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QUANTIFICATION AND TRANSMISSION OF SEED-BORNE

XANTHOMONAS CAMPESTRIS PV. PHASEOLI

presented by

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QUANTIFICATION AND TRANSMISSION OF SEED-BORNE *XANTHOMONAS CAMPESTRIS* PV. *PHASEOLI*

By

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ABSTRACT

QUANTIFICATION AND TRANSMISSION OF SEED-BORNE *XANTHOMONAS CAMPESTRIS* PV. *PHASEOLI*

By

Lee Alan Rossmaessler

Common blight of beans, caused by *Xanthomonas campestris* pv. *phaseoli* (E.F. Smith) Dawson (XCP) and the brown pigment-producing fuscans variant, is a disease of major importance in production of dry beans in Michigan and on a world wide basis. Seed-borne XCP appears to be the most important source of primary inoculum in Michigan.

Experiments were conducted to develop a protocol to measure the quality (externally infested vs. internally infected) and quantity of bacteria associated with naturally contaminated seed. Recovery of XCP on a semi-selective medium (mSSM) from naturally infected seed was compared using various regimes of: seed grinding, surface disinfestation, and suspending conditions (including type of suspending solution, length of shaking time, and shaking

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temperature). The protocol utilized to quantify external XCP consisted of suspending seeds in aqueous suspension, shaking, and plating a sample on mSSM agar plates. Total number of colony forming units (cfu) of XCP per seed sample was determined by grinding the seed to a flour before suspension. The numbers of internal cfu of XCP were estimated by subtraction.

Numbers of XCP cfu recovered from seed samples taken from naturally infected seedlots was extremely variable. Some trends were apparent from the 1,761 individual seeds removed from various naturally infected seedlots and assayed for XCP: 1) No pathogen cells were recovered from most seeds. 2) Most of the seeds that harbored XCP bacteria appeared symptomless and contained relatively small numbers of the pathogen. 3) Most of the XCP cells in an infected seedlot were associated with a relatively small number of seeds, which usually had visual symptoms. 4) Most of the XCP bacteria, even in symptomless seeds, were borne internally.

Pathogen-free seed were artificially infected with XCP by vacuum infiltration followed by surface disinfestation. Seeds containing concentrations of XCP per seed similar to those concentrations recovered from naturally infected seed, were planted in two commercial fields located in two Michigan counties. It was determined that seeds containing fewer than 10^2 cfu of XCP per seed may produce infected plants and infected seed.

Dedicated to my merry Mary and T-n-T

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GENERAL INTRODUCTION AND LITERATURE REVIEW

Disease Description

Common blight of beans, caused by *Xanthomonas campestris* pv. *phaseoli* (E.F. Smith) Dawson (XCP) and the brown pigment-producing fuscans variant, is a bacterial disease of major importance in production of dry beans in Michigan and world-wide (77, 93, 128). The symptoms caused by the two bacterial types are indistinguishible in the field. However the causal agents may be easily distinguished in culture on the basis of pigment production; both produce a non-diffusible yellow pigment while only the fuscans variant produces a brown diffusible pigment in media containing tyrosine (27, 117). Although common blight was first described by Beach in 1892 (8), the disease caused by the fuscans variant was not described until 1930 (12). The causal organisms were described by E.F. Smith in 1897 and Burkholder in 1930 respectively (12, 95). Both variants are considered as XCP in this thesis.

Yield losses due to common blight have been reported at 35% in Colorado in 1919, 10 - 20% in Michigan in 1967, 38% in Ontario in the early 1970's, and 13 - 45% in Colombia in the mid-1970's (128, 130). Every year a high percentage of

bean seed samples submitted to the Michigan Department of Agriculture for blight testing are rejected, although these rejected samples may also be positive for halo blight of beans (23, personal communication from Steve McQuire, Director, Seed Laboratory, Michigan Department of Agriculture).

Symptomatology and plant infection have been amply documented by various workers (12, 128, 129, 130, 131, 132). Under natural conditions leaf symptoms first appear as water-soaked spots on the undersurface of leaves, which enlarge irregularly and coalesce. Affected regions of the leaf appear flaccid and are surrounded by a zone of lemon-yellow tissue, which later turns necrotic. The bacteria enter leaves through natural openings or wounds, where they may invade the intercellular spaces causing a gradual dissolution of the middle lamella. They may enter the stem through stomata and reach the vascular elements. They may also reach the vascular elements from infected leaves or cotyledons. If enough bacteria reach the xylem tissue, wilting may be caused by the plugging of vessels or disintegration of the cell walls. Pod lesions appear as water-soaked spots which enlarge slowly and become dark, red and sunken. If infection of the pod occurs early during pod and seed development, infected seed may shrivel or rot. XCP bacteria may occur within the seed (infected seed) or on the external surface of the seed (infested seed). Naturally

infected or contaminated seed, refers to seed that is both infested and infected.

The bactreria may enter the pod sutures from the vascular system of the pedicel and then travel through the raphe and into the funiculus. Once there, they remain under the seed coat until germination. Seed imbibiton results in a rupture of epidermal cells of the cotyledons, at which time the the pathogen invades the epidermal rifts and multiply in the intercellular spaces (131). Direct penetration by the bacteria through the seed coat has not been reported.

Seed infection is sometimes visible in white colored seeds. The symptoms may range from a darkening in the hilum region to complete butter-yellow discolorattion and shrivelling of the seed (120). Field symptoms of XCP in Michigan typically are not noticed until the plants are in bloom. This occurs because of the "umbrella effect" *viz.* by the time symptoms appear, the younger leaves have expanded and hide the older leaves (122).

Blight outbreaks are favored by warm humid weather (100, 120). Secondary spread of the bacteria may be influenced by; rain accompanied by wind (132), wind-blown soils (17), poor cultural practices (3), and non-host plants as well as susceptible and tolerant bean genotypes carrying inoculum (13, 91).

Disease Control Strategies

Strategies to control common blight on beans include, chemical control in the field, genetic control using resistant and tolerant cultivars, cultivation practices, and control by using pathogen-free seed etiher by eradicative treatment or certification schemes.

Chemical control in the field

Various chemical controls that have been found effective include: sprays of 50% copper hydroxide and 40% potassium (hydroxymethyl) methyl dithiocarbamate, Bordeaux mixture, 5% puratized spray and cuprox dust (copper oxychloride), and sprays of 1000 ppm streptomycin sulfate (10, 25, 121). However, Marlatt (64), found that streptomycin failed to control common blight when used as a spray. The following year Gray (35) demonstrated that the addition of a wetting agent, *i.e.*, glycerol, greatly increased the effectiveness of streptomycin sprays in controlling common blight in greenhouse plants. Generally, chemical field treatments for the control of the disease have not proven to be sufficiently effective and economical enough to be commercially useful.

Genetic control

Long term hope for controlling bean blights rests in the development of resistant and tolerant commercial cultivars of *Phaseolus* spp. (27). Some such cultivars do exist, however, their wide spread use is by no means assured, especially with reports that current tolerant and resistant cultivars support the epiphytic growth of the bacteria on plant parts and may produce infected seed (15, 16, 61, 91).

Control by cultural practices

Cultural practices such as remaining out of bean fields when plants are wet, and proper sanitation of farm machinery, are helpful in minimizing the spread of the disease. Crop rotation, weed control, and plowing bean refuse under after harvest are recommended practices (3, 42).

Control by planting pathogen-free seed

Planting pathogen-free seed is widely recognized as being of fundamental importance in the control of many seed-borne diseases (6, 70). Tillet (73), in 1755, was the first to prove the seed transmission of a plant pathogen, *i.e.*,

bunt of wheat. XCP was the first bacterial pathogen shown to be seed-borne (8, 73).

Eradicative seed treatments

Treatments to eliminate the pathogen from seed have historically been useful for many crops (2, 65, 70). In 1733, Jethro Tull was the first to observe that wheat seed that was salvaged from sea water was free from bunt (73). A slurry treatment containing streptomycin sulfate is routinely used on many Michigan seedlots (77). It is generally thought that this slurry treatment is helpful against external bacterial pathogens.

The most widely method used to surface disinfest seeds is submersion in 1-5% NaOCl followed by rinsing with sterile water (1, 79). However, Grogan and Kimble (38) found that by immersing infested bean seed for 15 minutes in 0.525% NaOCl did not effectively eradicate the external pathogen. The failure of immersion in NaOCl to adequately surface disinfest the seed was attributed to the bacteria being located in small rifts and splits in the seed coat. Apparently disinfestation was not successful because of lack of contact of the the disinfectant with the bacteria. Saurer and Burroughs found that rinsing seed in 95% ethanol, followed by immersion in NaOCl containing Tween 20, promoted contact of surface-borne pathogens with the disinfectant (79). However, it has been demonstrated (1) that even after rinsing seeds 8 times with sterile water, biologically active amounts of NaOCl remain.

Seed treatment that completely eradicates internal blight bacteria, without significantly affecting seedling emergence, is not available. Still there are many seed treatments that are helpful. Dihydrostreptomycin has been shown to act similarly to streptomycin sulfate (68, 69). Kreitlow (46) found the following four solutions effective in controlling bean blights: 1) 1:500 mercury bichloride in di-ethyl ether 2) 1:20,000 brilliant green in 50% ethyl alcohol plus 3% acetic acid 3) 1:500 mercury bichloride in 70% ethyl alchol plus 3% acetic acid 4) 1:20,000 gentian violet in 50% ethyl alcohol plus 3% acetic acid. After 25 years of seed treatment studies, Person and Edgerton (74) found that seeds treated for 12 - 14 minutes in a solution of 1:500 $HgCl_2$ in 70% ethyl alcohol plus 2% acetic acid provided the best eradicative treatment for blight. Treatment of seed with dry heat at 80C for 35 minutes followed by New Improved Ceresan for 24 hours was helpful (10). Adimihardja (2) recommended two different treatments based on the age of the seed: for seed less than one year old, 800 ppm tetracycline in methanol under partial vacuum for two minutes; for older seed, immersion in 800 ppm of tetracycline HCl and aureomycin in methanol for 30 minutes.

Seed Certification

When considering the use of pathogen-free seed in a certification scheme, two possible situations must be examined: 1) seed-borne diseases which are also soil- or

plant residue-borne, and 2) those diseases that are exclusively seed-borne.

Seed-transmitted diseases which are also soil-borne or plant residue-borne are affected by any reduction of the seed-borne inoculum in proportion to its relative importance in the cycle of the disease. Examples of such diseases are those caused by some species of *Fusarium*, *Septoria*, and *Cercospora* (70). Fernandez demonstrated that the tolerable percentage of seeds transmitting bean anthracnose in a seedlot is higher when the primary inoculum originating from crop residues of the previous season is high. This is especially important for tropical regions where beans are often planted twice a year, because the residual inoculum (which is greater after the rainy season crop) tends to mask the influence of the seed-borne primary inoculum (29).

Diseases exclusively seed-borne can be controlled or even eliminated by the use of healthy seed stock produced through appropriate certification schemes (37, 70).

Disease control in Michigan

Bacterial plant pathogens have been demonstrated to survive in various ways: in plant residues, associated with perennial hosts, as epiphytes on nonhosts, in the soil, or in association with insects (53, 54, 88, 89). However, in the case of XCP in Michigan, none of those survival strategies are the major source of primary inoculum. XCP

associated with the seeds is the major source of primary inoculum (122).

The inability of XCP to survive in plant residues to any significant degree has been demonstrated in New York (12), Ontario (116) and Australia (127). Saettler and co-workers have provided convincing evidence that the primary inoculum of common blight in Michigan is contaminated seed (78). Several attempts were made to recover 20 isolates of XCP from ten different bean genotypes ranging in susceptibility from resistant to susceptible over a 10-year period. Infected bean plant samples were either left standing, or were buried in the soil or were left on the soil surface at several sites in Michigan over several different winters. Bean blight bacteria were never recovered in any of the 191 trials. XCP has, however, been shown to survive from one growing season to the next in the field in some regions with less severe winters (87, 104).

The lack of the ability of foliar pathogens to survive long periods in the absence of a living host is often explained by what Garrett would call a low "competitive saprophytic ability" (31), *i.e.*, the lack of physiological attributes that enable successful colonization of dead organic substances (36, 43, 54). Also, reduced pathogenicity and lowering of numbers of recoverable antibiotic-resistant XCP has been demonstrated when infested leaves are repeatedly exposed to freezing and thawing (59), though no explanation as to why this occurred was provided.

The principle of controlling bean blight through the use of pathogen-free seed has been recognized and applied in many production areas of the United States and Canada (23, 92, 93). The production of certified bean seed in Michigan involves a three-step process (23, 60). 1) Newly developed breeder seed is provided to the Michigan Foundation Seed Association (MFSA). 2) The MFSA produces and maintains foundation seed by growing it in furrow-irrigated regions of western states. These climatic regions, along with the cultural practices employed, do not, theoretically, encourage the development of bean blights. 3) Certified seed is produced in Michigan from foundation seed. The certified seed-fields are visually inspected for blight symptoms, and seed samples are submitted to the Michigan Department of Agriculture (MDA) for a laboratory test for the presence of internal blight contamination (21). The MDA test consists of: a) surface sterilization of approximately 2.2 kg of seed for 10 minutes in 2.6% NaOCl; b) rinsing with sterile water; c) incubation of seed for 18-24 hours in sterile distilled water (SDW) containing approximately 10 g yeast extract / liter d) injection of the incubated suspension into the primary leaf nodes of at least 3 plants of a susceptible cultivar; e) visual inspection of the injected plants for symptoms up to four weeks after injection (21). Seeds harvested from fields which show no visual symptoms and which pass the MDA laboratory test may be sold as certified seed.

Often during the visual inspection symptoms are noted, but only in isolated areas of a field. The seed from the rest of the field may be still be certified if it passes the MDA laboratory test. In this case, the grower may not harvest the blighted and non-blighted areas at the same time. Theoretically, this prevents the mixing of clean and contaminated seed and the infesting of seed from contaminated threshing equipment (23).

Requisites of Successful Seed Certification

Despite the various recommended control practices, bean common blight remains a serious problem in Michigan (personal communication, Steve McQuire of the MDA). This may be attributable to a number of factors. Perhaps foremost is the lack of universal use of recommended control practices by Michigan bean growers. In a 1981 survey, only 56 out of 131 seed samples planted (42.7%) were certified seed, and 8.3% of the samples were known to have failed the MDA test.(22).

The process utilized in labeling seed as certified may itself be flawed. Visual inspection of fields for symptoms is not reliable especially since such inspections occur after defoliation when cool moist conditions frequently encourage pod and stem molding (61, 122). For example, the MDA inoculation test has caused rejection of as high as 25% of fields passing the visual inspection (23). The MDA tests

only for the presence of internal bacteria with the assumption that external bacteria will be eradicated by a streptomycin slurry treatment. Another important factor easily overlooked is the possibility for human error. Non-MCIA growers submit their own seed to the MDA for testing. The sample submitted may not be a random sample, or the sample may be smaller than that normally used by the MDA.

An additional problem with the inoculation technique used by the MDA is that the number of XCP cells necessary to result in a positive test may be higher than the number of XCP cells necessary to result in disease via seed transmission. The number of cells necessary for seed transmission is unknown, but should that number be lower than can be detected by the MDA, then there is a need for a more sensitive technique. Trujillo (108) developed a technique which involved using a liquid semi-selective medium to incubate seeds, followed by a Ouchterlony double diffusion test. Of 99 seed samples tested, 37 were positive for XCP with this technique while only 25 were positive with the MDA seedling injection technique. The detection limit was calculated to be 400 cells/ml at the beginning of incubation.

There are numerous recent reviews of the development of more sensitve techniques for detecting plant pathogenic bacteria (7, 28, 39, 44, 67, 70, 76, 80, 81, 88). Several detection techniques have been adapted for use in detecting XCP in bean seed. These include, enrichment techniques such

as a grow-out or dome test (112, 114); utilization of bacteriophage (26, 99); polyclonal antibody techniques such as enzyme-linked immunosorbent assay (ELISA) (63, 111), various immunofluorescent (IF) staining procedures (62, 110, 111), radioimmunoassay (RIA) and dot-immunobinding assay (DIA) (63); molecular hybridization using a DNA probe (33); and semi-selective media (18, 24, 61). There is a real need to standardize and compare the numerous techniques for detection of XCP to find one most suitable for the Michigan program.

In this regard, it is essential that the sensitivity of detection be known. It is well established that, with disease-conducive environmental conditions, the presence of low numbers of foliar bacterial plant pathogens in and on the above ground parts of a plant may multiply and spread rapidly to epidemic levels (16, 40, 43, 96, 115, 122). Weller (120) determined the mean *in situ* doubling time of two XCP isolates in Michigan plots to be 18.8 and 19.4 hours during the exponential growth phase. The question then remains, what type and level of seed contamination will allow transmission of the pathogen to the seedling? Baker and Smith in their comprehensive treatise on seed transmission say, "...it is often assumed without evidence that presence of the pathogen in or on seed assures transmission." (6). The need for information about seed to seedling transmission from natually occurring concentrations

of internal and external seed contamination has been made by several others (32, 43, 47, 62, 76, 81, 82, 88, 103, 115).

There are a lack of data indicating both the quantitative levels of XCP contamination per seed in a naturally contaminated seedlot, and the possible contribution to the primary inoculum of those quantitative levels. Similarly, data are lacking that indicate the qualitative type of contamination (infestation vs. infection) that occurs per seed in a naturally contaminated seedlot. Therefore, there is a need to know what may be termed the "contamination profile" of a seedlot. Such a profile would indicate the location (quality) and number (quantity) of XCP cells that occur per seed in a naturally contaminated seedlot. Once a seedlot contamination profile for common blight is established, the contributions that the quantity and quality of seed contamination make to the primary inoculum can be evaluated. Where and in what numbers XCP are found per seed in naturally contaminated seedlots, and the relative importance of the various levels and types of seed contamination in contributing to the primary inoculum is fundamental to understanding and ultimately controlling common blight in Michigan.

Tolerance Levels

The maximum proportion of contaminated seeds acceptable in a particular pathogen-crop-region system is the tolerance

level. Many tolerance levels have been established (6, 70), and many have been challenged (76, 81, 82, 88, 115). Nevertheless, since even very sensitive detection techniques with very large sample sizes cannot certify that there is absolutely no seed contamination, tolerance levels should be established, no matter how small (32, 37, 81). Determining the natural contamination profile would help in establishing an effective tolerance level.

Attempts to determine a tolerance level for halo blight of beans, caused by *Pseudomonas syringae* pv. *phaseolicola*, have given conflicting results. Walker and Patel (115) determined that adding only 0.02% infected seeds to an otherwise clean seedlot will result in an epidemic. Guthrie *et al.* (40) and Wharton (124) estimated that only 0.006% to 0.01% infected plants in a field may result in complete crop loss under epiphytotic conditions. The average number of infected seeds required to give rise to one infected seedling has been estimated at 10 (101), 8.6 (103) and 2 (102). The concentration of pathogen in infected seeds was estimated at over 10^5 cells in 80% of the infected seeds (101). Trigalet and Bidaud (107) assayed 476 growers' seedlots over a six year period and recommended a tolerance level of 0.005% infected seed. External seed contamination is the most common form of seed contamination by *Pseudomonas syringae* pv. *phaseolicola* in seed grown under furrow irrigation in low rainfall areas (38). None of the above studies actually measured the quantity and quality of

contamination per seed in order to establish a seedlot contamination profile.

Little research has been devoted toward establishing a tolerance level for the bean common blight pathogen. Wallen and Sutton (117) added visibly infected seed to clean seed at various rates, and they determined that 0.5% visibly infected seed was sufficient to incite an epiphytotic. This was the lowest level tested excluding the negative control. The possible importance of visibly infected seed as primary inoculum was discussed by Weller and Saettler (122). Seeds showing symptoms contained from 1.8×10^5 to approximately 10^9 colony forming units (cfu) of blight bacteria. Seeds with symptoms were classified in order to associate the severity of symptom with recoverable cfu. Most seeds with approximately 10^8 or more cfu died before they emerged from the soil (118, 120). The fact that high inoculum levels per seed leads to the pre-emergence death of the seedling has also been noted for other bacterial pathogens (71, 90, 131).

Pre-emergence death of seeds carrying carrying high concentrations of pathogen per seed seems to contratict other observations. For example, Wallen and Sutton (117) stated that the number of primary infection foci corresponded closely to the original percentage of visibly infected seed. Also, MCIA field inspectors have long noticed that XCP frequently occurs in isolated areas within a field (personal communication with Randy Judd of the

MCIA). This implies that the primary inoculum may have been only a few of the seeds planted.

Weller (120) infested healthy seed at different rates with dust from plants infected with antibiotic-resistant mutants of XCP. The minimum level of seed infestation required to produce blighted plants was determined to be an average of 10^3 - 10^4 cfu per seed for 100 g of seed. Levels of contamination similar to this are easily obtained during commercial threshing practices. However, average levels of pathogen cells per seed sample varied over a 10^5 fold range and replicate samples from the same seedlot varied considerably. Again, this implies that individual seed contamination may not be uniform. Therefore, only those seeds with pathogen concentrations required for transmission, should be considered in establishing the tolerance level.

Objectives

The objectives of this study were: 1) to develop a protocol to measure quality (internal vs. external) and quantity (concentration) of XCP contamination in naturally infected bean seedlots; 2) to establish a contamination profile for naturally infected seedlots, *i.e.*, to quantify the infestation and infection per seed in naturally infected seedlots; 3) to determine the minimum concentration of pathogen per seed required for disease transmission It is

hoped that the information obtained from this study will improve our understanding of, and provide further insight into, the initial establishment of XCP in bean fields. Furthermore it is hoped this information will contribute to establishing an acceptable seed tolerance level in Michigan.

PART II

SEED TO SEEDLING TRANSMISSION OF *XANTHOMONAS CAMPESTRIS* PV. *PHASEOLI*

MATERIALS AND METHODS

Seeds

Specific information about the seedlots used in this study is summarized in Table 1. All seedlots were commercial cultivars of white seeded navy beans. Foundation seedlots were grown under semi-arid conditions in the western United States which are not conducive to the development of common blight. These seedlots were acquired from the MFSA before slurry treatment with pesticides, and served as uninfested and uninfected controls in experiments. No XCP colonies were recovered from any samples taken from these seedlots.

Most naturally infected seedlots consisted of the excess seed not used in the MDA blight test. The seeds were sent to the MDA by growers in Michigan. All seedlots tested positive for blight by the MDA inoculation technique as described in the General Introduction.

Three naturally infected seedlots were produced experimentally at the Botany and Plant Pathology research farm, Michigan State University, East Lansing, Michigan. Naturally infected seed (provided by A.W. Saettler)

Table 1. Identity of seedlots utilized throughout study

Seedlot[a]	Source[b]	Cultivar
Foundation	MFSA	Seafarer
Foundation	MFSA	C-20
82382	MDA	unknown
83557	MDA	Seafarer
83869	MDA	unknown
83870	MDA	unknown
83873	MDA	C-20
83874	MDA	C-20
83933	MDA	C-20
84045	MDA	Exrico
84055	MDA	Seafarer
84059	MDA	Seafarer
84097	MDA	Fleetwood
84099	MDA	Kentwood
84114	MDA	Fleetwood
84122	MDA	Laker
84165	MDA	Midland
84187	MDA	Seafarer
84536	MDA	unknown
84595	MDA	C-20
84598	MDA	Seafarer
84633	MDA	C-20
84638	MDA	Seafarer
84689	MDA	Seafarer
84703	MDA	Seafarer
84704	MDA	Seafarer
84731	MDA	Midland
86148	MDA	Midland
87225	MDA	Seafarer
87243	MDA	unknown
91447	MDA	unknown
1983	A.W. Saettler	Seafarer
1984	experimental	Seafarer
1985	experimental	Seafarer

[a]Five digit numbers represent the Michigan Department of Agriculture's laboratory identification number. Four digit numbers represent the year the experimentally infected seedlots were produced.

[b]MFSA = Michigan Foundation Seed Association, MDA = Michigan Department of Agriculture

harvested in 1983 was planted and the next generation of infected seed was harvested in 1984. For the seedlot referred to as 1985, seed harvested in 1984 was mixed with an approximately equal amount of foundation seed of the same cultivar, planted and harvested. Care was taken with all naturally infected seedlots to minimize handling that might lead to added external contamination by XCP bacteria.

Seeds with visual symptoms of internal infection (*e.g.*, hilum spotting) were produced in following manner (120). Healthy plants were grown from foundation seed in a greenhouse. When pods had reached the half-filled stage of development, they were inoculated by scratching along the dorsal suture of the pod with a hypodermic needle attached to a syringe containing a suspension of cells prepared as described elsewhere.

Media

All chemicals, unless stated otherwise, were purchased from Sigma Chemical Corporation, St. Louis, MO. All media containing agar were steamed for 15 - 20 minutes before autoclaving for 20 minutes. Media were then cooled to 45 - 50C, and approximately 15 ml were poured into 9 cm diameter disposible plastic petri plates. All agar plates were allowed to cool to room temperature overnight, then were stored in plastic bags for up to 4 weeks before use.

NB: (Nutrient Broth) was prepared by dissolving 8 g of nutrient broth in 1 liter of distilled water and autoclaving.

SBS: (Sterile Buffered Saline) was prepared by mixing 70 ml of a stock solution of 0.1 M Na_2HPO_4 with 30 ml of a stock solution of 0.1 M KH_2PO_4 in a one liter flask. Nine hundred ml of distilled water and 8.5 g of NaCl were added yielding 0.01 M buffered saline, pH 7.2.

YCA: (Yeast extract Calcium carbonate Agar) was used as a standard medium for quantifying the recovery of non-XCP bacteria, unless otherwise noted. It was also used as the medium for XCP bacteria when non-XCP bacteria were not present, *i.e.*, when a pure culture was present. It contained (per liter of distilled water) 10.0 g yeast extract, 2.5 g calcium carbonate, 15.0 g Bacto-agar. Individual colonies of XCP were not visible until after at least 48 hours of incubation. Colonies of non-XCP bacteria were counted after 24 hours of incubation at 27 $\pm$ 1C, and plates were checked again for any additional non-XCP colonies after 48 hours. When YCA was used to determine colony forming units (cfu) of XCP, the colonies were counted after 72 hours of incubation, and checked for any additional colonies after 96 hours of incubation. XCP colonies first appeared as small yellow, circular, and convex colonies with entire margins. After several days of incubation, isolates of the fuscans variant were differentiated by the presence of a brown soluble pigment.

mSSM: (modified Semi-Selective Medium) was used to isolate and quantify XCP bacteria, unless otherwise stated. The basal liquid medium of mSSM was originally developed by Trujillo and Saettler (108, 109) and was later modified and made into a solid medium by Mabagala (61). Other studies, (61, 108, 109) have compared mSSM with different XCP detection and quantification methods. These studies have compared: ease of the method, efficiency in quantification, detection of a variety of isolates, recovery from sources containing low populations of the pathogen, and contaminant to pathogen ratios. The results of the above studies indicated that mSSM was suitable for use in this research.

The mSSM was prepared by dissolving 1.0 g of yeast extract, 15.0 g bacto-agar, and 8.0 g soluble potato starch in 970 ml 0.01 M phosphate buffer (pH 7.2). Six ul of a 1% aqueous solution of methyl green and three ul of a 1% solution of methyl violet 2B in 20% ethanol were added. The protocols for preparation of stock solutions were as outlined by Trujillo and Saettler (109). Stock solutions of antibiotics were filter-sterilized and stored for 3-4 weeks at 4 $\pm$ 1C. The final amount of antibiotics per liter of mSSM were: 25.0 mg cycloheximide, 2.0 mg nitrofurantoin, 1.0 mg nalidixic acid, and 0.5 mg gentamycin sulfate.

Some non-XCP bacteria grow on mSSM, however, the XCP bacteria are clearly differentiated on the basis of colony morphology. XCP colonies first become visible as small, light yellow, convex colonies with entire margins and a zone

of starch hydrolysis. With further incubation colonies become larger, darker yellow and slimy. The regions of starch hydrolysis expand and coalesce. Isolates that are fuscan variants do not produce the brown pigment in mSSM, however, usually these isolates are somewhat slower growing than the non-fuscans isolates.

Colonies of XCP growing on mSSM were counted after 48 hours of incubation at 27 $\pm$ 1C, and checked again for any additional XCP cfu after 72 hours of incubation.

XCP isolates

Bacterial isolates were routinely tested for pathogenicity in the following manner. To obtain rapidly growing cells, isolated colonies were spread on YCA and incubated for 24 hours. The bacteria were suspended in SBS and adjusted to an optical density of 0.05 - 0.07 at 620 nm (approximately 10^8 cfu). These suspensions were used to inoculate at least 3 seedlings of the susceptible cultivar Manitou that had fully expanded first trifoliolate leaves. The seedlings were inoculated by infiltrating 2 areas (each approximately 1.5 cm in diameter) on the adaxial side of each leaflet of the first true leaf. The infiltration was done using a 3 cc hypodermic syringe without a needle and appeared water-soaked (61). The plants were kept in a greenhouse at a temperature range of 20 - 30C and were

inspected for symptoms for up to three weeks after inoculation.

Short-term storage of bacterial cultures was accomplished by sealing the petri plate with Parafilm and storing it in an inverted position at 4 ± 1C. Long-term storage of XCP cultures was accomplished in infected seeds.

Factors affecting the recovery of XCP from naturally infected seed

Grinding regimes

In an effort to minimize the variability among replications, naturally infected seedlots were visually inspected to remove debris, damaged or broken seeds and seeds showing signs of internal infection. Seed samples of 100 g each were suspended in 300 ml of SBS and shaken on an oscillating shaker (68 cycles per minute), for 3 hours at room temperature (23 ± 2C). A two-factor factorial experiment was performed, using a completely randomized design (CRD). Ten replicate samples from various seedlots were ground before or after shaking, or were not ground. Grinding was done in a blender for 30 seconds. Recovery of bacteria was determined by making a serial dilution of the suspension, spreading 0.1 ml on mSSM and YCA, and counting cfu as described elsewhere.

Suspending solution

The effect of the suspending solution and seedlot on recovery of bacteria was determined in a two-factor factorial CRD experiment. Seeds from different infected seedlots were ground to a flour. Replicate flour samples were suspended in different aqueous solutions, *i.e.*, SDW, SBS, SBS containing 0.01% w/v Tween 20, SBS without the NaCl (buf), buf plus 0.01M $MgSO_4$ (SBM), or SBM containing 0.01% w/v Tween 20. Recovery of both XCP and non-XCP was determined as already described.

Shaking time

XCP cells in and on naturally infected mature bean seed are in a state of low moisture content and low metabolism. Cells in this condition are termed hypobiotic (53, 54). The length of time after rehydration required for hypobiotic XCP to begin to multiply (lag phase) was determined for isolates from several seedlots. Seed coats removed from 4 seeds showing visible symptoms of internal infection, were shaken in NB at 68 oscillations per minute for 30 minutes. The seed coats were removed from the suspension, and the OD_{620} was adjusted to approximately 0.05 ("initial OD_{620}"). The cfu of the "initial OD_{620}" suspension was determined. The turbidity was checked hourly to determine the time after rehydration that an increase in the turbidity occurred.

The "initial OD_{620}" of the hypobiotic suspension was due not only to viable XCP cells, but also to other organic material rinsed from the seed coats. Therefore, a "modified OD_{620}" was determined to account for suspended material that produced turbidity but not cfu of XCP. This was done by determining the cfu represented by various turbidities of a rapidly growing pure culture. A linear function was determined expressing cfu of a pure culture, as a function of OD_{620}. The cfu initially recovered from the suspension of hypobiotic XCP was used in the above function to determine the "modified initial OD_{620}".

The growth rates of XCP and non-XCP isolates recovered from the seedlots were also determined. Rapidly growing pure cultures were produced as previously indicated. A turbid suspension of bacteria was prepared in NB and shaken. The change in OD_{620} per hour represents the growth rate of the isolate under these conditions.

To determine the duration of the lag phase of XCP for isolates from several seedlots, two steps were utilized: 1) Determine the time required by the cfu of XCP producing the turbidity indicated as "modified initial OD_{620}", to multiply to a high enough concentration to produce the turbidity indicated as "initial OD_{620}". 2) Subtract this time from the time after rehydration that an increase in turbidity was first observed.

Shaking temperature

The recovery of XCP and non-XCP from infected seeds was further studied utilizing 2 seedlots, 2 shaking times and 2 temperatures in factorial experiments utilizing a CRD. Four replications of 50 g of flour from seedlots 1984 and 1985 were suspended and shaken in 150 ml of SBS for 3.0 or 6.0 hours at 1.5 or 23.0 $\pm$ 2C. Recoveries of XCP and non-XCP were determined as described above.

Factors affecting the recovery of external XCP

To determine the number of external XCP, the bacteria must be removed from the seed and disassociated from each other. Debris associated with naturally infected seedlots 1984 and 1985 was ground in a Wiley mill using a 20-mesh screen, then again using a 60-mesh screen. Samples of the debris (0.50 g) were suspended in 10 ml of SBS and shaken. Recovery of XCP was determined after various times.

Protocol used to assay seed

The method used to extract and suspend external XCP was to shake the seed for 3 minutes in a suspension of SBS. A sample of the suspension was removed from the seed and shaken for at least 10 minutes. Quantification was done utilizing mSSM as described.

The method used to quantify internal XCP was to determine the total XCP associated with the seed and subtract the number of external XCP. The total number of XCP was determined by grinding the seed to a flour and shaking a suspension of SBS for 3 hours. Quantification of cfu was done as described above.

Calibration of the method

Calibration of the total recovery method

The proportion of the total XCP present that are recovered by the method was determined. Twenty foundation seeds were individually inoculated by carefully placing 0.01 ml of an XCP suspension of known concentration on the seed. The seeds were allowed to dry for several hours. Each seed was assayed for total XCP. The ratio: cfu inoculated : cfu recovered, was determined.

Calibration of the external recovery method

The proportion of the external XCP that are recovered. To determine this, seed that was infested but not infected, was produced. Ground debris associated with seedlots was immersed in a 10-fold dilution of a suspension of rapidly growing XCP that had been adjusted to OD_{620} = 0.1. The excess liquid was filtered away and the infested debris was

allowed to thoroughly dry in a laminar flow hood. Replicate samples of foundation seed (75 g) were shaken in a closed container with the infested debris. The ratio of the cfu recovered by the external assay : the cfu recovered by the assay for total XCP, represents the proportion of external XCP recovered. Nineteen such samples of seed were assayed for external and total XCP.

The proportion of XCP recovered during external assay that have leached from inside the seeds. To determine this, the ratio of external recovery from seed samples that were only infested : infested and infected, was determined. To produce seeds that were both infested and infected, 75 g of foundation seed was vacuum infiltrated as described by Goth (34), with the following modifications. The XCP suspension used was adjusted to an OD_{620} of 0.56. The vacuum was released after 45 seconds and the seeds were allowed to stand for an additional 15 seconds. To produce seeds that were infested only, seed samples were immersed in a sub-sample of the same XCP suspension for 60 seconds. After treatment, seed samples were blotted dry with paper toweling and left in open trays under laboratory conditions overnight. Eight replicate samples of each treatment were assayed for external XCP.

Comparison of schemes used to estimate internal XCP

The internal concentration of XCP was estimated utilizing the subtraction method described above and two methods of surface disinfestation. Foundation seed samples artifically infested and infected were prepared as described above. Eight replicate samples were either: 1) Surface disinfested by rinsing for 10 seconds in 95% ethanol, followed by rinsing in a 1:1 dilution of commercial bleach (2.6% NaOCl), containing 0.01% Tween 20. The seeds were then rinsed three times in SDW, blotted to remove excess water and allowed dry for at least 3 hours in a laminar flow hood, 2) Surface disinfested by shaking the seed in an open tray 10 cm from an Hanovia type 30600 ultraviolet light source, 3) Not surface disinfested.

RESULTS

Factors affecting the recovery of XCP from naturally infected seed

Recovery of XCP on mSSM from naturally infected seed was compared using various grinding regimes and suspending conditions.

Grinding regimes

Preliminary experiments indicated that when a suspension of XCP cells in log phase was blended for 30 seconds there was no significant reduction in the number of cfu recovered compared to the number in the same suspension before blending. Recoveries of XCP from seedlot samples that were not ground, or ground prior to shaking, or ground after shaking, were compared (Table 2). The recovery from the seedlots varied, but grinding the seeds before shaking gave the highest recovery for seedlot 1984. The variability among samples of the seedlot 1985 was such that significant differences between the three grinding regimes were not demonstrated.

Table 2. Effect of grinding regime and seedlot on recovery of *Xanthomonas campestris* pv. *phaseoli*[y]

Seedlot	Mean number of colony forming units $x10^{-4}$		
	Seed ground prior to shaking	Seed ground after shaking	Seed not ground
1984	16.2 a[z]	4.7 b	2.2 b
1985	5.0 b	1.6 b	1.9 b

[y]Ten replications of seed, removed from naturally infected seedlots, were suspended in an aqueous solution and shaken at room temperature for 3 hours. Some samples were ground for 30 seconds in an blender prior to shaking. Other samples were ground after shaking, and others were not ground. Recovery of was determined by counting colonies on mSSM.

[z]Means followed by the same letter not significantly different according to Duncan's multiple range test at 5% level.

Since mSSM is only a semi-selective medium, and bacteria that are antagonistic *in vitro* to XCP are commonly isolated from bean seed (75), it is desirable to keep non-XCP bacteria at the lowest possible level. The effect of grinding regime on the recovery of non-XCP bacteria was determined using the same two naturally infected seedlots and one foundation seedlot (Seafarer). Neither the effect of the seedlot, nor the interaction of the seedlot with grinding regime were significant. Each of the grinding regimes resulted in a different recovery of non-XCP according to Duncan's multiple range test at the 5% level. The mean recoveries were 8.2×10^4, 1.3×10^6, and 3.9×10^2 cfu/sample for ground prior to shaking, ground after shaking and not ground respectively. Thus, grinding before shaking resulted in maximum recovery of XCP and mininized the number of contaminants.

Suspending solution

Various aqueous solutions were compared for recovery of XCP and non-XCP from infected seed flour. There were no significant differences in recovery of non-XCP among the solutions tested. For seedlot 1984, recovery was greatest with SBS; however there was no significant difference when the same buffer was used without the NaCl, or when Tween 20 was added to the SBS (Table 3). The recovery of XCP was significantly higher with SBS than with sterile distilled

Table 3. Effect of suspending solution on recovery of *Xanthomonas campestris* pv. *phaseoli* from infected bean flour[w]

Suspending solution[y]	Mean colony forming units $x10^{-6}$ recovered[x]	
	Seedlot	
	1984	1985
SBS	29.0 a[z]	1.3 c
buf	18.0 ab	1.2 c
SBS+T	12.0 abc	0.75 c
SBM	5.3 bc	0.93 c
SDW	5.3 bc	0.44 c
SBM+T	4.4 bc	1.0 c

[w]Flour samples from naturally infected seedlots were suspended in various aqueous solutions and shaken for 3 hours at room temperature. Recovery was determined by counting colonies on mSSM.

[x]Means represent 3 replications of 50 g flour suspended in 150 ml solution.

[y]Solutions tested were; sterile distilled water (SDW), 0.01M phosphate buffer, pH 7.2, (buf), buf plus 8.5 g/l NaCl (SBS), SBS plus 0.01% w/v Tween 20 (SBS+T), buf plus 10mM $MgSO_4$ (SBM), SBM plus 0.01% w/v Tween 20 (SBM+T).

[z]Means followed by the same letter are not significantly different according to Duncan's multiple range test at 5%.

water, or the when the osmoticum used was $MgSO_4$ instead of NaCl. The interaction of seedlot with solution was significant, because the extraction solution used had no effect seedlot 1985.

Shaking time

Preliminary results indicated that the recovery of XCP from infected seeds increased with increasing shaking time, however, the extraction time utilized must not allow the XCP cells to multiply. Hence, the duration of the lag phases were determined for XCP isolates from various seedlots. They were determined to be: 5.5, 7.8, 10.4, 10.7, and 11.6 hours for seedlots 82676, 81962, 1984, 84076, and 84068, respectively.

The growth rate of some non-XCP bacteria isolated from infected seedlots on mSSM were as much as ten times faster than XCP (data not shown). Often, shaking intact or ground infected seed samples for longer periods of time allowed so much growth of non-XCP bacteria that quantification of XCP was impossible.

Shaking temperature

It was hypothesized that by extracting the pathogen in the cold, a longer shaking time could be utilized. This would enhance recovery of XCP, and still limit growth of the

XCP and non-XCP bacteria. Flour samples from naturally infected seedlots were shaken in SBS at room temperature or in a cold room. Samples of the suspensions were dilution plated onto mSSM and YCA plates after 3 and 6 hours of shaking (Table 4). The results indicate that shaking infected seed flour at a cold temperature significantly lowered ($P = 0.05$) the recovery of XCP, when shaken for either 3 or 6 hours. The recovery of non-XCP bacteria was also significantly lower when flour samples were shaken in the cold.

Factors affecting the recovery of external XCP

Studies were conducted to determine the effect of Tween 20, rinse time, and the presence of internal XCP on recovery of external XCP. The effect of rinse time on recovery of XCP from contaminated dust and the effect of the presence of seeds in the suspension on the concentration of XCP was also studied.

Earlier results (Table 3) indicated that infected seed flour suspended in SBS yielded greater numbers of XCP on mSSM plates than when other suspending solutions were used. The addition of Tween 20 to the suspension had no significant effect on cfu recovered. To test whether the addition of Tween 20 significantly affected the recovery of external XCP, seed samples from infested seedlots, were shaken in SBS or SBS plus 0.01% w/v Tween 20. Serial

Table 4. Effect of time and temperature on recovery of *Xanthomonas campestris* pv. *phaseoli* from naturally infected navy bean flour[w]

Degrees (C)	Shaking Time (h)	Mean cfu ($x10^{-5}$) XCP[x]	Mean cfu ($x10^{-5}$) non-XCP[y]
1.5	3	1.2 c	0.4 c
	6	2.2 b	0.5 c
23	3	25.9 a	2.3 b
	6	42.1 a	24.0 a

[w]Flour samples from infected seedlots were shaken in an aqueous suspension at 1.5C or 23.0 ± 2C. Samples were taken after the indicated time and plated onto appropriate media. All values were log transformed before analysis.

[x]Each value is the mean of 4 replications x 2 seedlots. The main effect for seedlot and all other interactions are not significant.

[y]Each value is the mean of 4 replications using one seedlot.

[z]Means followed by the same letter within columns for each seedlot are not significantly different according to Duncan's multiple range test at the 5% level.

dilutions of the suspension were made and plated onto mSSM. There was no significant difference in recovery of XCP using SBS or SBS plus Tween 20 (data not shown).

Rinse time

Preliminary experiments indicated that the longer infested seeds were rinsed in an aqueous solution, the more XCP could be recovered. Wrinkling of seed coats, especially in the region around the hilum, was noted on all or nearly all submerged seeds between 3 and 5 minutes after submersion in aqueous solution. This indicates that imbibition had commenced.

Since it is unlikely that the XCP cells are multiplying in these relatively short time periods, *i.e.* 1 to 30 minutes, other possibilities were considered: 1) not all bacteria were removed from the surface of the seed when seeds were shaken for shorter time periods, 2) the bacteria were removed from the surface of the seeds, however, they remained in clumps that only formed one colony. If the latter explanation is correct, then when a suspension of infested debris is shaken without the presence of seeds, there should be an increase in recovery of XCP with time. Samples of ground debris associated with naturally infected seedlots were shaken in SBS. Samples of the suspension were taken after various times and assayed for XCP (Figure 1). The recovery increased for the first ten minutes of shaking.

Figure 1. Effect of shaking time on recovery of *Xanthomonas campestris* pv. *phaseoli* from infested debris. Debris associated with naturally infected seed was ground in a Wiley mill. Samples consisting of 0.50 g of debris suspended in 10 ml SBS, were assayed for the number of cfu XCP recovered after various minutes of shaking, by dilution plating onto mSSM. Recovery of XCP/ml represents the mean of 4 replications $\pm$ the standard error.

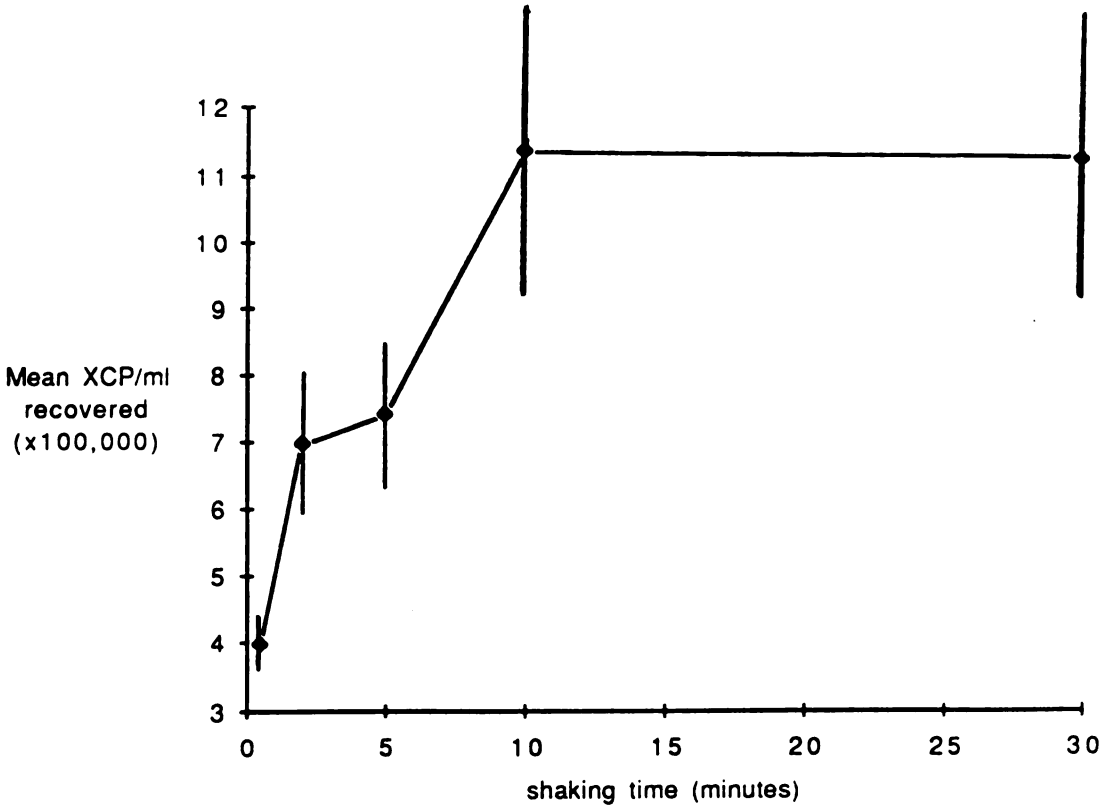
Mean XCP/ml
recovered
(x100,000)
12
11
10
9
8
7
6
5
4
3
0
5
10
15
20
25
30
shaking time (minutes)

Figure 1

In light of these results, infested debris should be shaken for at least ten minutes.

Presence of seeds

The imbibition of the suspension liquid by submerged seed may affect the concentration of XCP in the solution surrounding the seeds. For example, if the XCP cells are not imbibed along with the suspending solution, the concentration of XCP would increase. To study this possibility, ground debris associated with naturally infected seedlots was suspended in SBS and shaken 30 minutes to allow any clumps of XCP to disassociate. The suspension was sampled and plated on mSSM to determine the concentration of XCP bacteria. Immediately, seed from different foundation seedlots was added to samples of the suspension, shaken and resampled after various times. All or nearly all of the seeds showed signs of imbibition within 5 minutes after submersion, and appeared to be totally imbibed within 60 minutes. There was no appreciable change in XCP recovery after any of the times that samples were taken. The experiment was terminated after 60 minutes. Since the numbers of XCP per ml did not increase while seeds were imbibing the suspension, the suspended XCP must also be taken inside the seeds.

Calibration of the method

Calibration of the total recovery method

Individual foundation seeds were inoculated with a known volume of an XCP suspension. Each seed was assayed for total XCP. The mean ratio ± the standard error of: cfu recovered / cfu inoculated, was determined to be 0.35 ± 0.10. Thus, about 1/3 of the bacteria applied were recovered by the method used.

Calibration of the external recovery method.

If seeds are not rinsed long enough, many external XCP will not be recovered. Yet, the seed coat is not a very effective barrier to XCP, so by rinsing seeds too long internal XCP may be leached into the rinsing solution. Calibration experiments were performed on the recovery method to determine: 1) the proportion of the external XCP that are recovered, and 2) the proportion of XCP in the rinse solution that had leached.

Artifically infested samples of foundation seed were assayed for external XCP. Each sample was then ground and assayed for total XCP. Since this seed was surface-infested and not infected, no leaching of XCP could be occuring. Therefore, the ratio of: external / total recoveries, represents the proportion of external XCP recovered. The

mean ratio of external XCP recovered ± the standard error was 0.32 ± 0.04. Thus, the external recovery method utilized accounts for approximately 1/3 of the surface XCP.

To determine the proportion of leached XCP recovered during the external recovery assay, foundation seed samples were either: 1) infested and infected, or 2) infested only. The ratio XCP recovered from an external assay of: seed infested only / seed both infested and infected, represents the proportion of XCP recovered during the external assay that had leached. The mean proportion ± the standard error was 0.62 ± 0.08. Thus, only about 62% of the XCP recovered by the external assay were external, and about 38% were internal XCP that had leached.

Comparison of schemes used to estimate internal XCP

There are two basic schemes which can be used to extract and estimate the concentration of internal XCP. One is to eliminate external XCP by surface disinfestation and quantify the remaining pathogen. The other other scheme is to quantify the number of external and total (total includes both external and internal) number of XCP, and estimate the internal number by subtraction.

A comparison was made of the two schemes: 1) utilizing 2 different means of surface disinfestation, and 2) utilizing the subtraction method. The results (Table 5) indicated that the level of internal XCP estimated using the

Table 5. Comparison of three schemes to estimate levels of internal *Xanthomonas campestris* pv. *phaeoli*[y]

Estimation scheme	Mean estimate of number of internal cfu ($x10^{-4}$)
Total cfu recovered minus external cfu	6.8 a[z]
Total cfu recovered after ultraviolet surface disinfestation	9.1 a
Total cfu recovered after sodium hypochlorite surface disinfestation	0.9 b

[y]Foundation seeds were vacuum infiltrated with a turbid suspension of pathogen and allowed to dry overnight. Eight replicate seed samples were surface disinfested by ultraviolet light, or sodium hypochlorite or were not surface disinfested. Samples were assayed for external and total cfu of pathogen as described elsewhere.

[z]Means followed by the same letter are not significantly different according to Duncan's multiple range test at the 1% level

subtraction method was not significantly different from estimate utilizing ultraviolet light to surface disinfest. However, both of those estimates of internal XCP were significantly higher ($P = 0.01$) than the estimate made utilizing the sodium hypochlorite method of surface disinfestation. Thus, to avoid accidentally killing internal XCP, the subtraction method rather than surface disinfestation method was utilized in this study.

Quantification of infestation and infection in naturally infected Michigan seedlots

Naturally infected seed samples were acquired from the MDA or were produced at the Botany and Plant Pathology farm at Michigan State University. Three, 75-gram sub-samples were taken from each naturally infected seedlot, and assayed in 225 ml SBS for internal and external XCP. The results varied greatly. Estimates of infestation for the various seedlots ranged from no XCP recovered to 1.0×10^6 per ml ($\sim 0 - 6.0 \times 10^5$ cfu per seed). However, the variability between sub-samples of the same seedlot was so great that these estimates are not trustworthy. The average coefficient of variation for the infected seedlots was 125.6%. Most sub-samples had seed with cracked seed coats that often would would become completely loose or detached from the seed during the assay for external XCP.

Estimates of numbers of internal bacteria per seedlot also varied greatly. They ranged from zero to 3.4×10^7 per ml (~0 - 2.1×10^7 cfu per seed). Sub-samples from the same seedlots were also too variable to trust the estimate of internal infection. The average coefficient of variation of the internal infection estimates was 143.3%. Most sub-samples had some seeds showing visible symptoms of internal infection.

Variability in the number of XCP recovered from the 75-gram sub-samples (approximately 400 seeds) of the same seedlot, suggests that the bacteria may not be evenly distributed over all the seeds in a seedlot. To determine the proportion of visibly infected seeds that occur in naturally infected seedlots, 400 individual seeds from each lot were meticulously inspected for possible symptoms. Seeds that were even slightly suspected of showing visible symptoms, along with any seeds that were too dirty to determine if symptoms were present, were assayed. Seeds considered to show visible symptoms harbor 5×10^5 or more XCP cells (122). The proportion of seeds in a seedlot which exhibited visual symptoms varied from 0% to 2.5%. The mean percentage $\pm$ the standard error was 0.40% $\pm$ 0.12%. The majority of seeds that displayed visible symptoms exhibited a small brown spot, usually near the region of the hilum of the seed.

Many individual seeds were assayed because of being too dirty to determine or were suspected of showing symptoms.

Several of these seeds did harbor the pathogen, but the concentration of cfu per seed was too low to possibly produce true symptoms. The prevalence of symptomless contaminated seeds, and the concentration of the pathogen per seed was determined for several seedlots. The results indicated: 1) most symptomless seeds (86.4%) harbored no recoverable XCP (*i.e.* were not infected or infested), 2) most seeds that harbored any XCP, harbored relatively few cfu of XCP, 3) most XCP associated with a naturally infected seedlot are internal and are limited to a small proportion of seeds that usually display visual symptoms. The results from all the individual seeds assayed are summarized in Figure 2.

Figure 2. The percentage of seeds in naturally infected seedlots that harbored various concentrations of *Xanthomonas campestris* pv *phaseoli*. A total of 1,761 individual seeds from thirty naturally infected seedlots were assayed for external and internal pathogen. No pathogen was recovered from most of the seeds assayed (84.6%).

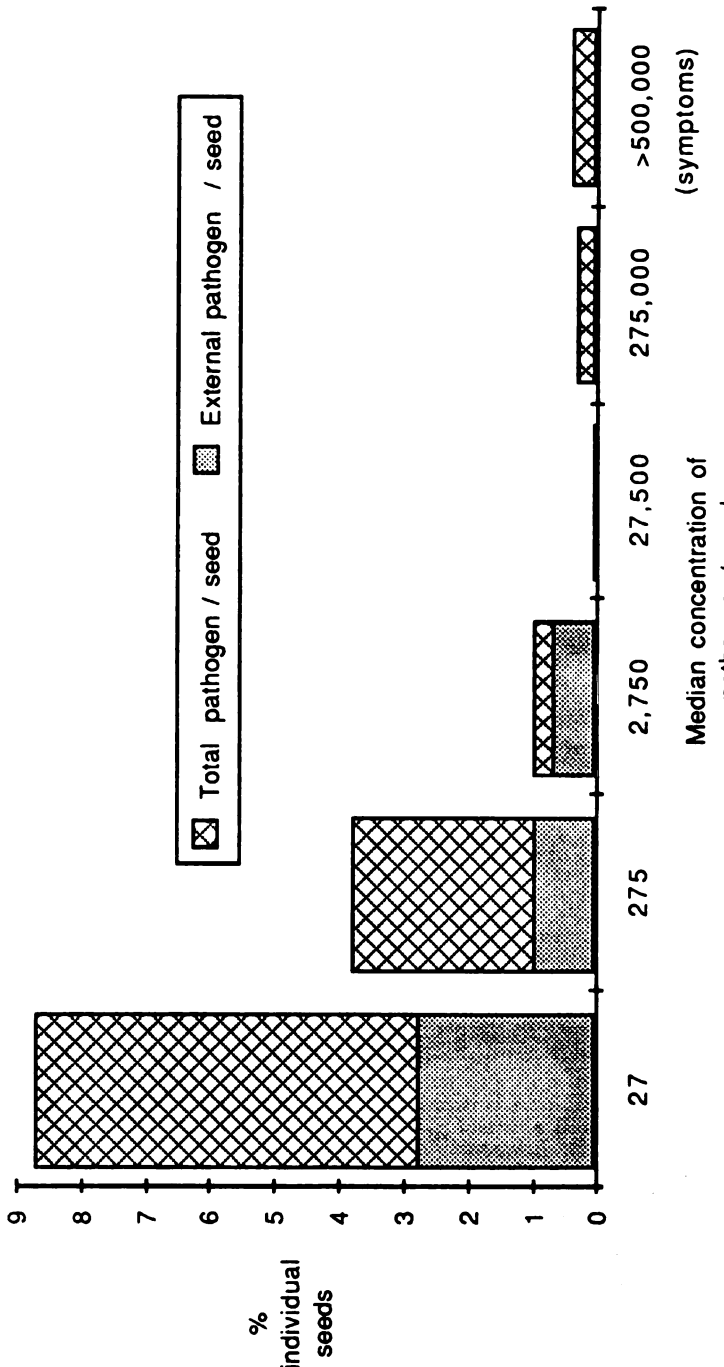
Total pathogen / seed
External pathogen / seed
% individual seeds
0
1
2
3
4
5
6
7
8
9
27
275
2,750
27,500
275,000
>500,000
(symptoms)
Median concentration of pathogen / seed

Figure 2

DISCUSSION

Experiments were conducted to develop a protocol to measure the quality (external vs. internal) and quantity of XCP in navy beans. The protocol developed was used to assay naturally infected seedlots.

Several factors were considered in the development of a protocol. The extracted XCP could be accurately, economically, and conveniently quantified using mSSM. The technique used to extract the pathogen should allow the maximum recovery possible of XCP from seed, without allowing multiplication of the viable XCP cells. The extraction technique should also not promote the growth of non-target bacteria. Antagonistic non-pathogenic bacteria are commonly isolated from bean seeds (75), as well as other types of seed infected with other pathogens (80).

The extraction procedures used by other workers have varied. For example, some workers (18, 26, 101, 105, 119,) have ground the seed, whereas others (23, 107) have soaked seeds intact prior to extracting the pathogen. In this study, the highest recovery of XCP was achieved by grinding the seeds. However, grinding the seeds also increased the recovery of non-XCP. Specifically, grinding the seeds before shaking yielded the most XCP, and grinding the seeds

after shaking yielded the most non-XCP. Reasons for this discrepancy between XCP and non-XCP are not clear. However, other studies on the effects of soaking intact bean seeds (72) have shown that during soaking, the cavity between the cotyledons has an excess of trapped water that results in an oxygen deficiency inside the seed. These anaerobic conditions are harmful to the seed, and result in reduced germination. The effect of soaking on germination is more apparent when conditions that promote bacterial activity are present (106). Perhaps the anaerobic conditions that occur inside submerged intact seeds allow the non-XCP bacteria to become metabolically active. The metaboloically active non-XCP may inhibit the recovery of XCP bacteria via antibiosis (75).

Extraction time used by various workers has also varied (15, 18, 83, 120). The basic problem is that by extracting for too short of a time too much pathogen goes undetected. However, by extracting for too long of a time: 1) the pathogen may begin to multiply, and/or 2) non-target bacteria may become metabolically active and inhibit the XCP or may multiply and grow on mSSM, preventing accurate quantification of XCP, and/or 3) internal XCP may leach into the aqueous solution during extraction of external XCP.

Recovery of XCP from infested debris increased when the debris was shaken for times too short to allow multiplication of the bacteria. This supports the hypothesis that extracted bacteria occur in clumps that

require at least ten minutes of shaking to thoroughly suspend the cells.produced by XCP. Moreover, XCP cells surrounded by dry extra-cellular polysaccharide (EPS) are released slowly when suspended in solution (56, 57). *In vitro* experiments dealing with EPS, have indicated that: 1) when cells are entering the stationary phase of the growth curve, such as when seeds dry and mature, EPS amounts are highest, 2) the amount of EPS produced by XCP is increased when carbohyrdates such as those available from the host plant are included in the medium, 3) the more EPS surrounding the XCP bacteria, the longer they survive under a variety of environmental conditions (48, 126). Microscopic wet mounts of seed coats with visible symptoms appear to have a lot of the EPS associated with the tightly packed XCP cells. The EPS is probably important in the mass survival of the pathogen, and enough time to dissolve it must be allowed during extraction from naturally dried host material.

Using the protocol to extract and quantify infection and infestation on sub-samples of the same seedlot commonly varied over a 10^3 or a 10^4 fold range, especially for estimates of total and internal bacteria. Some sub-samples contained seeds showing obvious symptoms of internal infection, and all sub-samples contained cracked and/or damaged seeds that lost their seed coats during the extraction of external XCP. This undoubtedly accounts for

part of the variability bacteria recovered from sub-samples of the same seedlot.

However, most of the variability is probably due to uneven distribution of bacteria on individual seeds in each seedlot. A total of 1,761 seeds from various infected seedlots were individually assayed. The major conclusions of the individual seed assay are: 1) no XCP bacteria are recoverable from most seeds (86.4%), 2) most of the seeds that do harbor XCP contain relatively few cfu, 3) the vast majority of the XCP associated with a seedlot (99.96%) are limited to a relatively small proportion of seeds (0.4%), and are internal.

When seeds were individually inoculated and assayed, a mean of 35% of the cfu inoculated were recovered. This proportion of recovery is probably low since inoculating the individual seeds involved drying the inoculum and some of the XCP cells probably died. Calibration of the external recovery method indicated that the cfu recovered were too high by an average of 62% due to internal XCP leaching out, and too low by an average of 32% due to external XCP not being extracted. If the cfu counted in this study were modified by these proportions, the three major conclusions indicated above would not be affected.

The subtraction method used in this study to estimate levels of internal XCP was compared to 2 other methods. Both of the other methods involved surface disinfestation, *i.e.* NaOCl and ultraviolet light. The NaOCl method gave

siginificantly lower estimates of internal XCP than either of the other methods. This may be explained by the NaOCl being imbibed by the seed and killing internal XCP. The ultraviolet light method produced higher estimates of internal XCP than the subtraction method, though the difference was not significant. This may be because the ultraviolet light does not kill all the external XCP. The protocol utilized in this study did not expose the seeds to any bactericidal agent before assay for these reasons.

One major conclusion of this study, *i.e.*, that most of the XCP in a Michigan grown seedlot are internal, differs from the conclusion of other studies. When beans are grown in low-rainfall areas where secondary spread during the growing season is rare, Grogan and Kimble (38) determined that seed infestation was the most important type of seed contamination. Weller (120) showed that infestation mainly occurs during the threshing process when bacteria from dried stems, pods, and to a lesser extent, leaves, become air-borne in bean dust. Thus, in the semi-arid western states, where foundation seed is produced utilizing furrow irrigation, infestation is the most common form of seed contamination. In Michigan however, environmental conditions are more favorable for secondary spread. Secondary spread of the pathogen is more apt to result in pod, and therefore internal seed infection. This probably explains why most of the XCP bacteria are internal in Michigan seedlots.

The findings reported here, *i.e.*, that most of the seeds that harbor any pathogen have a relatively low pathogen concentration / seed, contradict the findings of Taylor (101). Working with *Pseudomonas syringae* pv. *phaseolicola*, Taylor did not measure the actual pathogen numbers per seed, but rather estimated them statistically. Taylor's method was based on the assumption that the plot of the frequency of seeds vs. concentration of pathogen per seed (*e.g.* see Figure 2) conforms to the Poisson distribution. However, the Poisson distribution is based on independent events. Since the presence of one XCP cell invading a maturing seed would affect the probability of more cells invading the seed, the events are not independent. With his assumptions, Taylor estimated that most (>80%) of the infected seeds in a seedlot contained 10^5 bacteria or more. In this study, only a small proportion of infected seeds were found to harbor such large numbers of pathogen. Taylor also used these assumptions to estimate the tolerance level, *i.e.*, number of infected seeds per kilogram of required to transmit the disease, (102, 103, 107). Perhaps the assumption that most infected seeds contain large numbers of pathogen cells, partially explains the conflicting tolerance levels reported for halo blight of beans in the literature (40, 115, 107, 124).

The MDA laboratory test for internal XCP (as outlined elsewhere) may need modification in view of the results of this study. The MDA test involves NaOCl surface

disinfestation of seed samples followed by rinsing once with sterile water. The results of this study indicate that the number of internal XCP cfu recovered are significantly lowered by surface disinfestation with NaOCl. The NaOCl solution used in this study also contained Tween 20, and the seeds were rinsed in 95% ethanol prior to immersion. Still, the reliability of the MDA test may be reduced by the surface disinfestation step. This may explain why the MDA test has been determined to sometimes give false negatives (108).

After the surface disinfestation step, the MDA procedure calls for the extraction of the bacteria from intact seeds. The results of this study show that significantly more pathogenic bacteria were recovered when seeds were ground, than when left intact.

Another important implication of this study involves the procedure the MDA follows prior to performing the laboratory test. White seeded bean samples are visually inspected for the presence of seeds showing symptoms of internal infection. The suspected seeds are removed and forwarded to an expert for visual confirmation. The remaining seeds are tested for blight by the laboratory inoculation technique. On occasion, a seedlot that passes the MDA blight test based on inoculation procedures, will be labeled as blight contaminated on the basis of visual inspection only. This often leads to criticism from the individual that submitted the seed sample (personal

communication with Steve McQuire, director of the MDA seed laboratory). Apparently it is far more convincing to individuals submitting seed samples to have their product rejected because of a failed inoculation test, than a passed inoculation test and a failed visual inspection. By removing seeds with symptoms, the MDA is removing most of the XCP associated with the seedlot. Hence, it is not surprising that the inoculation part of the test may give false negatives under these circumstances.

PART I

QUANTIFICATION OF *XANTHOMONAS CAMPESTRIS* PV. *PHASEOLI* IN NATURALLY INFECTED NAVY BEAN SEED

INTRODUCTION

Kuan defines inoculum threshold as, "...the amount of seed infection or infestation with plant pathogens that will cause a disease in the field under a conducive environment and lead to economic losses" (47). It is well established that, with disease-conducive conditions, the presence of low numbers of foliar bacterial plant pathogens in and on the above ground parts of a plant, may multiply and spread rapidly to epidemic levels (16, 40, 43, 96, 115, 123). In Michigan, the mean *in situ* doubling time of two XCP isolates were determined to be 18.8 and 19.4 hours during the exponential growth phase (120). Seed to plant transmission of the pathogen does not necessarily mean that symptoms will be visible (13, 91, 120). Therefore, since numbers of XCP bacteria on bean foliage may increase at such a rapid rate without causing symptoms, any seeds that may transmit the pathogen to the seedlings and enable the bacteria to spread within a field, must be included in the establishment of a tolerance level of XCP in bean seeds.

The presence of the pathogen in a seedlot, however, does not necessarily mean it will be transmitted to the seedlings. Some wheat seed samples from which *Xanthomonas campestris* pv. *translucens* was recovered, did not transmit

the disease (84). Similar results with other bacterial plant pathogens have aided in the establishment of seed tolerance levels (86, 107). The minimum level of external XCP seed contamination required to produce blighted plants in Michigan was determined to be 10^3 to 10^4 cfu per seed (120).

The purpose of this research was to: 1) investigate the seed to seedling transmission of XCP and subsequent plant colonization from various infected seed samples, and 2) to measure the spread of XCP bacteria from a seed-borne point source in the field.

MATERIALS AND METHODS

Production of infested or infected seed

Production of seed free of bacteria

Foundation seeds were planted and grown in the greenhouse as previously described. When the pods reached the leathery stage, they were hand harvested, surface disinfested, and blotted dry with sterile cheese cloth. The pods were left undisturbed in open sterile trays under a closed microbiological transfer chamber at room temperature. When brittle, pods were aseptically broken open and seeds were removed and stored in closed sterile jars at room temperature until use. Incubating individual seeds in 5 ml of NB for 7 days on a shaker produced no turbidity, indicating the seeds were free from bacterial contaminants commonly occurring in the greenhouse and laboratory.

Aseptic production of "infested only" individual seeds

Sterile pod flour was prepared by grinding mature pods (after removal of seeds) in a Wiley mill using a 20-mesh screen, then again using a 60-mesh screen. Samples of flour

weighing 1 gram were autoclaved, cooled and inoculated by stirring the flour in a mixture of equal volumes (8 ml total) of a typical fuscans isolate and a typical non-fuscans isolate. Inoculum was prepared as previously described except the bacterial suspensions were adjusted to OD_{620} 0.10 (approximately 2 x 10^8 cfu/ml). Excess liquid was removed from the flour by filtering through a piece of Whatman #1 filter paper in a Buchner funnel. Flour was allowed to dry for 5 hours in a laminar flow hood, followed by grinding in a mortar. Infested flour was stored at 4 $\pm$ 1C until one hour before use. Individual bacteria-free seeds were infested by rolling them in the infested pod flour.

Aseptic production of "infected only" seeds with symptoms

Seeds bacteria-free except for internal XCP, were produced in the same manner as bacteria-free seeds with the following modifications. Pods were suture-inoculated, as previously described, when at the half-filled stage of development. Seeds showing the hilum spot symptom were surface disinfested with ethanol-NaOCl (described elsewhere) when they were removed from pods. Infected only seeds with symptoms were stored at 4 $\pm$ 1C until one hour prior to use.

Bulk production of "infected only" seeds without symptoms

To produce large quanities of seed infected with XCP at concentrations known to naturally occur, foundation seeds were inoculated by vacuum infiltration as previously described, with the following modifications. Suspensions of XCP included 2 fuscans and 2 non-fuscans isolates in equal proportions. One isolate of each variant originally isolated from seedlots 84165 and 84689 was used in equal proportions. The original suspension of cells was adjusted to OD_{620} = 0.1 and diluted to three different concentrations: high = $x10^{-1}$, medium = $x10^{-2}$, low = 2×10^{-3}. Each concentration was used to vacuum infiltrate seeds. After drying, the seeds were surface disinfested with ethanol-NaOCl. Seeds that appeared to have loose seed coats were removed. The remaining seeds were stored in sterile closed containers at 4 $\pm$ 1C until 1 hour before use. Individual seeds were assayed for XCP.

Colonization of seedlings grown *in vitro* from infected or infested seed

To determine what tissues of a young seedling are colonized by XCP cells, seed that was infested only or infected only was tested. A 2.5 cm x 15 cm test tube, containing 10 ml of distilled water and a rolled 10 cm x 75 cm piece of paper toweling, was autoclaved. After cooling,

one seed was planted and allowed to germinate and grow in the dark at 29 ± 1C. After various times (up to 96 hours) the seedling was recovered and aseptically dissected. The various dissected seedling parts were either surface disinfested, or not surface disinfested. Next, each seedling part was either; 1)shaken for up to 7 days in 1 ml NB and streaked onto YCA to detemine if XCP was present, or 2) ground in SBS and dilution plated directly onto YCA.

Seed to seedling transmission of XCP in the growth chamber

Plastic trays (19.4 cm x 5.6 cm x 8.7 cm, length x height x width) or clear plastic cups (5.6 cm x 5.6 cm, diameter x height) were surface disinfested and filled with autoclaved vermiculite. The occurrence of seed to seedling transmission and the colonization of seedlings were studied by planting individual seeds in plastic cups or in groups of 10 seeds in a plastic tray, respectively. Seeds used were foundation seed, or symptomless seed removed from naturally infected seedlots, or "infected only" seedlots. Seedlings were grown in a growth chamber (model # CEL 3714, Sherer-Gillett Co., Marshall, MI) under 24 hours of light for 6 days at 28.5 ± 1C. Plastic trays and cups were placed in the growth chamber so as to minimize the accidental spread of bacteria within the growth chamber. Seedlings were carefully watered daily with SDW.

Colonization of various seedling parts by XCP was determined by; excising all emerged seedlings in one tray at the soil level, and shaking for 30 seconds in SBS. The rinse solution, with external XCP suspended, was assayed for XCP with mSSM. The seedlings were then dissected into various parts, and each group of the same part was comminuted in SBS, and assayed for XCP with mSSM. All comminution was done with mortars and pestles that were washed, dried, rinsed with ethanol, flamed, and cooled immediately before use.

The occurrence of seed to seedling transmission was determined by excising individual shoots, and shaking in SBS for 30 seconds. The rinse solution, with external XCP suspended, was sampled for blight bacteria using mSSM. The entire seedling was then comminuted in SBS and assayed for XCP utilizing mSSM.

Transmission of seed-borne XCP in the field

"Infected only" seedlots, containing seeds with different concentrations of XCP per seed, were planted in 2 fallowed commercial fields. Each field was located in widely separated Michigan counties, *i.e.*, approximately 5 miles northwest of Byron, MI (Shiawassee County), and approximately 3 miles west of Applegate, MI (Sanilac County). Each seedlot was planted in three different areas (replicates) within each field. Each replicate was at least

45 meters from the others, and each was 2.4 x 2.4 meters. Row width, plant spacing, planting date, and planting depth were in agreement with standard cultivation practices. Each location was checked at least once every week to note the presence of symptoms, take samples, and/or to control the weeds.

Foliage was assayed for bacteria at two different times; 24 days after planting (establishment), and 44 days after planting (bloom). Therefore, the data were analysed as a three-factor factorial experiment, *i.e.*, as a CRD for location, with the concentration of XCP per seed, and days after planting split on location. Data were log normal-transformed before analysis.

Foliage samples were assayed for XCP and non-XCP populations on the surface as well as total cfu. The first trifoliolate leaf was removed from 5 randomly sampled plants in each replicate. Several procedures have been used in previous studies to dislodge surface bacteria from bean foliage. These include; "gentle shaking" (13), 2 minutes of shaking (98), 2 1/2 minutes of shaking (120), 30 minutes of shaking (61). In this study, surface populations of bacteria on leaves were quantified by weighing each sample, shaking in SBS for 30 seconds, followed by assay on the appropriate medium. Total numbers of bacteria recovered from the same samples were determined by blending the leaves in a commercial laboratory blender (Stomacher, model no.

STO-400, Tekmar Co., Cincinnati, OH) for 30 seconds, followed by assay on the appropriate medium.

Plants at both locations were hand-harvested at normal crop maturity, and stored in open, large plastic bags at room temperature until threshing. Plants from each plot were threshed manually in clean burlap bags. Three, 75-gram sub-samples of harvested seed were taken from each replicate, and assayed for total XCP utilizing mSSM.

Spread of XCP from a seed-borne point source in the field

The same Michigan locations used in the transmission study described above, were also utilized to study the spread of bacteria from a seed-borne point source. All assays and plant culturing were as indicated already, with the following modifications. Several hilum-spotted seeds were planted in the middle of each 2.6 x 2.6 meter replicate plot of foundation seed. Foliage and harvest samples were taken at various distances from the source within each replicate plot.

RESULTS

Recovery of XCP from infested or infected seed

Recovery of XCP from "infested only" seed

Infested seeds were artifically produced by rolling individual seeds free of bacteria in infested flour. To determine the numbers of external and internal XCP bacteria present, twelve individual seeds were ground, suspended in SBS, shaken for 3 hours and dilution plated onto YCA. The concentration of XCP per seed was determined to be $1.2 \times 10^4 \pm 2.3 \times 10^3$.

Recovery of XCP from "infected only" seed

"Infected only" seeds with symptoms were produced by suture inoculation of the pod with a suspension of XCP followed by surface disinfestation of the seeds. Twelve hilum-spotted seeds were individually assayed for XCP. All contained more than 5×10^5 cfu per seed (Figure 1); the mean $\pm$ the standard error was $6.5 \times 10^6 \pm 1.3 \times 10^6$.

"Infected only" seeds with lower concentrations of XCP per seed were produced by vacuum infiltation followed by surface disinfestation. Individual seeds infiltrated with

Figure 1. Colony forming units of *Xanthomonas campestris* pv. *phaseoli* recovered from artifically infected individual seeds. Foundation navy bean seeds were vacuum infiltrated in one of three suspensions of XCP prepared at known concentrations; low = 6.0×10^4, medium = 3.0×10^5, high = 3.0×10^6 cfu per ml. Hilum spotted seeds were produced by suture inoculation. Seeds were surface disinfested, ground to a flour and suspended in an aqueous solution for 3 hours, followed by dilution platings of the suspension made on mSSM. Number of seeds assayed were 144, 59, 62, and 12 for low, medium, high, and hilum spotted seed respectively.

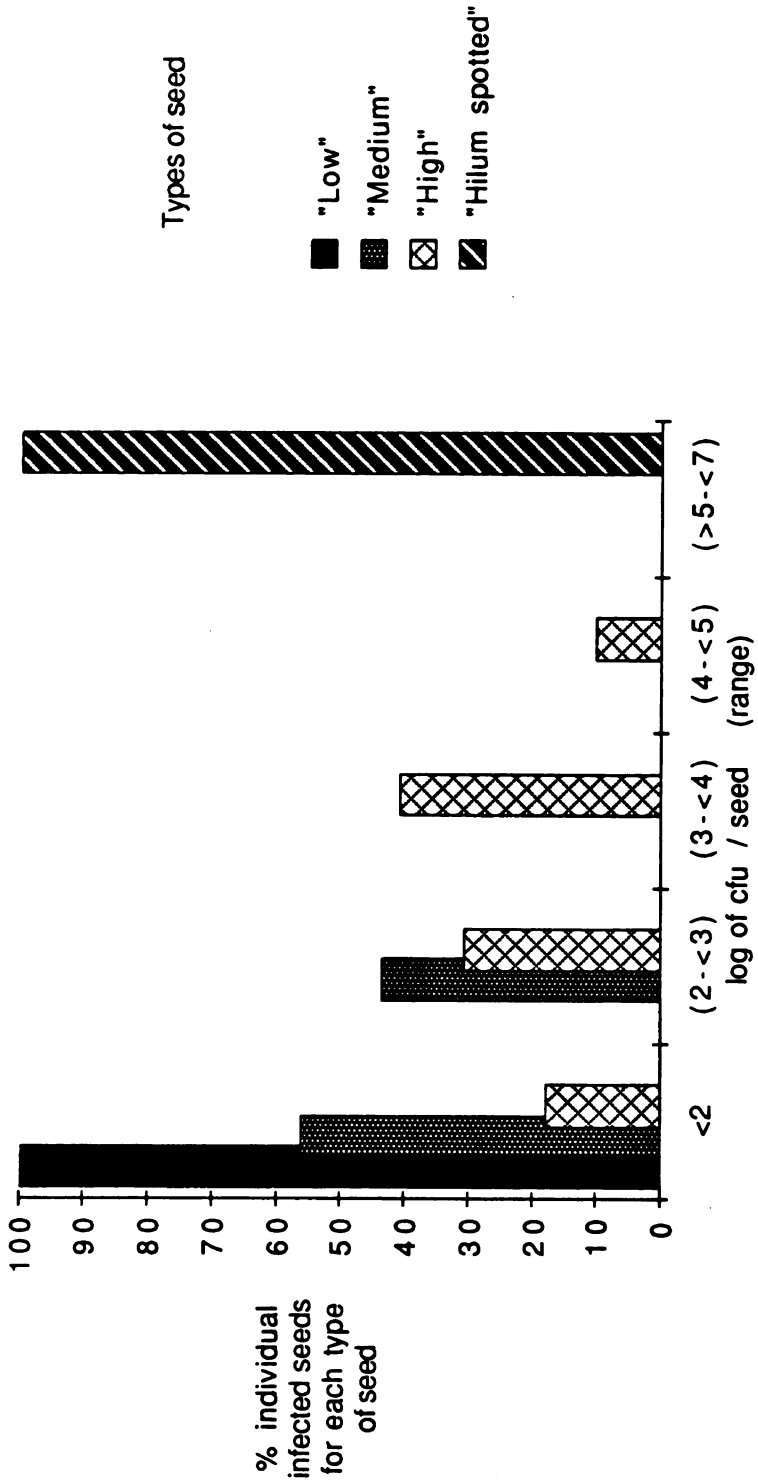
Types of seed
"Low"
"Medium"
"High"
"Hilum spotted"
% individual infected seeds for each type of seed
100
90
80
70
60
50
40
30
20
10
0
<2
(2-<3)
(3-<4)
(4-<5)
(>5-<7)
log of cfu / seed (range)

Figure 1

low, medium or high inoculum concentrations of XCP, or not infiltrated, were assayed to determine the level of internal infection. No XCP was recovered from the uninfiltrated seeds. All seeds infiltrated with the low concentration of XCP contained fewer than 10^2 cfu XCP per seed ($<10^2$), while all seeds infiltrated with the medium concentration contained fewer than 10^3 cfu per seed ($<10^3$). All seeds infiltrated with the high XCP concentration contained fewer than 10^5 cfu per seed ($<10^5$) (Figure 1). These levels of infection are consistent with levels of infection recovered from seed in naturally infected seedlots. That is, symptomless seeds in naturally infected seedlots may contain from zero to approximately 10^5 cfu of XCP per seed.

Colonization of seedlings grown *in vitro* from infected or infested seed

In preliminary trials, seedlings grown from bacteria-free seeds remained free of bacteria for at least 96 hours. After 96 hours, the seedlings elongated beyond the "hook stage". When seedlings were grown from infected only or infested only seed (Tables 1 and 2), colonization of all seedling parts occurred within the first 24 hours. Generally, longer periods of growth (up to 96 hours) resulted in a higher percentage of individual seedling parts colonized (Table 1), and higher numbers of XCP bacteria recovered from each seedling part (Table 2). Internal

Table 1. External and internal colonization of seedlings grown from seed internally infected with *Xanthomonas campestris* pv. *phaseoli*[a]

Location of XCP	Hours after planting seed	Percent of seedlings with XCP detected in:[b]				
		Cotyledons	Roots	Stem	Primary leaves	Seed coat
External and internal	24	91.6	8.3	8.3	0	100
	48	25	8.3	16.6	8.3	91.6
	96	50	66.7	58.3	25	75
Internal only	24	25	25	25	16.6	16.6
	48	25	25	25	16.6	16.6
	96	50	8.3	16.6	25	16.6

[a]Infected seeds, obtained by suture inoculation of pods, were surface disinfested and germinated in individual test tubes. After various times, 24 seedlings were removed from their respective test tubes. At each time, 12 seedlings were surface disinfested and 12 were not. The various parts of each seedling were assayed for the absence or presence of XCP.

[b]Cotyledons and primary leaves include 2 seedling parts per seedling. The remaining seedling parts include 1 seedling part per seedling.

Table 2. Colonization of seedlings grown from seed infested with *Xanthomonas campestris* pv. *phaseoli*[a]

Hrs[b]	Mean cfu ($x10^{-3}$) recovered per seedling part(s)					
	Cotyledons (both)	Roots	Stem	Primary leaves (both)	Seed coat	Total /seedling
	Internal + External Colonization					
24	0.1	0.0	0.1	0.1	14.8	15.2
48	7.3	0.0	0.7	0.1	793.3	726.6
96	306.6	289.2	786.7	7.91	>10000	>10000
	Internal Colonization Only					
24	0.0	0.0	0.0	0.0	0.0	0.0
48	0.0	0.0	0.0	0.0	0.0	0.0
96	130.2	0.0	1.4	0.1	0.0	131.6

[a]Seeds free of bacteria were individually infested and germinated in test tubes. After various times, 6 seedlings were removed, 3 of which were surface disinfested. The various parts of each seedling were assayed for the number of cfu of XCP present.

[b]Number of hours that had elapsed since planting the seed.

colonization was not detected in seedlings grown from seeds that were infested only until the seedlings were 96 hours old (Table 2). Much of the initial seedling colonization from internally infected seeds may be external since the percentage of seedling parts with internal XCP bacteria and the populations in these parts, except for cotyledons, were generally low (Tables 1 and 2).

Seed to seedling transmission of XCP in the growth chamber

Seedlings were grown for 6 days from symptomless seeds taken from foundation or naturally infected seedlots. No symptoms were seen on any of the seedlings. No XCP was recovered from any seedlings grown from foundation seed (Table 3). XCP bacteria were recovered from all parts of seedlings grown from symptomless seeds originating from naturally infected seedlots. In general, numbers of XCP recovered from the seedlings were highest for seedlot 1985, followed by 84689; there was very little recovery of XCP from lot 84165. XCP was also detected in: 1) the root masses of the emerged seedlings, 2) the seedlings that didn't emerge, and 3) the seeds that didn't germinate (data not shown).

Artificially infected seeds were grown and the seedlings were assayed in a similar fashion. The results (Table 4) indicate that seedlings grown from artifically infected seeds are colonized by XCP similarly to seedlings

Table 3. Recovery of *Xanthomonas campestris* pv. *phaseoli* from various parts of seedlings grown from symptomless seed removed from naturally infected seedlots[a]

	Mean cfu ($x10^{-3}$) recovered per seedling			
		External + Internal		
Seedlot[b]	External	Cotyledons	Primary leaves	Stem
Foundation	0	0	0	0
84165	0	0	0	0.1
84689	1.6	3.7	33	17
1985	40	367	2500	260

[a]Five replications of ten symptomless seeds were planted and grown in a growth chamber. After 6 days of growth the emerged seedlings were excised at the soil level and assayed for external XCP. The seedlings were then dissected further and each group of seedling parts was assayed for total XCP.

[b]The foundation seedlot was the negative control. The percentage of symptomless seeds harboring XCP were 4.0%, 15.8%, and 28.0% for seedlots 84165, 84689, and 1985 respectively.

Table 4. Recovery of *Xanthomonas campestris* pv. *phaseoli* from various parts of seedlings grown from artificially infected seed[a]

	Mean cfu ($x10^{-5}$) recovered per seedling			
		External + Internal		
Seedlot[b]	External	Cotyledons	Primary leaves	Stem
0	0	0	0	0
$<10^2$	0.3	5.7	26	2.4
$<10^3$	17	56	43	43
$<10^5$	24	321	210	340

[a]Four replications of ten artifically infected seeds were grown in a growth chamber. After 6 days of growth the emerged seedlings were excised at the soil level and assayed for external XCP. The seedlings were then dissected further and each group of seedling parts was assayed for total XCP.

[b]Numbers indicate the concentration of XCP per seed.

grown from naturally infected seeds.

Individual seedlings grown from symptomless seed taken from naturally or artificially infected seedlots were assayed for colonization by the pathogen. The proportion of colonized seedlings in each seedlot was generally similar to the proportion of seeds with detectable XCP (Table 5).

Transmissison of seed-borne XCP in the field

Field plots were planted with seedlots harboring different concentrations of XCP per seed. Disease symptoms observed on the leaves included water soaking, necrosis and lemon-yellow discoloration. In Shiawassee County, symptoms were first noted between 24 and 35 days after planting, and in Sanilac County between 35 and 44 days after planting. The mature pods were molded and discolored so no pod symptoms of XCP were observed.

Plants were assayed for the number of XCP cfu that could be easily rinsed off the surfaces of the leaves (Table 6). Plants grown in Sanilac County from seed containing $<10^2$ XCP per seed yielded no surface XCP 24 days after planting. However, surface XCP was recovered from all replicates 44 days after planting.

When total XCP per gram of tissue was determined (Table 7), the results were generally similar to the results of the assay for surface XCP. Twenty-four days after planting in Sanilac County, plants grown from seed containing $<10^2$ XCP

Table 5. Colonization of individual seedlings grown from symptomless seeds removed from seedlots infected with *Xanthomonas campestris* pv. *phaseoli*[a]

Seedlot	% of seedlings with[b]: Any XCP	External XCP	% of seeds with detectable XCP[c]
1985	18.6	7.1	28.0
$<10^2$	20.0	16.0	20.8
$<10^5$	56.7	54.0	62.9

[a]Symptomless seeds were removed from seedlots artifically infected (containing $<10^2$ or $<10^5$ cfu/seed) or naturally infected (seedlot 1985). Removed seeds were individually assayed for XCP, or were grown in a growth chamber for 6 days and excised at the soil line. The shoot portions were assayed for external XCP and the entire seedling was assayed for any detectable XCP. Assays were conducted using mSSM.

[b]The number of seedlings assayed were 70, 50, and 50 for seedlots 1985, $<10^2$, and $<10^5$ respectively.

[c]The number of seeds assayed were 225, 144, and 62 for seedlots 1985, $<10^2$, and 10^5 respectively.

Table 6. Surface populations of *Xanthomonas campestris* pv. *phaseoli* recovered from leaves of plants grown from seed containing different numbers of pathogen cells[y]

County \	Mean log cfu / g fresh weight	
	$<10^2$ / seed	$\sim10^6$ / seed
	24 Days after planting	
Shiawassee	5.8 ab	5.8 ab
Sanilac	0.0 c	6.2 ab
	44 Days after planting	
Shiawassee	7.0 ab	7.4 a
Sanilac	5.6 b	6.1 ab

[y]Field plots were planted with seed containing different concentrations of XCP cells per seed. Numbers of pathogen cfu on the plant surface were determined by rinsing 1st trifoliolate leaves and dilution plating onto mSSM. Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 7. Total populations of *Xanthomonas campestris* pv. *phaseoli* recovered from leaves of plants grown from seed containing different numbers of pathogen cells[y]

County \	Mean log cfu / g fresh weight	
	$<10^2$ / seed	$\sim10^6$ / seed
	24 Days after planting	
Shiawassee	7.4 b	7.8 ab
Sanilac	0.0 c	7.4 b
	44 Days after planting	
Shiawassee	8.1 ab	8.8 a
Sanilac	7.8 ab	7.9 ab

[y]Field plots were planted with seed containing different concentrations of XCP cells per seed. Numbers of pathogen cfu associated with the plants were determined by grinding 1st trifoliolate leaves and dilution plating onto mSSM. Means that are followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

cells yielded no pathogen. However, by 44 days after planting, the total number of XCP recovered from those plants was nearly the same as the other plants. This pattern of slow seed to seedling transmission for seeds harboring a low concentration of XCP was not observed in Shiawassee County.

Numbers of non-XCP bacteria that were easily rinsed off the surfaces of the leaves were determined 44 days after planting. The concentration of XCP per seed planted had no significant effect on the recovery of non-XCP from the foliage. However, the number of surface non-XCP bacteria recovered from all the Shiawassee County foliage was higher than the number recovered from all the Sanilac County foliage (data not shown).

The concentration of XCP per seed did not significantly affect the total non-XCP bacteria recovered from the foliage. However, the interaction of days after planting, with location was highly significant (Table 8). Only in Shiawassee County was the total number of non-XCP recovered 44 days after planting higher than 24 days after planting.

The harvested seeds were assayed for total XCP. Neither the concentration of XCP per seed planted, nor the interaction of location x concentration of XCP per seed had a significant effect on the recovery of XCP from the seed harvested. However, the amount of XCP recovered from the seed produced in Shiawassee County was higher than the

Table 8. Total populations of non-*Xanthomonas campestris* pv. *phaseoli* bacteria recovered from leaves of plants grown from seed containing various numbers of pathogen cells[z]

	Mean log cfu / g fresh weight	
County \	24 days	44 days
Shiawassee	4.8 b	6.2 a
Sanilac	5.3 b	5.6 ab

[z]Field plots were planted with seed containing different concentrations of XCP cells per seed. Plots located in two different counties were assayed for the total number of non-XCP bacteria 24 and 44 days after planting. Total number of cfu were determined by grinding 1st trifoliolate leaves in SBS and dilution plating onto YCA. Means represent 2 levels of seed infection x 3 replications. Means followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.01). All other interactions and the effect of concentration of XCP per seed are not significant.

amount recovered from the seed produced in Sanilac County (data not shown).

Spread of XCP from a seed-borne point source in the field

The spread of XCP from a seed-borne point source of inoculum was measured by collecting and assaying samples of leaves from areas located various distances from the source. Populations of XCP and non-XCP bacteria were determined 24 and 44 days after planting.

Disease symptoms observed on the leaves included water soaking, necrosis and lemon-yellow discoloration. In Shiawassee County, symptoms were first noted on plants grown from hilum-spotted seed between 24 and 30 days after planting, and in Sanilac County 35 days after planting. Symptoms on plants 0.6 or 1.2 meters away from the source were first noted between 30 and 56 days after planting in Shiawassee County and between 44 and 63 days after planting in Sanilac County. Mature pods were molded and discolored, hence no symptoms of XCP were observed.

The recovery of surface XCP (Figure 2) generally decreased as foliage samples were taken further away from the source, and increased when samples were taken later in the growing season. Shiawassee County yielded more surface XCP than did Sanilac County. Also the spread of surface XCP from the source occurred more rapidly in Shiawassee County.

Figure 2. Recovery of surface *Xanthomonas campestris* pv. *phaseoli* from bean foliage located at various distances from a point source of the pathogen. Seeds showing symptoms of infection were planted in the center of a plot of foundation seeds. Three such plots were planted in two counties. The number of pathogen cfu on the plant surface were determined by rinsing first trifoliolate leaves and dilution plating onto mSSM. Data are the averages of three replications.

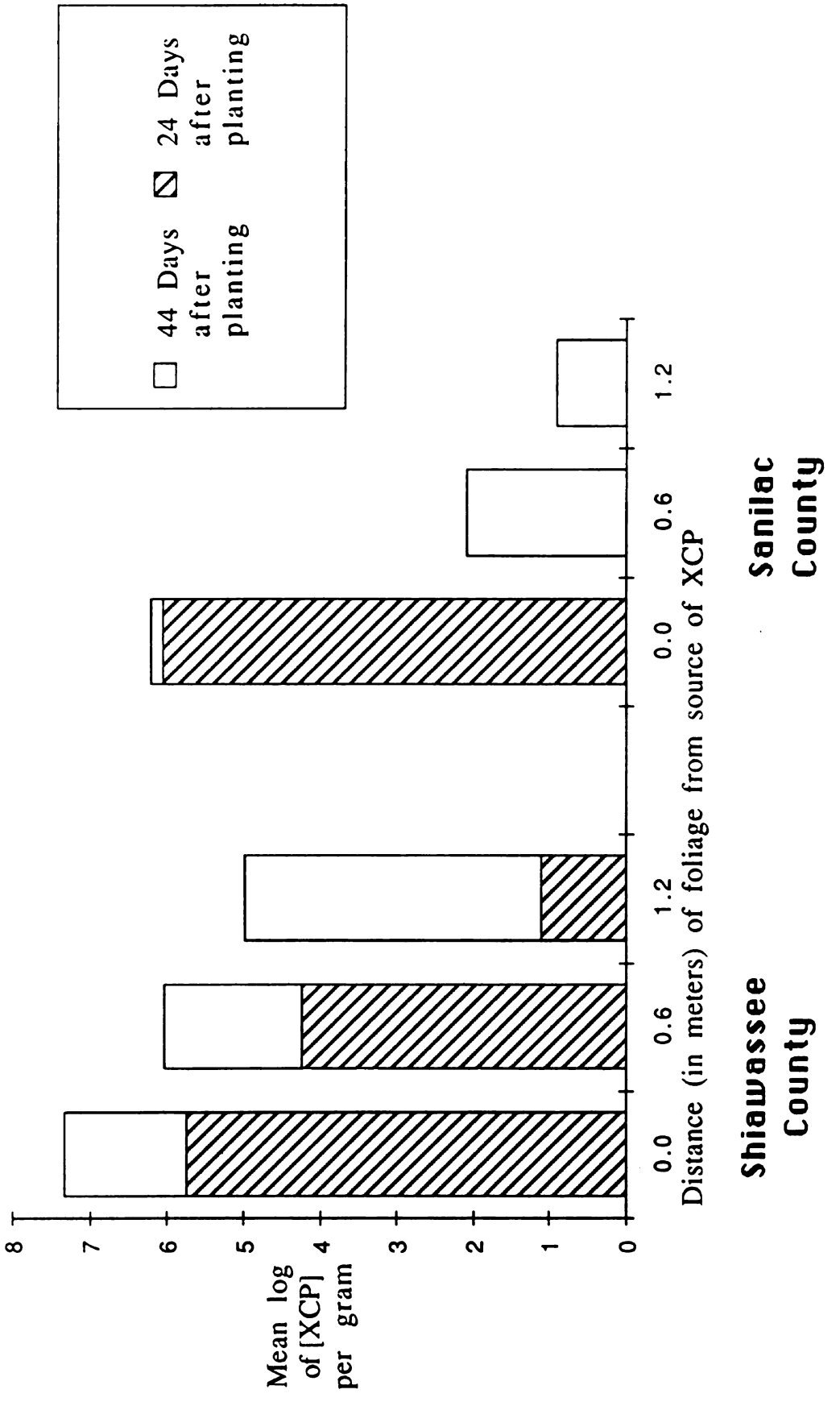
44 Days after planting
24 Days after planting
Mean log of [XCP] per gram
0
1
2
3
4
5
6
7
8
0.0
0.6
1.2
0.0
0.6
1.2
Distance (in meters) of foliage from source of XCP
Shiawassee County
Sanilac County

Figure 2

The recovery of total XCP, shown in Figure 3, was generally higher than the recovery of surface XCP, shown in Figure 2. The recovery of total XCP generally decreased as foliage samples were taken further away from the source, and increased when samples were taken later in the growing season. Shiawassee County yielded more XCP than did Sanilac County. Also the spread of surface XCP from the source occurred more rapidly in Shiawassee County.

Numbers of total and surface non-XCP bacteria were also determined at each distance from the seed-borne point source of XCP. Distance from the source did not affect the recovery of surface or total non-XCP. However, generally higher numbers of non-XCP were recovered: 1) in Shiawassee County than in Sanilac County, and 2) 44 days after planting than 24 days after planting (data not shown).

Harvested seeds were assayed for XCP. Neither distance from the source, nor the interaction of location and distance from the source, had a significant effect on the recovery of XCP from the seed harvested. However, the amount of XCP recovered from the seed produced in Shiawassee County was higher than the amount recovered from the seed produced in Sanilac County (data not shown).

Figure 3. Recovery of total *Xanthomonas campestris* pv. *phaseoli* from bean foliage located at various distances from a point source of the pathogen. Seeds showing symptoms of infection were planted in the center of a plot of foundation seeds. Three such plots were planted in two counties. The number of pathogen cfu associated with the plants were determined by grinding first trifoliolate leaves in SBS and dilution plating onto mSSM. Data are the averages of three replications.

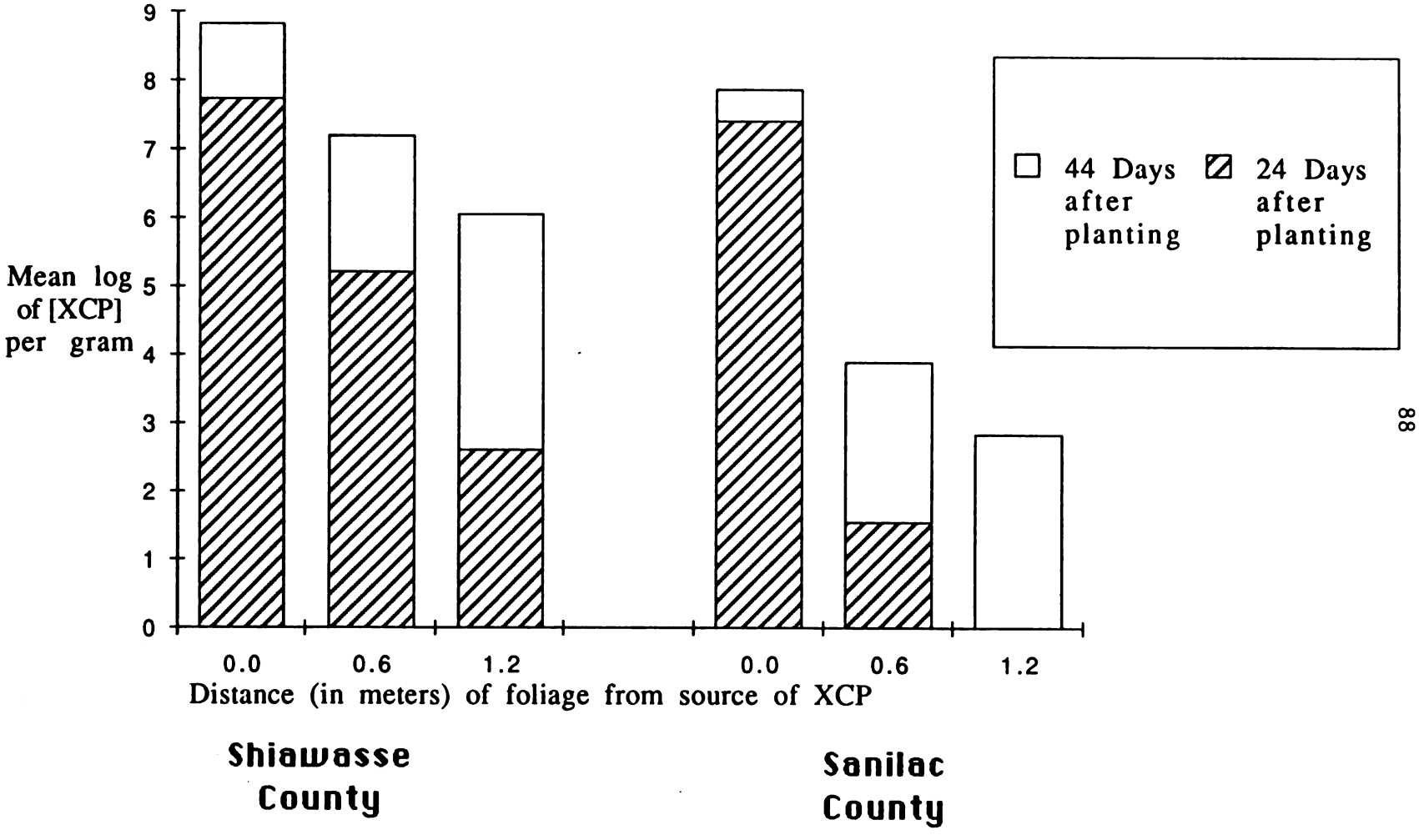
9
8
7
6
5
4
3
2
1
0
Mean log of [XCP] per gram
44 Days after planting
24 Days after planting
0.0
0.6
1.2
0.0
0.6
1.2
Distance (in meters) of foliage from source of XCP
Shiawasse County
Sanilac County

Figure 3

DISCUSSION

The first report that symptomless navy bean seed may contain blight bacteria was made ten years ago by Weller (120). Since that time, there have been no studies on the ability of symptomless infected seed to transmit the bacteria to the seedling, or to the next generation of seed. The results of Part I indicated that the majority of seeds harboring XCP are symptomless, and harbor relatively low concentrations of XCP per seed. The results of Part II show that symptomless seeds harboring very low concentrations of XCP per seed are able to transmit the bacteria to the seedling and to the next generation of seed.

The bacterial colonization of seedlings grown under laboratory or growth chamber conditions has been examined with numerous other crops including, cucumber (49, 52, 55), tomato (55), maize (55), soybean (55, 66), and wheat (30). The results shown in Tables 1 through 4 may be interpreted to mean that both infested seed and infected seed: 1) give rise to seedlings that are colonized by XCP when the seedlings are grown under artifical conditions, 2) all portions of the seedlings are colonized by XCP, 3) the bacteria are located on both inside and on the surface of the seedling. Internal colonization of the seedling

occurred earlier in seedlings raised from infected seed than it did in seedlings raised from infested seed. However, since XCP eventually did colonize the internal tissues of the young seedlings grown from infested seed, both types of seed infection result in similar colonization of the young seedling.

For several years most seeds used as planting stock in Michigan have been slurry treated to eradicate infestation (22, 23, 77). Therefore, infested seed is probably not epidemiologically important in commmerical bean production in Michigan. Since colonization of young seedlings in the growth chamber is similar from infected and infested seeds, it is likely that blight remains a chronic problem in Michigan mostly because of infected seeds.

The three seedlots of artifically infected bean seed used in the growth chamber studies, *i.e.* seeds containing $<10^2$, $<10^3$, or $<10^5$ cfu of XCP per seed, are fundamentally different from those used in studies conducted by others on bacterial colonization of plants grown under artifical conditions. Often other studies are not quantitative (55). The concentrations of XCP per seed in the artifically infected seeds were representive of the concentrations of XCP per seed recovered from symptomless seeds removed from naturally infected seedlots (see Part I). The percentage of symptomless seeds that actually were infected in each particular seedlot was similar to the percentage of XCP-colonized seedlings produced for that seedlot (Table 5).

Futhermore, most colonized seedlings had external XCP. The external XCP would probably be easily spread within the field. In Part I, it was determined that most of the infected seeds contain a relatively low concentration of XCP per seed. Therefore, these growth chamber results support the hypothesis that seeds with low concentrations of XCP may be very important in the epidemiology of the disease.

Seedlings grown in a growth chamber from naturally infected seedlots (Table 3) generally were colonized by fewer cfu of XCP than were seedlings grown from seedlots artifically infected with $>10^2$ cfu per seed, Table 4. This is probably because most symptomless seeds in a naturally infected seedlot do not harbor any detectable XCP. There is no way of determining which symptomless seeds are infected and which are not infected without destroying the seed and / or removing the XCP in the assay. However, most of the difference is probably due to the fact that most of the symptomless seeds that are naturally infected contain $<10^2$ XCP per seed. Therefore, during the first few days after germination, the concentration of XCP per seed may affect the extent to which the resulting seedling in the growth chamber is colonized.

The fact that individual infected seeds carrying low numbers of XCP bacteria transmit the pathogen to the resulting seedlings in the growth chamber does not necessarily mean that transmission occurs in the field to the degree necessary to incite the disease. However, seeds

containing low concentrations of XCP ($<10^2$) gave rise to XCP bacteria on the resulting foliage and to infected seed in the next generation.

Fewer XCP cfu were recovered from Shiawassee County than from Sanilac County. Though data was not taken on the weather, the general weather pattern observed may account for this difference, *i.e.* Sanilac County plots experienced much drier conditions throughout the growing season than did the Shiawassee County plots. The number of XCP bacteria recovered from plants near a source increased to a greater extent, and did so more rapidly, in the field that had a generally wetter growing season (Shiawassee County).

Seed to seedling transmission of some other bacterial pathogens has been shown to be favored under conditions of high compared to low humidity (30, 52). The inability of *Pseudomonas syringae* pv. *coronafaciens* to be transmitted from seed to seedling in infected seedlots, and its inability to move on young wheat seedlings has been attributed to the lack of significant amounts of rainfall (30, 43).

The interaction of effects of location with days after planting and with concentration of XCP per seed planted had a significant effect on the seed to seedling transmission. Plants raised from seed containing relatively few XCP, in a location with less disease-condusive conditions, transmitted the pathogen slower and to a lesser degree than did seeds containing a relatively high concentration of XCP per seed,

or raised under more disease-conducive conditions. That is, seeds with a low concentration of XCP per seed transmitted the pathogen slower than did seeds with a high concentration of XCP per seed only in some environments. Ralph (76) agreed in general that the environment plays a much more important role in seed to seedling transmission when the seed inoculum level is low.

Several authors have indicated that there is a need for seed tolerance levels to be modified to account for the environment in which the seeds will be planted (6, 70). The findings of this study do not support the need for flexible tolerance levels. Though the concentration of XCP per seed planted initially affected the seed to seedling tranmission, the seed to seed transmission of XCP was not affected. The important issue to a bean grower is whether or not the seeds harvested are infected. The recovery of XCP from the seed harvested was affected by neither the concentration of XCP per seed planted, nor the interaction of location with concentration of XCP per seed. Seed to seed transmission of XCP occurs regardless of location and concentration of XCP per seed planted.

Epidemics caused by foliar phytopathogenic bacteria generally occur when inoculum is dispersed by rainsplash (43, 96, 115). This study showed that the numbers of total and external XCP decreased as plants were located at increasing distances from the source of inoculum. The XCP available for secondary spread from the primary sources of

inoculum are most likely to be those bacteria that can be easily rinsed from the foliage. Growth chamber studies indicated that symptomless infected seeds may give rise to seedlings that have external XCP. In the field, the numbers of surface XCP on plants raised from seed with low concentrations of XCP (Table 6), were comparable to the numbers of surface XCP on plants acting as a focus of primary infection (Figure 2). This supports the conclusion that infected seed containing low concentrations of XCP per may serve as primary inoculum of the disease.

It has been suggested (43, 51, 61) that resident non-pathogenic bacteria may be important in the control of foliar diseases. It was hypothesized that the colonization of plants by XCP would affect the colonization by non-XCP. However, this study provided no evidence to support that hypothesis for the following reasons: 1) There was no effect of concentration of XCP per seed planted on the colonization of plants by non-XCP bacteria. 2) Foliage that was located various distances from the source of the pathogen, and which did not show any significant differences in non-XCP recovery, did show significant differences in the recovery of XCP. One explanation for this may be that the non-XCP bacteria present were not species that affect development of the disease (61, 75). Another explanation may be that the numbers of XCP bacteria present from infected seeds, or plants growing near a source of inoculum,

were large enough to obscure any effect that the non-XCP bacteria were having.

The plants in Shiawassee County yielded more non-XCP bacteria than the plants in Sanilac County, and the total number of non-XCP bacteria significantly increased between 24 and 44 days after planting only in Shiawassee County. Since population levels of resident bacteria are higher under climatic conditions where moisture is prevalent (5), these recoveries are consistent with the general weather pattern observed.

The conclusions of other workers regarding the tolerance levels for bacterial blights have been based on studies involving seeds infected with a very large number of pathogen propagules (40, 107, 117, 124). The findings of this study suggest that rate of seed to seedling transmission, and the eventual quantitative and qualitative colonization of the plant are similar for seeds infected with low or high concentrations of XCP. Establishing a tolerance level in terms of the proportion of highly infected seed is not useful because seeds with low numbers of XCP per seed are common in naturally infected seedlots and may serve as primary foci of infection.

Other workers have noted (13, 91, 120) that XCP may be present in and on the plants in a field without the appearance of symptoms. Weller and Saettler determined that at least 5×10^6 cfu/20 cm^2 leaf tissue are required for symptom development (123). The lowest number of total XCP

cells recovered from a plot displaying symptoms was 3.5 x 10^6 cfu per gram fresh weight of first trifoliolate leaf. This is in general agreement with Weller and Saetter since one gram fresh weight equals approximately 20 cm^2.

Michigan certification standards permit the presence of 0.005% blighted plants during field inspections. Fields with isolated areas of blighted plants may be accepted if the grower agrees to keep blighted seed separate from the rest of the seed (21). However, this study has shown that there may now be many more initial foci of infections than were previously known. In light of this, and the fact that XCP may be present in and on the plants without the appearance of symptoms, the aforementioned practices of the MDA should be modified. None of the seed harvested from a field with blight symptoms should be certified as "disease-free" seed.

In conclusion: 1) Most of the infected seeds in a naturally infected seedlot are symptomless and contain relatively few XCP per seed. 2) These symptomless seeds may transmit the pathogen to the seedling in manner that ultimately will result in infected seeds in the next generation. These symptomless infected seeds are currently the most important source of common blight primary inoculum for Michigan bean growers. Until seed treatments and/or detection and rejection techniques are able to eradicate these seeds, the most important source of primary inoculum for common blight will continue to be present in Michigan.

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