Population Dynamics of *Xanthomonas* Associated with Bacterial Spot of Tomato and Pepper during 27 Years across Taiwan

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Abstract

Bacterial spot caused by *Xanthomonas* spp. is the second most important bacterial disease after bacterial wilt of tomato and pepper in Taiwan. To determine the species composition of the *Xanthomonas* population over 27 years (1989 to 2016) across the country, a large collection of strains from tomato ($n = 292$) and pepper ($n = 198$) were examined. In the 1989 to 1999 population, all strains ($n = 147$) from pepper and 95% strains ($n = 198$) from tomato were *X. vesicatoria*. In a 2000 to 2009 population from tomato ($n = 36$), 22% of the strains were *X. perforans* and the remaining 78% strains were *X. euvesicatoria*. In the 2010 to 2016 population, 92% of the strains ($n = 50$) from pepper were still *X. euvesicatoria* and the remaining 8% of the strains were *X. perforans*; however, 99% ($n = 58$) of the strains from tomato were *X. perforans*. All of the evaluated ($n = 25$) strains of *X. euvesicatoria* collected during 1990 to 2006 were tomato race T1. Four pepper races (P1, P2, P7, and P8) were identified in the *X. euvesicatoria* population. The strains of *X. vesicatoria* collected during 1989 to 1999 ($n = 8$) were tomato race T2 and strains of

X. perforans from tomato collected during 2010 to 2016 ($n = 12$) were race T4 (83%) and race T3 (17%). Four strains of *X. perforans* from pepper were race T4. All of the strains of *X. vesicatoria* and *X. perforans* caused a hypersensitive response in all pepper differentials. Biochemical characterization of representative strains ($n = 48$) showed that strains of *X. euvesicatoria* were negative on amylolytic test and positive on lipase and oxidative-fermentative (OF) tests. The strains of *X. vesicatoria* were positive on amylolytic and OF tests and were negative on the lipase test. All *X. perforans* strains showed positive reactions on three tests. Evaluation of the same 48 strains for the sensitivity to copper sulfate (50, 100, 200, 300, and 400 mg liter⁻¹) revealed that the majority of *X. euvesicatoria* (86%) and *X. perforans* (94%) strains in the 2010 to 2016 population were tolerant to copper sulfate. The findings suggest that management strategies and breeding programs should consider the new *X. perforans* species and their new races. The increased number of copper-sulfate-tolerant strains in the 2010 to 2016 population further shows the need for alternative options to copper for managing bacterial spot of tomato and pepper.

Tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum* spp.) are major vegetable crops and widely grown in temperate, tropical, and subtropical regions around the world. Historically, bacterial spot is an economically important disease of tomato and pepper worldwide (European and Mediterranean Plant Protection Organization 2013; Potnis et al. 2015). The disease incidence has increased recently in North America (Abbasi et al. 2015; Horvath et al. 2012), South America (Araújo et al. 2017), Africa (Kebede et al. 2014), Asia (Hamza et al. 2010; Osdaghi et al. 2016), and Europe (Beran et al. 2014). Bacterial spot symptoms can appear on all aboveground parts of the plants and include necrotic lesions and, sometimes, chlorotic halos surrounding the necrotic spots. As the disease progresses, spots coalesce, turn yellow, and may lead to defoliation, causing significant yield losses. Warm temperatures, high humidity, and prolonged leaf wetness due to either rain or dew are favorable weather conditions for the disease epidemics (Araújo et al. 2010; McInnes et al. 1988). *Xanthomonas* spp. causing bacterial spot are externally seed-borne and can also survive in the infected plant residue in soil or epiphytically on tomato plants during latent infection. Therefore,

contaminated seed and transplants can be important sources for disseminating the pathogen (European and Mediterranean Plant Protection Organization 2013; McGuire et al. 1991). Management of bacterial spot of tomato and pepper includes the use of healthy certified seed and pathogen-free seedlings; seed treatment with hot water; removal of weeds, volunteer plants, and infected host plants; and field rotations with nonhost crops (Ritchie 2000). Foliar sprays of copper-based fungicides combined with mancozeb or maneb are recommended to manage bacterial spot of tomato and pepper (Conover and Gerhold 1981). However, copper-insensitive strains of *Xanthomonas* spp. have been widely distributed in several vegetable-growing regions, including Canada (Abbasi et al. 2015), the United States (Ma 2015; Mixon 2012), and Australia (Martin et al. 2004).

Colony morphology and biochemical characteristics such as starch hydrolysis, lipase activity, oxidative-fermentative (OF) metabolism of carbohydrates, and carbon source utilization, among others, are commonly used to characterize the bacterial spot pathogen (European and Mediterranean Plant Protection Organization 2013; Jones et al. 2004; Lue et al. 2010). In recent years, molecular techniques such as genomic fingerprinting revealed by pulse-field electrophoresis, species-specific molecular markers, multilocus sequence analysis (MLSA), and whole-genome studies have also been used for the characterization and taxonomic classification of bacterial spot pathogens (Jones et al. 2004; Schwartz et al. 2015; Timilsina et al. 2015). The causal agent of bacterial spot of tomato was first named as *Bacterium vesicatorium*, (Doidge 1920) and the pathogen was renamed as *Xanthomonas campestris* pv. *vesicatoria* (Dye 1978). Based on pathogen genetic and biochemical characteristics, four distinct groups (A, B, C, and D) were reported (Jones et al. 2004; Stall et al. 1994). Jones et al. (2004) reclassified the groups as four distinct species: *X. euvesicatoria* (group A), *X. vesicatoria* (group B), *X. perforans* (group C), and *X. gardneri* (group D).

Compared with bacterial wilt (*Ralstonia solanacearum*), host resistance for bacterial spot in tomato and pepper was not widely exploited; therefore, modes of inheritance of disease resistance in early cultivars were not well understood. However, both major resistance (*R*)-gene-mediated (hypersensitive) and quantitative resistance

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governed by multiple genes were reported (Stall et al. 2009). Based on hypersensitive reactions (HR) and susceptible reactions on tomato differentials, four tomato races (T1 to T4) of *Xanthomonas* were identified. In tomato, several sources for bacterial spot resistance were identified. Three recessive resistant genes (*rx1*, *rx2*, and *rx3*) were identified in Hawaii 7998 of *S. lycopersicum* (Yang et al. 2005), resistant to race T1. However, strains of races T2 and T3 overcome the resistance in Hawaii 7998. The resistant gene *Xv3* identified in PI128216 (*S. pimpinellifolium*) and Hawaii 7981 (*S. lycopersicum*) causes a HR and resistance to strains of race T3 but not against strains of race T4 (Stall et al. 2009). The resistant gene *Xv4* was identified in *S. pennellii*, which causes an HR against strains of races T3 and T4. Similarly, 11 pepper races (P0 to P10) were identified using pepper differentials. Five dominant genes (*Bs1*, *Bs2*, *Bs3*, *Bs4*, and *BsT*) and two recessive genes (*bs5* and *bs6*) were identified in three *Capsicum* spp. (*Capsicum annuum*, *C. chacoense*, and *C. pubescens*) (Stall et al. 2009).

Different species of *Xanthomonas* associated with bacterial spot of tomato and pepper have been reported from temperate, tropical, and subtropical countries (Araújo et al. 2017; Hamza et al. 2010; Horvath et al. 2012; Kebede et al. 2014). *X. euvesicatoria* and *X. vesicatoria* were reported worldwide historically (Potnis et al. 2015). In recent years, the frequency of *X. perforans* has increased rapidly in several tomato-producing regions of the world, including the United States (Ma 2015; Horvath et al. 2012), Canada (Abbasi et al. 2015), Brazil (Araújo et al. 2017), and Ethiopia (Kebede et al. 2014). *X. gardneri* has also been reported in these countries (Abbasi et al. 2015; Araújo et al. 2017; Kebede et al. 2014; Ma 2015).

In Taiwan, tomato and sweet pepper are widely cultivated during the dry season (from October to April). Tomato and sweet pepper are also grown from July to October as an off-season crop in highland areas, whereas chili pepper is commonly grown in the rainy months of March to September. Because warm, humid, and wet weather conditions prevail during most of the vegetable production period in Taiwan, bacterial diseases are common production constraints on both crops. Among the bacterial diseases of tomato and pepper, bacterial spot caused by *Xanthomonas* spp. is the second most important disease after bacterial wilt caused by *R. solanacearum* (Lin et al. 2014; Lue et al. 2010). Although bacterial spot infection is common in Taiwan, very limited studies have been conducted for disease survey, pathogen characterization to species level, and host resistance. In addition, little is known about the temporal shift of the species of *Xanthomonas* and copper sensitivity to the bacterial strains. In this study, we characterized a large population of *Xanthomonas* spp. associated with bacterial spot of tomato and pepper over 27 years (1989 to 2016) from major production areas in Taiwan. The objectives were to (i) identify species of *Xanthomonas* associated with bacterial spot of tomato and pepper in Taiwan and understand the dynamics in the species composition over two and a half decades across diverse regions, (ii) characterize the race structure and biochemical properties of these strains, and (iii) assess the shift in the sensitivity to copper sulfate among the strains over time.

Materials and Methods

Bacterial strains. Bacterial strains were isolated from leaf, stem, and fruit samples of tomato and pepper infected with bacterial spot from diverse locations across Taiwan (Table 1; Fig. 1). Samples were collected during 1989 to 2016 from production fields during routine field visits and disease surveys as well as experimental plot areas established by the World Vegetable Center. The samples were collected from a total of 82 locations across 13 counties, which cover northern, central, southern, and eastern regions of Taiwan (Table 1; Fig. 1). The infected tissues were washed with sterile water, surface sterilized with 70% ethanol, and dried. Thereafter, small pieces of symptomatic tissue were cut, kept on a sterile glass slide, and milled with a glass rod, adding a few drops of sterile water. The suspensions from the milled tissue were streaked onto nutrient agar (NA) media (15 g of agar, 3 g of beef extract, 5 g of peptone, and 1 liter of distilled water) with a sterile loop, and the plates were incubated at 30°C for 48 to 72 h. The suspected colonies (pale or bright yellow, circular, and mucoid) were transferred to NA and, thereafter, to 523 media

(Kado and Heskett 1970) for further purification of the bacterial colonies. All bacterial strains were preserved in 30% glycerol at –80°C for further use.

Molecular characterization for species identification. Bacterial strains ($n = 490$) (Table 1) preserved at –80°C were revived in NA media. Freshly grown (24 to 48 h) bacterial strains were suspended with sterile water and a suspension of approximately 5×10^8 CFU ml^{–1} (optical density at 600 nm [OD₆₀₀] = 0.3) were prepared. Boiled bacterial cells were used as a DNA template for polymerase chain reaction (PCR) amplification with species-specific primers (Table 2). Bacterial suspensions (5 µl, approximately 5×10^8 CFU ml^{–1}) were kept in a PCR tube and boiled for 5 min in a water bath for 5 min. The boiled bacterial suspensions were kept on ice and PCR reagents were added. In addition, strains of *X. campestris* pv. *campestris* from leafy mustard, cabbage, pak-choi, and choy-sum were also included. A total volume of 20 µl of reaction mixture of each sample was prepared for PCR. Each 20-µl reaction mixture consisted of 2.0 µl of 10× PCR buffer, 2.0 µl of 15 mM MgCl₂, 0.2 µl of *Taq* DNA polymerase at 1 U/ml, 1.6 µl of 2.5 mM dNTP, 0.5 µl of each forward and reverse primer (total volume of 4 µl) at 20 pmol µl^{–1} targeted for four *Xanthomonas* spp. (Table 2), 5.0 µl of bacterial suspension (approximately 5×10^8 CFU ml^{–1}), and 5.2 µl of deionized water. All PCR reagents were purchased from Yeastern Biotech Co. Ltd. All reactions were amplified using touchdown multiplex PCR. The amplification cycle consisted of one cycle of 5 min initial denaturation at 94°C; 10 cycles of 30 s at 94°C, 30 s at 65°C with a touchdown of 1°C per cycle, and 30 s at 72°C; 20 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and a final extension of 10 min at 72°C. Amplified products were separated in 1.5% agarose gels in 1× TAE (Tris-Acetate-EDTA) buffer, stained with ethidium bromide, and then visualized under UV light. A 100-bp DNA ladder was used to estimate the size of the amplified products.

Race characterization. In total, 49 strains, including *X. euvesicatoria* ($n = 25$), *X. vesicatoria* ($n = 8$), and *X. perforans* ($n = 12$) isolated from tomato and *X. perforans* ($n = 4$) isolated from pepper (Table 3), were selected for pathogenicity tests on tomato and pepper genotypes (Stall et al. 2009). Tomato genotypes were Walter (no *R* genes), Hawaii 7998 (genes *rx1*, *rx2*, and *rx3*), Hawaii 7981 (*Xv3*), and LA716 (*Xv4*). Pepper genotypes included Early Calwonder (ECW) (no *R* genes), ECW-10R (gene *Bs1*), ECW-20R (*Bs2*), ECW-30R (*Bs3*), and PI 234057 (*C. pubescens*, gene *Bs4*). Pathogenicity trials were conducted in the greenhouse under natural light, with a mean temperature of 27 to 29°C. The experiments were arranged in a split-plot design with three replications and six plants per differential host per replication. Bacterial strains were main-plot factors and host differentials were subplot factors. Both tomato and pepper seedlings with four fully expanded leaves were used to inoculate bacterial strains. Freshly grown bacterial strains (24 h old) on NA media were used to inoculate plant seedlings. A bacterial suspension of approximately 5×10^8 CFU ml^{–1} (OD₆₀₀ = 0.3) of each strain was infiltrated on the abaxial surface of the leaf near the midrib. Plants were evaluated at 24, 48, and 72 h after inoculation to record no reaction, HR, and susceptible reaction. HR was indicated by a rapid necrotic collapse of the infiltrated areas and was observed at 24 and 48 h postinoculation. A susceptible reaction was indicated by necrotic spots and, sometimes, chlorotic halos surrounding the necrotic spots. The evaluation of the plants continued for a week to confirm the susceptible reactions. Pepper races (P0 to P10) and tomato races (T1 to T4) were categorized based on hypersensitive or susceptible reactions on differential genotypes of pepper and tomato, respectively (Stall et al. 2009).

Biochemical characterization. In total, 48 strains ($n = 32$ from tomato and 16 from pepper), representative of the three *Xanthomonas* spp. collected during different time periods, were used for biochemical characterization (Table 4). Three biochemical assays were conducted: starch hydrolysis, lipase activity, and OF metabolism of carbohydrates (OF test). Experiments were repeated three times for each test. For all three biochemical tests, each bacterial strain was categorized as a positive or negative based on the chemical reactions.

Starch hydrolysis. Bacterial strains were characterized for amylolytic activity based on their reactions to starch agar medium (5 g of starch, 2.5 g of peptone, 1.5 g of beef extract, 7.5 g of agar, and 500 ml of distilled water) (Bouzar et al. 1994). Freshly grown (24-h-old) bacterial strains were spotted on plates. The plate was divided into four quarters and each quarter was used to test one sample. The bacterial strains on starch agar plates were incubated for 45 h at 30°C and observed for the formation of an opaque zone surrounding the bacterial colony. To confirm starch hydrolysis, the plates were flooded with Lugol's iodine (1% iodine and 2% potassium iodide) and observed within 5 min. A clear, unstained hydrolyzed zone in the stained colony indicates positive hydrolysis (Supplementary Fig. S2) whereas colonies changing to black indicates the negative reaction (Bouzar et al. 1994; Lelliott and Stead 1987).

Lipase activity. Lipase activity of the bacterial strains was tested on sterilized Tween 80 agar plates (peptone at 10.0 g liter⁻¹, sodium chloride at 5.0 g liter⁻¹, calcium chloride at 0.1 g liter⁻¹, agar at 15.0 g liter⁻¹, and 0.2 ml of Tween 80). To prepare media, Tween 80 was autoclaved separately and mixed before plating. Bacterial strains were spotted on the media plates and incubated for 5 days at 30°C. The plates were wrapped with parafilm to avoid moisture loss from the media. The plates were then observed for the development of a milky-white precipitate surrounding the colony, which indicates the positive lipase activity of the bacterial strains (Lelliott and Stead 1987).

OF test. Bacterial strains were inoculated in the OF semisolid medium (peptone at 1.0 g liter⁻¹, NH₄H₂PO₄ at 1.0 g liter⁻¹, KCl

at 0.2 g liter⁻¹, MgSO₄ · 7H₂O at 0.2 g liter⁻¹, bromothymol blue at 0.08 g liter⁻¹, and 90 ml of distilled water). To prepare the media, 0.27 g of agar was added to 90 ml of the abovementioned solution and pH was adjusted to 7.0 by adding 20% NaOH solution. Glucose solution (10% wt/vol) was prepared in a separate bottle. Glucose solution and peptone media were autoclaved separately and mixed thereafter. The media (180 µl) was transferred to a 200-µl microcentrifuge PCR tube and a bacterial strain was streaked on the media (adding one drop of mineral oil on one tube as a negative control) and incubated at 30°C. The PCR tubes were examined daily up to 3 days to observe color change. A change in color from green to yellow indicated the positive reaction.

Sensitivity to copper. Bacterial strains from *X. euvesicatoria* (*n* = 25), *X. vesicatoria* (*n* = 2), and *X. perforans* (*n* = 21) were evaluated for sensitivity to copper sulfate (CuSO₄ · 5H₂O). These strains represent all three periods (1989 to 1999, 2000 to 2009, and 2010 to 2016), hosts (*n* = 6 from pepper and *n* = 42 tomato), and diverse geography of Taiwan (Supplementary Table S1). The tolerant strain XVP-65 was used as a positive control and the sensitive strain XVP 40 as a negative control. These two strains were previously evaluated in the bacteriology unit of the World Vegetable Centre (data unpublished). Both strains were *X. euvesicatoria* and their sensitivity to copper sulfate was previously determined. Copper sensitivity of 48 bacterial strains was determined on NA media. Each strain was replicated three times per experiment and the experiment was arranged in a completely randomized block design. Experiments were

Table 1. Sampling details and species composition of xanthomonads associated with tomato and pepper in Taiwan collected during 1989 to 2016

Year, crop, region ^b	Name of counties	Number of		Frequency of <i>Xanthomonas</i> spp. ^a		
		Locations	Strains	Xeu ^v	Xves	Xper
1989–1999						
Pepper						
C	Chuanguhua, Yunlin, Nantou	8	46	46	0	0
S	Chiayi, Tainan, Pingtung	5	70	70	0	0
E	Yilan, Hualien, Taitung	7	31	31	0	0
Subtotal	...	20	147	147	0	0
Tomato						
N	Hsinchu	2	8	8	0	0
C	Taichung, Chuanguhua, Yunlin, Nantou	8	62	53	9	0
S	Chiayi, Tainan, Pingtung	13	53	53	0	0
E	Yilan, Hualien, Taitung	17	75	75	0	0
Subtotal	...	40	198	189	9	0
2000–2009						
Pepper						
S	Tainan	1	1	0	0	1
Subtotal	...	1	1	0	0	1
Tomato						
N	Taoyuan, Hsinchu	3	7	3	0	4
C	Miaoli, Yunlin, Nantou	4	10	8	0	2
S	Chiayi, Tainan	5	9	7	0	2
E	Yilan, Hualien	3	10	10	0	0
Subtotal	...	15	36	28	0	8
2010–2016						
Pepper						
N	Hsinchu	1	1	0	0	1
C	Changhua, Yunlin, Nantou	6	16	14	0	2
S	Tainan, Kaohsiung, Pingtung	5	18	18	0	0
E	Hualien, Taitung	4	15	15	0	0
Subtotal	...	16	50	47	0	3
Tomato						
N	Taoyuan	1	2	0	0	2
C	Nantou	9	35	1	0	34
S	Tainan, Pingtung	4	17	0	0	17
E	Hualien	1	4	0	0	4
Subtotal	...	15	58	1	0	57
Total	...		490	412	9	69

^a Xeu^v = *Xanthomonas euvesicatoria*, Xves = *X. vesicatoria*, and Xper = *X. perforans*.

^b Regions: N, C, S, and E = north, central, south, and east Taiwan respectively.

repeated twice and strains that showed inconsistent results in two experiments were repeated a third time. The bacterial strains preserved at -80°C were revived on NA. Bacterial suspensions of approximately 5×10^8 CFU ml^{-1} ($\text{OD}_{600} = 0.3$) were prepared from 24-h-old bacterial strains. Copper sulfate (Sigma-Aldrich) was amended in NA media before pouring it on plates to make final concentrations of 50, 100, 200, 300, and 400 mg liter^{-1} (0, 0.2, 0.4, 0.8, 1.2, and 1.6 mmol liter^{-1}). The media plates were divided to four quadrants, and 20 μl of bacterial suspension of approximately 5×10^8 CFU ml^{-1} was dropped in each quadrant. The spotted cultures were kept in the laminar flow for few minutes to allow them to air dry before

incubation. The bacterial cultures were incubated at 30°C for 3 days and bacterial growth was recorded. Bacterial strains able to grow in ≥ 200 mg liter^{-1} were categorized as tolerant and those did not grow in that concentration were categorized as sensitive to copper

Results

Species composition of bacterial strains. In total, 490 bacterial strains were isolated from the bacterial-spot-infected foliage and fruit of tomato ($n = 292$) and pepper ($n = 198$) from 1989 to 2016 across the four regions of Taiwan (Table 1; Figs. 1 and 2). Among the bacterial strains, 345, 37, and 108 strains were collected during the periods of 1989 to 1999, 2000 to 2009, and 2010 to 2016, respectively. The strains collected in three periods were defined as 1989 to 1999, 2000 to 2009, and 2010 to 2016 populations, respectively. All of these bacterial strains were visually identified as *Xanthomonas* spp. based on colony morphology (pale or bright yellow, circular mucoid) in NA and 523 media. Strains of *X. euvesicatoria*, *X. vesicatoria*, and *X. perforans* were identified using molecular markers, which amplified 560-, 197-, and 300-bp band sizes, respectively (Supplementary Fig. S1). None of the strains were *X. gardneri*. In the 1989 to 1999 population, 100% of strains from pepper ($n = 147$) and 95% of strains from tomato ($n = 198$) were *X. euvesicatoria* (Table 1; Fig. 2). Only 5% of strains (nine strains) collected from tomato from Nantou County of central Taiwan were *X. vesicatoria*.

In the bacterial population from 2000 to 2009 ($n = 36$) isolated from tomato, 78% of the strains were *X. euvesicatoria* and 22% were *X. perforans*. The frequency of *X. perforans* in the 2010 to 2016 population from tomato increased dramatically (99%, $n = 58$) (Table 1; Fig. 2). Only one strain isolated from Nantou county in 2016 was *X. euvesicatoria*. However, the vast majority of the bacterial strains from pepper in the 2010 to 2016 population were *X. euvesicatoria* (92%, $n = 50$). Only four strains—one strain from Tainan (south Taiwan) in 2007, one strain from Hsinchu county (north Taiwan) in 2015, and two strains from Nantou county (central Taiwan) in 2016—were *X. perforans*. These four strains were isolated from sweet pepper.

Race characterization. All eight strains of *X. vesicatoria* were identified as race T2 and all caused an HR on pepper (Table 3). Among the strains of *X. euvesicatoria* ($n = 25$), 5, 3, 5, and 12 were tomato race T1 and pepper race P1 (T1P1), T1P2, T1P7, and T1P8, respectively. Among the 12 strains of *X. perforans* isolated from tomato, 2 strains were race T3 and 10 strains were T4. The four strains of *X. perforans* isolated from pepper were race T4. All strains of *X. perforans* isolated from tomato and pepper produced HR 24 to 36 h after inoculations on all pepper genotypes, including susceptible cultivar ECW.

Biochemical characterization. All strains of *X. euvesicatoria* ($n = 25$), except one strain from pepper from the 1989 to 1999 population, showed a negative reaction on the amyolytic test (Table 4). All 25 strains showed positive reactions on lipase activity and the OF test. The *X. vesicatoria* strain ($n = 2$) showed positive reactions on starch hydrolysis and the OF test but a negative reaction on lipase activity. *X. perforans* strains ($n = 21$) showed positive reactions on all three biochemical tests. All of the strains of *X. euvesicatoria* isolated from tomato and pepper during different time periods (1989 to 1999, 2000 to 2009, and 2010 to 2016) showed similar biochemical reactions, which was also true among strains of *X. perforans*.

Fig. 1. Sampling sites of *Xanthomonas* spp. isolated from tomato and pepper across Taiwan.

Table 2. Polymerase chain reaction primer, sequence, and amplified product size used in this study to identify four species of *Xanthomonas* associated with bacterial spot of tomato and pepper

Targeted species	Primer name	Sequence	Product size (bp)	Reference
<i>Xanthomonas perforans</i>	HpaF-f	5'-GTG GCA GGC AGG CAA TCG ACG-3'	300	Ning 2012
	HpaF-r	5'-CCG GCA CGT CGA CGC CTG GAA ACC-3'	...	...
<i>X. euvesicatoria</i>	RST13	5'-TGG TTC CAG CCG TCC AGC AGG G-3'	560	Jones et al. 1993; Lue et al. 2010
	RST14	5'-CCC TAA AGG CAC TGG GCG TCG G-3'	...	...
<i>X. vesicatoria</i>	Xv-1f	5'-CCA ATT TAA CGA GCT GGC AT-3'	197	Lue 2003
	Xv-1r	5'-CCA TGG CGA TTT ACC AAG AT-3'	...	...
<i>X. gardneri</i>	BS-XgF	5'-TCA GTG CTT AGT TCC TCA TTG TC-3'	154	Koenraad et al. 2009
	BS-XgR	5'-TGA CCG ATA AAG ACT GCG AAA G-3'	...	...

Copper sensitivity test. Strains of xanthomonads showed variability in copper sensitivity (Table 4). All strains grew on media with copper sulfate at 50 mg liter⁻¹ (0.2 mmol liter⁻¹) but none of them grew on media with copper sulfate at 400 mg liter⁻¹ (1.6 mmol liter⁻¹). The strains that grew on copper sulfate at 100 mg liter⁻¹ were also able to grow at 200 mg liter⁻¹. The strains that did not grow on media with copper sulfate at 200 mg liter⁻¹ were categorized as sensitive strains. All of the sensitive strains did not grow

Table 3. Race characterization of strains of *Xanthomonas vesicatoria*, *X. euvesicatoria*, and *X. perforans* isolated from tomato and *X. perforans* isolated from pepper

Strains	Year	Region ^a	County	Town	Race
From tomato					
<i>X. vesicatoria</i>					
XVT-45	1991	C	Nantou	Chun Yang	T2
XVT-46	1991	C	Nantou	Chun Yang	T2
XVT-146	1994	C	Nantou	Pu li	T2
XVT-147	1994	C	Nantou	Chin Gin	T2
XVT-148	1994	C	Nantou	Chin Gin	T2
XVT-149	1994	C	Nantou	Chin Gin	T2
XVT-150	1994	C	Nantou	Chin Gin	T2
XVT-310	1994	C	Nantou	Chin Gin	T2
<i>X. euvesicatoria</i>					
XVT-28	1990	S	Tainan	Chiah Lih	T1P1
XVT-49	1991	C	Nantou	Chung Yang	T1P2
XVT-51	1991	C	Nantou	Shin Yi	T1P8
XVT-102	1993	C	Nantou	Hsin Yi	T1P8
XVT-106	1993	C	Nantou	Hsin Yi	T1P8
XVT-110	1993	C	Nantou	Chin Gin	T1P1
XVT-151	1994	C	Nantou	Hsin Yi	T1P2
XVT-77	1992	S	Tainan	Shanhua	T1P8
XVT-161	1994	S	Tainan	Shanhua	T1P8
XVT-163	1995	S	Tainan	Ching Liao	T1P8
XVT-236	1998	S	Tainan	Lung Tien	T1P7
XVT-245	1999	S	Tainan	Shan Shang	T1P7
XVT-88	1993	S	Chiayi	Ming Shung	T1P8
XVT-87	1993	S	Chiayi	Ming Shung	T1P8
XVT-176	1995	S	Chiayi	Shui Shang	T1P8
XVT-224	1997	S	Chiayi	Tai Pao	T1P7
XVT-117	1994	S	Pintung	Kao Shu	T1P8
XVT-121	1994	S	Pintung	Li Kang	T1P8
XVT-123	1994	S	Pintung	Li Kang	T1P8
XVT-221	1997	E	Yilan	Chuang Wei	T1P7
XVT-233	1998	S	Tainan	Chung Chei	T1P7
XVT-243	1999	E	Haulien	Rueysuey	T1P2
XVT-247	2000	S	Tainan	Kuan Tien	T1P1
XVT-248	2000	S	Tainan	Kuan Tien	T1P1
XVT-308	2006	S	Chiayi	Lioujeau	T1P1
<i>X. perforans</i>					
XVT-280	2008	S	Tainan	Tainan	T4
XVT-312	2011	S	Chiayi	Lioujeau	T4
XVT-283	2013	S	Tainan	Shanhua	T3
XVT-285	2013	C	Nantou	Hsin shiang	T4
XVT-290	2013	C	Nantou	Hsin shiang	T4
XVT-293	2013	C	Nantou	Rona, Sinyi	T4
XVT-298	2013	C	Nantou	Rona, Sinyi	T4
XVT-300	2013	C	Nantou	Tung Pu	T4
XVT-302	2013	C	Nantou	Tung Pu	T4
XVT-303	2014	C	Pingtung	Li Kang	T4
XVT-306	2015	N	Taoyuan	Yang Mei	T3
XVT-307	2015	N	Taoyuan	Yang Mei	T4
From pepper					
<i>X. perforans</i>					
XVP-205	2007	S	Tainan	Shanhua	T4
XVP-208	2015	N	Hsinchu	Hsiun Glin	T4
XVP-244	2016	C	Nantou	Rona Sinyi	T4
XVP-245	2016	C	Nantou	Rona Sinyi	T4

^a Regions: N, C, S, and E = north, central, south, and east Taiwan, respectively.

on media with copper sulfate at ≥ 100 ppm (0.4 mmol liter⁻¹). Bacterial strains that were grown at 200 mg liter⁻¹ were categorized as tolerant strains, based on the threshold described by Stall et al. (1986).

In both 1989 to 1999 and 2000 to 2009 populations of *X. euvesicatoria*, the majority of the strains (73 and 80%, respectively) were sensitive to copper sulfate (Table 4). The sensitivity of *X. euvesicatoria* strains changed substantially in the 2010 to 2016 population, where only one strain was sensitive to copper sulfate ($n = 7$) and the remaining six strains were tolerant. Two of five strains of *X. perforans* from the 2000 to 2009 population and 14 of 16 strains from the 2010 to 2016 population were tolerant to copper sulfate.

Discussion

This is the first comprehensive study in Taiwan to characterize the large population of xanthomonads ($n = 490$) associated with bacterial spot of tomato and pepper over two and a half decades. Host specificity was observed in the species composition of xanthomonads. Among the four species of xanthomonads, *X. euvesicatoria*, *X. vesicatoria*, and *X. perforans* were identified from tomato, whereas *X. euvesicatoria* and a few strains of *X. perforans* were identified from pepper. Strains of *X. gardneri* were not found in either host in our study, although this species has been reported in Canada (Abbasi et al. 2015), the east north-central region of the United States (Kim et al. 2010; Ma 2015), Brazil (Araújo et al. 2017), former Yugoslavia and Costa Rica (Jones et al. 2000), Ethiopia (Kebede et al. 2014), the southwest Indian Ocean region (Hamza et al. 2010), and Malaysia (Rashid et al. 2016). Araújo et al. (2010) reported that lower temperature is favorable for strains of *X. gardneri* to cause infection on tomato. In Taiwan, hot and humid weather typically exists during the tomato and pepper production seasons, which may not be very suitable for *X. gardneri*.

In tomato, a substantial shift in the species composition occurred over time. In the 1989 to 1999 population, 95% of the strains were *X. euvesicatoria* and the remaining 5% of strains ($n = 9$) were *X. vesicatoria*. *X. vesicatoria* strains were detected from tomato samples collected from Chin Gin ($n = 6$), Chun Yang ($n = 2$), and Pu li ($n = 1$), which are located in highland areas (approximately 1,200 to 1,700 m altitude above sea level) in Nantou County of central Taiwan. Historically, *X. vesicatoria* strains were reported worldwide (Potnis et al. 2015) and strains of *X. vesicatoria* were found to be pathogenic only on tomato (Bouzar et al. 1994; Hamza et al. 2010). Hamza et al. (2010) also reported *X. vesicatoria* from tomato in the southwest Indian Ocean countries of Madagascar and Réunion. In our study, none of the 1989 to 1999 population strains were *X. perforans*. Strains of *X. perforans* ($n = 2$) were first detected from tomato samples in Hsinchu County from North Taiwan in 2005. Thereafter, one strain of *X. perforans* each from Yunlin County, central Taiwan and Tainan County, southern Taiwan were identified in 2006. All of the strains collected after 2007, except for one strain from Nantou County in 2016, were *X. perforans* in tomato. Lue et al. (2010) also reported eight strains of *X. perforans* in Taiwan, the majority of which were *X. euvesicatoria* (40 of 53 samples); Lue et al. (2010) did not describe the years of bacterial collection and, therefore, it is not known when the *X. perforans* was collected and identified. Our study and other studies indicate a worldwide spread of *X. perforans* in tomato and subsequent displacement of *X. euvesicatoria*, including in Canada (Abbasi et al. 2015), the United States (Horvath et al. 2012), Brazil (Araújo et al. 2017), and Ethiopia (Kebede et al. 2014). In Florida in the United States, *X. perforans* strain T3 was first detected in 1991. *X. euvesicatoria* was the predominant species causing bacterial spot in tomato in the southern United States during that period (Jones et al. 1995). In contrast, 100% of the strains ($n = 377$) collected from 20 fields in 2006 in Florida were *X. perforans* (Horvath et al. 2012). In Brazil, 92% of strains collected during 2009 and 2012 from commercial tomato fields from 33 counties were *X. perforans* (Araújo et al. 2017).

Race characterization of tomato strains also showed a shift in race structure between the 1989 to 1999 population (*X. euvesicatoria*, $n = 25$ and *X. vesicatoria*, $n = 8$) and the 2010 to 2016 population (*X. perforans*, $n = 16$). All of the strains of *X. euvesicatoria* were T1. These

strains were also pathogenic on pepper differentials, with four pepper races (P1, P2, P7, and P8) identified. All of the *X. vesicatoria* strains were T2 and produced HR in pepper differentials. Similar to our finding, Kebede et al. (2014) also identified that *X. vesicatoria* from tomato in Ethiopia were T2 but these strains were P2. Interestingly, all of the *X. perforans* strains were T4 (87%) and T3 (13%). All *X. vesicatoria* and *X. perforans* strains showed HR in all pepper differentials, indicating that these strains were not pathogenic on pepper. Stall et al. (2009) reported four tomato races (T1 to T4) and 11 pepper races globally. Similar to our findings, strains of *X. euvesicatoria* race T1 were only present in Florida prior to 1991 (Jones et al. 1995). In the 2006 survey, about 77% of the strains were *X. perforans* race T4 and the remainder were race T3 (Horvath et al. 2012). The 2012 survey found only *X. perforans* race T4 ($n = 175$) (Timilsina et al. 2015). In Brazil, Araújo et al. (2017) also reported race T3 (97%) and race T4 (3%) among the 188 strains of *X. perforans* collected from 32 counties during 2009 to 2012. In central Ethiopia, Kebede et al. (2014) found about 59% of *X. perforans* strains from bacterial spot infected tomato samples and 93% of them were race T3.

Unlike tomato, we did not find a shift in the species composition of xanthomonads on pepper over time (1989 to 2016) in Taiwan. All of the strains from pepper in the 1989 to 1999 population ($n = 147$) and 92% of the strains in the 2010 to 2016 population ($n = 50$) were identified as *X. euvesicatoria*. *X. euvesicatoria* was also reported as a predominant species in pepper from Iran (Osdaghi et al. 2016), the northeast United States (Ma 2015), and Brazil (Areas et al. 2014). Osdaghi et al. (2016) identified all 20 strains as *X. euvesicatoria*, which were collected from 30 pepper fields in three regions (Hejavan, Bonab, and Koshksaray) of Iran, where severe outbreaks of bacterial spot occurred for the first time. Timilsina et al. (2016) found that all *X. euvesicatoria* strains from pepper collected over a 20-year period in the United States were clustered in one phylogenetic group (group 1), indicating that genetic homogeneity existed among the *X. euvesicatoria* strains from pepper. Future MLSA of *X. euvesicatoria* strains from pepper and tomato collected over time in Taiwan may reveal a different genetic structure. Four *X. perforans* strains were collected from sweet pepper in this study. The first strain of *X. perforans* observed on pepper came from Tainan County of south

Taiwan in 2007. The other three strains were detected in Hsinchu County in 2015 and Nantou County in 2016 (both counties are in central Taiwan). All four strains of *X. perforans* were race T4. Few strains of *X. perforans* were detected from pepper in our study and all of them produced HR on pepper genotypes, including susceptible pepper cultivar ECW, strongly suggesting that pepper may not be preferred host for the *X. perforans*. It is logical to raise the question of why the four strains of *X. perforans* were isolated from sweet pepper foliage if these strains are not causing susceptible reactions on ECW. A possibility is that these four pepper *X. perforans* strains were isolated from atypical lesions caused by the interaction between *avrBsT* and *BsT* (Minsavage et al. 1990), and the presence *avrBsT* in the *X. perforans* could be responsible for causing HR on ECW. Timilsina et al. (2016) also reported the presence of *avrBsT* in the strains of *X. perforans*. Previous studies also reported that strains of *X. perforans* that were pathogenic only in tomato (Bouzar et al. 1994; Hamza et al. 2010) were nonpathogenic in pepper (Kebede et al. 2014).

The specific reasons for the replacement of *X. euvesicatoria* by *X. perforans* on tomato in Taiwan and globally are not well understood. Several researchers speculated that global trade of *Xanthomonas*-contaminated vegetable seed could be responsible for the global spread of *X. perforans* and other new species (Araújo et al. 2017; Hamza et al. 2010; Osdaghi et al. 2016; Potnis et al. 2015). Hybrid tomato seed were produced and distributed widely in Southeast Asia, China, and India (Peter Hanson, tomato breeder, World Vegetable Center, personal communication). *X. perforans* may have been introduced into Taiwan from infected seed or seedlings. Interestingly, *X. perforans* strains ($n = 4$) were isolated only from sweet pepper, and sweet pepper hybrid seed are also imported in Taiwan. In laboratory experiments, strains of *X. perforans* produced bacteriocins that inhibited the growth of *X. euvesicatoria* (Hert et al. 2005; Lue et al. 2010). Therefore, *X. perforans* may have a competitive advantage over *X. euvesicatoria* in tomato. Further studies are required to understand the ability of *X. perforans* strains to produce bacteriocins in tomato and pepper plants and their possible interaction with strains of *X. euvesicatoria*. Genetic studies using amplified fragment length polymorphism markers (Hamza et al. 2010) and MLSA using house-keeping genes (Hamza et al. 2010; Timilsina et al. 2016) of strains of

Table 4. Characterization of representative strains of *Xanthomonas* spp. for copper sensitivity and biochemical activities

Collection period, species	Host origin	N ^c	Sensitivity to copper sulfate ^a		Biochemical characterization ^b		
			S	T	Amy	Lip	OF
1989–1999							
<i>Xanthomonas euvesicatoria</i> ^d	Pepper	6	5	1	–	+	+
<i>X. euvesicatoria</i>	Tomato	7	4	3	–	+	+
<i>X. vesicatoria</i>	Tomato	2	2	0	+	–	+
Subtotal	...	15	11 (73)	4 (27)	...	...	...
2000–2009							
<i>X. euvesicatoria</i>	Tomato	5	4	1	–	+	+
<i>X. perforans</i>	Tomato	4	2	2	+	+	+
<i>X. perforans</i>	Pepper	1	1	0	+	+	+
Subtotal	...	10	7 (70)	3 (30)	...	...	...
2010–2016							
<i>X. euvesicatoria</i>	Pepper	6	1	5	–	+	+
<i>X. euvesicatoria</i>	Tomato	1	0	1	...	...	...
<i>X. perforans</i>	Pepper	3	0	3	+	+	+
<i>X. perforans</i>	Tomato	13	2	11	+	+	+
Subtotal	...	23	3 (13)	20 (87)	...	...	...
Total	...	48	21 (44)	27 (56)	...	...	...

^a S = sensitive; bacterial strains did not grow in concentrations of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) of 200 mg liter⁻¹ or higher; T = tolerant; bacterial strains grew in concentrations of 200 mg liter⁻¹ or higher. Values in parenthesis are percentage of strains.

^b Amy = amylolytic activity, Lip = lipase activity, and OF = oxidative-fermentative metabolism of carbohydrate test; + and – indicate positive and negative reactions, respectively.

^c Number of bacterial strains.

^d One strain of *X. euvesicatoria* from pepper showed positive amylolytic activity on starch media.

four *Xanthomonas* spp. from multiple continents showed that strains of *X. euvesicatoria* were genetically close to *X. perforans* and provided evidence of interspecies recombination between the two species. In addition, Timilsina et al. (2016) revealed evidence of specific recombination between *X. perforans* and *X. euvesicatoria*. Using whole-genome studies, Schwartz et al. (2015) also found

pathogen diversity among the strains of *X. perforans* and *X. euvesicatoria* from the United States. These findings suggest that changes in the genetic composition of *X. euvesicatoria* strains could lead to the evolution of *X. perforans* in Taiwan; however, further research on the pathogen genetics using MLSA of housekeeping genes and other molecular markers are required to verify the hypothesis. In

Fig. 2. Frequency distribution of three species of *Xanthomonas* isolated from symptomatic foliage and fruit of tomato and pepper from 1989 to 2016 in Taiwan. **A**, *Xanthomonas* spp. from tomato; **B**, *Xanthomonas* spp. from pepper; and **C**, frequency of *Xanthomonas* spp. from both crops in every 5- to 6-year period.

addition, next-generation sequencing techniques can be also utilized for further study of pathogen variation at a genomic level.

Strains of three different species showed variability in biochemical activities. All of the strains of *X. euvesicatoria*, except one strain from pepper, showed negative amylolytic reaction, and all of the strains showed positive reactions on lipase activity and OF tests. All of the strains of *X. vesicatoria* showed positive reactions on amylolytic and OF tests and negative lipase activity, whereas strains of *X. perforans* showed positive reactions on all three biochemical tests. Lue et al. (2010) also found similar results from Taiwanese strains of *X. euvesicatoria*, *X. vesicatoria*, and *X. perforans*. Negative amylolytic activity is a common trait of *X. euvesicatoria* (Jones et al. 2004); however, *X. euvesicatoria* strains isolated from pepper in Iran (Osdaghi et al. 2016) and Brazil (Areas et al. 2014) showed strong amylolytic activity. Similar to our findings, strains of *X. perforans* and *X. vesicatoria* from Florida showed positive amylolytic activity.

We also found a shift in the copper sulfate sensitivity among the evaluated strains of *Xanthomonas* ($n = 48$) over time. For example, more than two-thirds of *X. euvesicatoria* strains from the 1989 to 1999 population and 60% of *X. perforans* strains from the 2000 to 2009 population were sensitive to copper sulfate. However, only one strain of the *X. euvesicatoria* and two strains of *X. perforans* population from 2010 to 2016 were sensitive to copper sulfate and the remaining strains were tolerant. Among the strains tested, two tolerant strains isolated in 1991 from Changhua County of central Taiwan and Tainan County of south Taiwan were initially detected. The frequency of tolerant strains slightly increased in the 2000 to 2009 population and substantially increased in the 2010 to 2016 population, indicating that the copper tolerance problem has increased in Taiwan in recent years. Similar to our findings, Wu et al. (2008) also found copper tolerance for 25% of the *Xanthomonas* strains ($n = 88$) collected from counties of east, central, and south Taiwan during 1990 to 2001, and for 55% of the strains ($n = 31$) collected in 2006. Wide distribution of copper-tolerant strains of *Xanthomonas* spp. have also been reported from several countries, including Canada (Abbasi et al. 2015), the United States (Ma 2015; Mixon 2012), and Australia (Martin et al. 2004). Copper-based fungicides have been routinely applied to manage bacterial spot and other foliar diseases of tomato and pepper in Taiwan (Fei and Wang 2007). The huge shift in sensitivity of xanthomonads to copper fungicides suggests that alternative strategies are required to manage copper-tolerant pathogen populations. Integrated approaches, including use of certified or clean seed and transplants, seed treatments, and cultivation of resistant cultivars, can be used. Tank mixing or rotating of copper-based fungicides with mancozeb or maneb may improve copper activity by increasing copper solubility (Jones et al. 1991). However, Martin et al. (2004) found similar numbers of copper-tolerant strains of *Xanthomonas* from the plots treated with copper and copper combined with mancozeb. Exploitation of other compounds, including antibiotics, defense elicitors, plant-growth-promoting rhizobacteria, and so on, that can be combined with copper-based fungicides, could be also useful for the management of copper resistance. Worthington et al. (2012) revealed that foliar sprays of small-molecule 2-aminoimidazole mixing with copper hydroxide (Kocide 3000) were useful to reduce bacterial spot of bell pepper and the numbers of copper-tolerant strains of *X. euvesicatoria*.

Overall, we revealed a substantial shift in the species composition, race structure, and copper sensitivity for xanthomonads associated with bacterial spot of tomato in Taiwan over 27 years (1989 to 2016). There are few practical alternatives to copper for the management of bacterial spot of tomato and pepper, stressing the need of improved host resistance. Most of the tomato and pepper breeding programs worldwide, except for a few breeding programs in the United States, do not actively breed for bacterial spot resistance. The World Vegetable Center, working for several developing countries in Asia and Africa, has a strong resistance breeding program for bacterial wilt caused by *R. solanacearum* as well as other fungal and viral diseases in tomato and pepper (Hanson et al. 2016; Lin et al. 2013); however, limited research has been done to identify sources of resistance to bacterial spot. The worldwide increase in the

incidence of bacterial spot of tomato and pepper and changes in the pathogen population suggest that there is a need for exploitation of host resistance in the World Vegetable Center's breeding program. Our findings suggest that tomato breeding programs should target races T3 and T4 of *X. perforans*. Monitoring of race structure of *X. perforans* strains across the island of Taiwan and other relevant countries is also suggested. Both T3 and T4 races of *X. perforans* overcame the resistance governed by recessive genes *rx1*, *rx2*, and *rx3* (Hawaii 7998, *S. lycopersicum*), and elicited an HR on the tomato genotype 716 (*S. pennellii*), which has resistance gene *Xv4* (Astua-Monge et al. 2000) or *RXopJ4* (Sharlach et al. 2013); therefore, it is suggested to incorporate this gene into new breeding lines or cultivars.

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Literature Cited

- Abbasi, P. A., Khabbaz, S. E., Weselowski, B., and Zhang, L. 2015. Occurrence of copper-resistant strains and a shift in *Xanthomonas* spp. causing tomato bacterial spot in Ontario. *Can. J. Microbiol.* 61:753-761.
- Araújo, E. R., Costa, J. R., Ferreira, M. A. S. V., and Quezado-Duval, A. M. 2017. Widespread distribution of *Xanthomonas perforans* and limited presence of *X. gardneri* in Brazil. *Plant Pathol.* 66:159-168.
- Araújo, E. R., Pereira, R. C., Moita, A. W., Ferreira, M. A. S. V., Café-Fiho, A. C., and Quezado-Duval, A. M. 2010. Effect of temperature on pathogenicity components of tomato bacterial spot and competition between *Xanthomonas perforans* and *X. gardneri*. III International symposium on tomato diseases. *Acta Hortic.* 914:39-42.
- Areas, M. S., Goncalves, R. M., Soman, J. M., Sakate, R. K., Gioria, R., da Silva, T. A. F., Junior, and Maringoni, A. C. 2014. Prevalence of *Xanthomonas euvesicatoria* on pepper in Brazil. *J. Phytopathol.* 163:1050-1054.
- Astua-Monge, G., Minsavage, G. V., Stall, R. E., Vallejos, C. E., Davis, M. J., and Jones, J. B. 2000. *Xv4-AvrXv4*: A new gene-for-gene interaction identified between *Xanthomonas campestris* pv. *vesicatoria* race T3 and the wild tomato relative *Lycopersicon pennellii*. *Mol. Plant-Microbe Interact.* 13:1346-1355.
- Beran, P., Mraz, I., Kokoskova, B., and Bohata, A. 2014. Monitoring the occurrence of bacterial spot of tomato and pepper in the Czech Republic and development of new PCR primers for detection of *Xanthomonas vesicatoria*. *Eur. J. Plant Pathol.* 141:617-621.
- Bouzar, H., Jones, J. B., Minsavage, G. V., Stall, R. E., and Scott, J. W. 1994. Proteins unique to phenotypically distinct groups of *Xanthomonas campestris* pv. *vesicatoria* revealed by silver staining. *Phytopathology* 84:39-44.
- Conover, R. A., and Gerhold, N. R. 1981. Mixtures of copper and maneb or mancozeb for control of bacterial spot of tomato and their compatibility for control of fungus diseases. *Proc. Fla. State Hortic. Soc.* 94:154-156.
- Doidge, E. M. 1920. A tomato canker. *J. Dep. Agric. Union S. Afr.* 1:718-721.
- Dye, D. W. 1978. Genus IX. *Xanthomonas*. Dowson 1939. *N. Z. J. Agric. Res.* 21: 153-177.
- European and Mediterranean Plant Protection Organization. 2013. PM 7/110 (1) *Xanthomonas* spp. (*Xanthomonas euvesicatoria*, *Xanthomonas gardneri*, *Xanthomonas perforans*, *Xanthomonas vesicatoria*) causing bacterial spot of tomato and sweet pepper. *Bull. OEPP/EPPO Bull.* 43:7-20.
- Fei, W. C., and Wang, Y. 2007. Pages 48-49 in: *Plant Protection Manual—Vegetable Articles*. Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture, Taichung, Taiwan.
- Hamza, A., Robene-Soustrade, I., Jouen, E., Gagnevin, L., Lefeuvre, P., Chiroleu, F., and Pruvost, O. 2010. Genetic and pathological diversity among *Xanthomonas* strains responsible for bacterial spot on tomato and pepper in the southwest Indian Ocean region. *Plant Dis.* 94:993-999.
- Hanson, P., Lu, S.-F., Wang, J.-F., Chen, W., Kenyon, L., Tan, C.-W., Tee, K. W., Wang, Y.-Y., Hsu, Y.-C., Schafleitner, R., Ledesma, D., and Yang, R.-Y. 2016. Conventional and molecular marker-assisted selection and pyramiding of genes for multiple disease resistance in tomato. *Sci. Hortic. (Amsterdam)* 201:346-354.
- Hert, A., Roberts, P., Momol, M., Minsavage, G., Tudor-Nelson, S., and Jones, J. 2005. Relative importance of bacteriocin-like genes in antagonism of *Xanthomonas perforans* tomato race 3 to *Xanthomonas euvesicatoria* tomato race 1 strains. *Appl. Environ. Microbiol.* 71:3581-3588.
- Horvath, D. M., Stall, R. E., Jones, J. B., Pauly, M. H., Vallad, G. E., Dahlbeck, D., Staskawicz, B. J., and Scot, J. W. 2012. Transgenic resistance confers effective field level control of bacterial spot disease in tomato. *PLoS One* 7:e42036.
- Jones, J. B., Minsavage, G. V., Stall, R. E., Kelly, R. O., and Bouzar, H. 1993. Genetic analysis of a DNA region involved in expression of two epitopes associated with lipopolysaccharide in *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Plant Pathol.* 83:551-556.
- Jones, J. B., Bouzar, H., Stall, R. E., Almira, E. C., Roberts, P., Bowen, B. W., Sudberry, J., Strickler, P., and Chun, J. 2000. Systematic analysis of

- xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. *Int. J. Syst. Bacteriol.* 50:1211-1219.
- Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., and Schaad, N. W. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Syst. Appl. Microbiol.* 27:755-762.
- Jones, J. B., Stall, R. E., Scott, J. W., Somodi, G. C., Bouzar, H., and Hodge, N. C. 1995. A third tomato race of *Xanthomonas campestris* pv. *vesicatoria*. *Plant Dis.* 79:395-398.
- Jones, J. B., Woltz, S. S., Jones, J. P., and Portier, K. L. 1991. Population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets with copper bactericides. *Phytopathology* 81:714-719.
- Kado, C. H., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. *Phytopathology* 60:969-976.
- Kebede, M., Timilsina, S., Ayalew, A., Admassu, B., Potnis, N., Minsavage, G. V., Goss, E. M., Hong, J. C., Strayer, A., Paret, M., Jones, J. B., and Vallad, G. E. 2014. Molecular characterization of *Xanthomonas* strains responsible for bacterial spot of tomato in Ethiopia. *Eur. J. Plant Pathol.* 140:677-688.
- Kim, S. H., Olson, T. N., Pepper, N. D., Nikolaeva, E. V., Park, S., and Kang, S. 2010. First report of bacterial spot of tomato caused by *Xanthomonas gardneri* in Pennsylvania. *Plant Dis.* 94:638.
- Koenraad, H., van Betteray, B., Germain, R., Hiddink, J. B., Jones, J. B., and Oosterhof, J. 2009. Development of specific primers for the molecular detection of bacterial spot of pepper and tomato. *Acta Hort.* 808:99-102.
- Lelliott, R. A., and Stead, D. E. 1987. *Methods for the Diagnosis of Bacterial Diseases of Plants*, 1st ed. Blackwell Scientific Publication, Oxford, UK.
- Lin, C. H., Tsai, K. C., Prior, P., and Wang, J. F. 2014. Phylogenetic relationships and population structure of *Ralstonia solanacearum* isolated from diverse origin in Taiwan. *Plant Pathol.* 63:1395-1403.
- Lin, S. W., Chou, Y. Y., Shieh, H. C., Ebert, A. W., Kumar, S., Mavlyanova, R., Rouamba, A., Tenkouano, A., Afari-Sefa, V., and Gniffke, P. A. 2013. Pepper (*Capsicum* spp.) germplasm dissemination by AVRDC—The World Vegetable Center: An overview and introspection. *Chron. Hort.* 53:21-27.
- Lue, Y. S. 2003. Identification and detection of polymerase chain reaction of *Xanthomonas vesicatoria*, a bacterial spot pathogen of Solanaceae. Master's thesis, National Institute of Plant Pathology, National Chung Hsing University.
- Lue, Y. S., Deng, W. L., Wu, Y. F., Cheng, A. S., Hsu, S. T., and Tzeng, K. C. 2010. Characterization of *Xanthomonas* associated with bacterial spot of tomato and pepper in Taiwan. *Plant Pathol. Bull.* 19:181-190.
- Ma, X. 2015. Characterization and management of bacterial leaf spot of processing tomato in Ohio. Ph.D. thesis, The Ohio State University.
- Martin, H. L., Hamilton, V. A., and Kopittke, R. A. 2004. Copper tolerance in Australian populations of *Xanthomonas campestris* pv. *vesicatoria* contributes to poor field control of bacterial spot of pepper. *Plant Dis.* 88:921-924.
- McGuire, R. G., Jones, J. B., Stanley, C. D., and Cszinsky, A. A. 1991. Epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* and bacterial spot of tomato as influenced by nitrogen and potassium fertilization. *Phytopathology* 81:656-660.
- McInnes, T. B., Gitaitis, R. D., McCarter, S. M., Jaworski, C. A., and Phatak, S. C. 1988. Airborne dispersal of bacteria in tomato and pepper transplant fields. *Plant Dis.* 72:575-579.
- Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene for gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions. *Mol. Plant-Microbe Interact.* 3:41-47.
- Mixon, J. T. 2012. Prevalence of copper resistance among foliar bacterial pathogens of tomato in Tennessee. Master's thesis, the University of Tennessee, Knoxville.
- Ning, F. Y. 2012. Identification and detection of *Xanthomonas perforans* by the polymerase chain reaction technique and characterization of *X. perforans* strains in Taiwan by DNA polymorphism. Master's thesis, Department of Plant Pathology, National Chung Hsing University, Taiwan.
- Osdaghi, E., Taghavi, S. M., Hamzehzarghani, H., and Lamichhane, J. R. 2016. Occurrence and characterization of the bacterial spot pathogen *Xanthomonas euvesicatoria* on pepper in Iran. *J. Phytopathol.* 164:722-734.
- Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., Vallad, G. E., and Jones, J. B. 2015. Bacterial spot of tomato and pepper: Diverse *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge. *Mol. Plant Pathol.* 16:907-920.
- Rashid, S., Kamaruzaman, S., Golkhandan, E., Nasehi, A., and Awla, H. K. 2016. First report of *Xanthomonas gardneri* causing bacterial spot of tomato in Malaysia. *Plant Dis.* 100:854.
- Ritchie, D. F. 2000. Bacterial spot of pepper and tomato. Online publication. *Plant Health Instruct.* doi.org/10.1094/PHI-I-2000-1027-01
- Schwartz, A. R., Potnis, N., Timilsina, S., Wilson, M., Patané, J., Martins, J., Jr., Minsavage, G. V., Dahlbeck, D., Akhunova, A., Almeida, N., Vallad, G. E., Barak, J. D., White, F. F., Miller, S. A., Ritchie, D., Goss, E., Bart, R. S., Setubal, J. C., Jones, J. B., and Staskawicz, B. J. 2015. Phylogenomics of *Xanthomonas* field strains infecting pepper and tomato reveals diversity in effector repertoires and identifies determinants of host specificity. *Front. Microbiol.* 6:535.
- Sharlach, M., Dahlbeck, D., Liu, L., Chiu, J., Jiménez-Gómez, J., Kimura, S., Koenig, D., Maloof, J. N., Sinha, N., Minsavage, G. V., Jones, J. B., Stall, R. E., and Staskawicz, B. J. 2013. Fine genetic mapping of *RXopJ4*, a bacterial spot disease resistance locus from *Solanum pennellii* LA716. *Theor. Appl. Genet.* 126:601-609.
- Stall, R. E., Beaulieu, C., Egel, D., Hodge, N. C., Leite, R. P., Minsavage, G. V., Bouzar, H., Jones, J. B., Alvarez, A. M., and Benedict, A. A. 1994. Two genetically diverse groups of strains are included in *Xanthomonas campestris* pv. *vesicatoria*. *Int. J. Syst. Bacteriol.* 44:47-53.
- Stall, R. E., Jones, J. B., and Minsavage, G. V. 2009. Durability of resistance in tomato and pepper to xanthomonads causing bacterial spot. *Annu. Rev. Phytopathol.* 47:265-284.
- Stall, R. E., Loschke, D. C., and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 76:240-243.
- Timilsina, S., Abrahamian, P., Potnis, N., Minsavage, G. V., White, F. W., Staskawicz, B. J., Jones, J. B., Vallad, G. E., and Goss, E. M. 2016. Analysis of sequenced genomes of *Xanthomonas perforans* identifies candidate targets for resistance breeding in tomato. *Phytopathology* 106:1097-1104.
- Timilsina, S., Jibrin, M. O., Potnis, N., Minsavage, G. V., Kebede, M., Schwartz, A., Bart, R., Staskawicz, B., Boyer, C., Vallad, G. E., Pruvost, O., Jones, J. B., and Goss, E. M. 2015. Multilocus sequence analysis of xanthomonads causing bacterial spot of tomato and pepper plants reveals strains generated by recombination among species and recent global spread of *Xanthomonas gardneri*. *Appl. Environ. Microbiol.* 81:1520-1529.
- Worthington, R. J., Rogers, S. A., Huigens, R. W., III, Melander, C., and Ritchie, D. F. 2012. Foliar-applied small molecule that suppresses biofilm formation and enhances control of copper-resistant *Xanthomonas euvesicatoria* on pepper. *Plant Dis.* 96:1638-1644.
- Wu, Y. F., Chen, S. C., Peng, R. J., Huang, S. H., and Zheng, A. X. 2008. Study on resistance to copper of pathogenic bacteria in Solanaceae bacterial leaf spot disease. In: Tainan District Agricultural News Issue 64. Tainan District Agricultural Research and Extension Station, Council of Agriculture, Taiwan. (In Chinese.)
- Yang, W., Sacks, E. J., Lewis Ivey, M. L., Miller, S. A., and Francis, D. M. 2005. Resistance in *Lycopersicon esculentum* intraspecific crosses to race T1 strains of *Xanthomonas campestris* pv. *vesicatoria* causing bacterial spot of tomato. *Phytopathology* 95:519-527.