Occurrence in Seeds and Potential Seed Transmission of *Xanthomonas vasicola* pv. *vasculorum* in Maize in the United States

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Accepted for publication 12 March 2020.

ABSTRACT

This paper reports original evidence regarding the potential role of seed transmission of *Xanthomonas vasicola* pv. *vasculorum* in the epidemiology of bacterial leaf streak (BLS) in maize. We evaluated the occurrence of the pathogen on seeds from diseased fields and its subsequent transmission to seedlings. In 2016 and 2017, *X. vasicola* pv. *vasculorum* was detected by TaqMan PCR from 22 of 41 maize seed lots harvested from naturally infected fields in Colorado, Nebraska, and Iowa. However, many of the PCR-positive samples did not yield culturable *X. vasicola* pv. *vasculorum* colonies. The highest levels of seed contamination were detected in dent maize and popcorn from NE and CO. Seed transmission was evaluated in greenhouse grow-outs from eight seed lots, totaling more than 14,000 plants. Putative seed transmission events from naturally contaminated seed lots, estimated from PCR results, occurred at a frequency between 0.1 and 0.5% in 10-seedling pooled samples and at a frequency of 2.7%

from individual plant assays. However, no seedling symptoms were observed during these assays and live *X. vasicola* pv. *vasculorum* colonies were not recovered from PCR-positive seedlings. In contrast, seed transmission was readily demonstrated from artificially contaminated seed lots, including typical symptoms and recovery of live bacteria. Seed transmission consistently occurred from seeds soaked in bacterial suspensions with concentrations of $\geq 10^6$ CFU/ml, suggesting that a threshold population of the bacterium is necessary for the development of BLS symptoms and recovery of live bacteria. The low bacterial populations on naturally contaminated seeds apparently were not sufficient to result in diseased seedlings.

Keywords: bacterial leaf streak, bacteriology, ecology and epidemiology, etiology, maize, seed transmission, *Xanthomonas vasicola* pv. *vasculorum*

The emerging bacterial leaf streak (BLS) disease of maize, caused by *Xanthomonas vasicola* pv. *vasculorum* (Lang et al. 2017) (syn. *X. campestris* pv. *vasculorum* [Dye 1978]) (Young et al. 1978), was first reported in the United States in 2016. However, symptoms of the disease were observed in Nebraska as early as 2014 (Korus et al. 2017). Symptoms of BLS include initially water-soaked or greasy interveinal streaks that eventually become necrotic. Lesions can be yellow, brown, or tan in color and, in severe cases, may coalesce to form large necrotic areas (Robertson et al. 2016). BLS has reached near-epidemic proportions in some counties in Nebraska, Colorado, and Kansas, with disease incidence levels greater than 90% in several fields and disease severities of more than 50% leaf area infected. The disease has been reported from several other U.S. states including Iowa, Illinois, Minnesota, Oklahoma, South Dakota, Wisconsin, and Texas (Damicone et al. 2018; Jamann et al. 2019; Korus et al. 2017; Smith et al. 2018). The BLS disease and its causal agent were previously known only in South Africa, as *X. campestris* pv. *zeae* (Coutingo and Wallis 1991; Qhobela et al. 1990), with the first report in 1949 (Dyer 1949). However, BLS was recently reported both from Argentina (Plazas

et al. 2018) and from Brazil (Leite et al. 2018), leading to questions regarding the mechanism of spread and the phylogenetic relationships among *X. campestris* and *X. vasicola* isolates from different geographic regions and hosts, particularly maize, sorghum, and sugarcane (Perez-Quintero et al. 2019).

Lang et al. (2017) presented an extensive analysis of pathogenicity and genomic comparisons among historical and recently collected *Xanthomonas* isolates from these three crops. They showed that the U.S. strains of *X. vasicola* pv. *vasculorum* were most similar (99.9% genomic identity) to the historical South African *X. campestris* pv. *zeae* strain SAM119, isolated from maize leaves more than 25 years earlier (Qhobela et al. 1990). The U.S. isolates were less similar, both phylogenetically and in host plant aggressiveness, to *X. vasicola* pv. *holcicola*, the causal agent of BLS of sorghum, which occurs both in the United States and South Africa (Qhobela et al. 1991), or to *X. vasicola* pv. *vasculorum* isolates from sugarcane.

The BLS disease has been confirmed on many cultivars of popcorn and field (dent) maize, sweet maize, as well as volunteer maize. The conditions leading to severe outbreaks in the United States seem to be favored by the use of overhead irrigation, which is common in semiarid regions, and by continuous maize production (Broders 2017).

The origin of the BLS pathogen in the United States is uncertain but based on the 99% genomic similarity to *X. vasicola* pv. *vasculorum* from South Africa (Lang et al. 2017) and to isolates from Argentina (Plazas et al. 2018), introduction of the pathogen via seeds is suspected. The role of seed as a pathway for movement of *Xanthomonas* spp. in maize has not been studied previously, but seed transmission is well known for *Xanthomonas* species on other crops, including *Brassica* spp., carrot, common bean, onion, pepper,

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Funding: This project was funded by USDA-Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Plant Pest and Disease Management and Disaster Prevention Program, under agreement 16-8130-0731-CA.

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and supplementary tables are published online.

The author(s) declare no conflict of interest.

squash, tomato, and wheat (Bockus et al. 2010; ISF 2019). Maize residue and weeds are other potential sources of primary inoculum. Recently in the United States, *X. vasicola* pv. *vasculorum* was shown to survive in corn residue on the soil surface from the previous growing season (Ortiz-Castro et al. 2018). The pathogen was also isolated from naturally infected host plants in or near BLS-infected maize fields including big bluestem (*Andropogon gerardii* Vitman) and bristly foxtail (*Setaria verticillata* (L.) Beauv.) (Hartman et al. 2020). Such hosts could serve as potential inoculum reservoirs for the pathogen.

The goal of this research was to improve our understanding of the potential role of seeds in the epidemiology of BLS of maize. Specific objectives were (i) to assess the frequency and extent of *X. vasicola* pv. *vasculorum* contamination in seed from diseased plants and (ii) to assess the potential for transmission of *X. vasicola* pv. *vasculorum* from maize seeds to plants. Two approaches were evaluated for the detection of *X. vasicola* pv. *vasculorum* from maize seeds: dilution plating onto semiselective agar media and PCR testing, by both conventional and TaqMan PCR. Traditional dilution plating of the seed extract on semiselective or selective media continues to be a standard method for detection of *Xanthomonas* in seeds (Roberts and Koenraad 2015). Conventional and quantitative real-time PCR assays have recently been developed for rapid, specific, and sensitive detection of *X. vasicola* pv. *vasculorum* in maize (Lang et al. 2017; Stulberg et al. 2020). Both types of PCR were used in order to ensure sensitivity and specificity and to take advantage of the throughput capabilities of TaqMan PCR.

MATERIALS AND METHODS

Bacterial isolates. *X. vasicola* pv. *vasculorum* strain Har-1 was isolated from symptomatic maize leaves from Harcourt, Iowa in 2016. Pathogenicity was confirmed by plant inoculation, including side-by-side comparison with known *X. vasicola* pv. *vasculorum* strains NE-S1 and NE-P3-2y, which were isolated from Nebraska maize leaves by Charles Block in 2015 (Iowa State University). The strain *X. vasicola* pv. *vasculorum* CO-5 originated in Colorado. U.S. stock cultures of all strains were preserved on silica beads (Microbank, Austin, TX) by shaking beads in a cell suspension, removing excess fluid, and stored in 1-ml cryovials at -80°C . The stock cultures were also preserved in a 50:50 mix of 10% sterile skim milk/15% glycerol in 1-ml cryovials at -80°C .

Maize seed sources. The frequency and extent of seed contamination by *X. vasicola* pv. *vasculorum* was investigated from 2016 to 2018 on seed collected from BLS-diseased fields in three U.S. states: Nebraska (NE; 12 fields), Colorado (CO; seven fields), and Iowa (IA; nine fields). Each field was given a qualitative BLS disease severity rating in accordance with the percentage of leaf area affected (Robertson et al. 2016). Disease ratings ranged from low (+) severity, with $<5\%$ of the leaf area affected, to severe (++++), with 50% or more extensive lesions (Table 1). In 2016, two types of ear samples were collected: (i) preharvest ears harvested from individual plants that showed moderate to severe BLS disease symptoms, and (ii) at-harvest grain samples collected from a subset of the same fields during the combining operation. Preharvest samples were harvested at the R5.5 to R6 (midvent to physiological maturity) reproductive growth stage. Samples consisting of 20 to 30 ears from each dent maize and popcorn fields (28 fields) were dried at 25 to 30°C until the seeds reached 11 to 13.5% moisture (approximately 4 days), shelled and stored at 10°C . The at-harvest samples consisted of approximately 10,000 dent maize kernels each, from three IA fields and six CO fields, collected during machine harvest.

In 2017, ear samples were hand-harvested at maturity from four NE fields (two dent and two popcorn). Also in 2017, we collected ears from inoculated IA field plots of 'Incredible' sweet corn and Schlessman hybrid SH3077 popcorn at the Iowa State University

Bass Farm in Boone County, IA. Leaves had been inoculated by spraying to runoff at growth stage V8 and again at growth stage VT with a suspension of *X. vasicola* pv. *vasculorum* IA strain Har-1 at 10^7 CFU/ml. Additional contaminated seed lots were generated from inoculated field plots in IA in 2018. Potential *X. vasicola* pv. *vasculorum*-contaminated seed was obtained from pinprick and/or double sprayed popcorn (SH3077) and dent corn (Syngenta hybrid N61P-3000GT) (Table 1). Pinprick inoculations were done on the leaves with a sterile needle by dipping in bacterial suspension (Block et al. 1998). Ears were collected at maturity, dried, shelled, and stored as previously described.

Detection of *X. vasicola* pv. *vasculorum* from maize seeds. Two approaches were evaluated for the detection of *X. vasicola* pv. *vasculorum* from maize seeds. Pathogen extraction was based on soaking 400-seed subsamples in 50 to 75 ml of sterile $1\times$ phosphate buffered saline (PBS; pH 7.4) containing 0.02% Tween 20 plus thiabendazole at 20 mg/liter. Seeds were placed in 500 ml Erlenmeyer flasks on a shaker at 120 rpm and soaked for 4 h at 4 to 8°C . The resulting suspension was divided into two subsamples, one for DNA extraction and the other for dilution plating onto semiselective agar media. DNA extraction of seed wash aliquots was followed by PCR testing with conventional and Taqman qPCR.

PCR test (Taq-Man and conventional PCR). Approximately 10 ml-seed extracts were concentrated by centrifugation (Allegra 25R Centrifuge, Beckman Coulter Inc., Brea, CA) at low speed (400 relative centrifugal force [RCF] for 5 min) to clear the subsample of particulate debris. The supernatant was decanted into a sterile tube and further centrifuged at 15,300 RCF for 10 min to precipitate bacteria. The supernatant was discarded and the pellet resuspended by vortexing with 567 μl of Tris-EDTA buffer at pH 7.5. Genomic DNA was extracted with a CTAB extraction protocol (Wilson 2001) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Science Inc., MA, U.S.A.). TaqMan PCR was performed using a standard TaqMan PCR protocol on an Applied Biosystems (Foster City, CA) Step One Plus. The 10- μl PCR mixture included 2 μl (20 to 25 ng/ μl) of DNA template, 5 μl of $2\times$ PrimeTime Gene Expression Master Mix (P/N: 1055772, Applied Biosystems), 0.2 μl of TaqMan probe (56-FAM/ATC CAC AAA/ZEN/GCC ATC CGA AGC G/3IABkFq), 0.8 μl of forward primer (GCA AAC CAA ATA CAG CCG ATC) and 0.8 μl of reverse primer (AAG CGT CAT TAT AGC CTC ACC). The reactions were incubated in a 96-well plate at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers and probe for this assay were designed using the PrimerQuest Tool (Integrated DNA Technologies) with default parameters. We targeted the 207-bp Xvv3 region identified by Lang et al. (2017) for primer design. The amplicon is 107 bp in length. An *X. vasicola* pv. *vasculorum*-DNA positive control and a no template control of PCR-grade water were included in all runs. To test for potential PCR inhibitors, qPCR was conducted with both spiked and nonspiked seed wash extracts.

An additional conventional PCR assay was used to verify that different primer sets amplify the target. This assay was performed as described by Lang et al. (2017). Briefly, the extracted DNA (2 μl , 20 to 25 ng/ μl) was added to a reaction mixture (18 μl) consisting of 10 μl of Go Taq Green Master Mix, $2\times$ (Promega, Madison, WI, U.S.A.), 0.8 μl of each Xvv3 primer at 10 μM (Xvv3F, CAAGCAGAGCATGGCAAAC; Xvv3R, CACGTAGAACCGG TCTTTGG) and 6.4 μl of water. Amplification was performed on an Eppendorf (Hamburg, Germany) Mastercycler Nexus Gradient PCR cycler. Samples were denatured at 95°C for 10 min; 30 cycles of amplification at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. The reaction was completed by a final 5 min extension at 72°C . Aliquots of amplification products were separated on 1.5% agarose gel in $1\times$ TBE buffer at 120 V for 60 min and visualized by SafeView Classic (Applied Biological Materials Inc., Richmond, BC, Canada) staining and UV transillumination. A 100-bp DNA ladder (EZ Load BioRad, CA, U.S.A.) was used as a molecular size marker.

Standard curves, detection limit, and specificity of TaqMan PCR. Standard curves were generated with 10-fold serial dilutions of cell suspensions (10^2 to 10^8 CFU/ml) of freshly cultured *X. vasicola* pv. *vasculorum* strain Har-1 according to the procedure described by Stulberg et al. (2020). The serial dilutions of cell suspensions were made in autoclaved water and in 4-h maize seed wash extract to evaluate the matrix effect. Limit of detection, linearity, and amplification efficiency were determined. The experiment was repeated three times.

To evaluate the specificity of the Xvv3 primers, 20 to 25 ng/μl of purified genomic DNA was tested from *X. vasicola* pv. *vasculorum*

strains isolated from maize leaf/seed from multiple states of the United States and 3 ng/μl of purified DNA of the historical South African *X. campestris* pv. *zeae* strain SAM119. Specificity testing included the closely related strains of *X. vasicola* pv. *holcicola* from sorghum and non-*Xanthomonas* strains that can be mainly found contaminating maize such as *Pantoea*, *Acidovorax*, and *Pseudomonas* (Supplementary Table S1). Assay specificity was also determined by using 13 DNA extracts from maize leaf samples with BLS-like symptoms and 25 DNA extracts from pure cultures that represented several *Xanthomonas* spp. obtained from the National

TABLE 1. Evaluation of natural contamination of *Xanthomonas vasicola* pv. *vasculorum* on maize seeds collected from fields with bacterial leaf streak (BLS) disease symptoms for the years 2016 and 2017

Field number	State	County	Field name	BLS disease severity ^a	Targeted PCR amplification of <i>X. vasicola</i> pv. <i>vasculorum</i> ^b	Ct value (positive range)
Preharvest sample, 2016						
1	IA	Tama	Lincoln 1	+	0/3 subsamples	
2	IA	Grundy	Lincoln 2	+	0/3 subsamples	
3	IA	Boone	Harcourt-1	++	0/3 subsamples	
4	IA	Boone	Harcourt-2	+	0/3 subsamples	
5	IA	Tama	Lincoln 3	+	0/3 subsamples	
6	IA	Tama	Lincoln 4	++	0/3 subsamples	
7	IA	Carroll	Carroll-1	++	1/3 subsamples	27.5
8	IA	Carroll	Carroll-2	+	0/3 subsamples	
9	IA	Carroll	Carroll-3	++	1/3 subsamples	29.7
10 ^c	NE	Holt (SE)	NE-1	++++	0/3 subsamples	
11	NE	Holt (C)	NE-2	++	0/3 subsamples	
12	NE	Clay	NE-3	+++	0/3 subsamples	
13 ^c	NE	Valley	NE-4	++++	1/3 subsamples	29.5
14	NE	Dawson	NE-5	++	1/3 subsamples	28.9
15	NE	Dawson	NE-6	+++	0/3 subsamples	
16	NE	Dawson	NE-7	++	1/3 subsamples	27.8
17	NE	Dawson	NE-8	++	1/3 subsamples	29.4
18	NE	Lincoln	NE-9	++	0/3 subsamples	
19	NE	Perkins (SW)	NE-10	++	1/3 subsamples	27.5
20 ^{c,d}	NE	Perkins (SE)	NE-11	++	2/3 subsamples	25.6–27.8
21 ^c	NE	Chase	NE-12	++	2/3 subsamples	26.5–27.9
22	CO	Yuma	Eckley	+++	0/3 subsamples	
23	CO	Yuma	Wray-1	++	1/3 subsamples	28.8
24	CO	Yuma	Wray-2	+	0/3 subsamples	
25	CO	Yuma	Alvin	+++	2/3 subsamples	30.0–30.2
26	CO	Phillips	Holyoke-1	+++	1/3 subsamples	28.2
27	CO	Yuma	Yuma-1	++	0/3 subsamples	
28	CO	Yuma	Yuma-2	++	2/3 subsamples	29.8–30.5
At-harvest samples, 2016						
29	IA	Tama	Lincoln 1	++	0/3 subsamples	
30	IA	Tama	Lincoln 3	++	1/3 subsamples	28.4
31	IA	Boone	Harcourt-1	++	0/3 subsamples	
32	CO	Yuma	Eckley	+++	2/3 subsamples	30.4–30.9
33	CO	Yuma	Alvin	+++	2/3 subsample	30.1–30.4
34	CO	Phillips	Holyoke-1	+++	0/3 subsamples	
35	CO	Phillips	Holyoke-2	++	2/3 subsamples	29.4–29.7
36	CO	Yuma	Yuma-1	++	1/3 subsamples	28.8
37 ^d	CO	Yuma	Yuma-2	++	1/3 subsamples	28.9
At-harvest samples, 2017						
38 ^d	NE	Concord (NE)	NE-A-17	+	1/3 subsamples	30.6
39	NE	Clay Center (SC)	NE-B-17	++	2/3 subsamples	30.7–30.9
40 ^c	NE	North Loup (C)	NE-POP-A	++	0/3 subsamples	
41 ^c	NE	North Loup (C)	NE-POP-B	++	3/3 subsamples	29.5–30.1
42 ^{c,e}	IA	Boone	Pop-IA	++	3/3 subsamples	28.7–30.5
43 ^c	IA	Boone	IA-Sweet	++	3/3 subsamples	28.8–30.4
At-harvest samples, 2018						
44 ^{c,e}	IA	Boone	Pop-IA	++	0/3 subsamples	
45 ^{c,f}	IA	Boone	Pop-IA	++	0/3 subsamples	
46 ^f	IA	Boone	Dent-IA	++	0/3 subsamples	

^a Qualitative rating of BLS disease severity: +, low; ++, low to moderate; +++, moderate to severe; and +++, severe.

^b Number of 400-seed subsamples that tested positive by TaqMan PCR, defined as a cycle threshold (Ct) value ≤31 from three subsamples per field. PCR assay controls provided expected positive and negative results for each assay.

^c Popcorn samples. The rest of the samples are dent maize kernels.

^d Records in bold type indicate samples where live bacteria were recovered by dilution plating onto semiselective agar media.

^e Samples were harvested from artificially inoculated field plots. Inoculation method: double sprayed with 10^7 CFU/ml, *X. vasicola* pv. *vasculorum* Iowa stain HAR-1.

^f Samples were harvested from artificially inoculated field plots. Inoculation method: pinprick and double sprayed with 10^7 CFU/ml, *X. vasicola* pv. *vasculorum* Iowa stain HAR-1.

Collection of Plant Pathogenic Bacteria (NCPBP) (Stulberg et al. 2020) (Table 2).

Dilution plating on semiselective media. The original seed extract plus 10-, 100-, and 1,000-fold dilutions were plated in triplicate onto modified esculin hydrolysis agar medium. The medium was adapted from Arias et al. (2017) and consisted of esculin, 1 g; ferric ammonium citrate, 0.5 g; yeast extract, 5 g; anhydrous $\text{NH}_4\text{H}_2\text{PO}_4$, 0.5 g; $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$, 0.2 g; anhydrous K_2HPO_4 , 0.5 g; NaCl, 5 g; potassium bromide, 5 g; 16 g of Bacto-agar; and 400 μl of Botran (dicloran) from a stock solution of 250 mg/ml to 1 liter of deionized water. After autoclaving and cooling at 50°C, the medium was supplemented with thiabendazole (2 $\mu\text{g/ml}$), gentamicin (10 $\mu\text{g/ml}$), and trimethoprim (10 $\mu\text{g/ml}$). Plates were incubated for 5 to 7 days at 28°C. The strain HAR-1 was used as reference isolate of *X. vasicola* pv. *vasculorum* and was also plated in three dilutions onto the same medium. Plates were observed for the presence of brown glistening colonies similar in size and appearance to those of the reference isolate. Suspect colonies were subcultured onto yeast dextrose calcium carbonate (YDC) media to observe typical yellow mucoid *Xanthomonas* spp. colony appearance.

The identity of isolates was confirmed by PCR and by pathogenicity testing on susceptible maize seedlings. PCR was performed by the conventional PCR protocol of Lang et al. (2017), as described earlier. For pathogenicity, 10-day-old plants of maize inbred Mt42 were inoculated by injection and placed in a growth chamber at 25°C day/24°C night. One milliliter of inoculum ($\sim 10^8$ CFU/ml) in 1× PBS (pH 7.4) containing 0.02% Tween 20 was injected into plants near the base of leaf whorl with a 26 gauge tuberculin syringe. Control seedlings were injected with buffer alone. Seedlings were evaluated for symptoms 8 to 10 days after inoculation. Symptomatic seedlings were confirmed by dipping a cut leaf into sterile deionized water to examine for bacterial streaming, and confirmed as *X. vasicola* pv. *vasculorum* by PCR.

Detection of *X. vasicola* pv. *vasculorum* from seedling grow-outs. Seed transmission grow-out tests were conducted in the Iowa State University Department of Plant Pathology and Microbiology greenhouse facilities in Ames. Seed-to-plant transmission of *X. vasicola* pv. *vasculorum* was evaluated in eight seed samples from NE, CO, and IA fields that had exhibited moderate to high severity of BLS symptoms (Table 2). Seeds were planted in a commercial potting mix (Pro-Moss, Premier Horticulture Inc., Quakertown, PA, U.S.A.) and plants were grown under plastic tents in high humidity conditions with automated mist irrigation. The tents were constructed from PVC pipe frames (50 cm × 85 cm × 100 cm tall) and covered with clear plastic. Each frame was equipped with an overhead four-way mist nozzle and two plant trays (38 × 38 cm × 15 cm deep). The mist was set to run for 15 s every 6 h. Seedling grow-outs were conducted at 4-week intervals with 100 plants per tray. After 4 weeks, (i) the seedlings were visually examined for BLS symptoms; (ii) the first or second leaf of each plant was collected for PCR assay; and (iii) the fresh stems were cut for stem-printing onto agar to potentially recover live bacteria.

A stem-printing technique was used on greenhouse grow-outs to evaluate seed transmission of *X. vasicola* pv. *vasculorum* following the assay described by Block et al. (1998). Briefly, each plant was aseptically cut approximately 2 cm above the soil line and the cut cross-section gently pressed onto esculin agar medium to leave a stem imprint. All suspected *X. vasicola* pv. *vasculorum* colonies were subcultured onto YDC media at 28°C for 48 h and their identity was confirmed by PCR and pathogenicity testing as previously described.

Samples of the first or second leaf from each plant were pooled into 10-seedling groups for DNA extraction, except for seedlings from the at-harvest CO field Yuma-2 where individual seedlings were analyzed. The plant material was lyophilized in a freeze dryer. The lyophilized tissue was transferred to 15-ml conical plastic tubes with a sterile forceps and ground in a Geno-Grinder using four to six steel balls (4.5 mm) per tube. Samples that did not pulverize well were frozen in liquid nitrogen before grinding. DNA extraction was conducted on subsamples of 10 to 20 mg per tube, according to the methodology of Dietrich et al. (2002) or by automated DNA extraction using a KingFisher Flex (Thermo Fisher Scientific, Waltham, MA, U.S.A.). For the automated extraction, samples were prepared using the MagMAX Plant DNA Isolation kit (Thermo Fisher Scientific, MA, U.S.A.) according to manufacturer's recommended protocol. DNA quantification and PCR assays were performed as described earlier for detection of *X. vasicola* pv. *vasculorum* from maize seeds. Protocols were changed from a CTAB-based to a MagMAX total DNA purification method in order to increase sample throughput. The MagMAX method resulted in the same or better total DNA quality and quantity from leaf tissue.

Artificially contaminated seed lots. Inoculum was prepared by growing Har-1 and CO-5 *X. vasicola* pv. *vasculorum* strains for 48 h on Luria-Bertani (LB) agar plates and transferring those cells to LB broth for overnight incubation at 28°C. Bacterial suspensions were transferred to 50-ml polypropylene tubes and centrifuged at 15,300 RCF (Allegra 25R Centrifuge, Beckman Coulter Inc.) for 10 min at 21°C. The supernatant was discarded and the pellet was suspended in 1× PBS buffer (pH 7.4). The bacterial concentration of this material was determined by spectrophotometer absorbance at 600 nm and compared with a standard curve of absorbance versus bacterial concentration. The number of CFU/ml was standardized to an absorbance of 0.10 (approximately 10^8 CFU/ml). Tenfold dilutions in 1× PBS (pH 7.4) were made to create concentrations of *X. vasicola* pv. *vasculorum* inoculum, 10^5 , 10^6 , and 10^7 CFU/ml. Maize seeds were soaked for 8 h at 10°C with gentle agitation, followed by air drying to 10 to 11% moisture. The bacterial populations on the inoculated seeds were verified by dilution plating onto modified esculin hydrolysis agar (previously described). For each concentration, samples of 50 seeds were soaked in 10 ml of sterile 1× PBS (pH 7.4) containing 0.02% Tween 20 plus thiabendazole at 20 mg/liter for 4 h with gentle shaking at 10°C and 120 rpm. Aliquots of 100 μl of three serial 10-fold dilutions and the undiluted seed extract were spread onto agar in triplicate and incubated at 28°C for 4 to 6 days. The sample plates were examined

TABLE 2. Estimated seed-to-seedling transmission (based on PCR testing of seedlings) of *Xanthomonas vasicola* pv. *vasculorum* from naturally contaminated maize seed in greenhouse grow-out tests

State	County	Field name	Field season	BLS infection	Seedling survival	Seed transmission frequency ^a
NE	Perkins (SE)	NE-11	2016	Natural infection	3,720/4,000	0.55%
CO	Yuma	Yuma-2	2016	Natural infection	300/400	2.70%
CO	Yuma	Alvin	2016	Natural infection	1,521/1,600	0.40%
NE	Perkins (SW)	NE-10	2016	Natural infection	1,512/1,600	0.13%
NE	Concord (NE)	NE-A17	2017	Natural infection	1,405/1,600	0.14%
NE	North Loup	NE-POP-B	2017	Natural infection	1,504/1,600	0.20%
IA	Boone	IA-POP	2017	Spray-inoculated	1,531/1,600	0.20%
IA	Boone	IA-Sweet	2017	Spray-inoculated	929/1,600	0.00%

^a Estimates of seed transmission frequency using SeedCalc8. Pooled samples of 10 plants were tested by TaqMan PCR, except samples from Yuma-2 (CO), where individual plant samples were analyzed. Positive samples were defined as those with cycle threshold values of ≤ 31 .

for the presence of typical *X. vasicola* pv. *vasculorum* colonies by comparison with the positive control plates. Suspect colonies were confirmed by first subculturing onto YDC followed by PCR and pathogenicity testing. Colonies were counted and the average number of CFU/seed was calculated. Greenhouse grow-out tests were carried out under the same conditions as previously described. One hundred seeds per treatment were planted in the commercial potting mix and grown under high humidity. Four weeks after planting, symptoms of common BLS such as light brown-yellow streaks in the leaves, atypical symptoms, e.g., leaf scorching, wilt, nonspecific spots, and asymptomatic plants, were observed and recorded. The experiment was conducted twice. Seedlings also were assayed by stem-printing onto agar to recover live bacteria and by TaqMan PCR.

Statistical analyses. Estimates of seed transmission frequency from pooled seedling tissue samples were calculated using SeedCalc8. SeedCalc8 is a Microsoft Excel application available from the International Seed Testing Association website at https://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--3449--1102.html. SeedCalc uses binomial probability equations to estimate the percentage of contamination in a seed lot from results of pooled samples.

RESULTS

Occurrence of *X. vasicola* pv. *vasculorum* on maize seeds.

The occurrence of *X. vasicola* pv. *vasculorum* was determined in 45 samples of maize kernels from fields in IA, NE, and CO from the

2016 to 2018 growing seasons (Table 1). In 2016, 13 of 28 preharvest seed lots tested positive by qPCR (one or more, of three, subsamples). Positive samples were defined as those with cycle threshold (Ct) values of ≤ 31 . *X. vasicola* pv. *vasculorum* was more frequently detected from NE (7 of 12 seed lots) and CO (four of seven seed lots) seed samples than from IA samples (two of nine seed lots). The highest bacterial contamination, i.e., the most qPCR-positive subsamples, occurred in two NE popcorn seed lots (NE-11 and NE-12) and two dent maize seed lots from CO (Alvin and Yuma-2), all with two of three positive subsamples. However, live *X. vasicola* pv. *vasculorum* colonies were recovered by dilution plating only from the NE-11 seed lot. The estimated disease severity from the parent plants was not well-correlated with the molecular detection of *X. vasicola* pv. *vasculorum* from seed. The 2016 at-harvest samples demonstrated proportionally higher levels of contamination by *X. vasicola* pv. *vasculorum* than preharvest seeds; six of nine at-harvest samples had one or more qPCR-positive subsamples. Despite the pathogen being most frequently detected in CO seeds from Alvin, Eckley, and Holyoke-2 (two of three positive subsamples), live bacteria were isolated only from the Yuma-2 seed lot.

In 2017, *X. vasicola* pv. *vasculorum* was found in one or more subsamples from five of the six analyzed fields, with one negative seed lot (NE POP-A). The number of qPCR-positive subsamples was variable among NE fields, ranging from zero of three subsamples (NE POP-A) to three of three (NE POP-B); nevertheless, culturable bacteria were detected from only one seed lot, NE-A17. This isolate (Fig. 1) and previous seed isolates were confirmed as *X. vasicola* pv. *vasculorum* by qPCR and pathogenicity testing as described in the Materials and Methods. All six subsamples from IA fields Pop-IA (lot 42) and Sweet corn (lot 43) were qPCR-positive, but no culturable bacteria were recovered. In 2018, although two different inoculation methods were applied, none of the subsamples tested positive for the presence of the pathogen in or on seeds.

In summary, in 2016 and 2017, *X. vasicola* pv. *vasculorum* was detected by qPCR in one or more subsamples from 22 of 41 seed lots from naturally infected fields; the pathogen also was detected in two of five in seed lots from fields inoculated in 2017 and 2018. The highest numbers of contaminated subsamples were found in dent corn and popcorn from NE and CO that had low-to-moderate and moderate-to-severe BLS symptoms in the fields. Repeated isolation

Fig. 1. PCR and pathogenicity tests for confirmation of suspect colonies of *Xanthomonas vasicola* pv. *vasculorum* isolated from seeds from Concord, Nebraska (2017). **A**, Targeted amplification of *X. vasicola* pv. *vasculorum* by PCR assay. Lane L: DNA ladders (100 bp). Lanes 1, 2, and 3 (left to right): colonies 1, 2, and 3 for triplicate isolates from seed corn. Lane PC: *X. vasicola* pv. *vasculorum* positive control. Lane NC: no template control. The arrow indicates 200-bp fragment produced by the primers Xvv3F/Xvv3R (Lang et al. 2017). **B**, Maize leaves after 12 days postinoculation with *X. vasicola* pv. *vasculorum*. Symptoms of bacterial leaf streak are narrow, yellow-tan lesions with water-soaked appearance and necrotic areas.

Fig. 2. Confirmation by conventional PCR of *Xanthomonas vasicola* pv. *vasculorum* in Perkins, Nebraska seedlings (NE-11) grown from naturally infected seed lots. Samples from this field were detected by qPCR (see cycle threshold values) and by conventional PCR using different primer sets. Amplification of *X. vasicola* pv. *vasculorum* by conventional PCR assay. Lane L: DNA ladders (100 bp). Lanes 1, 2, 4, and 8 (left to right): corn seedlings 1, 2, 4, and 8 in duplicate. Lane PC: *X. vasicola* pv. *vasculorum* positive control. Lane NC: no template control. The arrow indicates 200-bp fragment produced by the primers Xvv3F/Xvv3R (Lang et al. 2017).

attempts from the majority of seed lots yielded no viable bacteria. Live bacteria were recovered in low numbers (e.g., one to three colonies detected per 400 kernel sample) from three fields (NE-11, 2016 preharvest sample; Yuma-2, 2016 at-harvest sample; and NE-A17, 2017 at harvest sample).

Seed transmission of *X. vasicola* pv. *vasculorum* in maize. Seed to seedling transmission of *X. vasicola* pv. *vasculorum* was investigated in greenhouse grow-out trials with eight seed lots: six from naturally infected maize fields in NE and CO, and two from inoculated field plots in IA (Table 2). In the initial grow-out study of 4,000 seeds from the NE-11 popcorn field, with a disease incidence of approximately 90% in the field, seedlings showed no disease symptoms during the 4-week period, but TaqMan PCR results suggested that *X. vasicola* pv. *vasculorum* was present in several of the symptomless seedling samples. The SeedCalc estimate of seed transmission frequency was 0.55% based on 20 of 372 pooled seedling samples (10 plants per pool) having Ct values of ≤ 31 . Of particular interest were three samples with Ct values of 21.46, 24.71, and 26.08, Ct values that were in the range of the positive bacterial controls. These samples also produced strong bands with conventional PCR, using the Xvv3F/Xvv3R conventional PCR primer set (Lang et al. 2017) (Fig. 2). We were unable to isolate *X. vasicola* pv. *vasculorum* bacteria from the lyophilized leaf tissues of these samples or from the fresh stems via stem-printing onto agar. The next two 2016 seed lots, NE-10 and Alvin, had 2 of 151 and 6 of 152 pooled samples with Ct values of ≤ 31 , corresponding to estimated seed transmission rates of 0.13 and 0.40%, respectively (Table 2). The highest seed transmission estimate came from Yuma-2, where 300 individual plants were tested. In total, 8 of 300 plants gave a putative positive qPCR signal, corresponding to a seed transmission rate of 2.7% (Table 2). Grow-outs were continued with 1,600-seed plantings of 2017 seed lots NE-A17, NE-POP-B, IA-POP, and IA-Sweet. NE-A17 had two pooled samples with Ct ≤ 31 , NE-POP-B and IA-POP had three each, and IA-Sweet had zero, but IA-Sweet showed a poor germination rate of 58%. No live bacteria were isolated from any seedling tissue samples. The estimated seed transmission rates from NE-A17, NE-POP-B, and IA-POP were similar at 0.14, 0.2, and 0.2%, respectively.

Seed transmission of *X. vasicola* pv. *vasculorum* from artificially contaminated maize seeds. Contaminated seeds from each of the three treated seed lots (seeds soaked in 10^5 , 10^6 ,

and 10^7 CFU/ml) carried average bacterial loads of 2.4×10^2 , 2.7×10^3 , and 0.8×10^4 CFU/seed, respectively (Table 3), as determined by dilution plating. Inoculated seeds were grown in the greenhouse as described previously. The estimated percentages of seedlings testing positive for *X. vasicola* pv. *vasculorum*, determined by qPCR assay of two-seedling pooled samples, were 44 and 46% from the 10^6 and 10^7 seed groups, respectively. Dramatically lower pathogen detection (2%) was observed in seedlings from the 10^5 CFU/ml group.

Symptoms of BLS were easily seen on seedling leaves between the third and fourth weeks after planting (Table 4). Typical BLS symptoms were thin yellow-brown leaf streaks, most apparent on the first true leaves (Fig. 3). Atypical symptoms such as leaf scorching or round to elongated leaf spots were observed, but not necessarily associated with infected plants, as they were found on noninfected control seedlings as well. With regard to seed transmission, the percentage of positive seedlings was predominantly associated with symptomatic samples. However, the pathogen was detected in 2, 9, and 12% of the asymptomatic seedlings from the 10^5 , 10^6 , and 10^7 groups, respectively (Table 4). All PCR-negative seedlings were symptomless.

Standard curves, detection limit and specificity of TaqMan PCR. Standard curves showed a linear correlation between Ct values and $\log_{10}$ bacterial cells mixed into water ($R^2 = 0.993$, slope = -3.623 , PCR efficiency = 93.69%) (Supplementary Fig. S1A) or mixed with negative corn seed wash water extract ($R^2 = 0.999$, slope = -3.4947 , PCR efficiency = 93%) (Supplementary Fig. S1B). No significant difference (P value = 0.48) was found between the slopes of two standard curves, indicating no inhibition due to seed constituents in the seed wash.

The qPCR Xvv3 assay was tested against DNA extracted from positive and negative pure bacterial cultures (Supplementary Table S1), on total DNA from leaf samples and *Xanthomonas* spp. from the NCPPB (Supplementary Table S2). Supplementary Table S1 showed that all seven *X. vasicola* pv. *vasculorum* strains were pathogenic on maize inbred Mt42 seedlings causing BLS symptoms and were correctly detected by qPCR Xvv3. Fourteen additional nontarget bacterial pathogens and saprophytes, mostly isolated from maize were undetected by qPCR. Supplementary Table S2 showed that qPCR Xvv3 assay correctly identified the positive leaf samples from different states. The culture collection consisted of 25

TABLE 3. Seed-to-seedling transmission of *Xanthomonas vasicola* pv. *vasculorum* from artificially contaminated maize seed in greenhouse grow-out tests

Seed soaking suspension (CFU/ml) ^a	Estimated bacterial population per seed (CFU/seed) ^b	Seedling germination ^c	Estimated seed transmission frequency (%) ^d
1×10^5	2.4×10^2	98/100	2%
1×10^6	2.7×10^3	96/100	44%
1×10^7	0.8×10^4	96/100	46%

^a Different levels of bacterial contamination were generated by soaking corn seeds in *X. vasicola* pv. *vasculorum* isolate CO-5 suspensions of 10^5 , 10^6 , and 10^7 CFU per ml for 8 h at 10°C.

^b Average bacterial concentration per seed as determined by seed wash and dilution plating.

^c Seedling survival after 4 weeks of greenhouse grow-out test.

^d Estimates of seed transmission frequency using SeedCalc8. Two-seedling pooled samples of each treatment were tested by TaqMan PCR. Positive samples showed cycle threshold values of ≤ 31 . Live bacteria were isolated from seedlings grown from 10^6 and 10^7 bacterial suspension levels.

TABLE 4. Relationship between bacterial leaf streak (BLS) symptoms and *Xanthomonas vasicola* pv. *vasculorum* PCR-positive seedlings from artificially contaminated maize seed

Seed soaking suspension (CFU/ml)	PCR-positive, total ^a	PCR-positive, typical BLS symptoms ^b	PCR-positive, atypical symptoms ^c	PCR-positive, no symptoms ^d	PCR-negative, no symptoms
1×10^5	4%	1%	1%	2%	96%
1×10^6	69%	40%	20%	9%	31%
1×10^7	71%	48%	11%	12%	29%

^a Percentage of seedlings from which *X. vasicola* pv. *vasculorum* was detected by TaqMan PCR.

^b Percentage of seedlings showing BLS symptoms.

^c Percentage of seedlings showing nonspecific symptoms of BLS.

^d Percentage of seedlings that were PCR-positive but symptomless.

Xanthomonas spp. strains (Supplementary Table S2). No off-target organisms were detected.

DISCUSSION

In the present study, we examined the role of maize seed in the epidemiology of BLS by *X. vasicola* pv. *vasculorum* and the potential transmission of the bacterium from seeds to seedlings. Low levels of *X. vasicola* pv. *vasculorum* contamination were found in seeds harvested from BLS-diseased fields; i.e., more than half of the samples collected had one or more 400-seed subsamples that tested positive by qPCR. However, culturable colonies were recovered from only three seed lots, suggesting that the level of bacterial seed contamination was minimal, even on seed from fields with high disease severity. This observation differs from that of Tubajika et al. (1998), who concluded that detection of *X. translucens* in wheat seeds was correlated with a threshold level (~15 to 35%) of BLS severity on the parent plants. In another wheat study, Schaad and Forster (1985) readily isolated *X. translucens* from naturally contaminated seeds and suggested that a minimum concentration of 10^3 CFU/ml of the bacterium in a seed wash was needed for disease to develop in the field. However, wheat seeds are more exposed in situ than maize seed, which is protected by the husk. BLS symptoms have not been observed on husk tissue.

There has been no evidence shown for systemic plant colonization, and thus we believe that seed contamination by *X. vasicola* pv. *vasculorum* is mainly superficial. Because corn husks typically cover the kernels during much of ear development, there seems to be little chance for kernel exposure until late in the season when husks loosen. Even when seeds were presoaked in bacterial concentrations ranging from 10^5 to 10^8 CFU/ml, a 1-min seed treatment in 1% sodium hypochlorite (NaOCl) resulted in no detectable *X. vasicola* pv. *vasculorum* from any seed lot compared with the control seeds (data not shown).

More than half of the subsamples tested positive by qPCR, but live bacteria were isolated from only three, which leads to uncertainty as to whether the bacteria were simply present in such low numbers that they were lost in dilution, poorly competitive, dead but still detected by PCR, or in a viable but nonculturable (VBNC) state. During the agar media development, we saw no drop-off in *X. vasicola* pv. *vasculorum* recovery from spiked samples on semiselective esculin media compared with non-selective LB media and do not believe that lack of competitiveness was a major problem. The VBNC state has been demonstrated in other xanthomonads such as *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* (Del Campo et al. 2009; Ghezzi and Steck 1999; Oliver 2010). A VBNC state would result in underestimation or false negative detection of the pathogen by a culture-based method.

Putative seed transmission events from naturally contaminated seeds were identified by qPCR, occurring at a frequency of 0.1 to 0.5% from pooled seedling data and 2.7% from individual plants. We were not able to isolate live bacterial colonies from any seedlings grown from naturally contaminated seeds, although the Ct values in the low 20s for two samples from seed lot NE-11 strongly suggests that bacteria were present. These findings are in line with those recorded by Tubajika et al. (1998) in wheat, who reported a low disease incidence ($\leq 3\%$) and high disease severity on plants grown from seeds harvested from diseased plants.

In this work, however, seed transmission and bacterial isolations were easily demonstrated from artificially contaminated seed lots, proving that seed transmission was indeed possible. The artificially contaminated seeds carried average bacterial loads of 10^3 to 10^4 CFU/seed resulting in seed transmission rates of 44 and 46%, respectively (Table 3). Seeds that were contaminated at level of 10^2 CFU/seed showed a seed transmission rate of just 2% (Table 3). These findings suggest that a threshold level of 10^2 CFU/seed may be needed for seed transmission to occur. Host resistance and

environment may also have a considerable influence on the frequency of seed transmission. Milus and Mirlohi (1995) reported *X. translucens* seed transmission from artificially contaminated wheat seeds under growth chamber conditions ranged from 4.0 to 24.5% for a susceptible cultivar and 0.0 to 0.3% for moderately resistant cultivar, but seed transmission was scarce in the susceptible cultivar under field conditions.

There is limited information regarding *X. oryzae* pv. *oryzicola* seed transmission of BLS in rice, but that pathosystem may be informative. Rao (1987) observed BLS symptoms in rice plants grown from seeds of severely affected cultivars during one summer season, without establishment of the disease during winter weather. Shekhawat and Srivastava (1972a) proposed that *X. oryzae* pv. *oryzicola* was carried upward from artificially contaminated seeds to the aerial parts of the seedlings when the first leaf emerged from the elongating plumule. However, the researchers (Shekhawat and Srivastava 1972b) could not isolate the pathogen from naturally infected seeds. In that study, BLS transmission was evaluated by planting naturally contaminated seeds of a mixture of rice varieties under field and laboratory conditions. Seedlings developed disease symptoms and the pathogen was isolated on a nonselective medium and its pathogenicity confirmed. Our seed transmission results would support the hypothesis that the first leaf might carry the bacteria to the aerial parts, as the first leaf was the most frequently and consistently symptomatic. However, to our understanding, the seed transmission studies in rice were not conclusive, and further investigations were recommended on pathogen identification and the influence of host plant resistance.

Interestingly, *X. vasicola* pv. *holcicola*, the causal agent of BLS of sorghum, shares hosts with *X. vasicola* pv. *vasculorum*, in that both bacteria can cause disease in maize, sugarcane and sorghum, with varying levels of severity (Lang et al. 2017). However, seed-related aspects, especially seed transmission, have not been shown for BLS in sorghum, and the potential importance of seed transmission is not clear.

A common theme throughout these studies is that plant disease severity and the likelihood for seed infection and seed transmission were variable, dependent on cultivar, bacterial populations, and environmental conditions. Epidemiological models for *Xanthomonas* and other foliar pathogens have shown that epiphytic bacterial populations are predictive of disease (Rouse et al. 1985; Stromberg 1998). Stromberg (1998) observed a strong increase in BLS symptoms in wheat when the *X. translucens* populations grew from 10^4 to 10^5 CFU/leaf, which may be due to density-dependent colonization or epiphytic pathogen survival on the phyllosphere. In this study, it was shown that transmission from artificially contaminated seeds was highly affected by *X. vasicola* pv. *vasculorum* population density, a phenomenon associated with bacterial quorum sensing (QS). This observation is similar to a previous study by Johnson and Walcott (2013), who showed that the

Fig. 3. Foliar symptoms of bacterial leaf streak on corn seedlings from artificially contaminated seeds with *Xanthomonas vasicola* pv. *vasculorum* under greenhouse conditions.

QS phenomenon contributes to seed transmission of *Acidovorax citrulli* in watermelon. *Xanthomonas* spp., including *X. vasicola* pv. *vasculorum*, produce exopolysaccharides that may be critical for biofilm formation and bacterial epiphytic survival. Similarly, in other plant pathogenic bacteria, exopolysaccharides, extracellular enzyme production, and other virulence factors are regulated for QS (Dow 2008; Johnson and Walcott 2013; Pirhonen et al. 1993). Future research should explore whether QS is associated with seed transmission of *X. vasicola* pv. *vasculorum*.

To our knowledge, this is the first study to investigate the occurrence and potential dissemination of *X. vasicola* pv. *vasculorum* on maize seeds. The work provides relevant insights regarding the likelihood for seed transmission of *X. vasicola* pv. *vasculorum*, demonstrating that maize seeds from natural disease outbreaks can be contaminated with low levels of *X. vasicola* pv. *vasculorum*, but that most seed samples did not contain culturable colonies. Levels of seed contamination that occurred under field conditions appeared to be too low to result in symptom development in seedlings. Seeds from a small number of the diseased fields produced qPCR-positive seedlings but they did not become symptomatic. This demonstrates the theoretical potential for seed transmission of *X. vasicola* pv. *vasculorum* but the risk of disease development from these PCR-positive seedlings seems unlikely, and fields with low disease severity appear to pose negligible risk of seed transmission.

ACKNOWLEDGMENTS

We thank Jillian Lang for providing primers for conventional PCR.

LITERATURE CITED

- Arias, S. L., Block, C. C., Mayfield, D. A., Jackson-Ziems, T. A., Broders, K. D., and Munkvold, G. P. 2017. Occurrence of *Xanthomonas vasicola* on maize seeds in the United States. (Abstr.) APS Annual Meeting. <https://apsnet.confex.com/apsnet/2017/meetingapp.cgi/Paper/5369>
- Block, C. C., Hill, J., and McGee, D. 1998. Seed transmission of *Pantoea stewartii* in field and sweet corn. *Plant Dis.* 82:775-780.
- Bockus, W., Bowden, R., Hunger, R., Morrill, W., Murray, T., and Smiley, R. 2010. Compendium of Wheat Diseases and Pests, 3rd ed. American Phytopathological Society, St. Paul, MN.
- Broders, K. 2017. Status of bacterial leaf streak of corn in the United States. In Proceedings of the Integrated Crop Management Conference, Iowa State University, Digital Press.
- Coutingo, T. A., and Wallis, F. M. 1991. Bacterial streak disease of maize (*Zea mays* L.) in South Africa. *J. Phytopathol.* 133:112-120.
- Damicone, J., Cevallos, F., and Olson, J. 2018. First report of bacterial leaf streak of corn caused by *Xanthomonas vasicola* pv. *vasculorum* in Oklahoma. *Plant Dis.* 102:437.
- Del Campo, R., Russi, P., Mara, P., Mara, H., Peyrou, M., De León, I. P., and Gaggero, C. 2009. *Xanthomonas axonopodis* pv. *citri* enters the VBNC state after copper treatment and retains its virulence. *FEMS Microbiol. Lett.* 298:143-148.
- Dietrich, C. R., Cui, F., Packila, M. L., Li, J., Ashlock, D. A., Nikolau, B. J., and Schnable, P. S. 2002. Maize Mu transposons are targeted to the 5' untranslated region of the gl8 gene and sequences flanking Mu target-site duplications exhibit nonrandom nucleotide composition throughout the genome. *Genetics* 160:697-716.
- Dow, M. 2008. Diversification of the function of cell-to-cell signaling in regulation of virulence within plant pathogenic xanthomonads. *Sci. Signal.* 1:pe23.
- Dyer, R. A. 1949. Botanical surveys and control of plant diseases. Farming in South Africa. Annu. Rep. Dep. Agric. S. Afr. 275:119-121.
- Ghezzi, J. I., and Steck, T. R. 1999. Induction of the viable but non-culturable condition in *Xanthomonas campestris* pv. *campestris* in liquid microcosms and sterile soil. *FEMS Microbiol. Ecol.* 30:203-208.
- Hartman, T., Tharnish, B., Harbour, J., Yuen, G. Y., and Jackson-Ziems, T. A. 2020. Alternative hosts in the families Poaceae and Cyperaceae for *Xanthomonas vasicola* pv. *vasculorum*, causal agent of bacterial leaf streak of corn. *Phytopathology* 110:1147-1152.
- ISF. 2019. International Seed Federation regulated pest list database. <https://pestlist.worldseed.org/public/pestlist.jsp>
- Jamann, T., Plewa, D., Mideros, S., and Bissonnette, S. 2019. First report of bacterial leaf streak of corn caused by *Xanthomonas vasicola* pv. *vasculorum* in Illinois. *Plant Dis.* 103:1018.
- Johnson, K. L., and Walcott, R. R. 2013. Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. *J. Phytopathol.* 161:562-573.
- Korus, K., Lang, J., Adesemoye, A., Block, C., Pal, N., Leach, J., and Jackson-Ziems, T. 2017. First report of *Xanthomonas vasicola* causing bacterial leaf streak on corn in the United States. *Plant Dis.* 101:1030.
- Lang, J., DuCharme, E., Ibarra Caballero, J., Luna, E., Hartman, T., Ortiz-Castro, M., Korus, K., Rascoe, J., Jackson-Ziems, T., and Broders, K. 2017. Detection and characterization of *Xanthomonas vasicola* pv. *vasculorum* (Cobb 1894) comb. nov. causing bacterial leaf streak of corn in the United States. *Phytopathology* 107:1312-1321.
- Leite, R., Jr., Custódio, A., Madalosso, T., Robaina, R., Duin, I., and Sugahara, V. 2018. First report of the occurrence of bacterial leaf streak of corn caused by *Xanthomonas vasicola* pv. *vasculorum* in Brazil. *Plant Dis.* 103:145.
- Milus, E., and Mirolohi, A. 1995. Survival of *Xanthomonas campestris* pv. *translucens* between successive wheat crops in Arkansas. *Plant Dis.* 79:263-265.
- Oliver, J. D. 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* 34:415-425.
- Ortiz-Castro, M. C., Jacobs, J. M., Plazas, M. C., Lang, J. M., Jackson-Ziems, T. A., Leach, J. E., Robertson, A. E., and Broders, K. D. 2018. Understanding the mechanisms of infection and survival of the maize pathogen *Xanthomonas vasicola* pv. *vasculorum*. (Abstr.) *Phytopathology* 108:S1.1.
- Perez-Quintero, A. L., Ortiz-Castro, M., Wu, G., Lang, J. M., Liu, S., Chapman, T. A., Chang, C., Ziegler, J., Peng, Z., and White, F. F. 2019. Genomic acquisitions in emerging populations of *Xanthomonas vasicola* pv. *vasculorum* infecting corn in the US and Argentina. *bioRxiv* 587915.
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO J.* 12:2467-2476.
- Plazas, M., De Rossi, R., Brücher, E., Guerra, F., Vilaró, M., Guerra, G., Wu, G., Ortiz-Castro, M., and Broders, K. 2018. First report of *Xanthomonas vasicola* pv. *vasculorum* causing bacterial leaf streak of maize (*Zea mays*) in Argentina. *Plant Dis.* 102:1026.
- Qhobela, M., Claflin, L., and Nowell, D. 1990. Evidence that *Xanthomonas campestris* pv. *zeae* can be distinguished from other pathovars capable of infecting maize by restriction fragment length polymorphism of genomic DNA. *Can. J. Plant Pathol.* 12:183-186.
- Qhobela, M., Leach, J., Claflin, L., and Pearson, D. 1991. Characterization of strains of *Xanthomonas campestris* pv. *holcicola* by PAGE of membrane proteins and by REA and RFLP analysis of genomic DNA. *Plant Dis.* 75:32-36.
- Rao, P. 1987. Across-season survival of *Xanthomonas campestris* pv. *oryzicola*, causal agent of bacterial leaf streak (BLS). *Int. Rice Res. Newsl.* 12:27.
- Roberts, S. J., and Koenraadt, H. 2015. 7-019a: Detection of *Xanthomonas campestris* pv. *campestris* on *Brassica* spp. Pages 1-16 in: International Rules for Seed Testing. International Seed Testing Association (ISTA), Bassersdorf.
- Robertson, A., Broder, K., French, R., Jackson-Ziems, T., Jardine, D., Korus, K., Lang, J., and Leach, J. 2016. Bacterial leaf streak. Crop Protection Network, CPN2008. <https://cropprotectionnetwork.org/resources/articles/diseases/bacterial-leaf-streak-of-corn>
- Rouse, D., Nordheim, E., Hirano, S., and Upper, C. 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. *Phytopathology* 75:505-509.
- Schaad, N., and Forster, R. 1985. A semiselective agar medium for isolating *Xanthomonas campestris* pv. *translucens* from wheat seeds. *Phytopathology* 75:260-263.
- Shekhawat, G., and Srivastava, D. 1972a. Mode of seed infection and transmission of bacterial leaf streak of rice. *Jpn. J. Phytopathol.* 38:4-6.
- Shekhawat, G., and Srivastava, D. 1972b. Epidemiology of bacterial leaf streak of rice. *Jpn. J. Phytopathol.* 38:7-14.
- Smith, D., Groves, C., Hudelson, B., and Lueloff, S. 2018. Bacterial leaf streak of corn confirmed for the first time in Wisconsin. Wisconsin Crop Manager Newsletter.
- Stromberg, J. 1998. Dynamics of Fremont cottonwood (*Populus fremontii*) and saltcedar (*Tamarix chinensis*) populations along the San Pedro River, Arizona. *J. Arid Environ.* 40:133-155.
- Stulberg, M. J., Santillana, G., Studholme, D. J., Kasiborski, B., Ortiz-Castro, M., Broders, K., Arias, S., Block, C., Munkvold, G., and Rascoe, J. 2020. Genomics-informed molecular detection of *Xanthomonas vasicola* pv. *vasculorum* strains causing severe bacterial leaf streak of corn. *Phytopathology* 110:1174-1179.
- Tubajika, K., Tillman, B., Russin, J., Clark, C., and Harrison, S. 1998. Relationship between flag leaf symptoms caused by *Xanthomonas translucens* pv. *translucens* and subsequent seed transmission in wheat. *Plant Dis.* 82:1341-1344.
- Wilson, K. 2001. Preparation of genomic DNA from bacteria. *Curr. Prot. Mol. Biol.* 56:2-4.
- Young, J., Dye, D., Bradbury, J., Panagopoulos, C., and Robbs, C. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. *N.Z. J. Agric. Res.* 21:153-177.