Plant Pathol. J. 32(5) : 431-440 (2016)
http://dx.doi.org/10.5423/PPJ.OA.01.2016.0016
pISSN 1598-2254 eISSN 2093-9280

Research Article Open Access

The Plant Pathology Journal

Xanthomonas euvesicatoria Causes Bacterial Spot Disease on Pepper Plant in Korea

Min-Seong Kyeon[1], Soo-Hyeong Son[1], Young-Hee Noh[1], Yong-Eon Kim[1], Hyok-In Lee[2], and Jae-Soon Cha[1]*

[1]*Department of Plant Medicine, Chungbuk National University, Cheongju 28644, Korea*

[2]*Animal and Plant Quarantine Agency, Anyang 39600, Korea*

(Received on January 17, 2016; Revised on March 25, 2016; Accepted on April 4, 2016)

In 2004, bacterial spot-causing xanthomonads (BSX) were reclassified into 4 species—*Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*. Bacterial spot disease on pepper plant in Korea is known to be caused by both *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*. Here, we reidentified the pathogen causing bacterial spots on pepper plant based on the new classification. Accordingly, 72 pathogenic isolates were obtained from the lesions on pepper plants at 42 different locations. All isolates were negative for pectolytic activity. Five isolates were positive for amylolytic activity. All of the Korean pepper isolates had a 32 kDa-protein unique to *X. euvesicatoria* and had the same band pattern of the *rpoB* gene as that of *X. euvesicatoria* and *X. perforans* as indicated by PCR-restriction fragment length polymorphism analysis. A phylogenetic tree of 16S rDNA sequences showed that all of the Korean pepper plant isolates fit into the same group as did all the reference strains of *X. euvesicatoria* and *X. perforans*. A phylogenetic tree of the nucleotide sequences of 3 housekeeping genes—*gapA*, *gyrB*, and *lepA* showed that all of the Korean pepper plant isolates fit into the same group as did all of the references strains of *X. euvesicatoria*. Based on the phenotypic and genotypic characteristics, we identified the pathogen as *X. euvesicatoria*. Neither *X. vesicatoria*, the known pathogen of pepper bacterial spot, nor *X. perforans*, the known pathogen of tomato plant, was isolated. Thus, we suggest that the pathogen causing bacterial spot disease of pepper plants in Korea is *X. euvesicatoria*.

Keywords **:** bacterial spot disease, pepper plants, re-identification, *Xanthomonas euvesicatoria*

*Corresponding author.
Phone) +82-43-261-2554, FAX) +82-43-271-4414
E-mail) jscha@chungbuk.ac.kr

Bacterial spot disease occurs on pepper (*Capsicum annum* L.) and tomato (*Solanum lycopersicum* L.) in warm, humid areas worldwide and causes lesions on the leaves, stems, and fruits (Jones et al., 2000; Stall et al., 1994). Yellow haloes appear around the lesions; smaller lesions coalesce into larger ones. Leaf infection results in blight, necrosis, and early leaf fall. These cause a reduction in photosynthesis and fruit infection, resulting in direct economic loss (Jones et al., 1991; Obradovic et al., 2004; Stall et al., 1994). Contaminated seeds and plant debris are common inoculum sources, and the disease is also transmitted by rain splash (Jones et al., 1991).

The pathogens causing bacterial spot disease were originally identified as *Bacterium vesicatoria* (Doidge, 1921) and *B. exitiosum* (Gardner and Kendrick, 1921). The 2 bacteria were later classified as *Xanthomonas vesicatoria* and then as *X. campestris* pv. *vesicatoria* by Young et al. (1978). Based on DNA homology by Vauterin et al. (1995), *X. campestris* pv. *vesicatoria* was separated into 2 species—*X. vesicatoria* and *X. axonopodis* pv. *vesicatoria*. *Pseudomonas gardneri* was first reported as the pathogen causing bacterial spot on tomato (Šutic, 1957) but was later reclassified as *X. gardneri* (De Ley, 1978; Dye, 1966). Jones et al. (2004) reported that all of the bacterial spot-causing xanthomonads (BSX) were reclassified as 4 species—*X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*. Among them, *X. euvesicatoria* and *X. vesicatoria* cause diseases on both pepper and tomato, while *X. perforans* and *X. gardneri* are known to

infect only tomato. Recently, however, *X. perforans* was isolated from the pepper plant (Potnis et al., 2015).

X. vesicatoria and *X. perforans* have strong amylolytic and pectolytic activity, but *X. euvesicatoria* and *X. gardneri* do not (Bouzar et al., 1994; Jones et al., 2000, 2004). *X. euvesicatoria* has a unique 32 kDa protein, while the other BSX have a 27 kDa protein (Bouzar et al., 1994; Jones et al., 2004). In addition, there are differences in carbon source utilization among BSX species (Jones et al., 2004; Stoyanova et al., 2014; Vauterin et al., 1995). *RpoB* based restriction fragment length polymorphism (RFLP) (Ferreira-Tonin et al., 2012), amplified fragment length polymorphism (AFLP) (Hamza et al., 2012) and multilocus sequence analysis (Almeida et al., 2010; Hamza et al., 2012; Kebede et al., 2014; Timilsina et al., 2015) were used to differentiate 4 species of BSX.

Bacterial spot is a common disease on pepper plants in Korea (Kim, 2004; Lee and Cho, 1996; Lee et al., 1999; Myung et al., 2005, 2006), and *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria* are listed as the causative pathogens (Yoo, 2009). *X. perforans* was reported as the causal agent of bacterial spot on tomato for the first time from a nursery farm in Korea (Myung et al., 2009). It is not clear as to which pathogens cause bacterial spot disease of pepper in Korea since *X. axonopodis* pv. *vesicatoria* is no longer included in the list of BSX, and since *X. perforans*, which was known to cause the disease on pepper plant, has been isolated only from tomato. The correct identification of the bacterial spot pathogen on pepper is important for plant quarantine, disease management, and breeding for resistance. In this study, the pathogen causing bacterial spot disease of pepper was reidentified by the isolation and identification of bacterial spot disease pathogens throughout Korea. To ensure correct identification, several phenotypic and genotypic characteristics were used, including amylolytic activity, pectolytic activity, unique protein band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), *rpoB* based RFLP, phylogenetic analysis with 16S rDNA sequences, and sequences of 3 housekeeping genes.

Materials and Methods

Isolation and pathogenicity test. Pepper leaves showing typical bacterial spot lesions were collected throughout Korea during 2013–2015. Small pieces of leaf lesions were macerated in sterile water, and the resulting suspension was streaked on nutrient agar (NA) (Difco™; BD, Sparks, MD, USA). After incubation at 27°C for 3–5 days, distinct single colonies were purified by subculturing. Isolates were stored in a deep freezer. Bacterial suspensions, optical density measured at a wavelength of 600 nm (OD_{600}) = 0.1 (ca. 1.0×10^8 cfu/ml) were prepared on NA in sterile water using 3-day-old cell cultures, and the suspensions were sprayed on pepper and tomato seedlings. The inoculated plants were saturated and maintained in a humid environment for 48 h and then in the greenhouse. Bacterial spot symptoms were observed 3 weeks post-inoculation.

Reference strains. Twenty-nine different strains from 4 BSX species were used as reference strains in this study (Table 1).

Amylolytic and pectolytic assays. Amylolytic and pectolytic assays were carried out according to the method of Bouzar et al. (1994). Bacteria were streaked on brilliant cresyl blue-starch (BS) agar and incubated at 27°C for 2 days. Haloes around the colonies indicated that the strain was positive for amylolytic activity. For pectolytic assay, bacterial cells were spotted on crystal violet pectate (CVP) agar and incubated at 27°C for 2 days. Dents around the colonies indicated that the strain was positive for pectolytic activity.

Observation of unique proteins by SDS-PAGE. SDS-PAGE for the observation of proteins unique to BSX species was carried out according to the method of Bouzar et al. (1994). Bacteria were cultured in 3 ml NA (BD Difco™) at 27°C for 18 h. Two milliliters of bacterial culture were harvested by centrifugation (> 13,000 × *g*) for 10 min, and the bacterial cells were washed twice in sterile water. The cell pellet was resuspended in 180 µl of 10% sorbitol and the bacterial suspension was mixed with an equal volume of 2 × sampling buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% β-mercaptoethanol, 0.04% bromophenol blue, and 4% SDS). After heating at 100°C for 10 min, 10 µl of suspension were electrophoresed in 12% resolving gel. The gel was stained with Coomassie R250 staining solution (0.1% Coomassie Blue R250 in 10% acetic acid, 45% methanol, 45% H_2O) for more than 1 h and destained for more than 2 h.

***rpoB* gene based RFLP.** The *rpoB* based RFLP was carried out according to the method of Ferreira-Tonin et al. (2012). The *rpoB* gene was amplified with rpoB2F (5′-TCA AGG AGC GTC TGT CGA T-3′) and rpoB3R (5′-TCT GCC TCG TTG ACC TTG A-3′) primers. PCR amplification was performed in PCR reaction mixture (25 µl) of Takara Ex Taq PCR kit (Takara Co., Shiga, Japan) containing 1 µl of each primer (10 pmol/µl) and 10 µl of genomic DNA (20 ng/µl). The PCR conditions were as follows: an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for

Table 1. List of bacterial spot-causing xanthomonads strains used in this study

Species	Strain*	Host	Origin	Year
Xanthomonas euvesicatoria	75-3	SL	USA	NK
	85-10	SL	USA	1985
	155	SL	USA	1985
	E3	SL	USA	NK
	LMG667	SL	NK	1976
	LMG905	NK	India	NK
	NCPPB936	CA	USA	1939
	NCPPB941	CA	USA	1939
	NCPPB2968^T	CA	USA	1947
X. vesicatoria	ATCC11551	SL	USA	1943
	LMG916	SL	New Zealand	1955
	LMG924	SL	Hungary	1957
	NCPPB422^T	SL	New Zealand	1955
	NCPPB424	SL	New Zealand	1955
	NCPPB509	SL	Zimbabwe	1956
	NCPPB701	SL	Zimbabwe	1956
	NCPPB1431	SL	Hungary	1957
X. perforans	GEV1026	SL	USA	2012
	KACC16356	SL	Korea	2007
	KACC16357	SL	Korea	2007
	NCPPB4321^T	SL	USA	1991
	NCPPB4322	SL	USA	1993
	TB15	SL	USA	2013
	Xp10-13	SL	USA	2006
	Xp19-10	SL	USA	2006
X. gardneri	444	SL	Costa Rica	1991
	NCPPB881^T	SL	Yugoslavia	1953
	NCPPB4323	SL	Costa Rica	1991
	NCPPB4324	SL	Costa Rica	1991

SL, *Solanum lycopersicum*; NK, not known; CA, *Capsicum annuum*.
*LMG, Collection of the laboratorium voor Microbiologie en Microbiele Genetica, Ghent University, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, United Kingdom; ATCC, American Type Culture Collection, USA; KACC, Korean Agricultural Culture Collection, Rural Development Administration, Korea; 75-3, 85-10, 155 from Stall; GEV1026, TB15, Xp10-13, Xp19-10, 444 from Jones; E3 from Hert.

1 min, with a final extension at 72°C for 5 min. The PCR product was purified and 300 ng of purified PCR product was restricted with *Hae*III (FastDigest-Thermo Fisher Scientific Inc., Waltham, MA, USA). The resulting DNA bands were observed after electrophoresis on a 4% agarose gel.

Phylogenetic tree with 16S rDNA sequences. The 16S rDNA was amplified with 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The amplicons were sequenced Macrogen Co. (Seoul, Korea). Phylogenetic analysis was carried out using the MEGA 6.0 program with neighbor-joining tree, Kimura 2-parameter model, and 3,000 bootstrap value.

Phylogenetic tree with multilocus sequences. Multilocus sequence analysis (MLSA) was carried out using 3 housekeeping genes—*gapA*, *gyrB*, and *lepA*. The PCR were carried out according to the method of Almeida et al. (2010). PCR primers were gap-1-F (5′-GGC AAT CAA GGT TGG YAT CAA CG-3′) and gap-1-R (5′-ATC TCC AGG CAC TTG TTS GAR TAG-3′) for *gapA*, gyrB-F (5′-AAG TTC GAC GAC AAC AGC TAC AA-3′) and gyrB-R (5′-GAM AGC ACY GCG ATC ATG CCT TC-3′) for *gyrB*, and lepA-F (5′-AAG CSC AGG TGC TCG ACT CCA AC-3′) and lepA-R (5′-CGT TCC TGC ACG ATT TCC ATG TG-3′). PCR reactions were performed in reaction mixture (25 μl) of Takara Ex Taq PCR kit containing 1 μl of each primer (10 pmol/μl) and 10 μl of

genomic DNA (10 ng/μl). The PCR conditions were as follows: an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The amplicons were sequenced at Macrogen Co. The concatenated sequence was 444 bp of *gapA*, 411 bp of *gyrB*, and 390 of *lepA*. Phylogenetic analysis was carried out using the MEGA 6.0 program with neighbor-joining tree, Kimura 2-parameter model, and 3,000 bootstrap value.

Results

Pathogen isolation. All isolates from the bacterial spot lesions of pepper plants were tested for pathogenicity on both pepper and tomato plants. About 5–10 days after inoculation, water soaked spots started to appear on the lower epidermis of leaves. Circular dark brown and black spots appeared, followed by yellow haloes around some of the spots (Fig. 1). A total of 72 isolates caused typical bacterial spot symptoms on both pepper and tomato plants. Our tests indicated that all of the isolates are pathogenic to both pepper and tomato plants despite the fact that all of them were isolated from only pepper plants. The pathogens were isolated from isolates collected from 42 different locations that cover all provinces of Korea, including Jeju Island (Table 2). The 2 pathogenic isolates CNUPBL 2030 and CNUPBL 2058 were deposited in Korean Agricultural Culture Collection as KACC18722 and KACC18723.

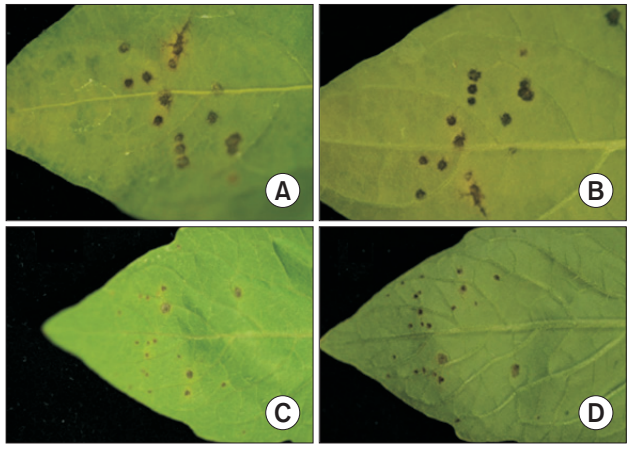

Fig. 1. Bacterial spot symptoms on pepper and tomato leaves inoculated with CNUPBL 2039, a pathogenic isolate from bacterial spot lesion of pepper plant. Lesions on the upper epidermis of pepper leaf (A), lower epidermis of pepper leaf (B), upper epidermis of tomato leaf (C), and lower epidermis of tomato leaf (D).

Table 2. List of the pathogenic isolates from bacterial spot lesion of the pepper plants in the Korea

Isolate*	Location	Year
CNUPBL 1984	Gundong, Gangjin	2013
CNUPBL 1985	Gundong, Gangjin	2013
CNUPBL 1986	Beopjeon, Bonghwa	2013
CNUPBL 1987	Jaesan, Bonghwa	2013
CNUPBL 1988	Yeonmu, Nonsan	2013
CNUPBL 1989	Gonggeun, Hoengseong	2014
CNUPBL 1990	Bokheung, Sunchang	2014
CNUPBL 1991	Gobu, Jeongeup	2014
CNUPBL 1992	Jocheon, Jeju	2014
CNUPBL 1993	Jocheon, Jeju	2014
CNUPBL 1994	Jocheon, Jeju	2014
CNUPBL 1995	Aewol, Jeju	2014
CNUPBL 1996	Dopyeong, Jeju	2014
CNUPBL 1997	Dopyeong, Jeju	2014
CNUPBL 1998	Dopyeong, Jeju	2014
CNUPBL 1999	Jocheon, Jeju	2014
CNUPBL 2000	Jocheon, Jeju	2014
CNUPBL 2001	Jocheon, Jeju	2014
CNUPBL 2002	Jocheon, Jeju	2014
CNUPBL 2003	Cheongso, Boryeong	2014
CNUPBL 2004	Inji, Seosan	2014
CNUPBL 2005	Inji, Seosan	2014
CNUPBL 2006	Inji, Seosan	2014
CNUPBL 2007	Eumam, Seosan	2014
CNUPBL 2008	Eumam, Seosan	2014
CNUPBL 2009	Bongsan, Yesan	2014
CNUPBL 2010	Bongsan, Yesan	2014
CNUPBL 2011	Oga, Yesan	2014
CNUPBL 2012	Oga, Yesan	2014
CNUPBL 2013	Oga, Yesan	2014
CNUPBL 2014	Myeoncheon, Dangjin	2014
CNUPBL 2015	Daesan, Gochang	2014
CNUPBL 2016	Samgye, Jangseong	2014
CNUPBL 2017	Samgye, Jangseong	2014
CNUPBL 2018	Hwangnyong, Jangseong	2014
CNUPBL 2019	Hwangnyong, Jangseong	2014
CNUPBL 2020	Myoryang, Yeonggwang	2014
CNUPBL 2021	Myoryang, Yeonggwang	2014
CNUPBL 2022	Sinbuk, Yeongam	2014
CNUPBL 2023	Sinbuk, Yeongam	2014
CNUPBL 2024	Sinbuk, Yeongam	2014
CNUPBL 2025	Eomda, Hampyeong	2014
CNUPBL 2026	Eomda, Hampyeong	2014
CNUPBL 2027	Munpyeong, Naju	2014
CNUPBL 2028	Munpyeong, Naju	2014
CNUPBL 2029	Hanbando, Yeongwol	2014
CNUPBL 2030†	Nam, Inje	2014
CNUPBL 2031	Inje, Inje	2014
CNUPBL 2032	Inje, Inje	2014

Table 2. Continued

Isolate*	Location	Year
CNUPBL 2033	Seo, Cheorwon	2014
CNUPBL 2034	Seo, Cheorwon	2014
CNUPBL 2035	Nam, Yangju	2014
CNUPBL 2036	Nam, Yangju	2014
CNUPBL 2037	Nam, Yangju	2014
CNUPBL 2038	Gwangjeok, Yangju	2014
CNUPBL 2039	Gwangjeok, Yangju	2014
CNUPBL 2040	Tanhyeon, Paju	2014
CNUPBL 2041	Tanhyeon, Paju	2014
CNUPBL 2042	Tanhyeon, Paju	2014
CNUPBL 2043	Munsan, Paju	2014
CNUPBL 2044	Hwasan, Yeongcheon	2014
CNUPBL 2046	Hayang, Gyeongsan	2014
CNUPBL 2047	Woldeung, Suncheon	2014
CNUPBL 2048	Gyeombaek, Boseong	2014
CNUPBL 2049	Miryeok, Boseong	2014
CNUPBL 2050	Deungnyang, Boseong	2014
CNUPBL 2058†	Jangseungpo, Geoje	2014
CNUPBL 2091	Dunnae, Hoengseong	2015
CNUPBL 2092	Simsheon, Youngdong	2015
CNUPBL 2093	Hallim, Jeju	2015
CNUPBL 2096	Pyoseon, Seogwipo	2015
CNUPBL 2098	Pyoseon, Seogwipo	2015

All hosts are *Capsicum annuum*.
*CNUPBL, Chunbuk National University Plant Bacteriology and Molecular Genetics Lab., Korea.
†Deposited in Korean Agricultural Culture Collection as KACC18722 (CNUPBL2030) and KACC18723 (CNUPBL2058).

Phenotypic characteristics. All of the *X. vesicatoria* and *X. perforans* reference strains were positive for amylolytic and pectolytic activities, whereas all of the *X. euvesicatoria* and *X. gardneri* reference strains were negative for both enzyme activities (Supplementary Fig. 1, Supplementary Fig. 2, Table 3). Five isolates (CNUPBL 1999, 2030, 2038, 2039, 2092) of the Korean pepper pathogens were positive for amylolytic activity and the rest were negative. All of the Korean pepper pathogens were negative for pectolytic activity (Table 3). As for the unique protein of BSX species, all of the *X. euvesicatoria* reference strains had a 32 kDa protein band and the other reference strains had a 27 kDa protein band (Supplementary Fig. 3, Table 3). All of the Korean pepper pathogens had a 32 kDa protein that is unique to *X. euvesicatoria* (Table 3).

Genotypic characteristics. In *rpoB* gene-based RFLP, all of the *X. euvesicatoria* and *X. perforans* reference strains had the same DNA band pattern with DNA bands of 339 bp, 154 bp, and 153 bp. *X. vesicatoria* and *X. gardneri* had DNA band patterns different from those of *X. euvesicatoria* and *X. perforans*, and also had different patterns from each other. *X. vesicatoria* had DNA bands of 216 bp, 123 bp, and 106 bp, and *X. gardneri* had DNA bands of 215 bp, 156 bp, 154 bp, and 123 bp (Fig. 2). All of the Korean pepper pathogens had the same DNA band pattern as that of *X. euvesicatoria* and *X. perforans* (Table 3).

A phylogenetic tree of the 16S rDNA sequences showed that all of *X. vesicatoria* and *X. gardneri* reference strains were grouped into their own clade. All *X. euvesicatoria*

Table 3. Characteristics of BSX reference strains and the Korean pepper isolates

BSX strain or pepper isolate		Amylolytic activity	Pectolytic hydrolysis	SDS-PAGE	*rpoB* gene based RFLP	Accession number			
						16S rDNA	*gapA*	*gyrB*	*lepA*
Xanthomonas euvesicatoria	75-3	–	–	32 kDa	*X.ev* or *X.p*	KU301873	KU939855	KU887562	KU939954
	85-10	–	–	32 kDa	*X.ev* or *X.p*	KU301875	KU939848	KU887555	KU939947
	155	–	–	32 kDa	*X.ev* or *X.p*	KU301874	KU939849	KU887556	KU939948
	E3	–	–	32 kDa	*X.ev* or *X.p*	KU301876	KU939847	KU887554	KU939946
	LMG667	–	–	32 kDa	*X.ev* or *X.p*	KU301877	KU939856	KU887563	KU939955
	LMG905	–	–	32 kDa	*X.ev* or *X.p*	KU301878	KU939857	KU887564	KU939956
	NCPPB936	–	–	32 kDa	*X.ev* or *X.p*	KU301879	KU939835	KU867863	KU939934
	NCPPB941	–	–	32 kDa	*X.ev* or *X.p*	KU301880	KU939836	KU887543	KU939935
	NCPPB2968^T	–	–	32 kDa	*X.ev* or *X.p*	KU301881	KU939837	KU887544	KU939936
X. vesicatoria	ATCC11551	+	+	27 kDa	*X.v*	KU301882	KU939860	KU887567	KU939959
	LMG916	+	+	27 kDa	*X.v*	KU301883	KU939858	KU887565	KU939957
	LMG924	+	+	27 kDa	*X.v*	KU301884	KU939859	KU887566	KU939958
	NCPPB422^T	+	+	27 kDa	*X.v*	KU301885	KU939838	KU887545	KU939937
	NCPPB424	+	+	27 kDa	*X.v*	KU301886	KU939839	KU887546	KU939938
	NCPPB509	+	+	27 kDa	*X.v*	KU301887	KU939840	KU887547	KU939939
	NCPPB701	+	+	27 kDa	*X.v*	KU301888	KU949841	KU887548	KU939940
	NCPPB1431	+	+	27 kDa	*X.v*	KU301889	KU939842	KU887549	KU939941

Table 3. Continued

BSX strain or pepper isolate		Amylolytic activity	Pectolytic hydrolysis	SDS-PAGE	*rpoB* gene based RFLP	Accession number			
						16S rDNA	*gapA*	*gyrB*	*lepA*
X. perforans	GEV1026	+	+	27 kDa	*X.ev* or *X.p*	KU301890	KU939850	KU887557	KU939949
	KACC16356	+	+	27 kDa	*X.ev* or *X.p*	KU301891	KU939861	KU887568	KU939960
	KACC16357	+	+	27 kDa	*X.ev* or *X.p*	KU301892	KU939862	KU887569	KU939961
	NCPPB4321^{T}	+	+	27 kDa	*X.ev* or *X.p*	KU301893	KU939843	KU887550	KU939942
	NCPPB4322	+	+	27 kDa	*X.ev* or *X.p*	KU301894	KU939844	KU887551	KU939943
	TB15	+	+	27 kDa	*X.ev* or *X.p*	KU301895	KU939851	KU887558	KU939950
	Xp10-13	+	+	27 kDa	*X.ev* or *X.p*	KU301896	KU939852	KU887559	KU939951
	Xp19-10	+	+	27 kDa	*X.ev* or *X.p*	KU301897	KU939853	KU887560	KU939952
X. gardneri	444	–	–	27 kDa	*X.g*	KU301898	KU939854	KU887561	KU939953
	NCPPB881^{T}	–	–	27 kDa	*X.g*	KU301899	KU939863	KU887570	KU939962
	NCPPB4323	–	–	27 kDa	*X.g*	KU301900	KU939845	KU887552	KU939944
	NCPPB4324	–	–	27 kDa	*X.g*	KU301901	KU939846	KU887553	KU939945
CNUPBL 1984		–	–	32 kDa	*X.ev* or *X.p*	KU301902	KU939864	KU887571	KU939963
CNUPBL 1985		–	–	32 kDa	*X.ev* or *X.p*	KU301903	KU939865	KU887572	KU939964
CNUPBL 1986		–	–	32 kDa	*X.ev* or *X.p*	KU301904	KU939866	KU887573	KU939965
CNUPBL 1987		–	–	32 kDa	*X.ev* or *X.p*	KU301905	KU939867	KU887574	KU939966
CNUPBL 1988		–	–	32 kDa	*X.ev* or *X.p*	KU301906	KU939868	KU887575	KU939967
CNUPBL 1989		–	–	32 kDa	*X.ev* or *X.p*	KU301907	KU939869	KU887576	KU939968
CNUPBL 1990		–	–	32 kDa	*X.ev* or *X.p*	KU301908	KU939870	KU887577	KU939969
CNUPBL 1991		–	–	32 kDa	*X.ev* or *X.p*	KU308457	KU939871	KU887578	KU939970
CNUPBL 1992		–	–	32 kDa	*X.ev* or *X.p*	KU308458	KU939872	KU887579	KU939971
CNUPBL 1993		–	–	32 kDa	*X.ev* or *X.p*	KU308459	KU939873	KU887580	KU939972
CNUPBL 1994		–	–	32 kDa	*X.ev* or *X.p*	KU308460	KU939874	KU887581	KU939973
CNUPBL 1995		–	–	32 kDa	*X.ev* or *X.p*	KU308461	KU939875	KU887582	KU939974
CNUPBL 1996		–	–	32 kDa	*X.ev* or *X.p*	KU308462	KU939876	KU887583	KU939975
CNUPBL 1997		–	–	32 kDa	*X.ev* or *X.p*	KU308463	KU939877	KU887584	KU939976
CNUPBL 1998		–	–	32 kDa	*X.ev* or *X.p*	KU308464	KU939878	KU887585	KU939977
CNUPBL 1999		+	–	32 kDa	*X.ev* or *X.p*	KU308465	KU939879	KU887586	KU939978
CNUPBL 2000		–	–	32 kDa	*X.ev* or *X.p*	KU308466	KU939880	KU887587	KU939979
CNUPBL 2001		–	–	32 kDa	*X.ev* or *X.p*	KU308467	KU950308	KU887626	KU939980
CNUPBL 2002		–	–	32 kDa	*X.ev* or *X.p*	KU308468	KU939881	KU887625	KU939981
CNUPBL 2003		–	–	32 kDa	*X.ev* or *X.p*	KU308469	KU939882	KU887624	KU939982
CNUPBL 2004		–	–	32 kDa	*X.ev* or *X.p*	KU308470	KU939883	KU887623	KU939983
CNUPBL 2005		–	–	32 kDa	*X.ev* or *X.p*	KU308471	KU939884	KU887622	KU939984
CNUPBL 2006		–	–	32 kDa	*X.ev* or *X.p*	KU308472	KU939885	KU887621	KU939985
CNUPBL 2007		–	–	32 kDa	*X.ev* or *X.p*	KU323669	KU939886	KU887620	KU939986
CNUPBL 2008		–	–	32 kDa	*X.ev* or *X.p*	KU323670	KU939887	KU887619	KU939987
CNUPBL 2009		–	–	32 kDa	*X.ev* or *X.p*	KU323671	KU939888	KU887618	KU939988
CNUPBL 2010		–	–	32 kDa	*X.ev* or *X.p*	KU323672	KU939889	KU887617	KU939989
CNUPBL 2011		–	–	32 kDa	*X.ev* or *X.p*	KU323673	KU939890	KU887616	KU939990
CNUPBL 2012		–	–	32 kDa	*X.ev* or *X.p*	KU323674	KU939891	KU887615	KU939991
CNUPBL 2013		–	–	32 kDa	*X.ev* or *X.p*	KU323675	KU939892	KU887614	KU939992
CNUPBL 2014		–	–	32 kDa	*X.ev* or *X.p*	KU323676	KU939893	KU887613	KU939993
CNUPBL 2015		–	–	32 kDa	*X.ev* or *X.p*	KU323677	KU939894	KU887612	KU939994
CNUPBL 2016		–	–	32 kDa	*X.ev* or *X.p*	KU323678	KU939895	KU887611	KU939995
CNUPBL 2017		–	–	32 kDa	*X.ev* or *X.p*	KU323679	KU939896	KU887610	KU939996
CNUPBL 2018		–	–	32 kDa	*X.ev* or *X.p*	KU323680	KU939897	KU887609	KU939997
CNUPBL 2019		–	–	32 kDa	*X.ev* or *X.p*	KU323681	KU939898	KU887608	KU939998
CNUPBL 2020		–	–	32 kDa	*X.ev* or *X.p*	KU323682	KU939899	KU887607	KU939999

Table 3. Continued

BSX strain or pepper isolate	Amylolytic activity	Pectolytic hydrolysis	SDS-PAGE	*rpoB* gene based RFLP	Accession number			
					16S rDNA	*gapA*	*gyrB*	*lepA*
CNUPBL 2021	–	–	32 kDa	*X.ev* or *X.p*	KU323683	KU939900	KU887606	KU940000
CNUPBL 2022	–	–	32 kDa	*X.ev* or *X.p*	KU323684	KU939901	KU887605	KU940001
CNUPBL 2023	–	–	32 kDa	*X.ev* or *X.p*	KU323685	KU939902	KU887604	KU940002
CNUPBL 2024	–	–	32 kDa	*X.ev* or *X.p*	KU323686	KU950309	KU887603	KU940003
CNUPBL 2025	–	–	32 kDa	*X.ev* or *X.p*	KU323686	KU939903	KU887602	KU940004
CNUPBL 2026	–	–	32 kDa	*X.ev* or *X.p*	KU323687	KU939904	KU887601	KU940005
CNUPBL 2027	–	–	32 kDa	*X.ev* or *X.p*	KU323688	KU939905	KU887600	KU940006
CNUPBL 2028	–	–	32 kDa	*X.ev* or *X.p*	KU323689	KU939906	KU887599	KU940007
CNUPBL 2029	–	–	32 kDa	*X.ev* or *X.p*	KU323690	KU939907	KU887598	KU940008
CNUPBL 2030	+	–	32 kDa	*X.ev* or *X.p*	KU323691	KU939908	KU887597	KU940009
CNUPBL 2031	–	–	32 kDa	*X.ev* or *X.p*	KU323692	KU939909	KU887596	KU940010
CNUPBL 2032	–	–	32 kDa	*X.ev* or *X.p*	KU323693	KU939910	KU887595	KU940011
CNUPBL 2033	–	–	32 kDa	*X.ev* or *X.p*	KU323694	KU939911	KU887594	KU940012
CNUPBL 2034	–	–	32 kDa	*X.ev* or *X.p*	KU323695	KU939912	KU887593	KU940013
CNUPBL 2035	–	–	32 kDa	*X.ev* or *X.p*	KU323696	KU939913	KU887592	KU940014
CNUPBL 2036	–	–	32 kDa	*X.ev* or *X.p*	KU323697	KU939914	KU887591	KU940015
CNUPBL 2037	–	–	32 kDa	*X.ev* or *X.p*	KU323698	KU939915	KU887590	KU940016
CNUPBL 2038	+	–	32 kDa	*X.ev* or *X.p*	KU323699	KU939916	KU887589	KU940017
CNUPBL 2039	+	–	32 kDa	*X.ev* or *X.p*	KU323700	KU939917	KU887588	KU940018
CNUPBL 2040	–	–	32 kDa	*X.ev* or *X.p*	KU323701	KU939918	KU887628	KU940019
CNUPBL 2041	–	–	32 kDa	*X.ev* or *X.p*	KU323702	KU939919	KU887629	KU940020
CNUPBL 2042	–	–	32 kDa	*X.ev* or *X.p*	KU323703	KU939920	KU887630	KU940021
CNUPBL 2043	–	–	32 kDa	*X.ev* or *X.p*	KU323704	KU939921	KU887631	KU940022
CNUPBL 2044	–	–	32 kDa	*X.ev* or *X.p*	KU323705	KU939922	KU887632	KU940023
CNUPBL 2046	–	–	32 kDa	*X.ev* or *X.p*	KU323706	KU939923	KU887633	KU940024
CNUPBL 2047	–	–	32 kDa	*X.ev* or *X.p*	KU323707	KU939924	KU887634	KU940025
CNUPBL 2048	–	–	32 kDa	*X.ev* or *X.p*	KU323708	KU939925	KU887635	KU940026
CNUPBL 2049	–	–	32 kDa	*X.ev* or *X.p*	KU323709	KU939926	KU887636	KU940027
CNUPBL 2050	–	–	32 kDa	*X.ev* or *X.p*	KU323710	KU939927	KU887637	KU940028
CNUPBL 2058	–	–	32 kDa	*X.ev* or *X.p*	KU323711	KU939928	KU887638	KU940029
CNUPBL 2091	–	–	32 kDa	*X.ev* or *X.p*	KU323712	KU939929	KU887639	KU940030
CNUPBL 2092	+	–	32 kDa	*X.ev* or *X.p*	KU323713	KU939930	KU887640	KU940031
CNUPBL 2094	–	–	32 kDa	*X.ev* or *X.p*	KU323714	KU939931	KU887641	KU940032
CNUPBL 2096	–	–	32 kDa	*X.ev* or *X.p*	KU323715	KU939932	KU887642	KU940033
CNUPBL 2098	–	–	32 kDa	*X.ev* or *X.p*	KU323716	KU939933	KU887643	KU940034

BSX, bacterial spot-causing xanthomonads; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RFLP, restriction fragment length polymorphism; *X.ev*, *Xanthomonas euvesicatoria*; *X.p*, *X. perforans*; *X.v*, *X. vesicatoria*; *X.g*, *X. gardneri*.

and *X. perforans* reference strains, however, were grouped into a different clade. All of the Korean pepper pathogens were grouped together with the reference strains of *X. euvesicatoria* and *X. perforans* (Fig. 3). In a phylogenetic tree of the concatenated sequences of *gapA*, *gyrB*, and *lepA*, all of the reference strains of each species were grouped into the same clade with strains of the same species. All of the Korean pepper pathogens were grouped together with the reference strains of *X. euvesicatoria* (Fig. 4).

Discussion

In this study, 72 pathogenic isolates were collected from bacterial spot lesions on pepper plants throughout Korea in order to reidentify the causative pathogen. The 3 phenotypic characteristics of the Korean pepper pathogens

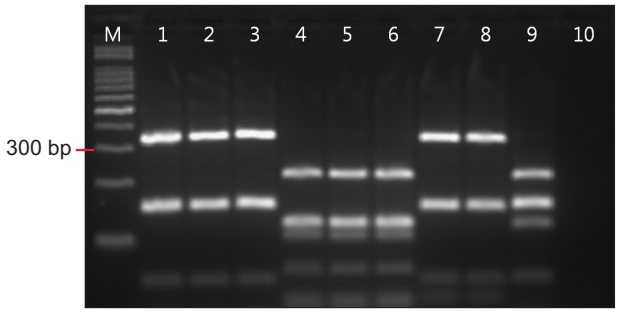

Fig. 2. Result of *rpoB* gene based restriction fragment length polymorphism. Lanes 1–3, *Xanthomonas euvesicatoria* 75-3, LMG667, LMG905; Lanes 4–6, *X. vesicatoria* LMG916, LMG924, ATCC11551; Lanes 7 and 8, *X. perforans* KACC16356, KACC16357; Lane 9, *X. gardneri* NCPPB881; Lane 10, negative control.

and the BSX reference strains were compared for correct identification. The 3 major characteristics were used to separate the 4 species of BSX referred to by Jones et al. (2004). All of the Korean pepper pathogens were negative for pectolytic activity, and all except 5 isolates were negative for amylolytic activity. These traits were identical to those of *X. euvesicatoria* and *X. gardneri*. The 5 amylolytic-positive isolates are not considered to be typical strains of *X. euvesicatoria* or *X. perforans*. Recently, Stoyanova et al. (2014) argued that some phenotype characteristics such as amylolytic activity and the utilization of *cis*-aconitic acid cannot be species-separating criteria of the BSX group. However, all of the Korean pepper pathogens have a 32 kDa protein that is unique to *X. euvesicatoria*. Thus, our results of the analysis of the 3 phenotypic characteristics suggest that all of the Korean pepper pathogens are *X. euvesicatoria*.

The result of *rpoB* based RFLP showed that all of the Korean pepper pathogens have DNA band patterns identical to those of *X. euvesicatoria* and *X. perforans*. A phylogenetic tree of the 16S rDNA sequences also showed that all of the Korean pepper pathogens were grouped together with *X. euvesicatoria* and *X. perforans*. These results suggest that *rpoB* based RFLP and 16S sequences are not enough to separate the 2 BSX species, *X. euvesicatoria* and *X. perforans*. These results also indicate that the 2 are very closely related to each other. MLSA generally gives more detailed genotypic information than does 16S rDNA sequencing. Several previous MLSA studies have also differentiated the 4 species of BSX (Almeida et al., 2010; Hamza et al., 2012; Kebede et al., 2014; Timilsnia et al., 2015). A phylogenetic tree of 3 housekeeping genes (*gapA*, *gyrB*, and *lepA*) showed that all of the Korean pepper isolates were grouped together into the same clade as that of the reference strains of *X. euvesicatoria*.

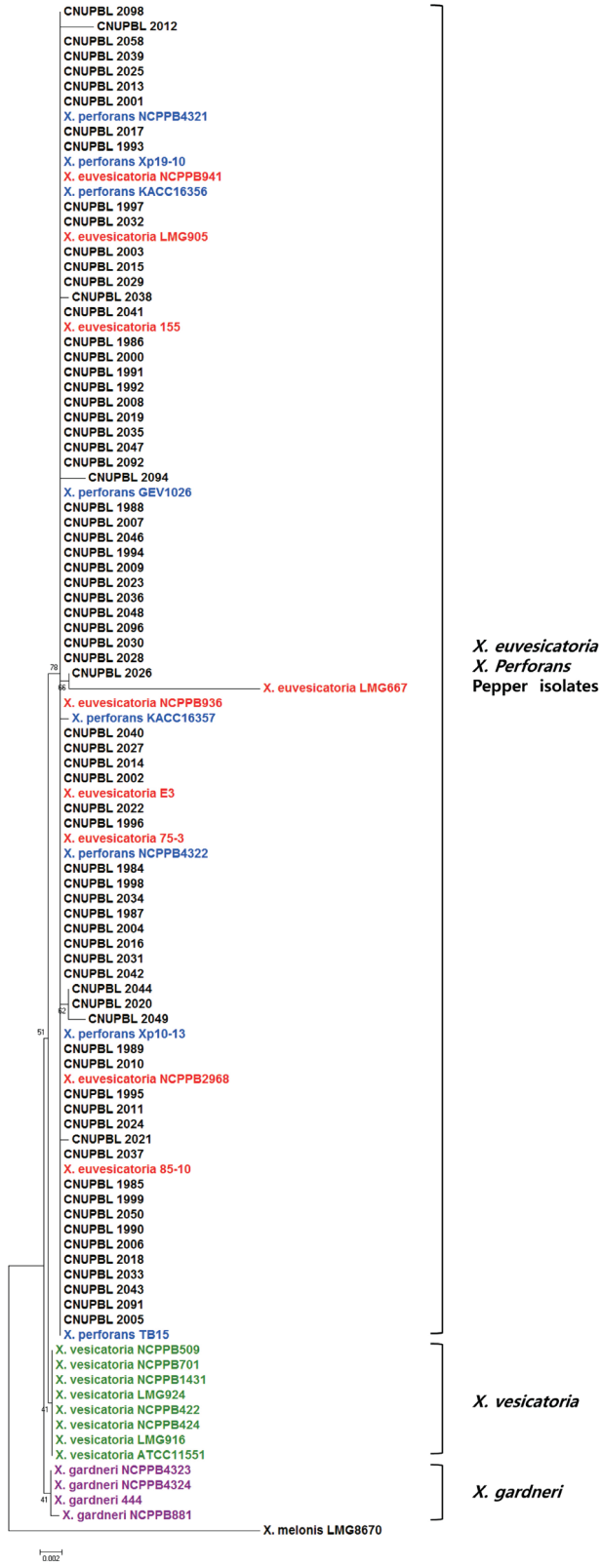

Fig. 3. Phylogenetic tree of 16S rDNA sequences of bacterial spot-causing xanthomonads strains and Korean pepper isolates using MEGA 6.0 program, neighbor-joining tree, Kimura 2-parameter model, and 3,000 bootstrap samples.

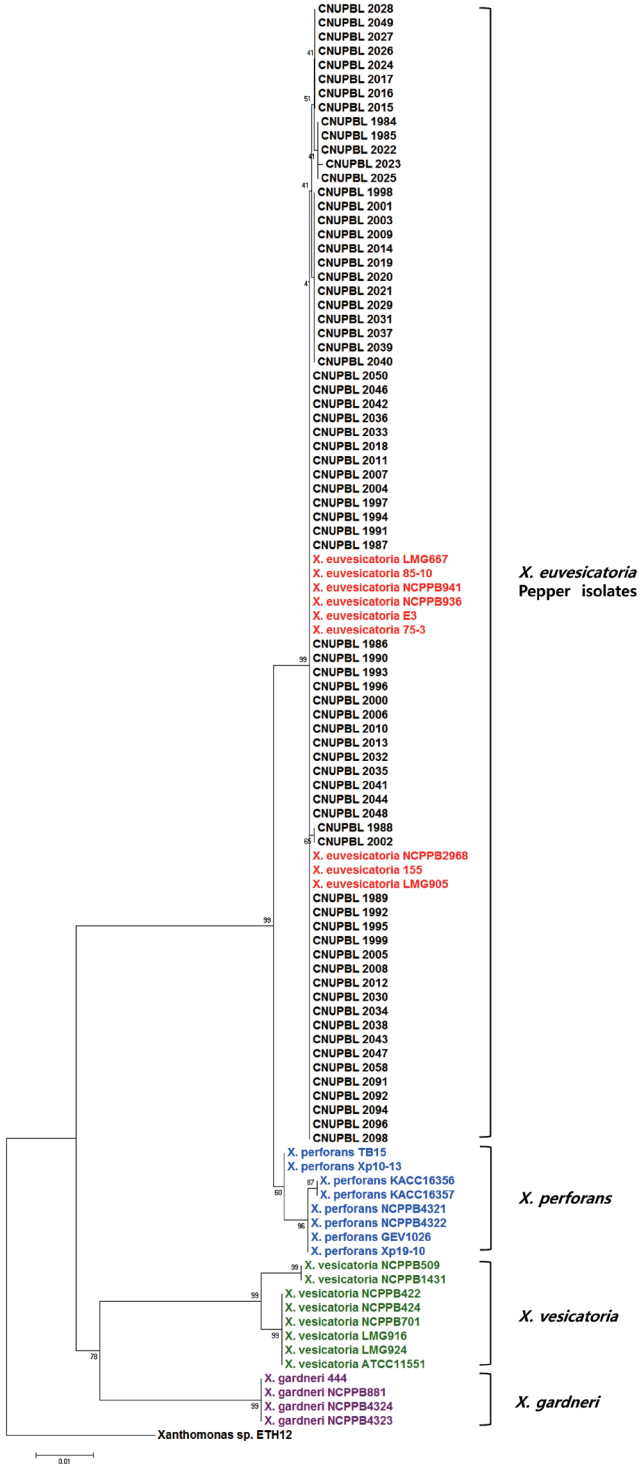

Fig. 4. Phylogenetic tree of a concatenated sequence of *gapA*, *gyrB*, and *lepA* of bacterial spot-causing xanthomonads strains and Korean pepper isolates using MEGA 6.0 program, neighbor-joining tree, Kimura 2-parameter model, and 3,000 bootstrap samples.

The phenotypic and genotypic characteristics of the Korean pepper pathogens suggest that all of those collected in this study are in fact *X. euvesicatoria*. Neither *X. vesicatoria*, which is considered the causative pathogen of pepper bacterial spot in the List of Plant Diseases in Korea (Yoo, 2009), nor *X. perforans*, which was recently reported as the causative pathogen of tomato bacterial spot, was isolated. It might be erroneous to designate *X. vesicatoria* as a causative pathogen of pepper bacterial spot in Korea since we could not find literature references for this. Although there is one study on the isolation of *X. perforans* from pepper plant in the United States, this species does not cause disease on pepper plant in Korea. Nevertheless, bacterial spot caused by *X. perforans* was reported on nursery-raised tomatoes in Korea, but not on field-grown tomatoes (Myung et al., 2009). *X. axonopodis* pv. *vesicatoria* is another species identified as a causative pathogen of pepper bacterial spot according to the List of Plant Diseases in Korea (Yoo, 2009). It was renamed as *X. euvesicatoria* following the reclassification of the 4 BSX species.

The results of the present study suggest that the bacterial spot of pepper plant in Korea is caused exclusively by *X. euvesicatoria*. Recently, Myung et al. (2015) reported that the latest strain of pepper bacterial spot disease in Korea is caused by *X. euvesicatoria*. Based on our study, the pepper bacterial spot reported as a new disease is in fact not new, but rather it is caused by the same pathogen whose scientific name was revised by Jones et al. (2004).

Acknowledgments

This study was supported by the Animal and Plant Quarantine Agency and by a research grant from Chungbuk National University in 2014. We thank Dr. Jeffery Jones for providing the reference strains of BSX.

References

Almeida, N. F., Yan, S., Cai, R., Clarke, C. R., Morris, C. E., Schaad, N. W., Schuenzel, E. L., Lacy, G. H., Sun, X., Jones, J. B., Castillo, J. A., Bull, C. T., Leman, S., Guttman, D. S., Setubal, J. C. and Vinatzer, B. A. 2010. PAMDB, a multilocus sequence typing and analysis database and website for plant-associated microbes. *Phytopathology* 100:208-215.

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.

De Ley, J. 1978. Modern molecular methods in bacterial taxonomy: evaluation, application, prospects. In: *Proceedings of the 4th International Conference on Plant Pathogenic*

Bateria, ed. by M. Ridé, pp. 347-357. Institut National de la Recherche Agronomique (INRA), Angers, France.

Doidge, E. M. 1921. A tomato canker. *Ann. Appl. Biol*. 7:407-430.

Dye, D. W. 1966. Cultural and biochemical reaction of additional *Xanthomonas* species. *N. Z. J. Sci*. 9:913-919.

Ferreira-Tonin, M., Rodrigues-Neto, J., Harakava, R. and Destéfano, S. A. 2012. Phylogenetic analysis of *Xanthomonas* based on partial *rpoB* gene sequences and species differentiation by PCR-RFLP. *Int. J. Syst. Evol. Microbiol*. 62:1419-1424.

Gardner, M. W. and Kendrick, J. B. 1921. Bacterial spot of tomato. *J. Agr. Res*. 21:123-156.

Hamza, A. A., Robene-Soustrade, I., Jouen, E., Lefeuvre, P., Chiroleu, F., Fisher-Le Saux, M., Gagnevin, L. and Pruvost, O. 2012. Multilocus sequence analysis-and amplified fragment length polymorphism-based characterization of xanthomonads associated with bacterial spot of tomato and pepper and their relatedness to *Xanthomonas* species. *Syst. Appl. Microbiol*. 35:183-190.

Jones, J. B., Bouzar, H., Stall, R. E., Almira, E. C., Robert, P. D., Bowen, B. W., Sudberry, J., Strickler, P. M. and Chun, J. 2000. Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. *Int. J. Syst. Evol. Microbiol*. 50:1211-1219.

Jones, J. B., Jones, J. P., Stall, R. E. and Zitter, T. A. 1991. Compendium of tomato diseases. APS Press, St. Paul, MN, USA. p. 73.

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.

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, C. H. 2004. Review of disease incidence of major crop in 2003. *Res. Plant Dis*. 10:1-7.

Lee, S. D. and Cho, Y. S. 1996. Copper resistance and race distribution of *Xanthomonas campestris* pv. *vesicatoria* on pepper in Korea. *Korean J. Plant Pathol*. 12:150-155.

Lee, S. D., Yoon, C. M., Lee, Y. K., Choi, Y. C. and Cho, Y. S. 1999. Occurrence and distribution of bacterial canker of red pepper caused by *Clavibacter michiganensis* subsp. *michiganensis*. *Plant Dis. Agric*. 5:105-110.

Myung, I. S., Hong, S. K., Lee, Y. K., Choi, H. W., Shim, H. S., Park, J. W., Park, K. S., Lee, S. Y., Lee, S. D., Lee, S. H., Choi, H. S., Kim, Y. G., Shin, D. B., Ra, D. S., Yeh, W. H., Han, S. S. and Cho, W. D. 2006. Review of disease incidences of major crops of the South Korea in 2005. *Res. Plant Dis.* 12:153-157.

Myung, I. S., Moon, S. Y., Jeong, I. H., Lee, Y. K. and Ra, D. S. 2009. Bacterial spot of tomato caused by *Xanthomonas perforans*, a new disease in Korea. *Plant Dis*. 93:1349.

Myung, I. S., Park, K. S., Hong, S. K., Park, J. W., Shim, H. S., Lee, Y. K., Lee, S. Y., Lee, S. D., Lee, S. H., Choi, H. W., Heu, S. G., Shin, D. B., Ra, D. S., Yeh, W. H. and Cho, W. D. 2005. Review of disease incidence of major crops of the South Korea in 2004. *Res. Plant Dis*. 11:89-92.

Myung, I. S., Yoon, M. J., Lee, J. Y., Kim, Y. S., Kwon, J. H., Lee, Y. K. and Shim, H. S. 2015. Bacterial spot of hot pepper, caused by *Xanthomonas euvesicatoria*, a new disease in Korea. *Plant Dis*. 99:1640.

Obradovic, A., Mavridis, A., Rudolph, K., Janse, J. D., Arsenijevic, M., Jones, J. B., Minsavage, G. V. and Wang, J. F. 2004. Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from peppers and tomatoes in serbia. *Eur. J. Plant Pathol*. 110:285-292.

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.

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.

Stoyanova, M., Vancheva, T., Moncheva, P. and Bogatzevska, N. 2014. Differentiation of *Xanthomonas* spp. causing bacterial spot in Bulgaria based on biolog system. *Int. J. Microbiol*. 2014:495476.

Šutic, D. 1957. Bakterioze crvenog patlidzana [Tomato bacteriosis]. *Posebna Izd. Inst. Zasht. Bilja, Beograd [Spec. Edit. Inst. Plant Prot., Beograd]* 6:65.

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.

Vauterin, L., Hoste, B., Kersters, K. and Swings, J. 1995. Reclassification of *Xanthomonas. Int. J. Syst. Evol. Microbiol*. 45:472-489.

Yoo, S. H. 2009. List of plant diseases in Korea. 5th ed. The Korean Society of Plant Pathology, Suwon, Korea. p. 76.

Young, J. M., Dye, D. W., Bradbury, J. F., Panagopoulos, C. G. and Robbs, C. F. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. *N. Z. J. Agric. Res*. 21: 153-177.