown on YDC. Seedlings were inoculated by stabbing through the primary node at an angle of about 45° with a contaminated needle. Noninoculated control plants were wounded with a sterile needle. Plants were covered with transparent plastic bags to maintain humidity >90%. Symptom development was observed and recorded 10 days after inoculation. The pathogens were re-isolated from leaf tissues adjacent to the lesions by plating on XCP1 semi-selective medium. Suspect colonies from slightly infected seedlots were tested using this method and symptoms were recorded and evaluated.

Efficacy of extraction methods for target bacteria using inoculated seeds

White bean seeds (*Phaseolus vulgaris*) (cv. 'Derby', Harris Moran Seed Company, Modesto, CA, USA), free from contamination by *Xanthomonas axonopodis* pv. *phaseoli* or *X. axonopodis* pv. *phaseoli* var. *fuscans*, were used for these

experiments. The efficiency of extraction methods was compared for the recovery of *X. axonopodis* pv. *phaseoli* cells from artificially inoculated bean seed. The inoculum was made with phosphate-buffered saline solution from 2-day-old *X. axonopodis* pv. *phaseoli* isolate B1. Original bacterial concentrations were adjusted spectrophotometrically [$OD_{\lambda 600} = 0.6$ ($\approx 1 \times 10^9$ CFU/ml)]. Ten-fold dilution series (10^4 CFU/ml— 10^9 CFU/ml) were made from the original inoculum. Bean seed samples were artificially inoculated by vacuum-infiltration in bacterial suspension of each concentration for 5 min. Subsequently, seeds were air-dried in a laminar-flow hood until excess water had evaporated. In order to confirm inoculation dose, ten infested seeds from each concentration were soaked individually in 1 ml saline solution at 4°C overnight. One-hundred μ l seed wash was spread on MT media, two replicates for each seed. After incubation for 2 d, colonies were counted and actual infestation level (CFU/seed) was calculated.

Subsamples of 1,000 *P. vulgaris* seeds were used to test extraction method efficiency. One inoculated seed was mixed with 999 clean ones. Ten replicate subsamples were tested for each treatment and the comparisons were repeated three times. In one set of experiments, incubation time/temperature and vacuum extraction were evaluated with inoculum levels of 10^2 CFU/seed, 10^3 CFU/seed and 10^5 CFU/seed. Incubation treatments were: 4°C overnight, room temperature 3h and room temperature 3h with vacuum. Overnight incubation consisted of 18 h duration in a cold room programmed for 4° C ($\pm 2^\circ$). Room temperature ranged from 20 to 24° C. For the vacuum-extraction treatment, a vacuum pump aspirator (Nalgene, NY) was applied on flasks containing soaking seeds for half hour to create low air pressure and the pressure was kept for 3h. In another set of experiments, centrifugation was evaluated using inoculum levels of 10^1 CFU/seed and 10^2 CFU/seed. This was a factorial experiment

with two incubation conditions (room temperature for 3 h or 4°C overnight), with or without centrifugation of the seed extract. For the centrifugation step, the total seed wash was centrifuged at 7,000 rpm for 10 min immediately after incubation had finished. The pellet was resuspended in 5 ml 0.85% NaCl solution and 100 µl aliquots were cultured on selective medium. Seed wash was cultured on MT and XCP1 media and target colonies were counted to measure detection sensitivity and extraction efficiency. On MT, *X. axonopodis* pv. *phaseoli* colonies are yellow, distinguished by two zones of hydrolysis: a large clear zone of casein hydrolysis and a smaller milky zone of Tween 80 lysis. On XCP1, *X. axonopodis* pv. *phaseoli* colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Sheppard, 2007). Detection sensitivity was recorded as the percentage of subsamples testing positive with each treatment. Treatments were compared using SAS procedure GLM and mean separation by the Tukey's method.

Method evaluation on naturally infested seeds

Seedlots known to be naturally infested with common bacterial blight were obtained from various geographic origins (Table 1). Samples of varying sizes were divided into subsamples of 1000 seeds. An equal number of subsamples were tested by each of two treatments: 4°C overnight incubation without vacuum or centrifuge application (ISTA method); 3 h incubation at room temperature with vacuum and centrifuge application (modifications to ISTA method based on inoculated-seed experiments). Seed extracts were cultured on the semi-selective media. Presence of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* colonies was recorded and suspect colonies were confirmed by culturing on YDC medium and pathogenicity testing. Detection sensitivity (proportion of samples testing positive) of each treatment for all subsamples was compared by t-test.

Results

For any inoculum level, no significant difference was observed between 3-hour room temperature incubation without vacuum and overnight 4 °C incubation without vacuum. However, the latter showed a trend of higher mean detection sensitivity than the former at each inoculum level (Figure 1). Vacuum application during the extraction procedure typically increased detection sensitivity, but the difference was significant only for the 10^5 CFU/seed inoculum level ($P<0.05$) (Figure 1).

Centrifugation enhanced sensitivity for both incubation conditions, but the difference was significant ($P<0.05$) only for the 3-hour room-temperature incubation. Centrifugation step more than doubled the sensitivity. Room temperature incubation with centrifugation had the highest sensitivity, also significantly higher than the 4°C overnight incubation ($P<0.05$) (Figure 2).

Naturally infested seeds

The modified extraction treatment resulted in higher detection sensitivity than the original extraction method. For most of the naturally infested seedlots, the modified method identified a higher proportion of positive subsamples and thus showed improved detection sensitivity. Overall, 35 of 70 subsamples were positive by the ISTA-approved method, and 48 of 70 subsamples were positive by the modified method (Table 2). The difference was significant ($P<0.05$). Seed wash from seedlots 2, 3, 9 and 14 was cultured on nutrient agar (Figure 3). Suspect colonies were also confirmed by culturing on YDC and pathogenicity testing.

Pathogenicity test

Suspect colonies from seedlots 2,3,5,6,7,11 and 14 were inoculated into plants. There were 2-6 colonies picked from each seedlot. After 1 week, most plants inoculated with colonies recovered from naturally infested bean seeds had symptoms of leaf spots

surrounded by a yellow halo except two colonies from seedlot 5 and 6 respectively and one from seedlot 14; however, several other colonies from the same seedlots were pathogenic on the plants. Leaf washes with sterile distilled water were cultured on semi-selective XCP1 medium and *X. axonopodis* pv. *phaseoli* colonies were recovered from the leaf lesions.

Discussion

Short soaking times at room temperature for seed extraction are attractive because results can be obtained faster than with overnight soaking. According to our results, seed soaking for a shorter period of time (eg., 3 h) at room temperature with vacuum applied was as effective as overnight soaking at 4°C. Vacuum extraction usually is not practical for overnight extraction because of the low temperature requirement. With centrifugation of the seed extract, the 3 h room temperature extraction was the most effective method tested in this study. Most commercial labs use overnight incubation. Longer extraction times may have been favored because they allow the bacteria more time either to enter into suspension, or to resuscitate, or to reproduce. Since we were using healthy seeds which were kept in storage for more than a half year, our inoculated seed samples did not display heavy natural contamination of other microorganisms which can be a problem for some seedlots, even on these semi-selective media. However, most fungal and bacterial species are inhibited on the well-developed and -evaluated MT and XCP1 media. Some of the naturally infested seedlots that we tested were recently harvested and displayed a substantial amount of contamination by saprophytic organisms (Figure 3).

Vacuum application (Gitaitis and Walcott 2007) on seed soaking could force air from beneath the seed coat and also bring bacterial cells out of the seed. It may also force the seed coat to loosen and fall apart, allowing deeper extraction. When it was

used during 3 h incubation under room temperature, the results showed that it significantly increased recovery.

Centrifugation (Hadas et al. 2005) has been used to enhance test sensitivity when a low infestation dose was expected. It not only enriches target bacteria concentration, but also other microorganisms and debris/chemicals from seeds. Interestingly, the centrifuge step added much more to sensitivity for short incubation under room temperature than for cold overnight incubation. The reason might be that the overnight seed extract may contain more debris particles, dirt and inhibitors from the seeds; it might also cause more potential injury to bacterial cells.

Naturally infected seeds were from various origins and of different types. Infestation level of common bacterial blight varied widely and so did the accompanying microflora. However, *X. axonopodis* pv. *phaseoli* colonies were easy to distinguish from other gram-negative bacterium on semi-selective medium by its glossy and transparent colony appearance with yellow pigment and smooth edge. No *X. axonopodis* pv. *phaseoli* var. *fuscans* was found in these infected seedlots. Using modified method, in some seedlots, *X. axonopodis* pv. *phaseoli* was the most common organism and only a few other colonies appeared on plates; in other cases, plates had numerous *Pseudomonas*-like white colonies and *Xanthomonas* could hardly be seen and found.

In summary, we found that a 3-hour vacuum extraction followed by centrifugation resulted in the most sensitive detection of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* from contaminated seed. This procedure should be considered as a modification to the ISTA-approved method.

Literature Cited

- Asma, M. (2005) Proposal for a new method for detecting *Xanthomonas hortorum* pv. *carotae* on carrot seeds. *ISTA Method Validation reports* 2, 1-17.
- Dreo, T., T. Demsar & M. Ravnikar (2003) Quarantine of *Xanthomonas campestris* pv. *phaseoli* in beans. *Zbornik predavanj in referatov* 6. Slovenskega Posvetovanje o Varstvu Rastlin, Zrece, Slovenije, 4-6 marec 2003, 123-127.
- Gitaitis, R. & R. Walcott (2007) The epidemiology and management of seedborne bacterial diseases. *Annual Review of Phytopathology*, 45, 371-397.
- Goodwin, P. H. & C. R. Sopher (1994) Brown pigmentation of *Xanthomonas campestris* pv. *phaseoli* associated with homogentisic acid. *Canadian Journal of Microbiology*, 40, 28-34.
- Goszczynska, T. & J. J. Serfontein (1998) Milk-Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *Journal of Microbiological Methods*, 32, 65-72.
- Hadas, R., G. Kritzman, F. Kletman, T. Gefen & S. Manulis (2005) Comparison of extraction procedures and determination of the detection threshold for *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds. *Plant Pathology*, 54, 643-649.
- Kahveci, E. & S. Maden (1994) Detection of *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola* by bacteriophages. *Journal of Turkish Phytopathology*, 23, 79-85.
- Mabagala, R. B. (1992) An improved semiselective medium for recovery of *Xanthomonas campestris* pv. *phaseoli*. *Plant Disease*, 76, 443-446.
- Maddox, D. A. (1997) Regulatory needs for standardized seed health tests. *Plant pathogens and the worldwide movement of seeds*, 81-92.
- Maringoni, A. C., H. Kimati & C. Kurozawa (1998) Comparison between two extraction methods of *Xanthomonas campestris* pv. *phaseoli* from bean seeds. *Pesquisa Agropecuaria Brasileira*, 33, 659-664.
- Njeru, R., D. G. Ferris, R. A. C. Jones & M. G. K. Jones (1997) Studies on seed transmission of subterranean clover mottle virus and its detection in clover seed by ELISA and RT-PCR. *Australian Journal of Agricultural Research*, 48, 343-350.
- Opiyo, A. F., J. M. Teri & D. J. Allen (1993) Studies on seed transmission of *Xanthomonas campestris* pv. *phaseoli* in common beans in Uganda. *African Crop Science Journal*, 1, 59-67.
- Pataky, J. K., C. C. Block, P. M. Michener, L. M. Shepherd, D. C. McGee & D. G. White (2004) Ability of an ELISA-based seed health test to detect *Erwinia stewartii* in maize seed treated with fungicides and insecticides. *Plant Disease*, 88, 633-640.
- Remeus, P.M. and Sheppard, J.W. (2006) Proposal for a new method for detecting *Xanthomonas axonopodis* pv. *phaseoli* on bean seeds. *ISTA Method Validation Reports* 3, 1-11
- Roumagnac, P., L. Gagnevin & O. Pruvost (2000) Detection of *Xanthomonas* sp., the causal agent of onion bacterial blight, in onion seeds using a newly developed semi-selective isolation medium. *European Journal of Plant Pathology*, 106, 867-877.
- Sakthivel, N., C. N. Mortensen & S. B. Mathur (2001) Detection of *Xanthomonas oryzae* pv. *oryzae* in artificially inoculated and naturally infected rice seeds and

- plants by molecular techniques. *Applied Microbiology and Biotechnology*, 56, 435-441.
- Saettler, A.W. (1989) Common bacterial blight. *Bean Production Problems in the Tropics*. 261-184
- Sheppard, J., Kurowski, C. and Remeus, P.M. (2007) Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris*. *International Rules for Seed Testing* 7-021
- Tebaldi, N. D., R. d. C. Panizzi & R. Sader (2007) Detection, transmission and effect of *Xanthomonas campestris* pv. *campestris* in the physiological quality of broccoli seeds. *Summa Phytopathologica*, 33, 290-293.
- Valarini, P. J. & J. O. M. Menten (1992) Methodology for detection of *Xanthomonas campestris* pv. *phaseoli* in bean seeds. *Fitopatologia Brasileira*, 17, 373-383.
- Valarini, P. J., J. O. M. Menten & M. A. Lollato (1992) Incidence of the common bacterial blight in the field and transport of *Xanthomonas campestris* pv. *phaseoli* through bean seeds, obtained by different methods. *Summa Phytopathologica*, 18, 160-166.
- Venette, J. R., R. S. Lamppa, D. A. Albaugh & J. B. Naves (1987) Presumptive procedure (dome test) for detection of seed-borne bacterial pathogens in dry beans. *Plant Disease*, 71, 984-990.
- Vieira, B. d. A. H. & R. M. d. Souza (2000) Virulence of isolates of *Xanthomonas axonopodis* pv. *phaseoli* and its variant *fuscans*. *Ciencia e Agrotecnologia*, 24, 94-102.
- Vieira, R. F., J. E. S. Carneiro & J. P. Lynch (2008) Root traits of common bean genotypes used in breeding programs for disease resistance. *Pesquisa Agropecuaria Brasileira*, 43, 707-712.
- Wong, W. C. (1991) Methods for recovery and immunodetection of *Xanthomonas campestris* pv. *phaseoli* in navy bean seed. *Journal of Applied Bacteriology*, 71, 124-129.

Table 1. Seedlots from various geographical origins naturally infested with common bacterial blight and used in this study to compare seed extraction methods.

Seedlot	Origin	Type	Cultivar	Seedlots	Subsamples
1	IA	Cranberry	True Red Cranberry	1	12
2	IA	Pole	Purple Podded Pole	1	8
3	IA	Green	Bountiful 337	1	6
4	PNW ^b	Green	Thoroughbred	1	10
5-6	PNW ^b	Green	Valentino	2	10
7-8	PNW ^b	Green	Hercules	2	10
9	CA	Red Kidney	Montcalm	1	10
10	CO	Pinto	Poncho	1	20
11-13	WI	Red Kidney	NS ^a	3	4,6,8
14	NS ^a	French	NS ^a	1	16

^aNS = Not Specified

^bPNW=Pacific North West

Table 2. Evaluation of pathogen detection sensitivity following seed extraction using the ISTA-approved method and modified method on seedlots naturally infested with *Xanthomonas axonopodis* pv. *phaseoli*, evaluated by culturing on selective media. Each entry in the table is the proportion of positive subsamples over the total number of subsamples.

Seedlot	Extraction method	
	ISTA method ^a	Modified method ^b
1	2/6	4/6
2	1/4	4/4
3	1/3	2/3
4	4/5	4/5
5	3/5	5/5
6	3/5	3/5
7	2/5	3/5
8	4/5	5/5
9	4/5	3/5
10	6/10	8/10
11	1/2	0/2
12	0/3	1/3
13	2/4	2/4
14	2/8	4/8
Total	35/70	48/70 ^c

^a Overnight incubation at 4° C without vacuum or centrifugation

^b Three-hour incubation at room temperature with vacuum extraction and centrifugation of seed extract.

^c Significantly different ($P < 0.05$)

Figure 1. Pathogen detection sensitivity (% of samples testing positive) for seed extraction methods differing in incubation time, temperature and vacuum application for seed samples artificially inoculated with *X. axonopodis* pv. *phaseoli* B1. (* = significantly different at $P < 0.05$).

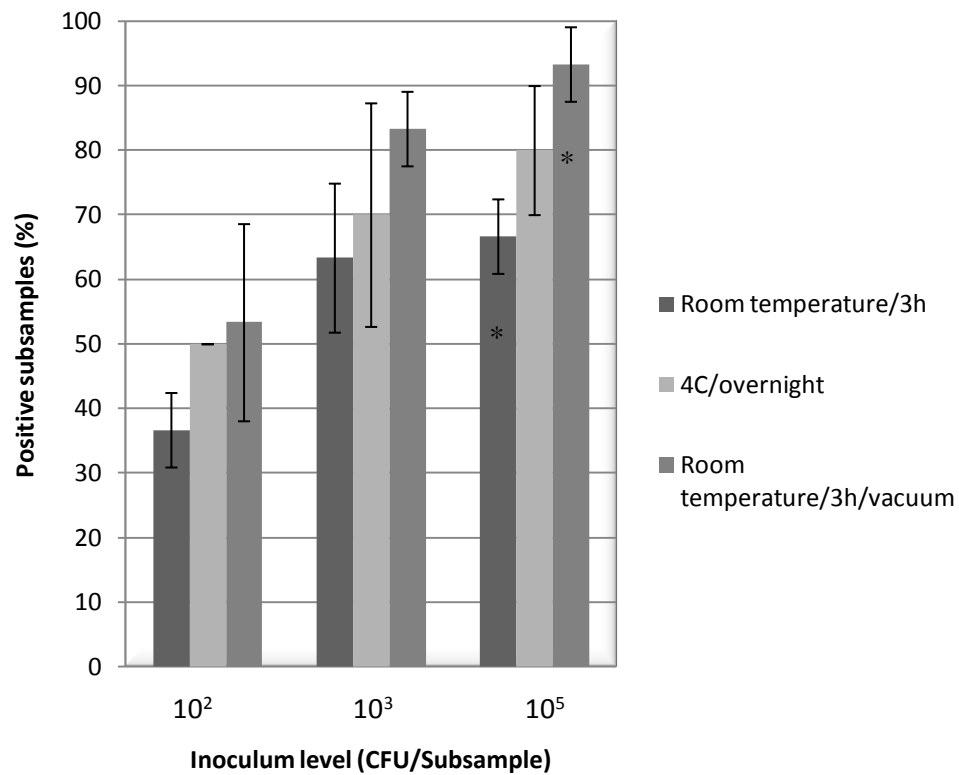


Figure 2. Pathogen detection sensitivity (% of samples testing positive) for seed extraction at room temperature (20-24° C) and 4°C incubation with or without centrifugation of seed extract for seed samples artificially inoculated with *X. axonopodis* pv. *phaseoli* B1 (*, ✕ = significantly different at $P < 0.05$)

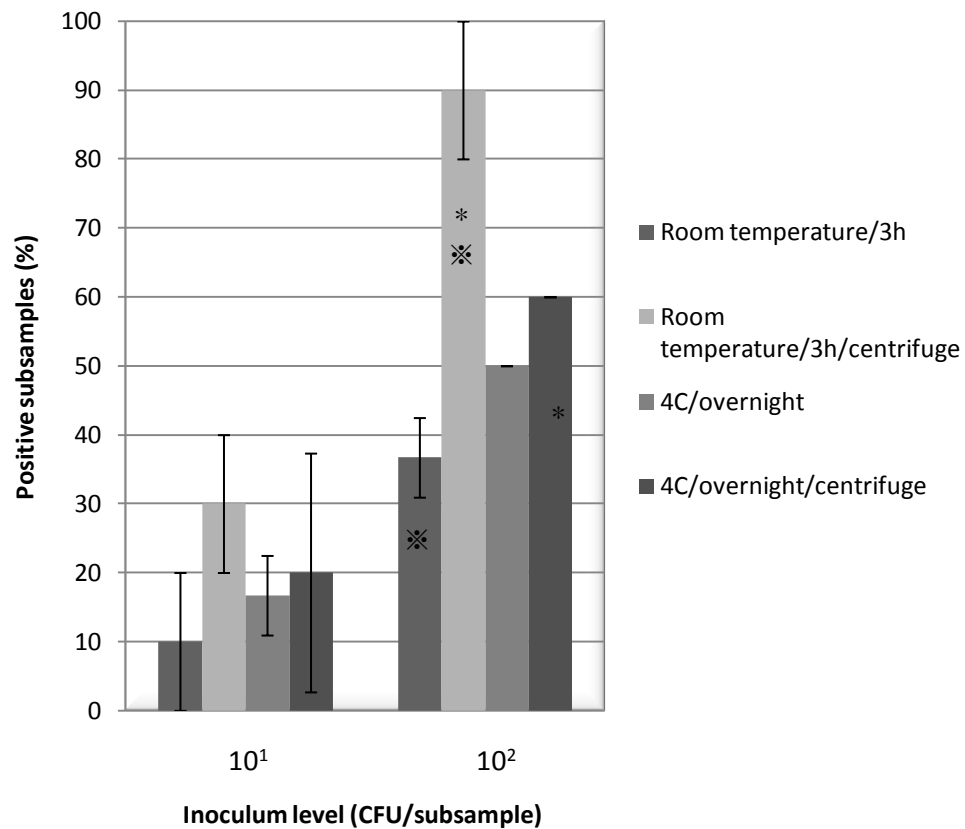
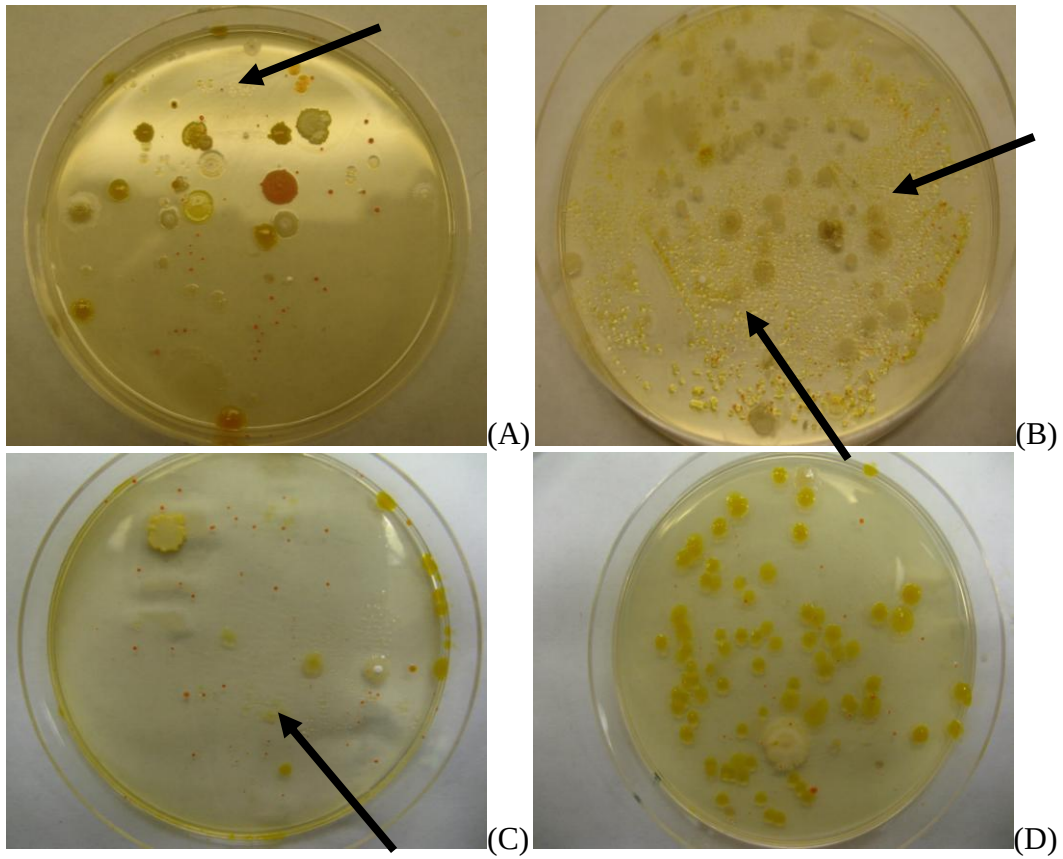


Figure 3. Seed extracts from seedlots naturally infected with *Xanthomonas axonopodis* pv. *phaseoli*, grown on nutrient agar. Arrows indicate *X. axonopodis* pv. *phaseoli* colonies (A: Purple podded pole from IA; B: Red kidney from CA; C: Bountiful from IA; D: French bean from Harris Moran).



CHAPTER 3. A DIAGNOSTIC REAL-TIME PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF *XANTHOMONAS AXONOPODIS* PV. *PHASEOLI* AND *XANTHOMONAS AXONOPODIS* PV. *PHASEOLI* VAR. *FUSCANS* IN SEEDS

A paper to be submitted to *European Journal of Plant Pathology*

Yiqing He, Anania Fessehaie, Lisa Shepherd and Gary Munkvold

Abstract:

Common bacterial blight of bean (*Phaseolus vulgaris*) is caused by *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. These seedborne, quarantined pathogens can cause up to 40% yield loss in susceptible cultivars, and disease-free seeds are required to prevent production losses. Seed health testing is one of the most significant steps to ensure a supply of healthy seeds for domestic planting and international seed trade. Based on sequence information from RAPD fragments generated from *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*, we developed real-time PCR methods for detection and quantification of the pathogens in or on seeds. Assay specificity was tested against DNA of several *Xanthomonas* species and pathovars of *X. axonopodis*. None of the closely related *Xanthomonas* strains were amplified using this PCR assay. The detection limit of the TaqMan assay for purified DNA and cells was 20 fg and 20 CFU per 25 µl PCR reaction mixture, respectively. A linear model was developed for seed contamination level in relation to amplification cycles based on sensitivity tests on spiked seeds. with *X. axonopodis* pv. *phaseoli* was extracted from naturally infested *Phaseolus vulgaris* seedlots using two different methods. Real-time PCR was more sensitive than a standard selective medium assay for both extraction methods.

Compared to culture plating and conventional PCR assays, real-time PCR showed higher sensitivity and delivered more rapid results. Real-time PCR should be useful as for rapid, highly sensitive and specific detection of these seedborne pathogens to ensure seed quality control and meet phytosanitary regulations. To our knowledge this is the first published real-time PCR assay developed for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*.

Introduction

Xanthomonas axonopodis pv. *phaseoli* (Smith) Vauterin and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder (Vauterin, 1995) cause common bacterial blight of bean (*Phaseolus vulgaris* L.), a destructive disease worldwide. They are aerobic, rod-shaped gram-negative bacteria with a polar flagellum. *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were first described as *X. campestris* pv. *phaseoli* (Smith) Dye and *X. campestris* pv. *phaseoli* var. *fuscans* by phenotypic approach, but later Vauterin et al. (1995) reclassified them to *X. axonopodis* pv. *phaseoli* due to their genomic homology with *X. axonopodis* (Vauterin et al. 1995). The two varieties can be differentiated using isoenzyme profiling (El-Sharkawy and Huisinigh 1971), plasmid profiling (Lazo and Gabriel 1987), or DNA-DNA hybridization (Hildebrand et al. 1990). The average genome sizes for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were 3850.6±48.9 and 3584.3±68.1 kb respectively (Chan and Goodwin 1999b). Pulsed-field gel electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP) using *Xba*I confirmed that the two bacteria were genetically different (Chan and Goodwin 1999b, Chan and Goodwin 1999c). *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* also can be distinguished by the production of a

diffusible brown pigment due to secretion and subsequent oxidation of homogentisic acid which is an intermediate in tyrosine catabolism for a number of bacteria.

Xanthomonas axonopodis pv. *phaseoli* var. *fuscans* appears to have a disrupted tyrosine catabolism pathway and cannot use tyrosine as a nutrient (Goodwin and Sopher 1994). A physical map of the *X. axonopodis* pv. *phaseoli* var. *fuscans* BXP65 chromosome has been constructed by PFGE and Southern hybridization (Chan and Goodwin 1999a).

Seed-borne bacteria are usually the primary inoculum for common blight (Saettler, 1989), but *X. axonopodis* pv. *phaseoli* also can survive for months in leaves and other plant debris on the soil (Saettler, 1989; Fourie 2002). The main route of spread is by planting infected seeds. Bacteria in/on the seed can survive longer than the seed itself (Dreo et al. 2003). Infection of approximately 1 in 10,000 seeds was capable of causing an outbreak of blight (Sutton and Wallen et al. 1970). The minimal population of *X. axonopodis* pv. *phaseoli* required to initiate infection in the field was 10 CFU/seed and there was a positive correlation between seed symptoms and the population of *X. axonopodis* pv. *phaseoli* per seed (Opio et al. 1993). A range of tolerances, ranging from 0 seeds infected in samples of 4,000-45,000, have been used in seed quality programs in US seed companies to prevent pathogen transmission by commercial seed lots (Maddox 1997).

Several different approaches have been used for detecting *X. axonopodis* pv. *phaseoli* in seeds, including a Dome Test (Venette et al. 1987), phage test (Kahveci and Maden 1994), selective media (Mabagala 1992, Reemeus and Sheppard 2006, Sheppard et al. 2007), PCR (Audy et al. 1994), and serology such as indirect immunofluorescence microscopy (Dias and Nalimova 1987) and ELISA (Wong 1991). Monoclonal antibodies were produced specifically against *X. axonopodis* pv. *phaseoli*

but cross-reacted with other *Xanthomonas* strains and certain other bacteria (Yucel et al. 1997). Commercial ELISA tests are available. Only a few of the published methods have been accepted as routine diagnostic tools.

The use of PCR for detection of *Xanthomonas* strains was first reported in 1994. A method to detect and identify phytopathogenic *Xanthomonas* strains by specific amplification of the *Hrp* gene in *X. axonopodis* pv. *vesicatoria* was reported and it was believed to be sensitive and specific to *Xanthomonads* (Leite et al. 1994). Primers based on the *hrp* gene sequence of nine different isolates of *X. axonopodis* pv. *phaseoli* amplified products in isolates pathogenic to beans (Nunes et al. 2008), but this method could not serve as a commercial seed health test which had to be specific and uniform only to all *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*.

ISXax1 was recently identified as a novel insertion sequence which was restricted to *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *phaseoli* var. *fuscans*, and *X. axonopodis* pv. *vesicatoria*. The sequence could be useful in identification procedures (Alavi et al. 2007). Suppression subtractive hybridization was also performed to isolate DNA fragments present in these bean pathogens and absent from closely related *Xanthomonads*. Some virulence gene candidates were identified, such as homologs of hemagglutinins, TonB-dependent receptors, zinc-dependent metalloproteases, type III effectors, type IV secretion system components and unexpectedly homologs of the type III secretion apparatus components (SPI-1 family) (Alavi et al. 2008). These also could contribute to isolate identification and characterization.

Several PCR methods have been reported to detect and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Audy et al. (1994) designed primers based on a 3.4-kb plasmid (p7) DNA fragment, which specifically directed the amplification of a 730-bp fragment from DNA of 27 pathogenic *X. axonopodis* pv. *phaseoli*; an additional

fragment of 550 bp was occasionally amplified from the DNA of *X. axonopodis* pv. *phaseoli* var. *fuscans* strains. Primers Xf1 and Xf2, based on a sequence conserved amplified region (SCAR) derived from RAPD PCR analysis of *X. axonopodis* pv. *phaseoli* var. *fuscans*, amplified a DNA fragment of 450 bp from all *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates and would not amplify any product from *X. axonopodis* pv. *phaseoli* isolates, or from any other DNAs tested (Toth et al. 1998). *Xanthomonas axonopodis* pv. *phaseoli* -targeted primers were then designed, but missed two virulent isolates out of 42 and amplified a fragment from the *X. axonopodis* pv. *vitians* genome (Halfeld-Vieira et al. 2001). RAPD PCR was used as a rapid approach to differentiate between isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. A RAPD PCR primer OP-G11 generated a 820bp PCR product from *X. axonopodis* pv. *phaseoli* var. *fuscans* and a 900-bp product from *X. axonopodis* pv. *phaseoli* (Birch et al. 1997). However, it was also reported that some *X. axonopodis* pv. *phaseoli* isolates did not always generate the 900-bp fragment. Rep-PCR has been used to generate DNA fingerprinting to classify other seed pathogens such as *Acidovorax avenae* subsp. *citrulli* on cucurbit (Walcott and Gitaitis 2000). Rep-PCR and PCR-RFLP of the ribosomal genes had been used to genotypically distinguish between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* and to calculate genetic diversity among populations (Mahuku et al. 2006).

Real-time PCR has been applied to successfully identify and quantify many phytopathogens with improved sensitivity compared to culture plate testing and conventional PCR (Schena et al. 2004). For plant pathogenic bacteria, Schaad first used it for *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers and it has become widely used as a diagnostic tool for many bacterial pathogens (Schaad et al. 1999). In selective detection of *Pseudomonas syringae* pv. *tomato*, primers and probes designed

according to the sequence of RAPD PCR products were used to make dot blot hybridization and real-time PCR which is highly specific and efficient (Fanelli et al. 2007). This approach can be applied to design specific TaqMan primers and probes to detect the existence of target pathogens such as *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Several real-time PCR methods for plant pathogen detection are in use, but their adoption in seed health testing has been slow. This is partially due to technical difficulties in consistently extracting pathogen DNA from seeds, and uncertainty in assessing disease transmission risk in relation to quantitative PCR results (Munkvold, 2009). Published PCR-based methods for *X. axonopodis* pv. *phaseoli* or *X. axonopodis* pv. *phaseoli* var. *fuscans* methods are used in the seed industry, but results are not always satisfactory and none of these has been approved as a standard seed health test by the International Seed Testing Association or International Seed Health Initiative (Munkvold 2009). One method has been tentatively recommended by the National Seed Health System in the USA, but its designation is “temporary standard” (www.seedhealth.org). There is a need to develop and apply real-time PCR tests in routine seed health testing to increase efficiency, specificity, and sensitivity. The aim of the present study was to develop a real-time PCR method for common bacterial blight pathogens to be used in bean seed health testing.

Materials and methods

Bacterial strains and growth conditions

Bacteria isolates used in this study were obtained from diverse geographical origins and sources (Table 1). Reference strain XP37 was received from the American Type Culture Collection (ATCC No.13464). Bacterial cultures were grown routinely on nutrient agar (Becton, Dickinson and Company, MD) or YDC medium at 28° C.

Isolation of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*

from naturally infested seed or inoculated plants was conducted on semiselective agar media MT (Goszczyńska and Serfontein, 1998; Sheppard et al. 2007) and XCP1 (McGuire *et al.*, 1986; Sheppard, 2007). Bacterial cultures were enriched on nutrient agar and YDC agar. Long-term, bacteria were stored on silica beads (Microbank, Austin, TX) by shaking beads in a cell suspension, removing excess fluid, and freezing at -80°C.

Pathogenicity Testing

Seeds of susceptible dark red kidney bean (*Phaseolus vulgaris*) (cv. Charlevoix) were planted in pasteurized soil in 20 cm pots in a greenhouse. Plants were allowed to grow until the first true leaf was fully established. Plants were watered 2 h before inoculation to ensure adequate moisture. A sterile needle was contaminated with bacteria from 2-day-old cultures grown on YDC. Seedlings were inoculated by stabbing a contaminated needle through the primary node at an angle of about 45°. Plants were covered with transparent plastic bags to maintain high humidity for one week. Symptom development was observed and recorded 10 days after inoculation. Noninoculated control plants were wounded with a sterile needle. The pathogens were re-isolated from leaf tissues adjacent to the lesions by culturing on XCP1 semi selective media.

DNA isolation from pure cultures and infected beans

For pure culture extraction, bacterial cultures were harvested at late-log phase from nutrient agar. Fresh cells were washed in 10mM TE and DNA isolated using the Promega DNA Extraction Wizard kit (Promega Corp. Madison, WI). For infected bean seed soak extracts, a simplified CTAB extraction method was used: extract concentration was incubated in CTAB solution for 1 hr at 62°C, followed by phenol/chloroform extraction and ethanol precipitation. For infected plants, leaf tissue

was first freeze-dried and then crushed by shaking with 0.5 mm glass beads (BioSpec Products, OK). Crushed powder was extracted by the CTAB method described above. DNA concentrations were measured with NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA samples were stored at -20 C until used.

Characterization of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates

Pathogenicity testing, PCR-RFLP and conventional PCR were used to characterize and differentiate isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Conventional PCR was performed in 25µl reaction mixture containing 50ng DNA, 200µM of each dNTP, 0.5µM of each primers, 1×PCR buffer, and 1.25U of *Taq* DNA polymerase (Promega, WI). Initial denaturation was at 95°C for 1min, followed by 35 repeated cycles of melting, annealing and extension at 95°C for 1 min, 65°C for 1 min, and 72°C for 2 min, respectively. X4c/X4e primer set as described in Audy et al. (1994) was used to amplify isolates. For PCR-RFLP, we used the conserved primer set Phill-1 and P322-Anti to amplify the 16S gene including IGS spacer region and used restriction endonuclease *HaeIII* to digest the PCR product as described in Mahuku et al. (2006). PCR amplifications were carried out in a 50µl reaction volume that contained 1X PCR buffer (pH 8.3), 30 ng of genomic DNA, 0.5 µM each primer, 0.2 mM dNTP, 2 mM MgCl₂ and 2U *Taq* polymerase (Promega,WI). Program was set for 35 cycles of 95°C for 1 min; 65°C for 1 min; 72°C for 3 min; and a final extension at 72°C for 10min. PCR products were digested with restriction endonuclease *HaeIII* (Mahuku et al. 2006). Restricted DNA was visualized by gel electrophoresis in 1.5% agarose gels and 100-bp ladder was used to estimate fragment size.

Isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were identified by RAPD PCR as described in Birch et al. (1997). RAPD-PCR was conducted using primer OP-G11 (Birch et al. 1997) in 25 µl reaction volume containing 50 ng of genomic DNA, 1U Taq DNA polymerase (Promega, Madison, WI), 0.6 µM primers, 1.5 µM MgCl₂, 100 µM dNTP, 1×PCR reaction buffer. PCR cycling conditions were as follows: initial denaturation at 95° C for 3 min followed by 40 cycles of 95° C for 1 min, 36°C for 2 min, and 72° C for 1.5 min, and a final extension step of 72° C for 5 min. PCR product was separated on a 1.5% agarose gel by electrophoresis, stained with ethidium bromide and the gel image was scanned with Bio-Rad gel image system. RAPD PCR amplified a 900 bp DNA fragment from all *X. axonopodis* pv. *phaseoli* isolates, and a 820 bp DNA fragment from all *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates.

The 900 bp amplification product obtained from *X. axonopodis* pv. *phaseoli* B1 and 820 bp from *X. axonopodis* pv. *phaseoli* var. *fuscans* PR8 were cloned in the PGEM-t vector (Promega, Madison, WI). Ligation was performed in 17 µl ligation system at 16°C for 5h. Transformation was conducted by heat shock for 2 min and then cells were recovered in 2YT broth media at 37°C for 1 h. Cells were grown on X-gal and under induction of IPTG (Isopropyl-beta-d- thiogalactopyranoside) at 37° C overnight. Putative transformant colonies were transferred and plasmids were extracted for sequencing. *X. axonopodis* pv. *phaseoli* -specific primers and probes were developed based on the exact 896 bp sequence for real-time PCR detection of *X. axonopodis* pv. *phaseoli* and based on the 819 bp sequence for *X. axonopodis* pv. *phaseoli* var. *fuscans*. Comparative sequence alignments revealed that the *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* sequences lacked homology. Both amplicons were also compared using a blast search on NCBI for

similar sequences in GenBank. Taxon-specific sequences were used to develop TaqMan primers and probes.

Development of TaqMan Real-Time PCR assay

New primers and probes were designed based on the sequence of RAPD PCR product fragments using Primer Express Software v2.0 (Applied Biosystems). The *X. axonopodis* pv. *phaseoli* probe was modified with a minor groove binder (MGB) ligand at the 3' end. The *X. axonopodis* pv. *phaseoli* probe was labeled with FAM and the *X. axonopodis* pv. *phaseoli* var. *fuscans* probe was labeled with HEX. Primers and probes were designed so that the fluorescence group could be set in a major groove to reach thermal equilibrium and the probe could bind tightly. Specificity of each of the TaqMan real-time PCR assays was tested against target and non-target bacterial DNA including *X. axonopodis* pv. *phaseoli* , *X. axonopodis* pv. *phaseoli* var. *fuscans*, other *Xanthomonas* pathovars and species, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *phaseolicola* and other phytopathogenic bacteria (Table 1).

To evaluate the sensitivity and linearity of each assay independently, limits of detection (LOD) were tested with purified *X. axonopodis* pv. *phaseoli* DNA, bacterial cell suspensions and seed soaks from artificially inoculated bean seeds using 7900HT Fast Real-Time PCR System (Applied Biosystems, CA). For purified DNA, a 10-fold serial dilution of *X. axonopodis* pv. *phaseoli* B1 DNA was made from 10 ng to 10 ag. For the bacteria cell suspension, a 10-fold serial dilution of *X. axonopodis* pv. *phaseoli* B1 suspension in water was made and ranged from 10^9 CFU/ml to 10^0 CFU/ml. Linear models of the relationships between Ct values and DNA amount/cell numbers were established. For seed wash from artificially inoculated bean seeds, seeds were inoculated by soaking in a *X. axonopodis* pv. *phaseoli* B1 suspension to a range of 10^2 CFU/seed to 10^5 CFU/seed and one contaminated seed was mixed in 1000-seed

subsamples. Five replicates of 10 subsamples were tested for contamination level of 10^5 and 10^3 CFU/seed. Three replicates of 10 subsamples were tested for contamination level of 10^4 and 10^2 CFU/seed. A modified linear model was established according to the mean Ct value of spiked seed samples. Sensitivity of detection was calculated as the mean percentage of subsamples testing positive for the appropriate pathogen.

Bean seeds known to be contaminated with common bacterial blight were provided by collaborators from different geographical locations (Table 2) and were used to determine the detection sensitivity of selective medium and PCR methods for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Both ISTA extraction method (4°C overnight) and modified extraction method (room temperature 20-24°C 3h with vacuum extraction and centrifugation of seed extract) were used to extract pathogens from seeds. Final extract was tested by both culture plate test and real-time PCR assay. Sensitivity of both extraction method and both assay from each seedlot was evaluated. Suspected *X. axonopodis* pv. *phaseoli* colonies were counted and confirmed by real-time PCR and pathogenicity test. A 96-well plate for real-time PCR was used to test all samples and positive subsample numbers were recorded. Sensitivity of the two methods was compared and statistically evaluated using t-tests.

Results

Characterization and pathogenicity test

In conventional PCR, a 730 bp fragment was consistently generated in all *X. axonopodis* pv. *phaseoli* (Figure 1A) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (Figure 1B) isolates which further confirmed that all isolates were *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. In RFLP analysis, isolates from different origins showed slight difference in profiles. After digestion with HaeIII, most *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* showed some

variation in band patterns. *X. axonopodis* pv. *phaseoli* always had two smaller distinct bands at around 300-400 bp while *X. axonopodis* pv. *phaseoli* var. *fuscans* had a larger band at approximate 700 bp which indicated that in *X. axonopodis* pv. *phaseoli* this fragment was digested by the restriction endonuclease (Figure 2A-B). Fifteen *X. axonopodis* pv. *phaseoli* isolates and 7 *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates were submitted to pathogenicity test and all of them were pathogenic isolates.

Primer OP-G11 generated a 900 bp band (Fig. 1C) in all *X. axonopodis* pv. *phaseoli* isolates and a 820 bp band (Fig. 1D) in all *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates. Comparative sequence alignments revealed that the *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* RAPD product sequences lacked homology and they belonged to different sites on the genome. The 894 bp fragment from *X. axonopodis* pv. *phaseoli* was homologous to part of an acetyltransferase gene present in some other *Xanthomonas* spp. The 819 bp fragment from *X. axonopodis* pv. *phaseoli* var. *fuscans* was not homologous or related to any known sequences in GenBank.

TaqMan Real-Time PCR assay specificity test

The novel *X. axonopodis* pv. *phaseoli* -specific primers and probe were forward primer: XAPF (5'-CGCAGATCACCATCAACGAA -3'), reverse: XAPR- (5'-CAACCCCGCGCTGTTC -3'), probe: Xap probe (5'-CAAGCAACGCGCTCA -3'). The new *X. axonopodis* pv. *phaseoli* var. *fuscans* primers and probe were forward: XAPF-F (5'-CCTTGTGGAACACGCTAGCA-3'), reverse: XAPF-R (5'-GCGAAGCATCAGCTTGATTG-3'), and probe: XAPF Probe (5'-CGCCTGCCTCAACAGAAAATGTGCA-3').

Specificity was assessed on DNA extracted from a collection of pure cultures (Table 1). All *X. axonopodis* pv. *phaseoli* isolates gave consistent positive results with

X. axonopodis pv. *phaseoli* primers and probe and most *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates gave negative results except 0794. All *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates gave consistent positive results with *X. axonopodis* pv. *phaseoli* var. *fuscans* primers and probe and most *X. axonopodis* pv. *phaseoli* isolates gave negative results except 269.30A, 269.30B. No cross amplification were observed from other close related *Xanthomonas* spp. DNA extracted from bean leaf tissue and bean seeds were included as negative controls and they all tested negative. Among other phyto bacteria species, all tested negative with both sets of primers and probes.

X. axonopodis pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* probes were both tested for detection sensitivity using pure genomic DNA, pure bacterial cells and naturally infected seeds. Limit of detection for the *X. axonopodis* pv. *phaseoli* probe was ~ 200 fg DNA/25 µl PCR reaction for pure DNA (Figure 3) and ~ 200 CFU/25 µl PCR reaction for cell suspension (Figure 4). Limit of detection for the *X. axonopodis* pv. *phaseoli* var. *fuscans* probe was ~ 20 fg DNA/25 µl PCR reaction for pure DNA (Figure 5) and ~ 20 CFU/25 µl PCR reaction for cell suspension (Figure 6). The correlation of Ct value and DNA/cell concentration fit well with the linear model which could be used for estimation of contamination levels. Detection sensitivity for spiked seeds by real-time PCR assay was also tested (Table 3) and the standard curve was calculated (Figure 7). Ct values of the detectable subsamples were analyzed and a modified linear model for spiked seeds was calculated. The modified linear model for spiked seed was $y = -3.3486x + 46.599$.

Infestation levels of *X. axonopodis* pv. *phaseoli* were greatly different among the seedlots. No *X. axonopodis* pv. *phaseoli* var. *fuscans* was found in any seedlots. The bacterial taxa present other than *X. axonopodis* pv. *phaseoli* was not diverse. The overall proportions of subsamples that were positive by the selective medium assay and

real-time PCR assay were 68.6% and 82.9% respectively. Statistical analysis for all seedlots showed that real-time PCR had higher detection sensitivity than culture plating assay ($P<0.05$) (Table 4)

Discussion

The use of real-time PCR to quantify microbial populations in complex environmental samples offers an alternative and more sensitive approach to previous, cultivation-based methods. It provided a more sensitive, reliable and fast approach to diagnosis of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* in seeds. We collected bacterial cultures from different locations within US (NE, CA, WI, ID, ND, MI, IA, PR) and also from other parts of the world. Some geographical origin variation among these isolates could be distinguished using RFLP analysis, but the fact that our primers and probes amplified all these isolates suggested they share these distinct sequences.

PCR-RFLP of the ribosomal genes had been used to genotypically distinguish between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* and to assess genetic diversity among populations. Sequence differences also could be identified and amplification sites could be designed based on sequence differences. However, the 16S gene including the IGS spacer region was highly conserved and subject to cross-reactions with other phytopathogens (Hauben et al. 1997). Despite this limitation, PCR-RFLP still offered another way to develop a specific amplification site (Junier et al. 2008). RAPD PCR primer OP-G11 (Birch et al. 1997) was effective for distinguishing *X. axonopodis* pv. *phaseoli* from *X. axonopodis* pv. *phaseoli* var. *fuscans* and our specificity tests showed that primers and probes designed from its amplification products did not amplify other *Xanthomonads* that we tested. They appear to be specific for *X. axonopodis* pv. *phaseoli* detection and characterization.

The sensitivity of this quantitative real-time PCR assay was higher than the traditional agar-plate test and took less effort and time. However, it still relies on operator skill for pathogen extraction. Careful extraction procedures should be emphasized to enhance high detection sensitivity. DNA extraction was necessary after seed soaking and pathogen collection. We used a brief SDS and CTAB method to assure all DNA was released and protected with minimal loss or degradation. According to the modified linear model for spiked seed, more amplification cycles were needed to cross the quantification threshold compared to the original model for pure cells at the same inoculum level. Sensitivity of any seed health test can be limited by constraints introduced during the pathogen and DNA extraction steps.

Interpretation of pathogen quantification by real time PCR can have important implications for seed health testing. It is possible that DNA from dead cells or viable but not culturable cells could be amplified, and thus the PCR method could overestimate cell counts (Reichert-Schwillinsky et al. 2009).

Comparing the modified model of spiked seeds with pure cell culture model, Ct value was always higher for spiked seeds at the same inoculum level, which indicated that the pathogen extraction procedure was not 100% efficient. Sensitivity of real-time PCR in the spiked seed samples was similar to or higher than that obtained with selective-medium assays using the same inoculum doses (He and Munkvold, 2010).

Results from naturally infested seedlots indicated that the real-time PCR method has better detection sensitivity than standard selective medium methods. Selective medium approaches can lack sensitivity at low levels of infestation because of the difficulty in distinguishing target colonies in the presence of other contaminants, or suppression of the growth of target colonies by other organisms or seed extract

components. The real-time PCR method described here could be useful as a routine seed health testing method, with distinct advantages over currently used culture-based and conventional PCR methods.

Literature Cited

- Aggour, A. R., D. P. Coyne, A. K. Vidaver & K. M. Eskridge (1989) Transmission of the common blight pathogen in bean seed. *Journal of the American Society for Horticultural Science*, 114, 1002-1008.
- Alavi, S. M., S. Poussier & C. Manceau (2007) Characterization of ISXax1, a novel insertion sequence restricted to *Xanthomonas axonopodis* pv. *phaseoli* (Variants fuscans and non-fuscans) and *Xanthomonas axonopodis* pv. *vesicatoria*. *Applied and Environmental Microbiology*, 73, 1678-1682.
- Alavi, S. M., S. Sanjari, F. Durand, C. Brin, C. Manceau & S. Poussier (2008) Assessment of the genetic diversity of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp. *fuscans* as a basis to identify putative pathogenicity genes and a type III secretion system of the SPI-1 family by multiple suppression subtractive hybridizations. *Applied and Environmental Microbiology*, 74, 3295-3301.
- Arsenijevic, M., A. Obradovic, D. Stevanovic & M. Ivanovic (1998) Antagonistic effect of some saprophytic bacteria to *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli*. *Bulletin OILB/SROP*, 21, 297-300.
- Audy, P., A. Laroche, G. Saindon, H. C. Huang & R. L. Gilbertson (1994) Detection of the bean common blight bacteria, *Xanthomonas-campestris* pv. *phaseoli* and *X-campeoli* var. *fuscans*, using the polymerase chain-reaction. *Phytopathology*, 84, 1185-1192.
- Berg, T., L. Tesoriero & D. L. Hailstones (2006) A multiplex real-time PCR assay for detection of *Xanthomonas campestris* from brassicas. *Letters in Applied Microbiology*, 42, 624-630.
- Birch, P. R. J., L. J. Hyman, R. Taylor, A. F. Opio, C. Bragard & I. K. Toth (1997) RAPD PCR-based differentiation of *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. *European Journal of Plant Pathology*, 103, 809-814.
- Cafati, C. R. & A. W. Saettler (1980) Transmission of *Xanthomonas-phaseoli* in seed of resistant and susceptible phaseolus genotypes. *Phytopathology*, 70, 638-640.
- Chan, J. & P. H. Goodwin (1999a) A physical map of the chromosome of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* BXP65. *Fems Microbiology Letters*, 180, 85-90.
- (1999b) Differentiation of *Xanthomonas campestris* pv. *phaseoli* from *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* by PFGE and RFLP. *European Journal of Plant Pathology*, 105, 867-878.
- (1999c) The molecular genetics of virulence of *Xanthomonas campestris*. *Biotechnology Advances*, 17, 489-508.
- Chavez-L, C. & G. A. Granada (1988) Survival of *Xanthomonas-campestris* pathovar *phaseoli* causal agent of bean common blight in the Cauca valley Colombia. *Fitopatologia Colombiana*, 12, 9-14.

- Correa, F. M., A. d. O. de Carvalho, M. Gorete & F. do Carmo (2008) Inoculation and survival of *Xanthomonas vesicatoria* on tomato seeds. *Summa Phytopathologica*, 34, 71-75.
- Darrasse, A., C. Bureau, R. Samson, C. E. Morris & M. A. Jacques (2007) Contamination of bean seeds by *Xanthomonas axonopodis* pv. *phaseoli* associated with low bacterial densities in the phyllosphere under field and greenhouse conditions. *European Journal of Plant Pathology*, 119, 203-215.
- Darsonval, A., A. Darrasse, K. Durand, C. Bureau, S. Cesbron & M. A. Jacques (2009) Adhesion and Fitness in the Bean Phyllosphere and Transmission to Seed of *Xanthomonas fuscans* subsp. *fuscans*. *Molecular Plant-Microbe Interactions*, 22, 747-757.
- Dias, J. & M. Nalimova (1987) Serological studies on *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye in Cuba. *Pochvoznanie, Agrokimiya i Rastitelna Zashchita*, 22, 77-81.
- Diaz, C. G., R. B. Bassanezi & A. Bergamin Filho (2001) Development and validation of a diagrammatic scale for *Xanthomonas axonopodis* pv. *phaseoli* in phaseolus bean. *Summa Phytopathologica*, 27, 35-39.
- Dreo, T., T. Demsar & M. Ravnikar (2003) Quarantine of *Xanthomonas campestris* pv. *phaseoli* in beans. *Zbornik predavanj in referatov 6. Slovenskega Posvetovanja o Varstvu Rastlin, Zrece, Slovenije, 4-6 marec 2003*, 123-127.
- El-Sharkawy, T. A. & D. Huisingh (1971) Differentiation among *Xanthomonas* species by polyacrylamide gel electrophoresis of soluble proteins. *Journal of General Microbiology*, 68, 155-&.
- Fanelli, V., C. Cariddi & M. Finetti-Sialer (2007) Selective detection of *Pseudomonas syringae* pv. *tomato* using dot blot hybridization and real-time PCR. *Plant Pathology*, 56, 683-691.
- Faria, J. C. D. & P. E. D. Melo (1989) Inoculation of common beans with *Xanthomonas-campestris* pathovar *phaseoli* under field condition. *Pesquisa Agropecuaria Brasileira*, 24, 987-990.
- Fininsa, C. (2003) Relationship between common bacterial blight severity and bean yield loss in pure stand and bean-maize intercropping systems. *International Journal of Pest Management*, 49, 177-185.
- Fourie, D. (2002) Distribution and severity of bacterial diseases on dry beans (*Phaseolus vulgaris* L.) in South Africa. *Journal of Phytopathology-Phytopathologische Zeitschrift*, 150, 220-226.
- Gent, D. H., J. M. Lang, M. E. Bartolo & H. R. Schwartz (2005a) Inoculum sources and survival of *Xanthomonas axonopodis* pv. *allii* in Colorado. *Plant Disease*, 89, 507-514.
- Gent, D. H., J. M. Lang & H. F. Schwartz (2005b) Epiphytic survival of *Xanthomonas axonopodis* pv. *allii* and *X axonopodis* pv. *phaseoli* on leguminous hosts and onion. *Plant Disease*, 89, 558-564.
- Gholve, V. M. & B. P. Kurundkar (2007) Transmission of *Xanthomonas axonopodis* pv. *Malvacearum* through infected seed. *Agricultural Science Digest*, 27, 41-43.
- Gitaitis, R. & R. Walcott (2007) The epidemiology and management of seedborne bacterial diseases. *Annual Review of Phytopathology*, 45, 371-397.
- Goodwin, P. H. & C. R. Sopher (1994) Brown pigmentation of *Xanthomonas-campestris* pv *phaseoli* associated with homogentisic acid. *Canadian Journal of Microbiology*, 40, 28-34.

- Goszczynska, T. & J. J. Serfontein (1998) Milk-Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *Journal of Microbiological Methods*, 32, 65-72.
- Groth, D. E. & E. J. Braun (1989) Survival, seed transmission, and epiphytic development of *Xanthomonas-campestris* pv *glycines* in the north-central United-States. *Plant Disease*, 73, 326-330.
- Gurtler, V. & H. D. Barrie (1995) Typing of staphylococcus-aureus strains by PCR-amplification of variable-length 16s-23s rDNA spacer regions - characterization of spacer sequences. *Microbiology-Uk*, 141, 1255-1265.
- Hadas, R., G. Kritzman, F. Kletman, T. Gefen & S. Manulis (2005) Comparison of extraction procedures and determination of the detection threshold for *Clavibacter michiganensis* ssp *michiganensis* in tomato seeds. *Plant Pathology*, 54, 643-649.
- Halfeld-Vieira, B. A., R. M. Souza, A. R. Figueira & A. De Jesus Boari (2001) Identification of *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* through the PCR technique. *Fitopatologia Brasileira*, 26, 737-740.
- Hauben, L., L. Vauterin, J. Swings & E. R. B. Moore (1997) Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology*, 47, 328-335.
- Hildebrand, D. C., N. J. Palleroni & M. N. Schroth (1990) Deoxyribonucleic-acid relatedness of 24 *Xanthomonad* strains representing 23 *Xanthomonas-campestris* pathovars and *Xanthomonas-fragariae*. *Journal of Applied Bacteriology*, 68, 263-269.
- Ibekwe, A. M., P. M. Watt, P. J. Shouse & C. M. Grieve (2004) Fate of *Escherichia coli* O157 : H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. *Canadian Journal of Microbiology*, 50, 1007-1014.
- Jiao, J. R., P. Goodwin & B. Grodzinski (1996) Photosynthesis and export during steady-state photosynthesis in bean leaves infested with the bacterium *Xanthomonas campestris* pv *phaseoli*. *Canadian Journal of Botany-Revue Canadienne De Botanique*, 74, 1-9.
- Jindal, K. K. (1991) Physical and chemical agents for the eradication of *Xanthomonas campestris* pv. *phaseoli* from bean seeds. *Plant Disease Research*, 6, 68-71.
- Junier, P., O. S. Kim, O. Hadas, J. F. Imhoff & K. P. Witzel (2008) Evaluation of PCR primer selectivity and phylogenetic specificity by using amplification of 16S rRNA genes from betaproteobacterial ammonia-oxidizing bacteria in environmental samples. *Applied and Environmental Microbiology*, 74, 5231-5236.
- Kahveci, E. & S. Maden (1994) Detection of *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola* by bacteriophages. *Journal of Turkish Phytopathology*, 23, 79-85.
- Khare, U. K. & M. N. Khare (1995) Studies on survival of *Xanthomonas campestris* pv. *glycines*. *Indian Phytopathology*, 48, 180-181.
- Lazo, G. R. & D. W. Gabriel (1987) Conservation of plasmid DNA-sequences and pathovar identification of strains of *Xanthomonas-campestris*. *Phytopathology*, 77, 448-453.
- Leite, R. P., G. V. Minsavage, U. Bonas & R. E. Stall (1994) Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of

- DNA-sequences related to the *hrp* genes of *Xanthomonas-campestris* pv *vesicatoria*. *Applied and Environmental Microbiology*, 60, 1068-1077.
- Liang, L. Z., J. M. Halloin & A. W. Saettler (1992) Use of polyethylene-glycol and glycerol as carriers of antibiotics for reduction of *Xanthomonas-campestris* pv *phaseoli* in navy bean-seeds. *Plant Disease*, 76, 875-879.
- Lopes, L. P., P. F. R. Alves, C. Zandona, M. P. Nunes & Y. R. Mehta (2008) A semi-selective medium to detect *Xanthomonas axonopodis* pv. *phaseoli* in bean seeds and its eradication through seed treatment with tolylfluanid (Meio semi-seletivo para detectar *Xanthomonas axonopodis* pv. *phaseoli* em sementes de feijoeiro e sua erradicacao atraves do tratamento de sementes com o fungicida tolylfluanid.). *Summa Phytopathologica*, 34, 287-288.
- Mabagala, R. B. (1992) An improved semiselective medium for recovery of *Xanthomonas-campestris* pv *phaseoli*. *Plant Disease*, 76, 443-446.
- Maddox, D. A. (1997) Regulatory needs for standardized seed health tests. *Plant pathogens and the worldwide movement of seeds*, 81-92.
- Mahuku, G. S., C. Jara, M. A. Henriquez, G. Castellanos & J. Cuasquer (2006) Genotypic characterization of the common bean bacterial blight pathogens, *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* by rep-PCR and PCR-RFLP of the ribosomal genes. *Journal of Phytopathology*, 154, 35-44.
- Malavolta Junior, V. A., M. A. R. de Oliveira & A. R. de Oliveira (2000) Identification and survival of *Xanthomonas translucens* pv. *cerealis* in seeds and crop debris of wheat. *Summa Phytopathologica*, 26, 20-23.
- Maringoni, A. C., H. Kimati & C. Kurozawa (1995) Presence of the *Xanthomonas campestris* pv. *phaseoli* in dry bean seeds and epidemiological consequences. *Fitopatologia Brasileira*, 20, 449-457.
- (1998) Comparison between two extraction methods of *Xanthomonas campestris* pv. *phaseoli* from bean seeds. *Pesquisa Agropecuaria Brasileira*, 33, 659-664.
- Marques, A. S. d. A., P. M. Guimaraes, J. P. d. Santos & T. M. Vieira (2005) Survival and viability of *Xanthomonas axonopodis* pv. *phaseoli* associated with bean seeds stored under controlled conditions. *Fitopatologia Brasileira*, 30, 527-531.
- Moyo, M., S. O. Amoo, M. W. Bairu, J. F. Finnie & J. Van Staden (2008) Optimising DNA isolation for medicinal plants. *South African Journal of Botany*, 74, 771-775.
- Munkvold, G. P. (2009) Seed pathology progress in academia and industry. *Annual Review of Phytopathology*, 47, 285-311.
- Mutlu, N., A. K. Vidaver, D. P. Coyne, J. R. Steadman, P. A. Lambrecht & J. Reiser (2008) Differential pathogenicity of *Xanthomonas campestris* pv. *phaseoli* and *X. fuscans* subsp *fuscans* strains on bean genotypes with common blight resistance. *Plant Disease*, 92, 546-554.
- Mutlu, N., A. K. Vidaver, D. P. Coyne, J. R. Steadman, J. Reiser & P. A. Lambrecht (2004) Identification of races of *Xanthomonas campestris* pv. *phaseoli* and establishment of the first common bacterial blight differential *Phaseolus vulgaris* lines. *Phytopathology*, 94, S73-S73.
- Nunes, W. M. d. C., M. J. Corazza, S. A. C. D. d. Souza, S. Tsai & E. E. Kuramae (2008) Characterization of *Xanthomonas axonopodis* pv. *phaseoli* isolates. *Summa Phytopathologica*, 34, 228-231.
- Ohata, K. I., S. Serizawa, K. Azegami & A. Shirata (1982) Possibility of seed transmission of *Xanthomonas-campestris*-var-*vitians* the pathogen of bacterial

- spot of lettuce. *Bulletin of the National Institute of Agricultural Sciences Series C (Plant Pathology and Entomology)*, 81-88.
- Opio, A. F., J. M. Teri & D. J. Allen (1993) Studies on seed transmission of *Xanthomonas campestris* pv. *phaseoli* in common beans in Uganda. *African Crop Science Journal*, 1, 59-67.
- Ovies, J. & L. Larrinaga (1988) Transmission of *Xanthomonas campestris* pv. *phaseoli* by a wild host. *Ciencia y Tecnica en la Agricultura, Proteccion de Plantas*, 11, 23-30.
- Pasquali, M., L. Marena, E. Fiora, P. Piatti, M. L. Gullino & A. Garibaldi (2004) Real-time polymerase chain reaction for identification of a highly pathogenic group of *Fusarium oxysporum* f.sp. *chrysanthemi* on *argyranthemum frutescens* L. *Journal of Plant Pathology*, 86, 53-59.
- Pastrik, K. H. & E. Maiss (2000) Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. *Journal of Phytopathology-Phytopathologische Zeitschrift*, 148, 619-626.
- Puchta, H. & H. L. Sanger (1989) Sequence-analysis of minute amounts of viroid RNA using the polymerase chain-reaction (PCR). *Archives of Virology*, 106, 335-340.
- Reichert-Schwillinsky, F., C. Pin, M. Dzieciol, M. Wagner & I. Hein (2009) Stress- and growth rate-related differences between plate count and real-time PCR data during growth of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 75, 2132-2138.
- Remeus, P.M. and Sheppard, J.W. (2006) Proposal for a new method for detecting *Xanthomonas axonopodis* pv. *phaseoli* on bean seeds. *ISTA Method Validation Reports* 3, 1-11
- Roberts, S. J., L. H. Hiltunen, P. J. Hunter & J. Brough (1999) Transmission from seed to seedling and secondary spread of *Xanthomonas campestris* pv. *campestris* in Brassica transplants: Effects of dose and watering regime. *European Journal of Plant Pathology*, 105, 879-889.
- Roumagnac, P., L. Gagnevin & O. Pruvost (2000) Detection of *Xanthomonas* sp., the causal agent of onion bacterial blight, in onion seeds using a newly developed semi-selective isolation medium. *European Journal of Plant Pathology*, 106, 867-877.
- Sakthivel, N., C. N. Mortensen & S. B. Mathur (2001) Detection of *Xanthomonas oryzae* pv. *oryzae* in artificially inoculated and naturally infected rice seeds and plants by molecular techniques. *Applied Microbiology and Biotechnology*, 56, 435-441.
- Schaad, N. W., Y. Berthier-Schaad, A. Sechler & D. Knorr (1999) Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. *Plant Disease*, 83, 1095-1100.
- Schena, L., F. Nigro, A. Ippolito & D. Gallitelli (2004) Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology*, 110, 893-908.
- Schuster, M. L. & R. M. Sayre (1967) A coryneform bacterium induces purple-colored seed and leaf hypertrophy of *phaseolus vulgaris* and other leguminosae. *Phytopathology*, 57, 1064-&.
- Seal, S. E., L. A. Jackson, J. P. W. Young & M. J. Daniels (1993) Differentiation of *Pseudomonas-solanacearum*, *Pseudomonas-syzygii*, *Pseudomonas-pickettii* and the blood-disease bacterium by partial 16s-ribosomal-RNA sequencing -

- construction of oligonucleotide primers for sensitive detection by polymerase chain-reaction. *Journal of General Microbiology*, 139, 1587-1594.
- Sharma, M., K. Agrawal & T. Singh (2002) Incidence and seed transmission of *Xanthomonas campestris* pv. *cajani* in pigeonpea. *Journal of Mycology and Plant Pathology*, 32, 1-5.
- Sheppard, J., Kurowski, C. and Remeus, P.M. (2007) Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris*. *International Rules for Seed Testing* 7-021
- Tadele, T. (2006) Effect of common bacterial blight severity on common bean yield. *Tropical Science*, 46, 41-44.
- Tebaldi, N. D., R. d. C. Panizzi & R. Sader (2007) Detection, transmission and effect of *Xanthomonas campestris* pv. *campestris* in the physiological quality of broccoli seeds. *Summa Phytopathologica*, 33, 290-293.
- Theodoro, G. d. F. & A. C. Maringoni (2002) Survival of *Xanthomonas axonopodis* pv. *manihotis* in manipueira under controlled conditions (Sobrevivencia de *Xanthomonas axonopodis* pv. *manihotis* em manipueira sob condicoes controladas.). *Summa Phytopathologica*, 28, 276-279.
- Torres, J. P. & A. C. Maringoni (1997) Reaction of dry bean of *Xanthomonas campestris* pv. *phaseoli* and seed transmission of the bacterium. *Fitopatologia Brasileira*, 22, 546-549.
- Torres, J. P., A. C. Maringoni & T. A. F. Silva (2009) Survival of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* in common bean leaflets on soil. *Journal of Plant Pathology*, 91, 195-198.
- Toth, I. K., L. J. Hyman, R. Taylor & P. R. J. Birch (1998) PCR-based detection of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* in plant material and its differentiation from *X. c.* pv. *phaseoli*. *Journal of Applied Microbiology*, 85, 327-336.
- Valarini, P. J., J. A. H. Galvao & D. D. A. Oliveira (1996) *Xanthomonas campestris* pv. *phaseoli*: Importance of seed inoculum in the epidemiology of the common bacterial blight of beans. *Fitopatologia Brasileira*, 21, 261-267.
- Valarini, P. J. & J. O. M. Menten (1991) Artificial inoculation of dry bean seeds with *Xanthomonas-campestris* pv. *phaseoli* and its effect on sanitary quality and the germination. *Summa Phytopathologica*, 17, 227-231.
- (1992) Methodology for detection of *Xanthomonas campestris* pv. *phaseoli* in bean seeds. *Fitopatologia Brasileira*, 17, 373-383.
- Valarini, P. J., J. O. M. Menten & M. A. Lollato (1992) Incidence of the common bacterial blight in the field and transport of *Xanthomonas campestris* pv. *phaseoli* through bean seeds, obtained by different methods. *Summa Phytopathologica*, 18, 160-166.
- Vauterin, L., B. Hoste, K. Kersters & J. Swings (1995) Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, 45, 472-489.
- Venette, J. R., R. S. Lamppa, D. A. Albaugh & J. B. Naves (1987) Presumptive procedure (dome test) for detection of seed-borne bacterial pathogens in dry beans. *Plant Disease*, 71, 984-990.
- Vieira, B. d. A. H. & R. M. d. Souza (2000) Virulence of isolates of *Xanthomonas axonopodis* pv. *phaseoli* and its variant *fuscans*. *Ciencia e Agrotecnologia*, 24, 94-102.

- Vieira, R. F., J. E. S. Carneiro & J. P. Lynch (2008) Root traits of common bean genotypes used in breeding programs for disease resistance. *Pesquisa Agropecuaria Brasileira*, 43, 707-712.
- Walcott, R. R. & R. D. Gitaitis (2000) Detection of *Acidovorax avenae* subsp *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. *Plant Disease*, 84, 470-474.
- Wong, W. C. (1991) Methods for recovery and immunodetection of *Xanthomonas campestris* pv *phaseoli* in navy bean seed. *Journal of Applied Bacteriology*, 71, 124-129.
- Yucel, F., E. Bermek & B. Cirakoglu (1997) Production of monoclonal antibodies specific for *Xanthomonas campestris* pv. *phaseoli*. *Turkish Journal of Biology*, 21, 125-131.
- Zaumeyer, W. J. & H. R. Thomas (1957) A monographic study of bean diseases and methods for their control. *Technical Bulletin. United States Department of Agriculture*, 868, 255 pp.
- (1958) New snap and Pinto beans resistant to several diseases. *Phytopathology*, 47, 454 p.

Table 1 Bacterial strains used for validation of real-time PCR assays for *Xanthomonas axonopodis* pv *phaseoli* and *Xanthomonas axonopodis* pv *phaseoli* var. *fuscans*, their host, origin and real-time PCR results.

Bacterial strains	Host	Origin	Real-time PCR result	
			<i>Xap</i> ^x	<i>Xap</i> ^f
<i>X. axonopodis</i> pv <i>phaseoli</i>	Bean			
PR1*		Puerto Rico, ‘Andean’	+	-
Xcp 34*		WI, USA,2005, ‘Andean’	+	-
Xcp 83*		WI, USA,2005, ‘Andean’	+	-
Xcp 209*		WI, USA,2005, ‘Andean’	+	-
Xcp280*		WI, USA,2006, ‘Andean’	+	-
Xcp 346*		WI, USA,2006, ‘Andean’	+	-
Xcp415*		WI, USA,2007, ‘Andean’	+	-
Xcp437*		WI, USA,2007, ‘Andean’	+	-
B1†		ID, USA	+	-
K3A, LB2, 9807 ^a		NE, USA, 1984	+	-
Michigan 3 Bean ^a		MI, USA, 1997, ‘K93623’	+	-
95-61 ^a		ND, USA	+	-
B705 ^a		Brazil(sent from NE)	+	-
1811NCPPB ^a		Romania	+	-
K3A ^a		Kimball, NE, 1985	+	-
LB2 ^a		Lincoln, NE, 1985	+	-
9807 ^a		Scottsbluff, NE, 1998	+	-
95-41		ND, USA	+	-
356.3.0 [#]		France	+	-
269.30A [#]		France	+	+
269.30B [#]		France	+	+
356.10B [#]		France	+	-
300.5.1 [#]		France	+	-
269.3-1 [#]		France	+	-
<i>X. axonopodis</i> pv <i>phaseoli</i> var. <i>fuscans</i>	Bean			
PR8*		Puerto Rico, ‘Middle American’ bean	-	+
90:11		ID, USA	-	+
94:202†		Netherlands, ‘PV539’	-	+
94:87†		CA, USA, ‘Granato’	-	+
XP37#(ATCC)		USA	-	+
Xpfs ^a		NE, USA, 1975	-	+
0794		Australia(Kingary), 1978	+	+
1158 NCPPB		UK	-	+
<i>P. syringae</i> pv. <i>syringae</i>	Bean			
97:M331†		ID, USA	-	-

Table 1 (continued)

06:41†		CA,USA	-	-
M332†		ID,USA	-	-
06:38†		ID,USA	-	-
<i>P. syringae</i> pv. <i>phaseolicola</i>	Bean			
97:28†		ID,USA	-	-
97:114A1-1†			-	-
04:M279†			-	-
<i>X. oryzae</i> pv. <i>oryzae</i>	Rice			
PXO99A‡			-	-
<i>X. campestris</i> pv. <i>vesicatoria</i>				
85-10‡	Citrus	Phillippines	-	-
<i>X.campestris</i> pv. <i>campestris</i>				
8004‡	Crucifer		-	-
<i>Acidovorax avenae</i> subsp. <i>citrulli</i>				
AAC00-1□	Citrus	-	-	-
94-21□	Watermelon	-	-	-
<i>Pseudomonas s. pv. glycinea</i>	Soybean			
Race 2§		-	-	-
Race 4§		-	-	-
<i>Pantoea s. subsp. stewartii</i>	Corn			
SS104■		Illinois,USA,1967	-	-
SW45■		Missouri, 1975	-	-
<i>Pantoea s. subsp. stewartii</i>	epiphyte			
175■		-	-	-
299■		-	-	-
<i>P. ananatis</i>	Onion			
EA-8366■		-	-	-
<i>P. ananas</i>	Corn			
DC130■		Missouri, 1976	-	-
<i>Erwinia amylovora</i>	Corn			
EA-2■		-	-	-

* contributed by Dr. Robert Gilbertson; † contributed by Elizabeth Vavricka; ‡

contributed by Dr. Adam Bogdanove; □ contributed by R.D.Gitatis; ■ contributed by

Dr. Charles Block; # from American Type Culture Collection. § contributed by

Dr. Edward Braun ; ^a contributed by Dr. Anne Vidaver. [#] contributed by Valerie

Grimault, GEVES/SNES, France. ^x: *Xanthomonas axonopodis* pv *phaseoli*; ^y:

Xanthomonas axonopodis pv *phaseoli* var. *fuscans*.

Table 2. Seedlots from various geographical origins naturally infested with common bacterial blight and used in this study to compare seed extraction methods.

Seedlot	Origin	Type	Cultivar	Seedlots	Subsamples
1	IA	Cranberry	True Red Cranberry	1	12
2	IA	Pole	Purple Podded Pole	1	8
3	IA	Green	Bountiful 337	1	6
4	PNW ^b	Green	Thoroughbred	1	10
5-6	PNW ^b	Green	Valentino	2	10
7-8	PNW ^b	Green	Hercules	2	10
9	CA	Red Kidney	Montcalm	1	10
10	CO	Pinto	Poncho	1	20
11-13	WI	Red Kidney	NS ^a	3	4,6,8
14	NS ^a	French	NS ^a	1	16

^aNS = Not Specified

^bPNW= Pacific North West

Table 3. Mean detection sensitivity and standard deviations for real-time PCR assay on 1000-seed common bean samples spiked with seeds artificially inoculated with *Xanthomonas axonopodis* pv. *phaseoli* isolate B1 (one inoculated seed per sample).
n=total number of samples

Inoculum dose	Sensitivity (Positive/Total)	Mean of Ct value
10^5 CFU/seed	96%±5.5% (n=50)	29.90
10^4 CFU/seed	97% ± 5.8%(n=30)	33.07
10^3 CFU/seed	66%±11.4% (n=50)	36.59
10^2 CFU/seed	63%±11.5% (n=30)	37.3

Table 4. Pathogen detection sensitivity for real-time PCR and a culture plate method (Sheppard et al. 2007) for common bean seedlots naturally infested with *Xanthomonas axonopodis* pv. *phaseoli* and extracted with two seed extraction methods (the ISTA-approved method and modified method [He and Munkvold, 2010]). Each entry in the table is the proportion of positive subsamples over the total number of subsamples.

Seedlot	ISTA method ^{a,c}		Modified method ^{b,c}	
	Culture plate	Real-time PCR	Culture plate	Real-time PCR
1	2/6	3/6	4/6	5/6
2	1/4	2/4	4/4	4/4
3	1/3	2/3	2/3	2/3
4	4/5	4/5	4/5	5/5
5	3/5	3/5	5/5	3/5
6	3/5	3/5	3/5	5/5
7	2/5	2/5	3/5	4/5
8	4/5	5/5	5/5	5/5
9	4/5	4/5	3/5	3/5
10	6/10	5/10	8/10	8/10
11	1/2	1/2	0/2	2/2
12	0/3	2/3	1/3	3/3
13	2/4	2/3	2/4	4/4
14	2/8	5/8	4/8	5/8
Total	35/70 ^d	43/70 ^d	48/70 ^e	58/70 ^e

a Overnight incubation at 4° C without centrifugation

b Three-hour incubation at room temperature with vacuum extraction and centrifugation of seed extract.

c, d, e: Significantly different ($P<0.05$)

Figure 1. Conventional PCR products generated by published primers (Audy et al. 1994, Birch et al 1997) for *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*. (A) 730 bp fragment amplified from *X. axonopodis* pv. *phaseoli* isolates by primers X4c/X4e. From left to right: isolates Xap B1, PR1, Xap89, Michigan3bean, 95-41, B705. (B) 730 bp fragment amplified from *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates by primers X4c/X4e. From left to right: isolates PR8, 94:202, 90:11, XP37, Xpfs, 94:87. (C) 900 bp fragment amplified from *X. axonopodis* pv. *phaseoli* isolates by OP-G11. From left to right: isolates Xap B1, PR1, Xap89. (D) 820 bp fragment amplified from *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates by OP-G11. From left to right: isolates PR8, 94:202, 90:11. DNA ladder was 1Kb plusTM from Invitrogen, CA.

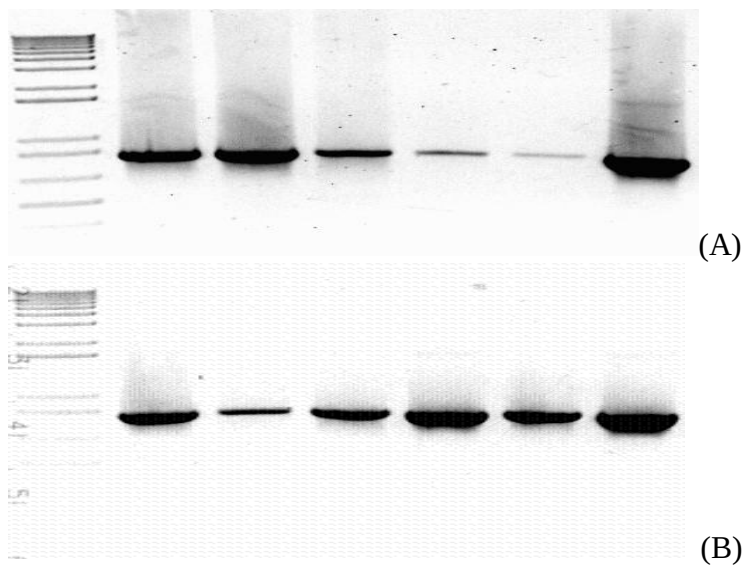
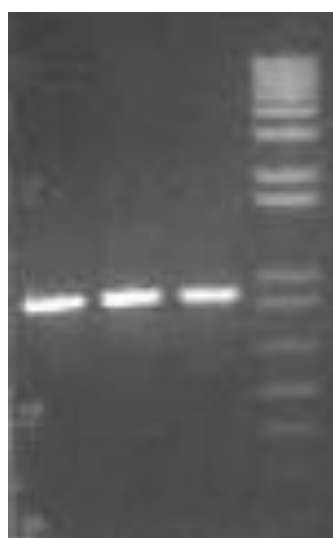
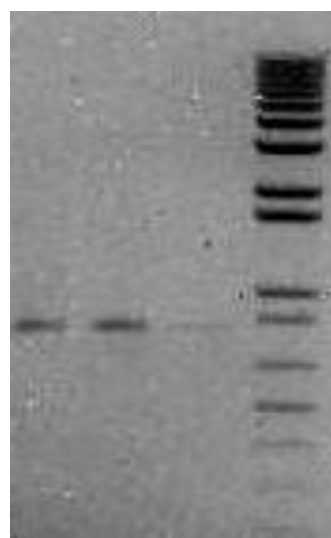


Figure 1.(continued)



(C)



(D)

Figure 2. PCR-RFLP results generated by published primers (Mahuku et al. 2006) for *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*. (A) PCR product amplified by Phill-1/P322-Anti from *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates on 16S gene including the IGS region.(1Kb plus DNA ladder) (B) RFLP profile digested by HaeIII(100bp DNA ladder). From left to right: isolates PR8, 94:202, XapB1, Xap89.

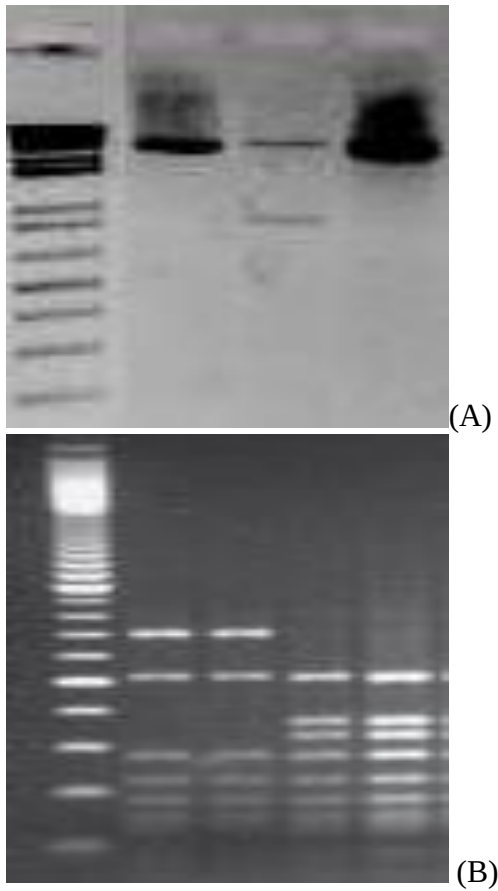


Figure 3. (A) Amplification plot of real-time PCR for *X. axonopodis* pv. *phaseoli* genomic DNA. The SDS software plots ΔR_n versus cycle number. (B) Linear model, with Ct plotted vs. DNA concentration.

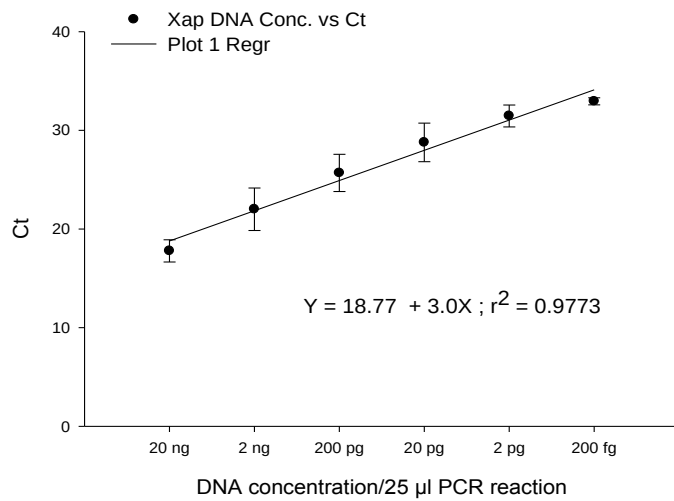
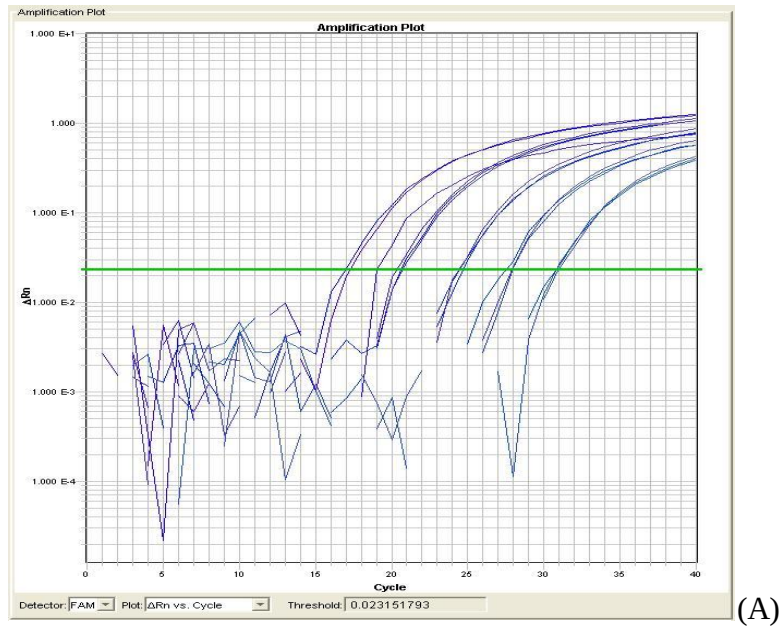


Figure 4. (A) Amplification plot of real-time PCR for *X. axonopodis* pv. *phaseoli* var. *fuscans* genomic DNA. The SDS software plots ΔRn versus cycle number. (B) Linear model, with Ct plotted vs. DNA concentration.

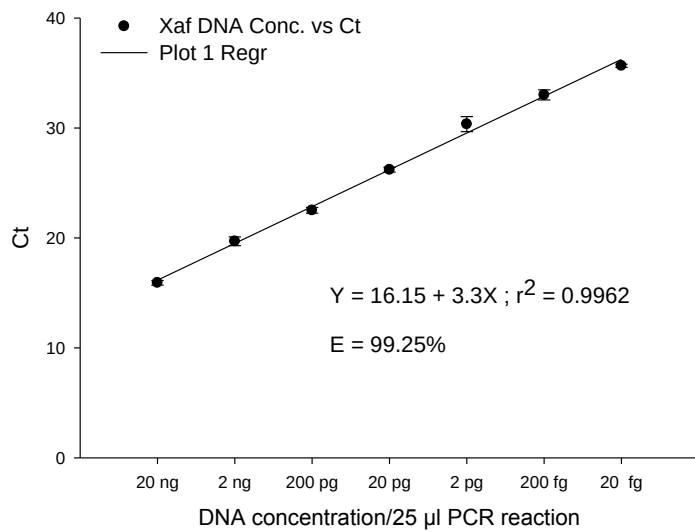
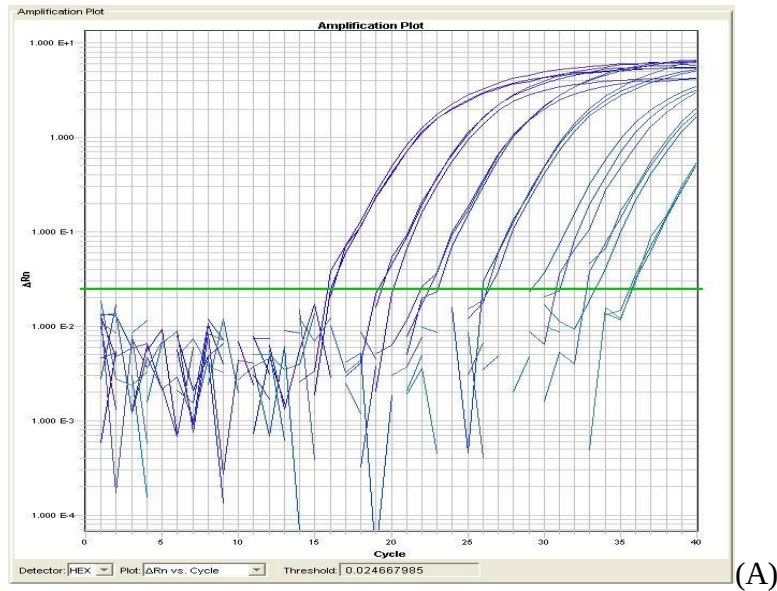


Figure 5. (A) Amplification plot of real-time PCR detection for pure *X. axonopodis* pv. *phaseoli* cells suspension. (B) Linear model, with Ct plotted vs. cell density.

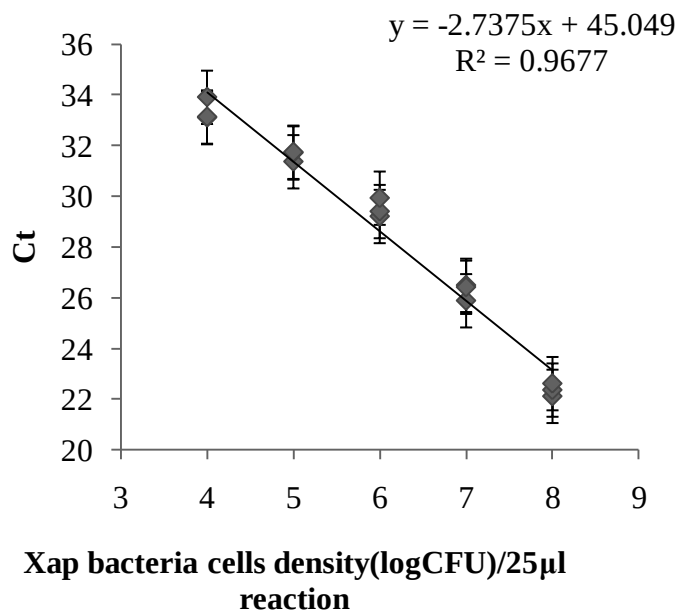
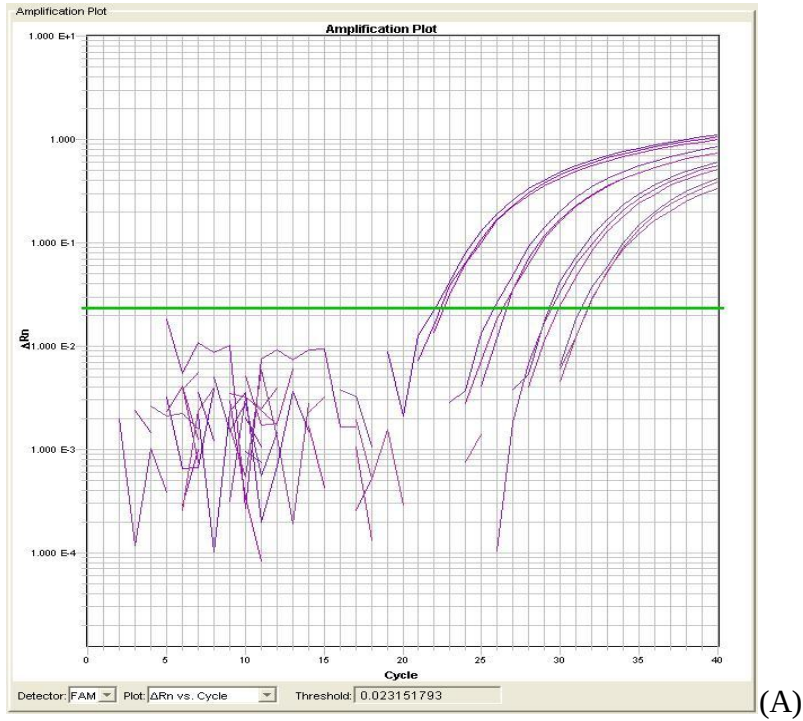
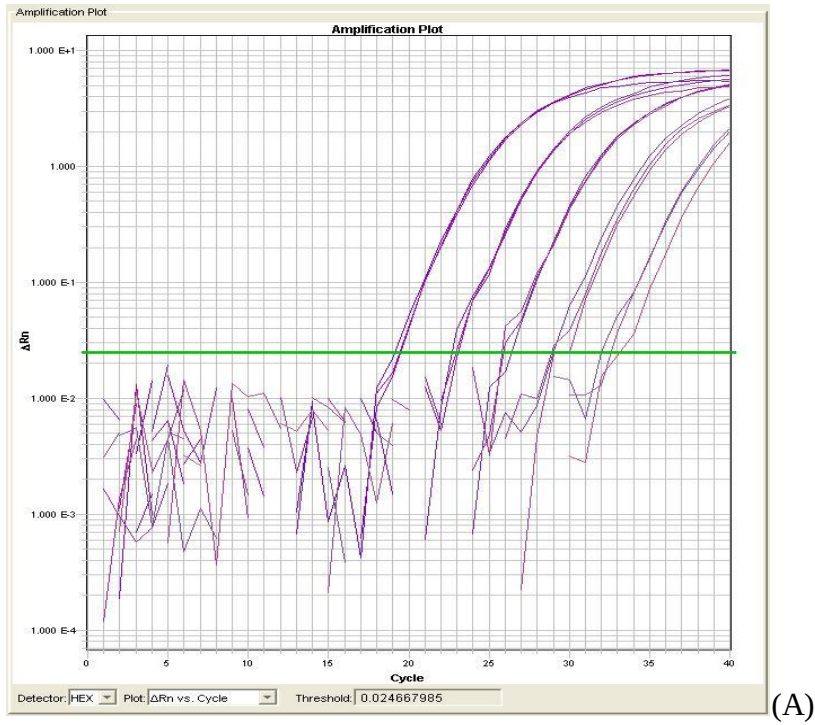
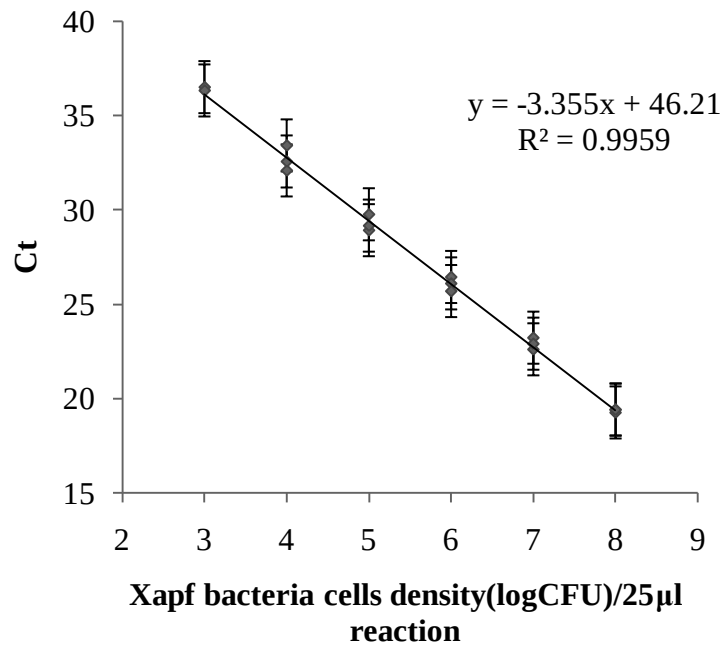


Figure 6. (A) Amplification plot of real-time PCR detection for pure *X. axonopodis* pv. *phaseoli* var. *fuscans* cells suspension. (B) Linear model, with Ct plotted vs. cell density.

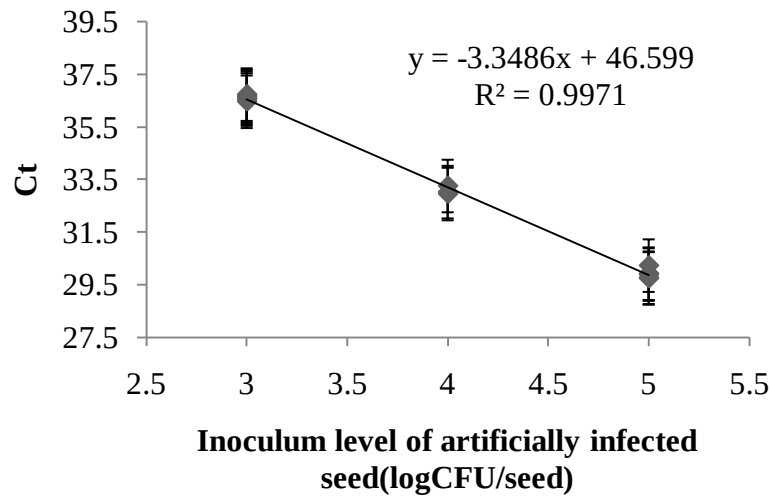


(A)



(B)

Figure 7. Linear model established according to the relationship of Ct values and inoculums level of spiked seeds by *X. axonopodis* pv. *phaseoli* var. *fuscans*.



**CHAPTER 4. EVALUATION OF SURVIVAL AND SEED TRANSMISSION OF
XANTHOMONAS AXONOPODIS PV. *PHASEOLI* AND *XANTHOMONAS*
AXONOPODIS PV. *PHASEOLI* VAR. *FUSCANS* BY QUANTITATIVE REAL-TIME
 PCR**

A paper to be submitted to *European Journal of Plant Pathology*

Yiqing He, Gary Munkvold

Abstract

Xanthomonas axonopodis pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* are the causal agents of common bacterial blight of bean (*Phaseolus vulgaris*). Seed transmission frequency and survival in storage were compared among five isolates of each variant from different geographic origins. Seeds of a susceptible bean genotype were inoculated and planted in a greenhouse or placed in an environmentally controlled seed storage facility. *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates had a higher percentage of seed-seedling transmission than *X. axonopodis* pv. *phaseoli* isolates ($P<0.001$), based on seedling emergence, seedling disease symptoms, and symptomless infection. Both variants reduced seedling emergence compare to the noninoculated control. *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* isolates reduced seedling emergence more than *X. axonopodis* pv. *phaseoli* ($P<0.001$) and the incidence of bacterial blight symptoms was higher in seedlings from *X. axonopodis* pv. *phaseoli* var. *fuscans* inoculated seeds than in seedlings from seeds inoculated with *X. axonopodis* pv. *phaseoli* ($P<0.001$). Real-time PCR was used to evaluate seedling infection and results showed that a higher percentage of seedlings were infected with *X. axonopodis* pv. *phaseoli* var. *fuscans* than *X. axonopodis* pv. *phaseoli*. PCR results also revealed symptomless infection of seedlings. Survival (population size) of both variants on stored seeds was monitored over time using real-time PCR. Survival did not differ significantly between the two

variants and real-time PCR gave higher population size estimates than a culture plating test after three months in storage ($P<0.05$).

Introduction

Xanthomonas axonopodis pv. *phaseoli* (Smith) Vauterin, and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder (Vauterin, 1995) cause common bacterial blight of bean (*Phaseolus vulgaris* L.). Seedborne inoculum is important in the survival and dissemination of these pathogens, and they can be transmitted from seed to growing plants, initiating damaging epidemics (Zaumeyer and Thomas 1957). The two strains can be differentiated using isoenzyme profiling (El-Sharkawy and Huislingh 1971), plasmid profiling (Lazo and Gabriel 1987), or DNA-DNA hybridization (Hildebrand et al. 1990). The average genome sizes for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* were 3850.6 ± 48.9 and 3584.3 ± 68.1 kb respectively (Chan and Goodwin 1999b). *X. axonopodis* pv. *phaseoli* var. *fuscans* also can be distinguished by the production of a diffusible brown pigment in culture due to secretion and subsequent oxidation of homogentisic acid which is an intermediate in tyrosine catabolism for a number of bacteria. *X. axonopodis* pv. *phaseoli* var. *fuscans* appears to have a disrupted tyrosine catabolism pathway and cannot use tyrosine as a nutrient (Goodwin and Sopher 1994). Five adhesin genes (*pilA*, *fhab*, *xadA1*, *xadA2*, and *yapH*) identified in *X. axonopodis* pv. *phaseoli* var. *fuscans* strain altered the ability of both variants to adhere to polypropylene or seed. Adhesins were implicated in various processes leading to host phyllosphere colonization and transmission to seeds (Darsonval et al. 2009).

It has been reported that *X. axonopodis* pv. *phaseoli* var. *fuscans* strains were more pathogenic than *X. axonopodis* pv. *phaseoli* strains from the same origin. African strains were most pathogenic on 13 common bean genotypes. *Xanthomonas axonopodis*

pv. phaseoli strains that originated in Caribbean and South American countries displayed the widest range in pathogenicity levels (Mutlu et al. 2008). Pathogenic variation was greater within *X. axonopodis pv. phaseoli* than within *X. axonopodis pv. phaseoli var. fuscans* (Mutlu et al. 2004). These studies were conducted by inoculating plant tissues. There are no published studies comparing aggressiveness of the two pathogenic variants following seed inoculation, or comparing their frequencies of seed transmission.

Survival of these pathogens in seed and crop residue has been studied, but it remains unclear whether there are consistent differences in survival characteristics between *X. axonopodis pv. phaseoli* and *X. axonopodis pv. phaseoli var. fuscans*. Seeds from fields with 100% of infected plants presented low pathogen incidences when stored for 3-4 years. Sun drying and different harvesting systems did not affect pathogen incidence on seeds (Valarini et al. 1992). In another study, the survival of seedborne *X. axonopodis pv. phaseoli* was reported to be reduced from 64 to 36-37% incidence during the first 6 months but the contamination rate was maintained after 30 and 60 months at both -18 and 5°C. The optimal temperature for bacterial survival and maintenance was 5°C and optimal conditions for seed conservation were the same as those for the maintenance of *X. axonopodis pv. phaseoli* (Marques et al. 2005). In crop residue, under rainy conditions *X. axonopodis pv. phaseoli* survived up to 3 weeks above and 1 week below ground in infected plant tissue. Under dry conditions it survived 8 weeks above and 5 weeks below ground in infected plant tissue (Chavez-L and Granada 1988). Crop residue also can provide a source for epiphytic colonization of other crop plants. In a dry bean - onion crop rotation scheme, epiphytic *X. axonopodis pv. phaseoli* was recovered from symptomless onion plants in fields cropped to dry beans the prior year, but not from fields cropped to plants other than dry

bean. Therefore, close rotation of onion and dry bean may allow *X. axonopodis* pv. *phaseoli* to persist epiphytically (Gent et al. 2005b). Some studies have shown that survival rate differed between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. *X. axonopodis* pv. *phaseoli* survival was higher in sterile than non-sterile soil, and was higher compared to that of *X. axonopodis* pv. *phaseoli* var. *fuscans*. Under mild temperatures and low rainfall, *X. axonopodis* pv. *phaseoli* var. *fuscans* survived for 2 to 6 months in leaflets on the soil surface, and for 1 to 4 months in those incorporated in the soil regardless of the depth. Under higher rainfall and temperatures, the survival was from 1.5 to 2 months in leaflets on the surface and from 1 to 1.5 months in those buried 10 or 15 cm deep (Torres et al. 2009).

Survival of closely related bacterial plant pathogens on seed and crop residue also has been studied. *Xanthomonas vesicatoria* populations on inoculated seeds dropped substantially within 30 days after inoculation in one study. Low seed moisture content also interfered with *X. vesicatoria* survival and transmission (Correa et al. 2008). In contrast, *Xanthomonas campestris* var. *vitians* survived at least 23 months after inoculation on lettuce seeds and the survival rates were higher in seeds stored at 10°C compared to room temperature (Ohata et al. 1982). In seeds of wheat and triticale, *X. translucens* pv. *cerealis* survival was only slightly reduced during the first two years after the harvest, but after 42 months the pathogen was not recoverable. Survival of *X. translucens* pv. *cerealis* in crop debris was longer than 30 months under laboratory conditions and shorter than 8 months under field conditions (Malavolta et al. 2000). Also on infected tissue, another closely related pathogen, *X. axonopodis* pv. *glycines* remained viable for 110 days in infected leaves on the soil surface and for 29 days in infected leaves buried at 15 cm depth, but did not survive at all at a depth of 30 cm (Khare and Khare 1995). *X. axonopodis* pv. *allii* could be recovered from infested

onion leaves 9 months after they were placed on the soil surface or buried to a depth of 25 cm, but culturable populations of the pathogen declined more rapidly in buried leaves (Gent et al. 2005a). *X. axonopodis* pv. *manihotis* survived for 48 h in manipueira of cassava incubated at 15°C, and less than one day at 20, 25, 30 and 35°C (Theodoro and Maringoni 2002).

Pathogen incidence on seeds is closely correlated with disease incidence in the seed production field (Valarini et al. 1992), and there is usually a positive correlation between seed symptoms and the population of bacteria per seed. In Uganda, the population of *X. axonopodis* pv. *phaseoli* in farmers', commercial and research seeds averaged 10^5 to 10^9 CFU/100 seeds and the incidence of seed infection was 0.3 to 16.1%.

Seedborne pathogen thresholds have been studied in relation to seed transmission and subsequent disease. Infection of approximately 1 in 10,000 seeds was capable of causing an outbreak of blight (Sutton and Wallen et al. 1970). In another study, the minimum population required to initiate disease in the field was 10 CFU/seed while a 0.2% seed infection level resulted in a serious disease outbreak (Opio et al. 1993). In Canada, 0.5% seed infection level has been shown to lead to disease epidemics (Zaumeyer and Thomas 1957). The fact that symptomless and slightly diseased seeds gave rise to severely infected seedlings demonstrated that the normal cleaning by removing colored and shriveled seeds would have limited effect on reducing the common bacterial blight infection of a seed stock if it was obtained from an infected field. Weller and Saettler (1980) showed that the minimum population to initiate infection depended on different bean genotypes and locations. Inoculum of *X. axonopodis* pv. *phaseoli* can be localized superficial or internally in the seed. Seed weight and germination can be significantly affected by incidence of *X. axonopodis* pv.

phaseoli in the field and on the seeds (Valarini and Menten 1991). However, up to 10% of infected seeds did not reduce significantly the emergence of seedlings, though infection levels of 5% and higher reduced bean yield due to disease transmission (Valarini et al. 1996). The bacterial inoculum recovered from plant tissue with visible symptoms of blight infection was always much lower in resistant and moderately resistant cultivars than in susceptible cultivars (Cafati and Saettler 1980). However, a later study showed that resistance to common blight foliar symptoms does not influence bacterial translocation to seeds (Torres and Maringoni 1997).

Similar studies of other seedborne *Xanthomonas* spp. have been conducted and results can be related to *X. axonopodis* pv. *phaseoli*. Cotton seeds inoculated with *X. axonopodis* pv. *malvacearum* transmitted disease in up to 68% of seedlings and 16.66% of the emerged seedlings wilted and died (Gholve and Kurundkar 2007). Epiphytic populations of *X. axonopodis* pv. *glycines* developed on both resistant and susceptible seedlings that grew from seeds with either external or internal populations. In the field the pathogen dispersed from a diseased source plant equally in all directions and at the same rate of either resistant or susceptible cultivars (Groth and Braun 1989).

The effects of inoculum load and watering regime on the transmission of *Xanthomonas campestris* pv. *campestris* from seed to seedlings of cauliflower were investigated (Roberts et al. 1999). Effects of watering regime treatments on symptoms and on the proportion of contaminated but symptomless plants were similar. Initially, they were influenced only by the dose of bacteria with little difference between the watering regimes, but later the proportion of plants with symptoms was greater for plants subjected to overhead watering, due to spread and secondary infection. For *X. campestris* pv. *cajani* in pigeonpea seed samples, heavy infection of seed caused failure

of seed germination. Moderate infection caused hypocotyl splitting , browning of the radicle and necrosis of cotyledonary tissues (Sharma et al. 2002). *X. campestris* pv. *campestris* in broccoli seeds did not affect either seed germination or vigor and there were no significant differences among genotypes regarding seed transmission (Tebaldi et al. 2007).

Real-time PCR has been used to detect and diagnose phyto pathogens and disease such as *Xanthomonas campestris* from brassicas (Berg, Tesoriero and Hailstones 2006, Reichert-Schwillinsky et al. 2009), *Fusarium oxysporum* f.sp. *chrysanthemi* on *Argyranthemum frutescens* L.(Pasquali et al. 2004), and a pathovar specific real-time PCR assay for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* detection was developed by He et al (He et al 2009).

The objectives of this study were to compare survival in stored seed and pathogenic seed transmission frequency for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* with help of a newly developed real-time PCR assay. Real-time PCR and a culturing method were compared for estimation of pathogen survival in seed.

Materials and methods

Bacterial isolates and growth conditions

Bacterial isolates used in this study were obtained from diverse geographical origins and sources (Table 1). Reference strain XP37 was received from ATCC (American Type Culture Collection). Bacterial cultures were grown routinely on nutrient agar (BD Difco™, MD) or YDC medium at 28°C. Isolation of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* from naturally infected seed

or inoculated plants was conducted on semiselective agar media MT (Goszczyńska and Serfontein 1998) and XCP1 (McGuire 1986). Bacterial cultures were enriched on nutrient agar or YDC agar. Long-term, bacteria were stored on silica beads (Microbank, Austin, TX) by shaking beads in a cell suspension, removing excess fluid, and freezing at -80°C.

Inoculation of seeds

Samples of 500 seeds of white bean (*Phaseolus vulgaris*) cultivar ‘Derby’ were inoculated with individual isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* (Table 1). Bacterial suspensions were adjusted to the concentration of 10^6 CFU/ml in phosphate buffered saline (PBS). Seeds were immersed in the suspension for 5 min with vacuum and air-dried in a biosafety cabinet. Seed contamination levels were confirmed by real-time PCR (He et al., 2009) and culturing on nutrient agar medium: from each one of the 10 inoculated samples, 10 seeds were sampled and immersed individually in 1 ml PBS at 4°C overnight. Seed wash was diluted to 10^{-3} and 10^{-4} and 100 µl was cultured on nutrient agar plates. One-hundred µl of the original seed wash was treated with 10% SDS /Proteinase K (20mg/ml) and CTAB solution to release DNA. The raw sample was used in real-time PCR after protein removal by phenol/chloroform/isoamylalcohol.

Seed-seedling transmission

Seeds were randomly sampled from the 500-seed inoculated samples immediately after drying and planted under greenhouse conditions. For each isolate, three replicate samples of 25 seeds were planted, one seed per container (3.8 by 21 cm) at 20°C without cover and cones were separated from each other to avoid cross contamination during watering. Emergence was assessed 4-5 d after planting, and one of the two primary leaves from plants that developed leaves was collected. Leaf tissue

was freeze-dried and crushed to powder by shaking with 0.5mm glass beads (BioSpec Products, OK). DNA was extracted from tissue powder by CTAB method (Moyo et al. 2008). Real-time PCR (He et al., 2009) was conducted to test for pathogen presence on each seedling. Seedlings were left to grow another week to allow symptom development. Symptoms of common blight such as blight spots, brown necrosis on cotyledons, failure of leaf development and leaf lesion were observed and recorded. Emergence, seed transmission frequency and incidence of common blight symptoms were compared between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* variants. The comparison between two variants was carried out using SAS procedure GLIMMIX and method was REML. Experimental design was RCBD (Complete Randomized Block Design) and means comparisons among individual isolates were made by the Tukey method ($P < 0.05$).

Storage survival test

Inoculated bean seeds were stored in an environmentally controlled seed storage room at 4°C and 50% relative humidity. Seeds were randomly sampled and tested by real-time PCR and culture medium assay for inoculum level at four points in time: initial, 1 month in storage, 3 months and 6 months. At each time point, 10 samples of 5 seeds were collected for each isolate and each sample was immersed in 5 ml PBS at 4°C for 16 h (Darrasse et al. 2007). One-hundred µl of the original seed wash, diluted to 10^{-3} and 10^{-4} , was cultured on nutrient agar medium and the remainder was treated with SDS extraction method as mentioned above for real-time PCR assay. Seeds were also planted in a greenhouse as described above to assess seed transmission for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates immediately after inoculation and after one months and three months in storage. Survival was assessed by estimating Log (CFU/seed) from colony counts on culture

plates or quantitative PCR results. Survival (population size) was compared between isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Data for emergence and incidence of symptoms over time were analyzed by SAS program using complete randomized design with repeated measures, and means were compared by Tukey's method. Comparison for plate count and real-time PCR results was carried out by t-test.

Real-time PCR assay

Real-time PCR was performed as described by He et al. (2009) on 96 wells plates using Quanta PerfeCTa qPCR supermix. In 25µl reaction mixture, 12.5 µl supermix buffer, 0.9 µl of both primers (25 µM) and 0.6 µl of probe (10 µM) were added. Reaction conditions were as followed: 95°C for 2min, 40 repeated cycles of 15s 95°C and 45s 60°C.

Results

Seed transmission

Emergence was significantly lower for inoculated seeds (52% to 70%) than for the noninoculated control treatment (86%) ($P<0.001$), and mean emergence for *X. axonopodis* pv. *phaseoli* var. *fuscans*-inoculated seed (58.1%) was lower than for *X. axonopodis* pv. *phaseoli*-inoculated seed (68.3%) ($P<0.001$) (Figure 1). Percentage of diseased plants was calculated as 100 x number of plants with symptoms divided by the number of emerged seedlings. There was a significant difference between *X. axonopodis* pv. *phaseoli* (55%) and *X. axonopodis* pv. *phaseoli* var. *fuscans* in percentage of symptomatic seedlings (68.6%) ($P<0.001$) (Figure 2). Emerged seedlings were tested by real-time PCR for the presence of the pathogens. Some apparently healthy seedlings were infected with the inoculated pathogen. The incidence of infection was significantly different ($P<0.001$) between the two variants: 73.2% of

seedlings were infected with *X. axonopodis* pv. *phaseoli* var. *fuscans* and 60.6% were infected with *X. axonopodis* pv. *phaseoli* of (Figure 3). However, one of the *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates had the lowest incidence of disease symptoms and infection among all the isolates. Non-inoculated seeds had a high emergence percentage and seedlings were all healthy except primary leaves failed to develop on 2 of 75 seedlings. Seedlings from inoculated seeds had various symptoms including lower emergence, failure of leaf development and common blight symptoms of leaves or stems (Figure 4).

Storage survival

Inoculum level decreased during the 6 months from 10^6 CFU/seed to approximately 10^3 CFU/seed. The initial inoculum level of each isolate was almost the same, but after three months, survival varied greatly among replicate samples (Figure 5). However, there were no significant differences in survival between the two *X. axonopodis* pv. *phaseoli* variants or among any of the isolates at any time point or over time. Comparing the culture plate test and real-time PCR test for estimation of bacterial populations, there was no difference initially. After one month, there was a slight trend that real-time PCR showed higher cell counts than culture plate test (data not shown). After three months, real-time PCR gave a significantly higher estimate of cells than the culture plate test ($P < 0.05$) (Figure 6). Stored seeds planted after one month showed higher emergence than those planted immediately after inoculation, which might be due to reduced inoculum survival on seeds. However, the emergence percentage decreased again in 3 months. Emergence over time for seeds inoculated with *X. axonopodis* pv. *phaseoli* was higher than for those inoculated with *X. axonopodis* pv. *phaseoli* var. *fuscans* ($P < 0.05$) (Figure 7). Incidence of diseased plants inoculated by *X. axonopodis* pv. *phaseoli* var. *fuscans* significantly decreased compared to seeds planted

immediately after inoculation; however there was no reduction for *X. axonopodis* pv. *phaseoli* inoculated seeds. The incidence of diseased plants over time was significantly different between variants ($P<0.05$) (Figure 8).

Discussion

Plants grown from inoculated seeds under greenhouse conditions showed various common blight symptoms in this study: reduced emergence, failure of leaf development, primary leaf lesions, brown necrosis of cotyledons, or blighted areas on leaves (Saettler, A.W. 1989). Plants with primary leaves were tested by real-time PCR for the presence of the pathogens, and some symptomless plants were found to be infected. The majority of infected plants showed symptoms; the overall mean percentage of symptomatic plants was 61.8% of all seedlings and 38.8% of all planted seeds, whereas the percentage of plant testing positive by PCR averaged 66.9% of all seedlings and 42.0% of all planted seeds. The results are consistent with previous reports of seed transmission percentages between 40% and 52% (Darrasse et al. 2007).

X. axonopodis pv. *phaseoli* may infect common bean plants systemically and invade into developing seeds through vascular system without producing visible symptoms. The newly developed real-time PCR for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* is useful for detection of infection in symptomless plant tissue due to its high sensitivity (He et al. 2009).

Leaf development failure was attributed to the bacteria and this phenomenon seldom appeared in noninoculated treatments (Zaumeyer and Thomas 1957). Seed and seedling infection may have disrupted cell differentiation. Typical symptoms such as brown spots and lesions on leaf tissue also appeared in infected seedlings, but they seldom developed into severe damage and stem cracks. Many seedlings from inoculated seeds had primary leaf damage either on the edge or in the middle of the

leaves compared to intact ones on seedlings from noninoculated seeds and these were also symptoms of infection (Zaumeyer and Thomas, 1957). Seedlings with these symptoms were all positive for infection according to real-time PCR results.

Isolates of *X. axonopodis* pv. *phaseoli* var. *fuscans* were overall more aggressive than *X. axonopodis* pv. *phaseoli* isolates, demonstrating a greater effect on emergence and higher frequencies of seed-seedling transmission and seedling disease. However, there was significant variation within the variants, and one of the *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates had a lower frequency of seed transmission and disease than the *X. axonopodis* pv. *phaseoli* isolates. These results are consistent with previous reports showing that isolates differed in pathogenicity on the same bean genotype regardless of susceptibility or resistance (Mutlu et al. 2008). In this study, only one susceptible bean genotype and five isolates of each variant were tested. Variation within variants was generally smaller than that between the two variants. Our results also were similar to the previous report of greater pathogenicity of *X. axonopodis* pv. *phaseoli* var. *fuscans* than *X. axonopodis* pv. *phaseoli* (Mutlu et al, 2008). In that study and other previous studies, aggressiveness was compared following plant inoculations (Mutlu et al, 2008), and our results indicate that the greater aggressiveness of *X. axonopodis* pv. *phaseoli* var. *fuscans* also is evident following seed inoculation. This is the first report indicating higher frequency of seed transmission by *X. axonopodis* pv. *phaseoli* var. *fuscans*.

After six months, pathogen survival on seeds varied to a great extent from 10 CFU/seed to 10^3 CFU/seed. It should be mentioned that the linear model that used to calculate population size from Ct value might not be precise enough when population size is around or lower than 10^2 CFU, so this may have contributed to variation in

results after six months in storage. The greatest reduction in population occurred during the first month of storage.

As inoculum level decreased on seeds during storage, the emergence rate increased but was still always lower than for noninoculated seeds. The reduction of population size might also diminish differences between *X. axonopodis* pv. *phaseoli* variants. Increased emergence compared to the initial test also might lead to higher disease incidence because of the emergence of diseased seedlings that may have failed to emerge if they had been planted immediately after inoculation. The disease incidence calculation was based on emerged seedlings.

Estimates of pathogen survival by real-time PCR were higher than those by culture plate counts. This may be due to greater sensitivity of the PCR method; however, the PCR method may overestimate populations due to amplification of DNA from nonviable cells, VBNC (Viable But Non-Culturable) cells or extracellular DNA. The decline in population estimates by PCR over time suggests that some cells died and were no longer detectable; however, the increasing difference between PCR and culture plate estimates over time suggests that some cells, no longer viable in culture, were being detected by PCR. A similar study of differences between plate count and real time PCR data on *Listeria monocytogenes* was carried out and overestimation of bacterial cell counts by real-time PCR was observed in the stationary phase under higher-stress conditions. The report suggested that real-time PCR data should be viewed with caution (Reichert-Schwillinsky et al., 2009). However considering the fast degradation of exposed DNA, damaged cells that failed to grow on semi-selective medium (but may be viable in plant tissue) and the advantage of fast and high throughput detection, real-time PCR should still be considered as a powerful tool to monitor and estimate survived populations.

Real-time PCR used in here in the seed transmission test served as a detection method instead of a quantitative test; however its use as a quantitative method in plant tissue could be developed and applied to estimate severity of infections.

Literature Cited

- Berg, T., L. Tesoriero & D. L. Hailstones (2006) A multiplex real-time PCR assay for detection of *Xanthomonas campestris* from brassicas. *Letters in Applied Microbiology*, 42, 624-630.
- Cafati, C. R. & A. W. Saettler (1980) Transmission of *Xanthomonas-phaseoli* in seed of resistant and susceptible phaseolus genotypes. *Phytopathology*, 70, 638-640.
- Chan, J. & P. H. Goodwin (1999) Differentiation of *Xanthomonas campestris* pv. *phaseoli* from *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* by PFGE and RFLP. *European Journal of Plant Pathology*, 105, 867-878.
- Chavez-L, C. & G. A. Granada (1988) Survival of *Xanthomonas-campestris* pathovar *phaseoli* causal agent of bean common blight in the cauca valley colombia. *Fitopatologia Colombiana*, 12, 9-14.
- Correa, F. M., A. d. O. de Carvalho, M. Gorete & F. do Carmo (2008) Inoculation and survival of *Xanthomonas vesicatoria* on tomato seeds. *Summa Phytopathologica*, 34, 71-75.
- Darrasse, A., C. Bureau, R. Samson, C. E. Morris & M. A. Jacques (2007) Contamination of bean seeds by *Xanthomonas axonopodis* pv. *phaseoli* associated with low bacterial densities in the phyllosphere under field and greenhouse conditions. *European Journal of Plant Pathology*, 119, 203-215.
- Darsonval, A., A. Darrasse, K. Durand, C. Bureau, S. Cesbron & M. A. Jacques (2009) Adhesion and fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp *fuscans*. *Molecular Plant-Microbe Interactions*, 22, 747-757.
- El-Sharkawy, T. A. & D. Huisingh, (1971) Differentiation among *Xanthomonas* species by polyacrylamide gel electrophoresis of soluble proteins. *Journal of General Microbiology*, 68, 155-&.
- Gent, D. H., J. M. Lang, M. E. Bartolo & H. R. Schwartz (2005a) Inoculum sources and survival of *Xanthomonas axonopodis* pv. *allii* in Colorado. *Plant Disease*, 89, 507-514.
- Gent, D. H., J. M. Lang & H. F. Schwartz (2005b) Epiphytic survival of *Xanthomonas axonopodis* pv. *allii* and *X axonopodis* pv. *phaseoli* on leguminous hosts and onion. *Plant Disease*, 89, 558-564.
- Gholve, V. M. & B. P. Kurundkar (2007) Transmission of *Xanthomonas axonopodis* pv. *Malvacearum* through infected seed. *Agricultural Science Digest*, 27, 41-43.
- Goodwin, P. H. & C. R. Sopher (1994) Brown pigmentation of *Xanthomonas-campestris* pv *phaseoli* associated with homogentisic acid. *Canadian Journal of Microbiology*, 40, 28-34.
- Goszczyńska, T. & J. J. Serfontein (1998) Milk-Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. *Journal of Microbiological Methods*, 32, 65-72.

- Groth, D. E. & E. J. Braun (1989) Survival, seed transmission, and epiphytic development of *Xanthomonas campestris* pv *glycines* in the north-central United-States. *Plant Disease*, 73, 326-330.
- He, Y., A. Fessehaie, L. Shepherd & G. Munkvold (2009) A diagnostic real-time PCR assay for the detection and quantification of *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. *Phytopathology*, 99, S34-S34.
- Hildebrand, D. C., N. J. Palleroni & M. N. Schroth (1990) Deoxyribonucleic-acid relatedness of 24 *Xanthomonas* strains representing 23 *Xanthomonas campestris* pathovars and *Xanthomonas fragariae*. *Journal of Applied Bacteriology*, 68, 263-269.
- Khare, U. K. & M. N. Khare (1995) Studies on survival of *Xanthomonas campestris* pv. *glycines*. *Indian Phytopathology*, 48, 180-181.
- Lazo, G. R. & D. W. Gabriel (1987) Conservation of plasmid DNA-sequences and pathovar identification of strains of *Xanthomonas campestris*. *Phytopathology*, 77, 448-453.
- Malavolta, V. A., Jr., M. A. R. de Oliveira & A. R. de Oliveira (2000) Identification and survival of *Xanthomonas translucens* pv. *cerealis* in seeds and crop debris of wheat. *Summa Phytopathologica*, 26, 20-23.
- Marques, A. S. d. A., P. M. Guimaraes, J. P. d. Santos & T. M. Vieira (2005) Survival and viability of *Xanthomonas axonopodis* pv. *phaseoli* associated with bean seeds stored under controlled conditions. *Fitopatologia Brasileira*, 30, 527-531.
- Moyo, M., S. O. Amoo, M. W. Bairu, J. F. Finnie & J. Van Staden (2008) Optimising DNA isolation for medicinal plants. *South African Journal of Botany*, 74, 771-775.
- Munkvold, G. P. (2009) Seed pathology progress in academia and industry. *Annual Review of Phytopathology*, 47, 285-311.
- Mutlu, N., A. K. Vidaver, D. P. Coyne, J. R. Steadman, P. A. Lambrecht & J. Reiser (2008) Differential pathogenicity of *Xanthomonas campestris* pv. *phaseoli* and *X. fuscans* subsp *fuscans* strains on bean genotypes with common blight resistance. *Plant Disease*, 92, 546-554.
- Mutlu, N., A. K. Vidaver, D. P. Coyne, J. R. Steadman, J. Reiser & P. A. Lambrecht (2004) Identification of races of *Xanthomonas campestris* pv. *phaseoli* and establishment of the first common bacterial blight differential *Phaseolus vulgaris* lines. *Phytopathology*, 94, S73-S73.
- Ohata, K. I., S. Serizawa, K. Azegami & A. Shirata (1982) Possibility of seed transmission of *Xanthomonas campestris*-var-*vitians* the pathogen of bacterial spot of lettuce. *Bulletin of the National Institute of Agricultural Sciences Series C (Plant Pathology and Entomology)*, 81-88.
- Opio, A. F., J. M. Teri & D. J. Allen (1993) Studies on seed transmission of *Xanthomonas campestris* pv. *phaseoli* in common beans in Uganda. *African Crop Science Journal*, 1, 59-67.
- Pasquali, M., L. Marena, E. Fiora, P. Piatti, M. L. Gullino & A. Garibaldi (2004) Real-time polymerase chain reaction for identification of a highly pathogenic group of *Fusarium oxysporum* f.sp. *chrysanthemi* on *argyranthemum frutescens* L. *Journal of Plant Pathology*, 86, 53-59.
- Reichert-Schwillinsky, F., C. Pin, M. Dzieciol, M. Wagner & I. Hein (2009) Stress- and growth rate-related differences between plate count and real-time PCR data

- during growth of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 75, 2132-2138.
- Roberts, S. J., L. H. Hiltunen, P. J. Hunter & J. Brough (1999) Transmission from seed to seedling and secondary spread of *Xanthomonas campestris* pv. *campestris* in Brassica transplants: Effects of dose and watering regime. *European Journal of Plant Pathology*, 105, 879-889.
- Sharma, M., K. Agrawal & T. Singh (2002) Incidence and seed transmission of *Xanthomonas campestris* pv. *cajani* in pigeonpea. *Journal of Mycology and Plant Pathology*, 32, 1-5.
- Tebaldi, N. D., R. d. C. Panizzi & R. Sader (2007) Detection, transmission and effect of *Xanthomonas campestris* pv. *campestris* in the physiological quality of broccoli seeds. *Summa Phytopathologica*, 33, 290-293.
- Theodoro, G. d. F. & A. C. Maringoni (2002) Survival of *Xanthomonas axonopodis* pv. *manihotis* in manipueira under controlled conditions (Sobrevivencia de *Xanthomonas axonopodis* pv. *manihotis* em manipueira sob condicoes controladas.). *Summa Phytopathologica*, 28, 276-279.
- Torres, J. P. & A. C. Maringoni (1997) Reaction of dry bean of *Xanthomonas campestris* pv. *phaseoli* and seed transmission of the bacterium. *Fitopatologia Brasileira*, 22, 546-549.
- Torres, J. P., A. C. Maringoni & T. A. F. Silva (2009) Survival of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* in common bean leaflets on soil. *Journal of Plant Pathology*, 91, 195-198.
- Valarini, P. J., J. A. H. Galvao & D. D. A. Oliveira (1996) *Xanthomonas campestris* pv. *phaseoli*: Importance of seed inoculum in the epidemiology of the common bacterial blight of beans. *Fitopatologia Brasileira*, 21, 261-267.
- Valarini, P. J. & J. O. M. Menten (1991) Artificial inoculation of dry bean seeds with *Xanthomonas-campestris* pv. *phaseoli* and its effect on sanitary quality and the germination. *Summa Phytopathologica*, 17, 227-231.
- Valarini, P. J., J. O. M. Menten & M. A. Lollato (1992) Incidence of the common bacterial blight in the field and transport of *Xanthomonas campestris* pv. *phaseoli* through bean seeds, obtained by different methods. *Summa Phytopathologica*, 18, 160-166.
- Zaumeyer, W. J. & H. R. Thomas (1957) A monographic study of bean diseases and methods for their control. *Technical Bulletin. United States Department of Agriculture*, 868, 255 pp.

Table 1. Bacterial isolates used for inoculation of *Phaseolous vulgaris* seeds to compare seed to seedling transmission and survival on stored seed.

Isolates	Host	Origin
<i>X. axonopodis</i> pv. <i>phaseoli</i>		
Michigan 3 Bean *	Bean	MI, USA, 1997, 'K93623'
B705 *	Bean	Brazil
9807 *	Bean	Scottsbluff, NE, USA, 1998
269.3-1 [✱]	Bean	France
Xcp 25 ^Δ	Bean	WI, USA, 2005, 'Andean'
<i>X. axonopodis</i> pv. <i>phaseoli</i> var. <i>fuscans</i>		
0794 *	Bean	Australia(Kingary), 1978
Xpfs *	Bean	NE, USA, 1975
94:202†	Bean	Netherlands, 'PV539'
1158a NCPPB *	Bean	UK
Xpf#5 ^Δ	Bean	Puerto Rico, 'Middle American'

* contributed by Dr. Anne Vidaver, Univ. NE.; [✱] contributed by Valerie Grimault,

GEVES/SNES, France; † contributed by Elizabeth Vavricka, ID Dept. of Agriculture;

^Δ contributed by Dr. Robert Gilbertson, Univ. CA, Davis.

Figure 1. Emergence of *Phaseolus vulgaris* seedlings after seed inoculation with *X. axonopodis* pv. *phaseoli* (five isolates on the left) and *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates (five isolates on the right) and noninoculated control seeds. Values are means of three replications of 25 seeds per isolate.

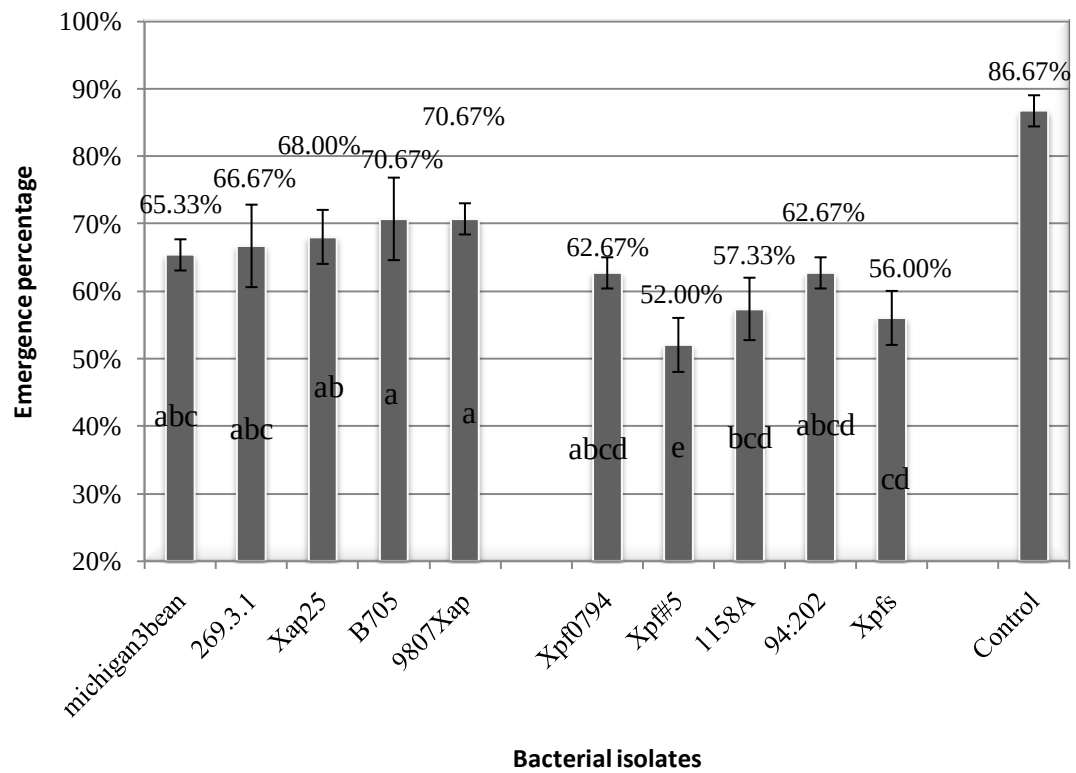


Figure 2. Incidence of common bacterial blight symptoms in *Phaseolus vulgaris* seedlings grown from seeds inoculated with isolates of *X. axonopodis* pv. *phaseoli* (five isolates on the left) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (five isolates on the right). Values are means of three replications, calculated as the percentage of emerged seedlings. No symptoms occurred in seedlings from noninoculated control seeds.

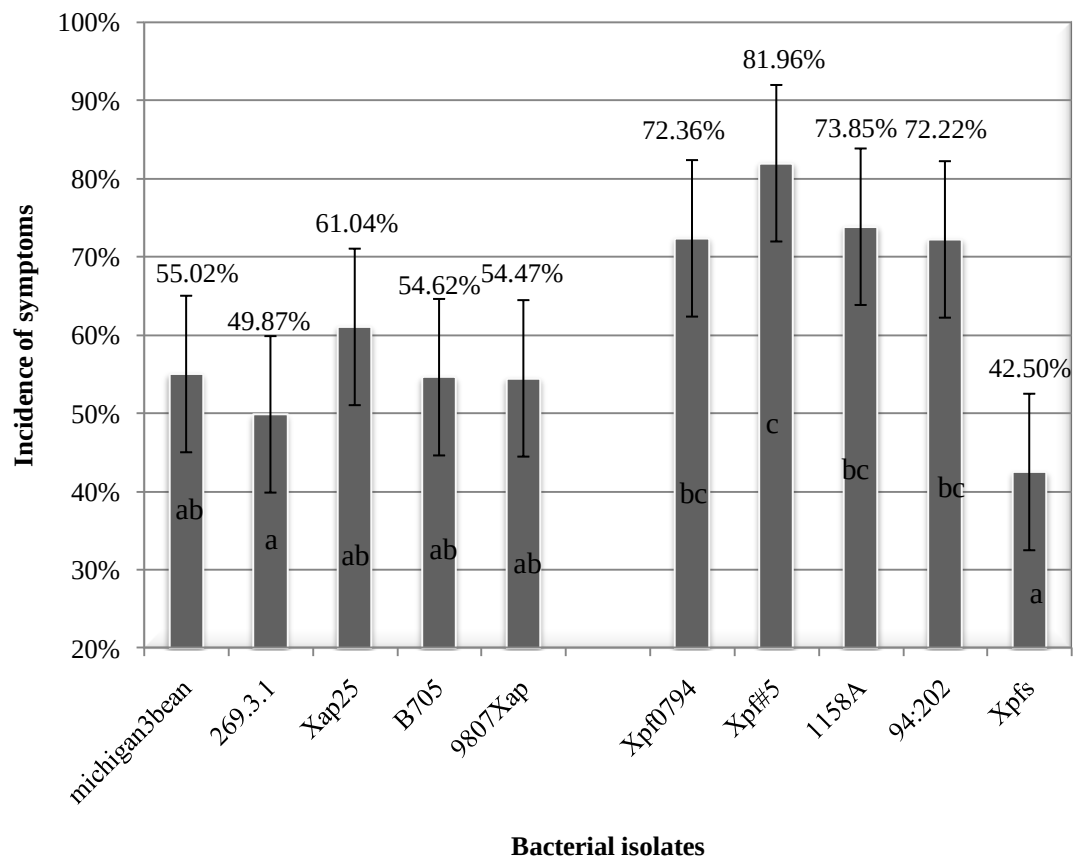


Figure 3. Incidence of infection by *X. axonopodis* pv. *phaseoli* (five isolates on the left) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (five isolates on the right) in *Phaseolus vulgaris* seedlings grown from inoculated seeds, according to real-time PCR. Values are means of three replications, calculated as the percentage of emerged seedlings. Pathogens were not detected in seedlings from noninoculated control seeds.

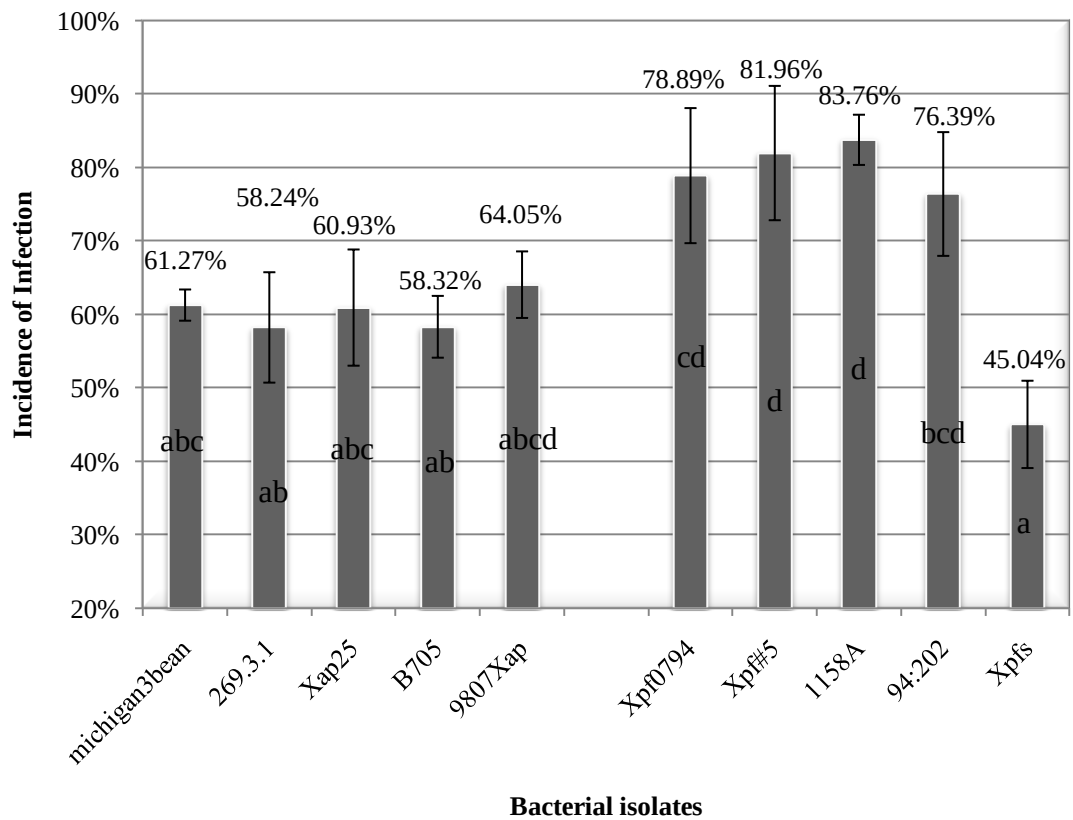
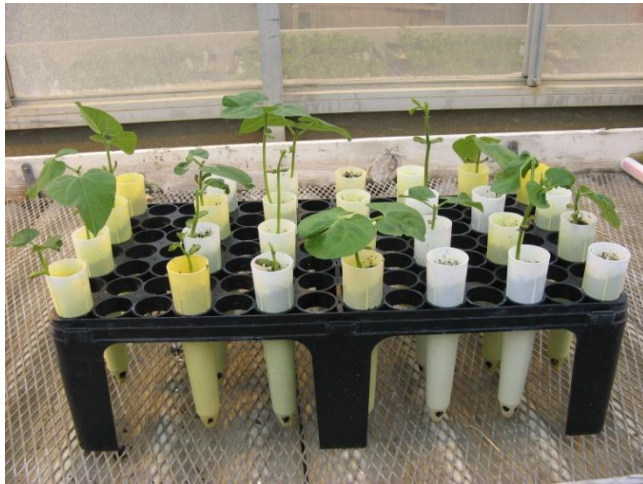


Figure 4. (A) Seedlings from *Phaseolus vulgaris* seeds inoculated with isolates of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* in cones seven days after planting; (B) Seedlings with bacterial blight symptoms; (C) germination failure; (D) leaf damage; (E) primary leaf development failure.



(A)



(B)

Figure 4. (continued)



(C)

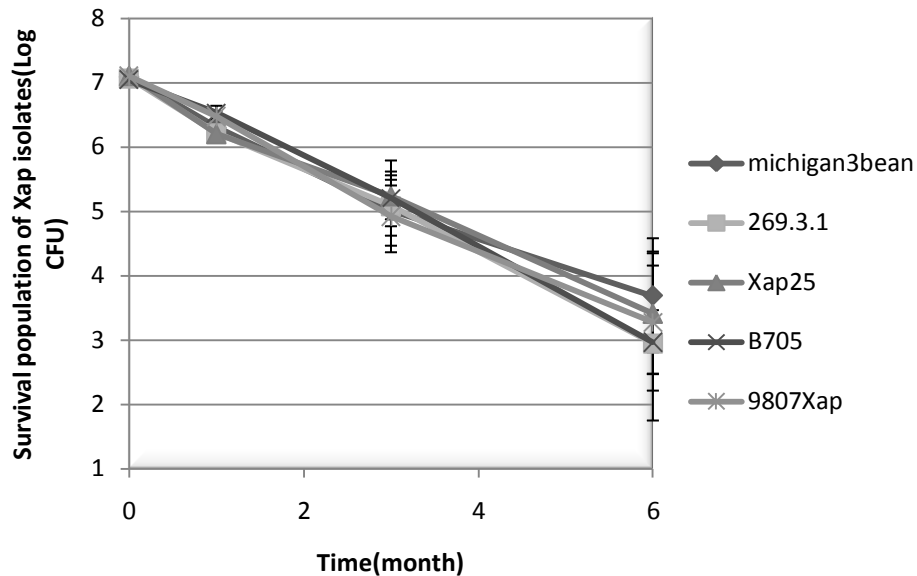


(D)

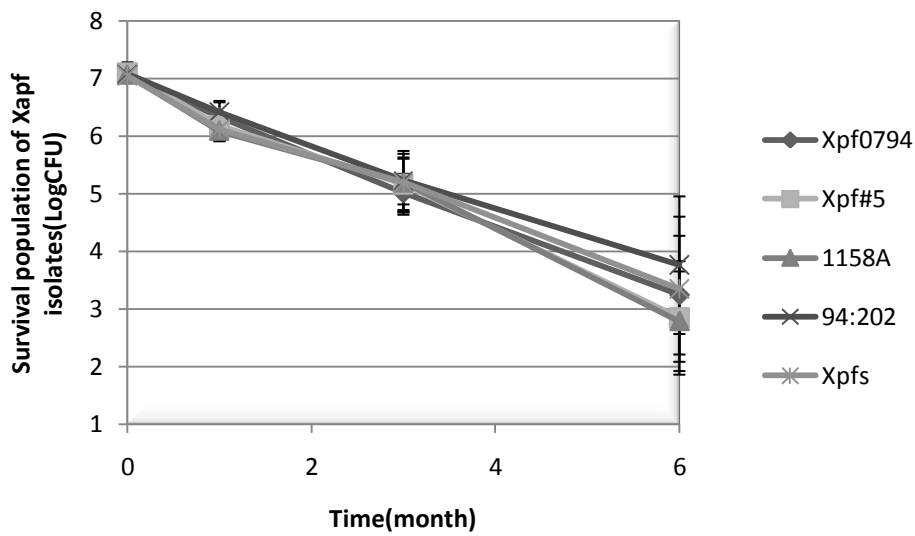


(E)

Figure 5. Estimates of bacterial population size (survival) over time in inoculated, stored seeds of *Phaseolus vulgaris*, based on real-time PCR: (A) *X. axonopodis* pv. *phaseoli* ; (B) *X. axonopodis* pv. *phaseoli* var. *fuscans*. Values are means of 10 replications of 5 seeds each isolate each time point.

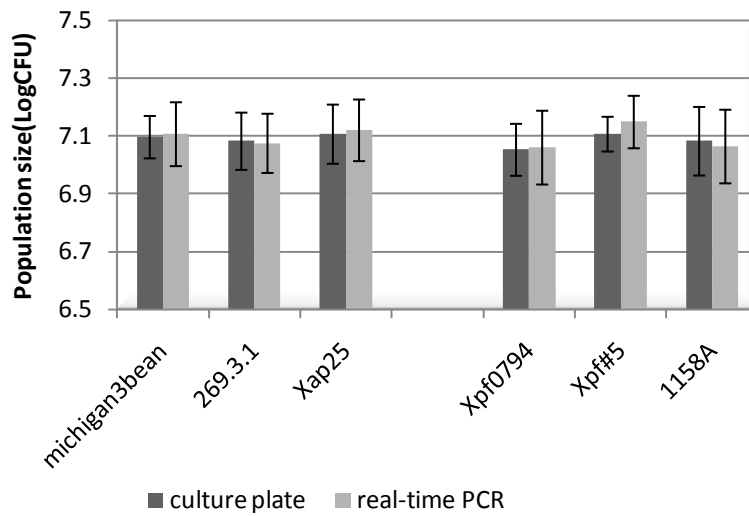


(A)

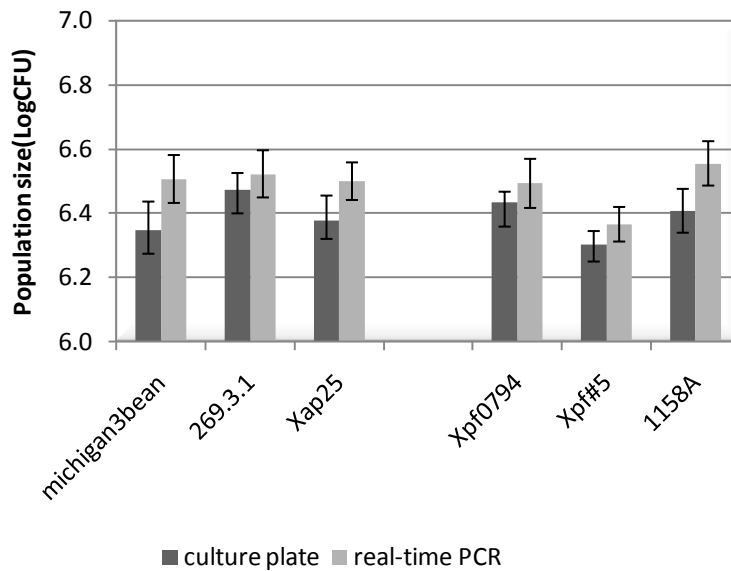


(B)

Figure 6. Estimates of *X. axonopodis* pv. *phaseoli* population size on stored *Phaseolus vulgaris* seeds, according to colony counts on artificial culture medium and real-time PCR; (A) initial populations immediately after inoculation; (B) after one month in storage; (C) after three months in storage. Values are based on 6 replications of 5 seeds for each isolate at each time point.

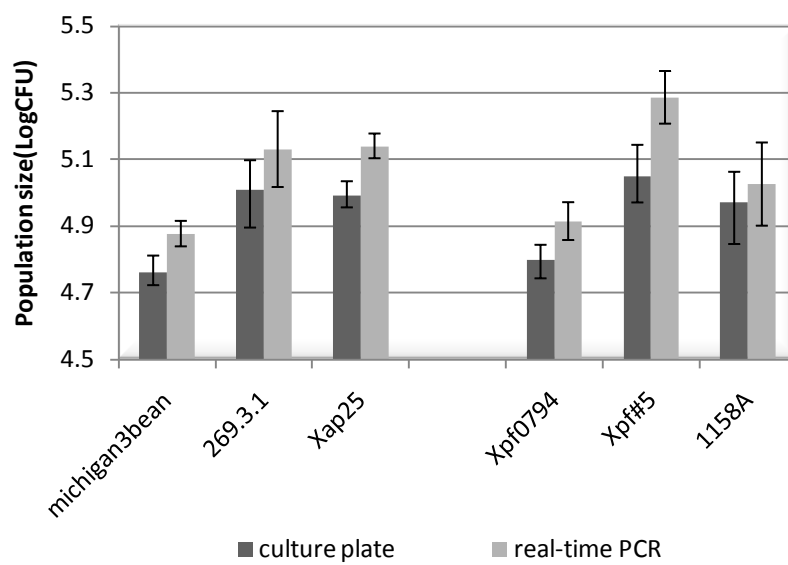


(A)



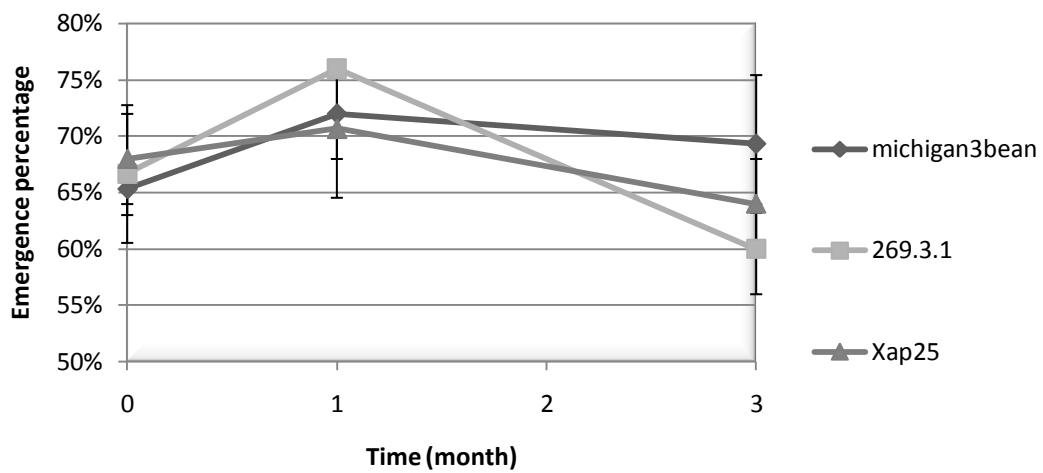
(B)

Figure 6. (Continued)

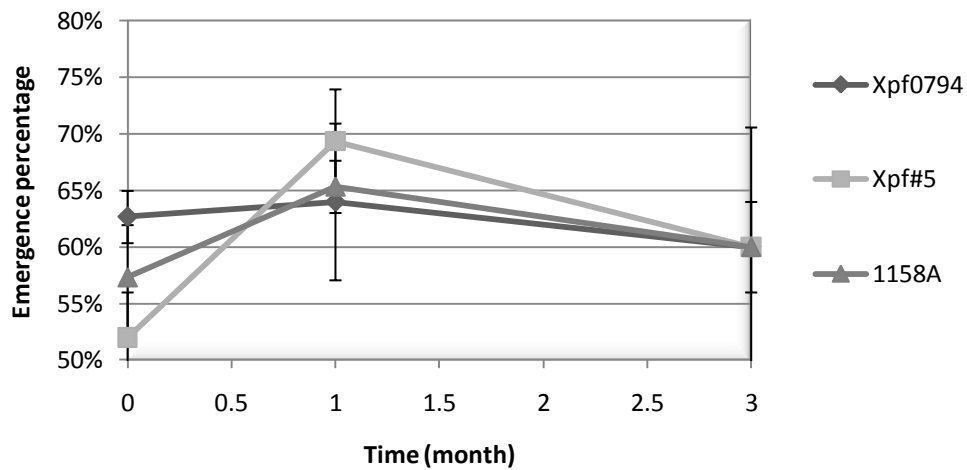


(C)

Figure 7. Emergence percentage of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* inoculated seeds over time. (A) seeds inoculated with *X. axonopodis* pv. *phaseoli* isolates; (B) seeds inoculated with *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates. Values are based on 3 replications of 25 seeds for each isolates and each time point.

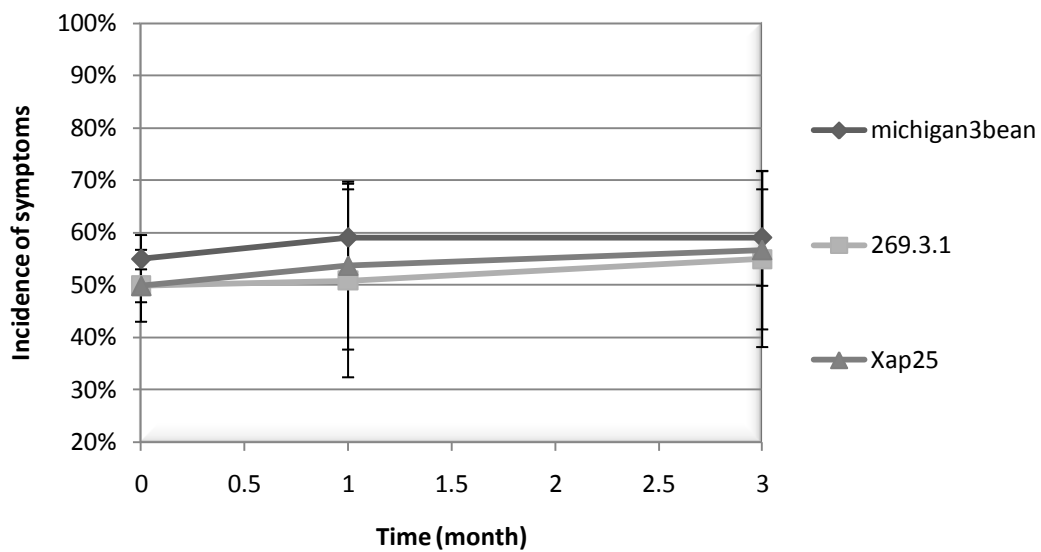


(A)

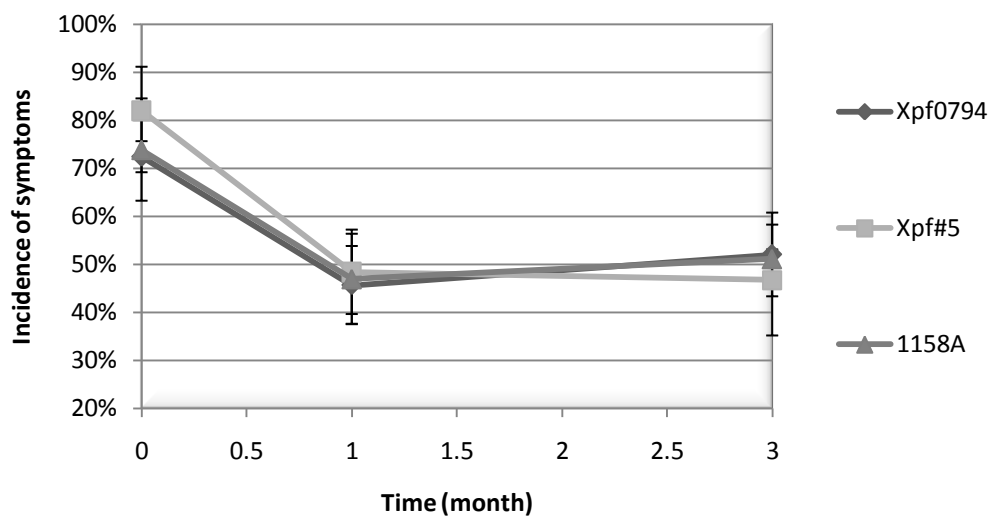


(B)

Figure 8. Incidence of common blight symptoms in seedlings grown from seeds inoculated with *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* and planted immediately or after one or three months in storage: (A) seeds inoculated with *X. axonopodis* pv. *phaseoli* isolates; (B) seeds inoculated with *X. axonopodis* pv. *phaseoli* var. *fuscans* isolates. Values are based on 3 replications of 25 seeds for each isolates and each time point.



(A)



(B)

CHAPTER 5. GENERAL CONCLUSIONS

The extraction methods evaluated in this research aimed at increasing pathogen extraction and detection sensitivity. Methods were chosen according to recommendations of a NSHS review and limited by available lab equipment and facilities. Results indicated that improvements can be made to increase the sensitivity of currently used culture plate assays for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*.

The real-time PCR method described here should be evaluated as a standard seed health test for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Other advances could be made to remedy some disadvantages of real-time PCR. Recently, IMS (immunomagnetic separation) has been used before PCR to capture target bacteria in detecting cucurbits seed pathogen *Acidovorax avenae* subsp. *citrulli* on a commercial-scale and showed a great potential as an effective enrichment step in pathogen extraction (Walcott, 2000). BIO-PCR has also been developed to avoid PCR inhibitors and ensure the presence of viable pathogen propagules on other phytopathogen diagnosis (Weller et al. 2000). These methods could be developed specifically for *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* extraction from common bean seeds and may provide a promising improvement on standard seed health test.

Besides designing primers according to RAPD PCR, primers could also be designed according to the DNA sequences of a protein which was unique in the target bacteria and consistent present in this *phaseoli* pathovar. However, these genes were normally present as a single copy per cell, while 16S rRNA as multiple copies in cell are always used (Patrik and Maiss 2000). The disadvantage of PCR primers from the 16S rRNA is that they have not been found to be highly specific (Seal et al. 1993);

Therefore, internal transcribed spacer (ITS) regions between the 16S and 23S ribosomal genes seem to be more promising as a target to design primers (Gurtler and Barrie 1995).

Suppression subtractive hybridization was performed to isolate DNA fragments present in these bean pathogens and absent from closely related *Xanthomonads*. Some virulence gene candidates were identified, such as homologs of hemagglutinins, TonB-dependent receptors, zinc-dependent metalloproteases, type III effectors, type IV secretion system components and unexpectedly homologs of the type III secretion apparatus components (SPI-1 family) (Alavi et al. 2008). These could also contribute to further research of pathogenic mechanism and resistance cultivar breeding. Moreover, these would help improve diagnostic tools to be able to distinguish between pathogenic and non-pathogenic isolates.

Difference in pathogenicity mechanisms of two variants could be subject to further research as they showed different transmission percentage in seed transmission and different impact in reducing emergence. The two variants were two genetically distinct groups and reported to harbor different pathogenicity on both resistant and susceptible bean genotypes (Mutlu et al 2008). Understanding of genes that contribute to pathogenicity and how they were acquired in the pathogen from the environment were important and helpful.

Literature Cited

- Alavi, S. M., S. Sanjari, F. Durand, C. Brin, C. Manceau & S. Poussier (2008) Assessment of the genetic diversity of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp *fuscans* as a basis to identify putative pathogenicity genes and a type III secretion system of the SPI-1 family by multiple suppression subtractive hybridizations. *Applied and Environmental Microbiology*, 74, 3295-3301.
- Gurtler, V. & H. D. Barrie (1995) Typing of *Staphylococcus-aureus* strains by PCR-amplification of variable-length 16s-23s rDNA spacer regions - characterization of spacer sequences. *Microbiology-Uk*, 141, 1255-1265.

- Mutlu, N., A. K. Vidaver, D. P. Coyne, J. R. Steadman, P. A. Lambrecht & J. Reiser (2008) Differential pathogenicity of *Xanthomonas campestris* pv. *phaseoli* and *X. fuscans* subsp *fuscans* strains on bean genotypes with common blight resistance. *Plant Disease*, 92, 546-554.
- Pastrik, K. H. & E. Maiss (2000) Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. *Journal of Phytopathology-Phytopathologische Zeitschrift*, 148, 619-626.
- Seal, S. E., L. A. Jackson, J. P. W. Young & M. J. Daniels (1993) Differentiation of *Pseudomonas-solanacearum*, *Pseudomonas-syzygii*, *Pseudomonas-pickettii* and the blood-disease bacterium by partial 16s-ribosomal-RNA sequencing - construction of oligonucleotide primers for sensitive detection by polymerase chain-reaction. *Journal of General Microbiology*, 139, 1587-1594.
- Walcott, R. R. & R. D. Gitaitis (2000) Detection of *Acidovorax avenae* subsp *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. *Plant Disease*, 84, 470-474.
- Weller, S. A., J. G. Elphinstone, N. C. Smith & D. E. Stead (2000) Detection of *Ralstonia solanacearum* from potato tissue by post-enrichment TaqMan PCR. *Bulletin OEPP*, 30, 381-383.

ACKNOWLEDGEMENTS

I would like to give my special thank to Dr. Gary Munkvold for guidance and help during these years. I am so grateful for being working with him in the lab and it is really an unforgettable experience in my whole life. His patience and wisdom inspire me for the ideas and help me to conquer the difficulties I was facing. His advices will accompany with me in the next stage of my career life.

I also want to thank Dr. Adam Bogdanove, Dr. Byron Stehm-Stecher and Dr. Anania Fessehaie for being my committee members. They also gave me many valuable advices and contributed tremendously to this paper.

I would like to give my acknowledgement to Lisa Shepherd who helped establish this project and those who contributed either isolates or seeds for this study: Dr. Adam Bogdanove, Dr. Anania Fessehaie, Dr. Ed Braun, Dr. Robert Gilbertson, Dr. Rob Duncan, Dr. Anne Vidaver, Liz Vavricka, and Valerie Grimault shared their bacterial isolates with us for this study. Laurel Carter from Seminis Vegetable Seeds, Elisabetta Vivoda from Harris-Moran Vegetable Seeds, Aaron Whaley from Seed Savers Exchange, Howard Schwartz & Mark Brick from CO State Univ., Robert Rand from Univ. of WI contributed naturally infested seeds to this study. Lu Liu from Dept. of Statistics helped with statistical analysis of datas.

Members of Dr. Munkvold's lab make me feel like I am in a warm family. They are Michele Beattie, Erin Bilsten, Jose Pablo Soto Arias, Maria Mercedes Diaz Arias, Marcos Da Silva, Tracy Bruns, and all the undergraduates. We help each other in the lab and our daily life and all of us become truly good friends.

In addition, I would like thank my many friends and family members, who provided a great deal of love and support during a very stressful time.

This research was funded and supported by ASTA Vegetable Seed Research Committee.