Multilocus Sequence Analysis Reveals Genetic Diversity in *Xanthomonas* Associated With Poinsettia Production

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Abstract

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Xanthomonas axonopodis pv. *poinsetticola* is traditionally identified as the primary causal agent of bacterial leaf spot on poinsettia (family Euphorbiaceae). Sixty-seven strains of *xanthomonads* isolated from lesions associated with several species within the family Euphorbiaceae were collected over a 64-year period. The pathogenicity of these strains was compared on several potential hosts and they were analyzed by multilocus sequence analysis (MLSA) using six housekeeping genes. The 67 *Xanthomonas* strains associated with poinsettia production were separated into three distinct clades based on MLSA. The first clade identified contained the *X. axonopodis* pv. *poinsetticola* reference strain (LMG849^{PT}). A second clade was more closely related to *X. hortorum* pv. *pelargonii* (LMG7314^{PT}) and the third clade contained the *X. codiae* type strain (LMG8678^T). This analysis indicated that there may also be other closely related pathovars or species of *Xanthomonas*

that can infect poinsettia. Strains from the three clades could not be distinguished by symptoms or virulence on poinsettia plants. Strains capable of infecting geranium were found in all three clades, although the extent of leaf spot formation and number of systemic infections were significantly less than those produced by *X. hortorum* pv. *pelargonii* strains, typically the main causal agent of bacterial leaf spot on geranium. Clade III also contained strains isolated from zebra plant (*Aphelandra squarrosa*, family Acanthaceae), which is a newly recognized host for *X. codiae* and *X. axonopodis* pv. *poinsetticola*. *Xanthomonas* leaf spot is a serious threat to poinsettia production that can be caused by several *Xanthomonas* spp. that can infect different ornamental plant hosts. It is imperative that growers maintain a strict sanitation program because reservoirs of inoculum can occur on a number of ornamental hosts.

Poinsettia (*Euphorbia pulchrimma*) is the number one flowering potted plant sold in the United States, with an annual wholesale value of over \$200 million (United States Department of Agriculture). Poinsettia cultivars have been produced and developed in the United States since the 1920s (Benson et al. 2002). Bacterial leaf spot of poinsettia, incited by *Xanthomonas axonopodis* pv. *poinsetticola*, was identified in U.S. poinsettia production in 1960 (McFadden and Mowry 1962). *X. axonopodis* pv. *poinsetticola* has since been described in every country where poinsettias have been cultivated.

Xanthomonads have also been associated with other members of family Euphorbiaceae. Crown-of-thorns (*Euphorbia milii*) (McFadden and Mowry 1962; Miller and Seymour 1972) and croton (*Codiaeum variegatum*) (Chase 1985) were described as hosts for *X. axonopodis* pv. *poinsetticola*. Both plant genera, like poinsettia, are popular ornamental crops. *Xanthomonas* strains isolated from croton were considered to be part of the pathovar *poinsetticola* based on biochemical tests and on their ability to cause similar symptoms on either poinsettia or croton (Chase 1985). Vauterin et al. (2000) later reclassified the strains from croton as *X. codiae*, based on further biochemical tests, metabolic profiles, and DNA-DNA homology.

Leaf spots on geranium (*Pelargonium hortorum*, family Geraniaceae) caused by *xanthomonads* are usually considered

to be caused by *X. hortorum* pv. *pelargonii*; however, we have isolated *X. axonopodis* pv. *poinsetticola* from geranium based on fatty acid methyl ester (FAME) analysis using the MIDI software (Sherlock ver. 4.5). Growers frequently report that symptoms of *Xanthomonas* sp.-caused diseases on geranium vary greatly from a few leaf spots to blights and wilts. They also report that the purchase of indexed *Xanthomonas* sp.-free plants does not preclude outbreaks of *Xanthomonas* leaf spot. Other ornamental crops within a given production facility are suspected to be reservoirs of pathogenic *xanthomonads*.

Within a phyto-bacterial species, host of origin is usually used as the primary factor in presumptive pathovar identification. Thus, there is a potential for misidentification and inaccurate inclusion of a strain within a pathovar. Phenotypic identification methods are frequently criticized for being less precise than genomic analysis. Phenotypic identification systems can be more precise if they are combined with genomic data to describe representative strains. Commonly used genomic methods such as repetitive extragenic palindromic sequence (Rep) PCR (Louws et al. 1994; Rademaker et al. 2000, 2005), amplified fragment length polymorphism (AFLP) (Bui Thi Ngoc et al. 2010; Janssen et al. 1996; Rademaker et al. 2000; Vos et al. 1995), and restriction fragment length polymorphism (RFLP) (Simões et al. 2007) were standard for *Xanthomonas* sp. determination until the late 1990s (Bui Thi Ngoc et al. 2010; Simões et al. 2007). DNA-DNA hybridization became the standard to identify species of *Xanthomonas* in the mid-1990s (Johnson 1994; Martens et al. 2008); however, DNA-DNA hybridization requires skilled technicians and standardization of techniques (Brenner 1973; Hildebrand et al. 1990; Johnson 1994; Marmur and Doty 1962).

In this study, we used multilocus sequence analysis (MLSA) (Almeida et al. 2010; Bui Thi Ngoc et al. 2010; Marcelletti et al. 2010; Martens et al. 2008; Young et al. 2008) to characterize the *Xanthomonas* strains associated with leaf spots on commercial poinsettias, other related euphorbias, and geranium. In MLSA, housekeeping genes

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conserved within a genus are sequenced and analyzed to determine phylogenetic relationships. When using MLSA to compare species within a genus, it is suggested to use at least five highly conserved genes that are universally distributed and are present in single copies at distinct locations within their respective chromosomes (Almeida et al. 2010; Martens et al. 2008). MLSA is considered to be superior to Rep-PCR, AFLP, and RFLP for species identification (Martens et al. 2008). For this study, we chose six housekeeping genes to examine *Xanthomonas* strains associated with poinsettia production.

Material and Methods

Bacterial strains. Sixty-seven *Xanthomonas* sp. isolated from poinsettia, crown of thorns, croton, zebra plant, geranium, nandina, and a number of type strains were utilized in this study (Table 1). These strains were isolated over a 64-year period, mainly from the Euphorbiaceae family. *Xanthomonas* strains isolated in 2010 from zebra plant (*Aphelandra squarrosa* in the family Acanthaceae) were also included in this study. Strains isolated from zebra plant were identified as *X. axonopodis* pv. *poinsetticola* by FAME analysis, with similarity coefficients >0.8. All strains were stored in 15% glycerol at -80°C and revived when needed.

Genomic DNA extraction, PCR protocols, and sequencing. Bacterial strains were revived from -80°C storage by streaking onto nutrient agar (NA; Becton, Dickinson, Sparks, MA) plates and incubating at 28°C . Single colonies were then restreaked twice to limit possible contamination. After 24 h of growth, genomic DNA was extracted using an illustra bacteria genomicPrep Mini Spin kit (GE, Pittsburgh, PA). Purified DNA was stored in elution buffer at -20°C .

Six gene loci (*fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA*) were sequenced using primers described by Almeida et al. (2010). Each amplification reaction contained 25 μl of GoTaq Green Master Mix (Promega Corp., Madison, WI) with 5.0 μl of both upstream and downstream primers, and 2 ng of DNA. Total volume was adjusted to 50 μl using sterile deionized water. Amplification of the DNA sequences was conducted in a thermocycler with the following thermal profile (Bio-Rad, Hercules, CA): a hot start of 95°C for 5 min; followed by 30 cycles of 95°C for 15 s, 54°C for 30 s, and 72°C for 60 s; and a 10-min extension at 72°C in the last cycle (Hong et al. 2012). The annealing temperature was adjusted according to the primers used. The PCR products were separated on a 1.0% agarose gel and visualized with UV light via SYBR Green Gel stain. Individual products were sequenced using a Sanger sequencer (Interdisciplinary Center for Biotechnology Research, ICBR, University of Florida, Gainesville).

MLSA analysis. To determine the phylogenetic relationships of the strains, the sequences of each of the six genes were analyzed separately as well as combined. Initially, raw sequence data were assigned Phred quality scores, trimmed, and assembled using CodonCode Aligner (ver. 4.0.3; CodonCode Corp., Centerville, MA). Sequences were aligned using CLUSTALW implemented in MEGA 5.05 (Tamura et al. 2011; Thompson et al. 1994). Maximum-likelihood searches were conducted using MEGA5 according to the general time-reversible model with γ -distributed invariant sites parameter suggested for the data matrix by Modeltest (Posada and Crandall 1998). Using the same data matrix that was analyzed by MEGA and the model suggested by MODELTEST, Bayesian Markov chain Monte Carlo phylogenetic analysis was conducted with MrBayes 3 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). Each Markov chain in the Bayesian search was started from a random tree and run for 106 cycles, sampling every 100th cycle from the chain, with each tree being rooted with *Stenotrophomonas maltophilia* as an outlier. Four chains were run simultaneously (three hot and one cold). Each simulation was run twice and the default settings for the priors on the rate matrix (0 to 100), branch lengths (0 to 10), and proportion of invariant sites (0 to 1) were used. Stationarity of the sum of the natural log of the likelihood values of the trees in each of the four chains, weighted according to the temperatures of the chains, was evaluated by

monitoring likelihood values graphically. The initial 100 trees in each run were discarded as burn-in. The remaining trees were used to construct majority-rule consensus trees. Bayesian posterior probabilities for each clade were derived from trees remaining after discarding the burn-in samples. For ease of visual comparison with bootstrap values, we present these probabilities as percentage whole numbers (0 to 100). Six additional strains and their sequences were added to the analysis as references (Almeida et al. 2010).

Host range test. A limited host range experiment was conducted using the 67 strains listed in Table 1. Inoculations were replicated three times for each of the following plants: poinsettia ('Eckespoint Prestige Red'), crown-of-thorns ('Red Splendens'), zonal geranium ('Patriot Bright Red'), zebra plant ('Dania'), and two croton cultivars ('Gold Dust' and 'Gold Star'). Three plants of each variety were arranged in a single block. Bacterial strains were incubated on NA plates at 28°C for 48 h. A suspension of each strain was spectrophotometrically adjusted (A600) in saline (NaCl at 8.5 g/liter) to 1×10^8 CFU/ml. Approximately 150 ml of this bacterial suspension was sprayed onto the entire group of plants. Immediately after inoculation, plants were bagged for 24 h to maintain a humid environment. After 24 h, the bags were removed and the plants were visually observed for symptoms daily for 30 days. The host range test was repeated as described above.

Leaf spots were enumerated, with plants exhibiting greater than 100 leaf spots recorded as >100. Average leaf spot counts were tabulated from poinsettia, crown-of-thorns, geranium, and croton (Table 2). Frequently, large necrotic zones developed on the zebra plants (Supplementary Fig. S1) with no distinct leaf spots; thus, an alternate rating scale was utilized as follows: 0 = no symptoms, 1 = 1 to 5 leaf spots 5 to 20 mm in size, and 2 = large necrotic zones developed on leaves followed by defoliation. Severity of plant symptoms for each species was statistically compared between strains in the three distinct MLSA clades using analysis of variance (ANOVA) and least significant difference (LSD) procedures (SigmaPlot 12.5).

MLSA clustering revealed three distinct clades of xanthomonads infecting poinsettia plants. In order to gain a better understanding of how these populations affected geranium production, additional virulence tests were conducted. In the first test, we selected six representative strains: *X. axonopodis* pv. *poinsetticola* (LMG849^{PT}, isolated from poinsettia, clade I), unknown *Xanthomonas* sp. (X808, isolated from poinsettia, clade II), *X. codiae* (LMG8678^T, isolated from croton, clade III), unknown *Xanthomonas* sp. (X1730, isolated from geranium, clade III), *X. hortorum* pv. *pelargonii* (LMG7314^{PT}, isolated from geranium), and *X. campestris* pv. *campestris* (LMG568^{PT}, isolated from *Brassica oleracea*). In this test, approximately 14 cm of high zonal geranium (Patriot Bright Red) was spray inoculated as previously described but, after inoculation, plants were incubated in an environmental growth chamber (Big Foot AC-60; BioChambers, Winnipeg, MB, Canada) maintained at 30°C and 50% relative humidity, with a 12-h light cycle ($310 \mu\text{mol mm}^{-2}\cdot\text{s}^{-1}$). After 2 weeks, the number of leaf spots were enumerated on each plant (10 replicates) and representative spots were used to reisolate and confirm infection. After 4 weeks, a cross section of the plant stem at the soil line was surface sterilized, macerated, and streaked onto NA agar to check for systemic infection. This experiment was repeated three times and included a noninoculated control. The numbers of leaf spots were statistically compared between strains using ANOVA and LSD procedures (SigmaPlot 12.5).

In order to mimic wet humid conditions commonly observed in commercial production and see the effect on virulence, a second test was done with *X. codiae* (LMG8678^T, isolated from croton, clade III). Geranium plants were inoculated as described above, with the exception that plants were incubated in a greenhouse and misted every 10 min for 3 s during daylight hours. Leaf spot development was visually monitored for 4 weeks, and plants were sampled and checked for systemic infections as previously described. Testing

was repeated three times and numbers of leaf spots was statistically compared between strains using ANOVA and LSD procedures (SigmaPlot 12.5).

Results

MLSA analysis. MLSA analysis of the 67 strains in this study revealed three *Xanthomonas* populations capable of infecting poinsettia (Fig. 1). Clade I contained the largest percentage of strains, including the pathotype strain for *X. axonopodis* pv. *poinsetticola* (LMG849^{PT}). Strains within this clade were recovered from poinsettia, crown-of-thorns, and nandina (*Nandina domestica*, family Berberidaceae). In Florida, Nandina is frequently infected with a nondescribed *Xanthomonas* sp. FAME analysis identified strain X1676 as *X. axonopodis* pv. *poinsetticola*. MLSA placed strain X1676 on the margin of clade I (Fig. 1), which suggests that it may be a separate pathovar. Further support for this premise is that X1676 produced no symptoms on plants tested in this study.

Clade II contained eight strains closely related by MLSA to *X. hortorum* pv. *pelargonii*. Seven of the eight strains were isolated from poinsettia. Four *X. hortorum* pv. *pelargonii* strains, including the pathotype strain (LMG7314^{PT}), were located in a separate clade above clade II.

Clade III contained strains originally isolated from poinsettia, croton, geranium, zebra plant, and aglaonema. Clade III contained the *X. codiae* pathotype strain (LMG8678^T) described by Vauterin et al. (2000). *X. codiae* has never been described as a pathogen of geranium or aglaonema. Two strains isolated from geranium (X1721 and X1730) grouped into clade III and were isolated from a greenhouse production facility that also cultivated poinsettia. Strain X1720 was also isolated at the same time from the same facility as X1721 but from an infected poinsettia plant. All *Xanthomonas* strains isolated in Florida in 2010 from zebra plants grouped into clade III.

An additional subclade containing two *Xanthomonas* strains (X870 and X874), both isolated in Florida in 1989, was separated from clade III. At the time of collection, these strains were presumptively identified as *X. axonopodis* pv. *poinsetticola* based on their host of origin (*Euphorbia* sp.). These strains were clearly separated in this MLSA study from other clades; however, no other information exists regarding host species or pathovar designation. Both strains produced symptoms on poinsettia like *X. codiae* and *X. axonopodis* pv. *poinsetticola* strains.

Host range test. Poinsettias were the most susceptible of all the plants, with disease symptoms developing with most strains tested in this study (Table 2; Fig. 2). Symptoms varied from a few spots to >100 spots per plant. Leaves with greater than 75 spots quickly

Table 1. *Xanthomonas* strains utilized in this study including original host, date of isolation, and source

Lab ID	Original ID	Original host (species)	Date of isolation ^a	Source ^b
X54	DPI-083-6248	Poinsettia (<i>Euphorbia pulcherrima</i>)	1983	JWM
X87	PDD-225-84	Croton (<i>Codiaeum variegatum</i>)	1984	DDB
X202	GWS 2705B-85	Poinsettia (<i>E. pulcherrima</i>)	1985	GWS
X340	DPI-P87-4567	Poinsettia (<i>E. pulcherrima</i>)	1987	JWM
X349	066-2894	Poinsettia (<i>E. pulcherrima</i>)	1966	RS
X350	070-4220	Crown-of-thorns (<i>Euphorbia milli</i>)	1970	RS
X351	C. po 2	Poinsettia (<i>E. pulcherrima</i>)	<1987	JBj
X352	071-425	Crown-of-thorns (<i>E. milli</i>)	1971	RS
X353	066-2936	(<i>Euphorbia</i> sp.)	1966	RS
X356	071-424	Crown-of-thorns (<i>E. milli</i>)	1971	RS
X357	071-559	Crown-of-thorns (<i>E. milli</i>)	1971	RS
X358	071-604	Crown-of-thorns (<i>E. milli</i>)	1971	RS
X386	NZTCC 3278	Poinsettia (<i>E. pulcherrima</i>)	1972	NZTCC
X387	NZTCC 3279	Poinsettia (<i>E. pulcherrima</i>)	1972	NZTCC
X394	NZTCC 5730	Euphorbia acalyphoides	1965	NZTCC
X395	NZTCC 5779	Poinsettia (<i>E. pulcherrima</i>)	1950	NZTCC
X419	...	Poinsettia (<i>E. pulcherrima</i>)	1988	MREC
X506	A2726	Poinsettia (<i>E. pulcherrima</i>)	<1988	AMA
X507	A2727	Poinsettia (<i>E. pulcherrima</i>)	<1988	AMA
X516	A2737	Poinsettia (<i>E. pulcherrima</i>)	<1988	AMA
X517	A2738	Poinsettia (<i>E. pulcherrima</i>)	<1988	AMA
X525	A2746	Poinsettia (<i>E. pulcherrima</i>)	<1988	AMA
X541	A2762	Poinsettia (<i>E. pulcherrima</i>)	<1988	AMA
X616	DPI-071-604	Crown-of-thorns (<i>E. milli</i>)	1971	JWM
X644	DPI-066-2936	Poinsettia (<i>E. pulcherrima</i>)	1966	JWM
X661	PDD-1772-88	'Maria' (<i>Aglaonema commutatum</i>)	1988	DDB

(continued on next page)

^a Symbol "<" indicates date strain acquired, isolation date unknown.

^b Strains were obtained from the following laboratories: AMA = Anne M. Alvarez, Department of Plant Pathology, University of Hawaii at Manoa, Honolulu, HI, 96822; ATCC = American Type Culture Collection, Manassas, VA 20108; BCCM/LMG = Belgian Coordinated Collections of Micro-organisms, Brussel, Belgium; DAC =; DCH = Donald C. Hildebrand, Department of Plant Pathology, University of California, Berkeley, CA 94704; DDB = D. D. Brunk, Plant Disease Diagnostics, Inc., Apopka, FL 32703; DLT = Darryl L. Thomas, Syngenta Flowers PO Box 1349, Gilroy CA 95021; GWS = Gary W. Simone, Department of Plant Pathology, University of Florida, Gainesville, FL 32611; JWM = J. W. Miller, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL 32602; JBj = Jeffrey B. Jones, Department of Plant Pathology, University of Florida, Gainesville, FL 32611; MREC = Mid-Florida Research and Education Center, University of Florida, Apopka, FL 32703; NZTCC = New Zealand Type Culture Collection; SK = Sven Keil, IDENTXX GmbH, Maybachstrasse 50, 70469 Stuttgart, Germany; and RS = Robert Stall, Department of Plant Pathology, University of Florida, Gainesville, FL 32611.

^c Pathotype strain *Xanthomonas campestris* pv. *campestris*.

^d Pathotype strain for *X. axonopodis* pv. *poinsetticola*.

^e Pathotype strain for *X. axonopodis* pv. *begonia*.

^f Pathotype strain for *X. hortorum* pv. *pelargonii*.

^g Type strain *X. codiae*.

turned chlorotic and abscised. X661 isolated from aglaonema (family Araceae) was highly virulent on poinsettia but produced little to no symptoms on other host plants (Table 2; Fig. 2). Interestingly, even the *X. campestris* pv. *campestris* (LMG568^{PT}) type strain produced leaf spots on poinsettia ($\bar{x}$ = 30). There were no significant differences in number of leaf spots produced on poinsettia between the three MLSA clades.

Two cultivars of croton were utilized in this study (Gold Dust and Gold Star) and only three strains—X1903, X1904, and the *X. codiae* type strain (LMG8678)—produced symptoms on these croton cultivars. These three strains had identical sequences for the six genes used in this study. Gold Dust and Gold Star were reported as being very susceptible to *X. axonopodis* pv. *poinsettiicola* in the 1980s (Chase 1985). We also have no information on the croton cultivar from which strains were isolated. However, symptoms in this study were limited, with an average of <11 leaf spots per plant. Other strains in clade III isolated from croton did not produce symptoms on croton. Only strains from clades I and II produced leaf spots on crown-of-thorns, with no symptoms being observed with strains from clade III. Strains from all clades produced symptoms on zebra plants. However, disease caused by strains from clade III was significantly (P = 0.05) more severe than for strains from other clades. Clade III also contained all strains originally isolated from zebra plant.

Many of these strains were highly virulent on zebra plant, forming large, coalescing, necrotic leaf spots that eventually resulted in leaf abscission.

Geraniums were not previously considered to be a host of *X. axonopodis* pv. *poinsettiicola*. However, strains within clades I, II, and III produced leaf spot on zonal geranium. The highest number of leaf spots was observed with strains in clade III. However, there were no significant differences between the number of spots observed on geranium between clades I and III. The two strains in clade III (*X. codiae* type strains LMG8678^T and X1730) produced a limited number of leaf spots when geranium plants were maintained at 30°C (Table 3). Leaf spots produced by these strains were difficult to count at times because they developed primarily on the leaf margins and frequently merged. The average number of leaf spots per plant for these two strains from cluster III was significantly lower (P = 0.05) when compared with the high numbers ($\bar{x}$ = 230) produced by the *X. hortorum* pv. *pelargonii* (LMG7314^{PT}) pathovar reference strain. All the geranium plants (30/30) sprayed with the reference strain LMG7314^{PT} became systemically infected whereas only 13% (4/30) and 10% (3/30) of the plants were systemically infected when inoculated with strains LMG8678^T and X1730, respectively. Plants inoculated with LMG8678^T (*X. codiae* type strains) and incubated under mist developed foliar blight with

Table 1. (continued from previous page)

Lab ID	Original ID	Original host (species)	Date of isolation ^a	Source ^b
X699	DPI-P88-4093-4	Poinsettia (<i>E. pulcherrima</i>)	1988	JWM
X700	DPI-P88-4093-2	Poinsettia (<i>E. pulcherrima</i>)	1988	JWM
X701	DPI-P85-2020	Poinsettia (<i>E. pulcherrima</i>)	1985	JWM
X749	PDD-539-89	Poinsettia (<i>E. pulcherrima</i>)	1989	DDB
X773	DPI-88-5463-4	Croton (<i>C. variegatum</i>)	1988	JWM
X808	GWS-2365B2-88	Poinsettia (<i>E. pulcherrima</i>)	1988	GWS
X822	DPI-89-3258-1	Crown-of-thorns (<i>E. milli</i>)	1989	JWM
X835	PDD-3199-89	(<i>Euphorbia</i> sp.)	1989	DDB
X842	...	Crown-of-thorns (<i>E. milli</i>)	1989	MREC
X852	PDD-3349-89	Poinsettia (<i>E. pulcherrima</i>)	1989	DDB
X870	DPI-P89-4476-3	<i>Euphorbia</i> sp.	1989	JWM
X874	DPI-P89-4476-2	<i>Euphorbia</i> sp.	1989	JWM
X879	DPI-P89-5239-2	Poinsettia (<i>E. pulcherrima</i>)	1989	JWM
X979	LMG568 ^{PTc}	Cabbage (<i>Brassica oleracea</i>)	1957	ATCC
X1144	XPI137-A	(<i>Euphorbia</i> sp.)	<1990	DCH
X1302	...	Croton (<i>C. variegatum</i>)	1991	MREC
X1505	...	Croton (<i>C. variegatum</i>)	1993	MREC
X1634	...	Poinsettia (<i>E. pulcherrima</i>)	1997	MREC
X1676	...	Nandina (<i>Nandina domestica</i>)	1998	MREC
X1720	...	Poinsettia (<i>E. pulcherrima</i>)	2000	MREC
X1721	...	Geranium (<i>Pelargonium</i> sp.)	2000	MREC
X1730	...	Geranium (<i>Pelargonium</i> sp.)	2001	MREC
X1763	...	Poinsettia (<i>E. pulcherrima</i>)	2002	MREC
X1807	...	Crown-of-thorns (<i>E. milli</i>)	2004	MREC
X1848	...	Poinsettia (<i>E. pulcherrima</i>)	2010	MREC
X1877	LMG849 ^{PTd}	Poinsettia (<i>E. pulcherrima</i>)	1964	BCCM/LMG
X1878	LMG7228	Geranium (<i>Pelargonium</i> sp.)	1986	BCCM/LMG
X1879	LMG7303 ^{PTe}	Begonia (<i>Begonia</i> sp.)	1986	BCCM/LMG
X1880	LMG7314 ^{PTf}	Geranium (<i>Pelargonium</i> sp.)	1986	BCCM/LMG
X1881	MEX 1	Geranium (<i>Pelargonium</i> sp.)	2011	DLT
X1890	MEX 11	Geranium (<i>Pelargonium</i> sp.)	2011	DLT
X1891	613	Poinsettia (<i>E. pulcherrima</i>)	2009	SK
X1892	614	Poinsettia (<i>E. pulcherrima</i>)	2009	SK
X1893	616	Poinsettia (<i>E. pulcherrima</i>)	2010	SK
X1894	617	Begonia (<i>Begonia</i> sp.)	<2011	SK
X1898	612	Poinsettia (<i>E. pulcherrima</i>)	2007	SK
X1899	615	Poinsettia (<i>E. pulcherrima</i>)	2009	SK
X1901	...	Zebra plant (<i>Aphelandra squarrosa</i>)	2011	MREC
X1903	...	Zebra plant (<i>A. squarrosa</i>)	2011	MREC
X1904	...	Zebra plant (<i>A. squarrosa</i>)	2011	MREC
X1906	LMG8678 ^{Tg}	Croton (<i>C. variegatum</i>)	1987	BCCM/LMG

extensive merged leaf spots (Supplementary Fig. S2). The number of systemically infected plants also increased to 23% (7/30).

Discussion

Ornamental plants are propagated and grown in close proximity, frequently under overhead irrigation. Within greenhouse structures, both high temperatures and humidity are common during production. In addition, to maintain profitability, many greenhouse facilities employ an intense multicrop production system, with many different genera of plants produced in close proximity. These environmental conditions are conducive to the development of many types of bacterial infections. When these infections occur, a presumptive disease diagnosis and pathogen identification is done based on symptoms observed on associated hosts. Further complicating this process is the fact that the many *Xanthomonas* spp. and pathovars have poorly defined host ranges. Thus, pathogens may be misidentified based on symptoms. This is further complicated when incorrectly identified strains are then used to develop phenotypic or diagnostic tests.

Whole bacterial genome sequencing will eventually help correctly identify and classify *Xanthomonas* spp. and pathovars, and aid in the development of new identification methods. However, until the cost of whole-genome sequencing is reduced, MLSA is an effective alternative (Almeida et al. 2010; Bui Thi Ngoc et al. 2010; Marcelletti et al. 2010; Martens et al. 2008; Young et al. 2008). In our study, we observed three distinct xanthomonad populations that can infect poinsettia, representing at least two described species (*X. axonopodis* pv. *poinsetticola* and *X. codiae*). One cannot distinguish these pathogen groups based on symptoms or disease severity. There are other potential *Xanthomonas* spp. that have the potential to infect and cause similar symptoms on poinsettia. This was demonstrated by the *X. campestris* pv. *campestris* type strain causing symptoms on poinsettia. Clades reported in this study are similar to those observed by Young et al. (2008). Young's MLSA study used four genes that clearly separated *X. codiae* from *X. campestris* pv. *campestris*, *X. hortorum* pv. *pelargonii*, and *X. axonopodis* pv. *begoniae*. However, Young did not include *X. axonopodis* pv. *poinsetticola* strains in his study. Further work should be done to compare phylogenetic relationship and host susceptibility of

Table 2. Multilocus sequence analysis (MLSA) clade designation and pathogenicity of 67 *Xanthomonas* strains on four hosts, and date of strain isolation

MLSA clade	Lab ID	Poinsettia (<i>Euphorbia pulcherrima</i>) leaf spot count ^a	Geranium (<i>Pelargonium</i> sp.) leaf spot count ^a	Crown-of-thorns (<i>Euphorbia milli</i>) leaf spot count ^a	Zebra plant (<i>Aphelandra squarrosa</i>) severity 0 to 2 ^b	Date of isolation ^c
I	X54	57	3	0	0.83	1983
I	X340	74	3	0	0.5	1987
I	X349	42	3	0	0	1966
I	X350	70	5	5	0	1970
I	X352	62	0	0	0.6	1971
I	X353	0	0	0	0.17	1966
I	X356	71	0	0	0	1971
I	X357	96	3	5	0	1971
I	X358	76	0	0	0	1971
I	X394	100	0	0	0	1965
I	X395	100	0	0	0.3	1950
I	X507	80	0	0	0	<1988
I	X516	88	0	0	0.67	<1988
I	X517	100	0	0	0.17	<1988
I	X525	100	0	0	0.3	<1988
I	X541	100	0	0	0	<1988
I	X616	28	0	0	0.3	1971
I	X644	62	1	0	0	1966
I	X822	100	0	9	0	1989
I	X835	90	0	0	0	1989
I	X842	58	3	13	0.5	1989
I	X1144	100	2	0	0	<1990
I	X1676	0	0	0	0	1998
I	X1807	25	0	0	0.17	2004
I	LMG849 ^{PTd}	100	0	0	0.17	1964
I	X1891	100	0	9	0.5	2009
I	X1892	100	0	0	0.17	2009
I	X1893	100	0	0	0	2010
I	X1898	100	0	1	0	2007
I	X1899	100	0	0	0	2009
		$\bar{x} = 76.0$	$\bar{x} = 0.77$	$\bar{x} = 1.4$	$\bar{x} = 0.18$	
II	X351	100	0	10	0.3	<1987
II	X386	92	0	0	0	1972
II	X387	92	0	0	0.3	1972

(continued on next page)

^a Average leaf spots per plant species (greater than 100 leaf spots recorded as >100). Wilt indicates developed wilt symptoms.

^b Rating scale: 0 = no symptoms, 1 = 1 to 5 leaf spots 5 to 20 mm in size, and 2 = large necrotic zones developed on leaves followed by defoliation.

^c Symbol "<" indicates date strain acquired, isolation date unknown.

^d Pathotype strain for *Xanthomonas axonopodis* pv. *poinsetticola*.

^e Type strain *X. codiae*.

^f Pathotype strain for *X. hortorum* pv. *pelargonii*.

^g Pathotype strain for *X. axonopodis* pv. *begoniae*.

^h Pathotype strain *X. campestris* pv. *campestris*.

X. axonopodis pv. *poinsetticola* strains with other closely related *X. axonopodis* pathovars, including pvs. *citri*, *fuscans*, *euvesicatoria*, *perforans*, and *alaifae*.

Xanthomonas leaf spot remains a serious threat to poinsettia production. The disease can be caused by a number of *Xanthomonas* spp., including *X. axonopodis* pv. *poinsetticola*, *X. codiae*, *X. hortorum*, and other undescribed xanthomonads. *Xanthomonas* leaf spot on croton was first described in 1985 (Chase 1985) as *X. axonopodis* pv. *poinsetticola*. Vauterin and colleagues (1997, 2000) reclassified strains from croton as *X. codiae*. The *X. codiae* type strain (LMG8678^T) in clade III was grouped with other strains isolated from croton, poinsettia, geranium, aglonema, and zebra plant. Interestingly none of the strains in clade III were isolated before 1984. This suggests that *X. codiae* was introduced into the United States and became established around this time. In this study, few symptoms were observed on croton even when inoculated with strains isolated from croton. To explain this observation, one must examine the history of croton production in the United States. In the 1980s, production occurred in the United States, with growers usually propagating from their own stock plants. As economic conditions changed, propagation of cuttings shifted to Central American countries, with propagative cuttings being harvested from plants grown in fields. Because there is a zero tolerance for leaf spots on imported ornamental

plants, there was extensive pressure to select more resistant plants. Subsequently, the commercially available crotons were more resistant than earlier varieties and *Xanthomonas* leaf spot became a minor concern in croton production. This is not the case with crops like poinsettia, which are produced in greenhouses where exclusion, not resistance, is the major mechanism of *Xanthomonas* disease management.

Our data suggest that *Xanthomonas* infections on geranium are not limited to *X. hortorum* pv. *pelargonii* but can also be caused by *X. codiae* and, to a lesser extent, *X. axonopodis* pv. *poinsetticola*. The virulence of *X. codiae* strains on geranium is related to environmental conditions, with high humidity, warm temperature, and overhead irrigation directly affecting leaf spot formation and vascular spread.

Greenhouse conditions along with intense multicropping of various ornamental plant species provides a unique environment for the adaptation and expansion of host range. It is possible that *Xanthomonas* spp. can adapt over time to infect new hosts that would not occur under natural conditions. Thus, one must not assume that species of *Xanthomonas* have distinct host ranges and do not pose a threat to other crops. Environmental factors, including raised temperature, humidity, and leaf wetness, contribute to the infection of new hosts. *Xanthomonas* strains isolated from poinsettia and geranium and separated by MLSA in this study provide evidence of more extensive and complex host susceptibility. Ornamental growers should always

Table 2. (continued from previous page)

MLSA clade	Lab ID	Poinsettia (<i>Euphorbia pulcherrima</i>) leaf spot count ^a	Geranium (<i>Pelargonium</i> sp.) leaf spot count ^a	Crown-of-thorns (<i>Euphorbia milli</i>) leaf spot count ^a	Zebra plant (<i>Aphelandra squarrosa</i>) severity 0 to 2 ^b	Date of isolation ^c
II	X701	91	0	0	0.3	1985
II	X749	63	0	0	1	1989
II	X808	55	5	0	0.17	1988
II	X1634	32	0	0	0	1997
II	X1848	20	0	0	0.17	2010
...	...	$\bar{x} = 80$	$\bar{x} = 0.62$	$\bar{x} = 1.3$	$\bar{x} = 0.28$	...
III	X87	75	0	0	1	1984
III	X202	69	0	0	1.7	1985
III	X419	96	0	0	1	1988
III	X506	100	2	0	1	<1988
III	X661	73	0	0	0.17	1988
III	X699	89	0	0	2	1988
III	X700	85	4	0	2	1988
III	X773	88	0	0	2	1988
III	X852	76	0	0	2	1989
III	X870	63	0	0	0.17	1989
III	X874	56	0	0	0	1989
III	X879	71	0	0	2	1989
III	X1302	91	0	0	2	1991
III	X1505	100	3	0	1.7	1993
III	X1720	83	2	0	2	2000
III	X1721	67	0	0	2	2000
III	X1730	49	11	0	2	2001
III	X1763	100	1	0	2	2002
III	X1901	75	0	0	2	2011
III	X1903	0	0	0	1.7	2011
III	X1904	0.33	0	0	1.8	2011
III	LMG8678 ^{Te}	24	0	0	1.7	1987
...	...	$\bar{x} = 69.5$	$\bar{x} = 1.0$	$\bar{x} = 0$	$\bar{x} = 1.5$	...
<i>pelargonii</i>	LMG7228	0	Wilt	0	0	1986
<i>pelargonii</i>	LMG7314 ^{PTf}	0	Wilt	0	0.33	1986
<i>pelargonii</i>	X1881	18	Wilt	0	0	2011
<i>pelargonii</i>	X1890	0	Wilt	2	0	2011
...	...	$\bar{x} = 4.5$	...	$\bar{x} = 0.5$	$\bar{x} = 0.08$	...
<i>begonia</i>	LMG7303 ^{PTg}	0	0	0	1	1989
<i>begoniae</i>	X1894	0	0	0	0.17	2011
...	...	$\bar{x} = 0$	$\bar{x} = 0$	$\bar{x} = 0$	$\bar{x} = 0.6$	...
<i>campestris</i>	LMG568 ^{PTb}	30	0	0	0	1957

treat xanthomonad outbreaks as a serious threat to their total production. Infected plants should be removed and destroyed. Other plants within the nursery must be treated as possible pathogen reservoirs. We have shown in this study that high-value crops of poinsettia and geranium can be infected with multiple species of *Xanthomonas*.

Thus, it would be advised not to cultivate these plants in the same facility. Minor symptoms should never be ignored on these crops because they may represent a pathotype that has the potential of spreading to other hosts or becoming more virulent in a disease-conducive environment.

Fig. 1. Bayesian tree and bootstrap values generated from the combined multilocus sequence analysis of six housekeeping genes for *Xanthomonas* strains associated with poinsettia production.

Fig. 2. Virulence of strains from major clades on five different ornamental hosts.

Table 3. Pathogenicity of six representative *Xanthomonas* strains on geranium under controlled environmental conditions

Species	ID number	Original host	MLSA clade ^a	Average geranium leaf spots ^b	Percent systemic infection (number)
<i>Xanthomonas axonopodis</i> pv. <i>poinsetticola</i>	LMG849 ^{PTc}	Poinsettia	I	0	0
<i>X. sp.</i>	X808	Poinsettia	II	0	0
<i>X. codiae</i>	LMG8678 ^{Td}	Croton	III	5.7 ^e	13 (4/30)
<i>X. sp.</i>	X1730	Geranium	III	2.23 ^e	10 (3/30)
<i>X. hortorum</i> pv. <i>pelargonii</i>	LMG7314 ^{PTf}	Geranium	...	230	100 (30/30)
<i>X. campestris</i> pv. <i>campestris</i>	LMG568 ^{PTg}	Cabbage	...	0	0

^a MLSA = multilocus sequence analysis.

^b Average leaf spots per plant.

^c Pathotype strain for *X. axonopodis* pv. *poinsetticola*.

^d Type strain *X. codiae*.

^e Leaf spots developed on leaf margins and merged; thus, there is a probable underestimation.

^f Pathotype strain for *X. hortorum* pv. *pelargonii*.

^g Pathotype strain *X. campestris* pv. *campestris*.

Acknowledgments

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