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Phenotypic and genotypic characterization of *Xanthomonas campestris* pv. *zinniae* strains

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Abstract

During 1997 and 1998, serious outbreaks of bacterial leaf spot disease were observed on zinnia plants grown in home and commercial gardens in Ohio, USA. Twenty-two strains of *Xanthomonas campestris* pv. *zinniae*, isolated from diseased zinnia plants and contaminated seeds, were identified based on morphological, physiological and biochemical tests, fatty acid methyl ester analyses and pathogenicity tests on zinnia cv. Scarlet. Host range studies indicated that all of the *X. campestris* pv. *zinniae* strains were pathogenic on zinnia and tomato, but not on cabbage, lettuce, pepper and radish. The phenotypic and genotypic relationships among the strains determined based on serological reaction pattern, fatty acid profiles, repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) fingerprints and sequence analysis of the 16S–23S rDNA spacer region suggested that *X. campestris* pv. *zinniae* strains were closely related to each other, but clearly distinct from other *Xanthomonas* species including *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians* tested in this study. The results also demonstrated that rep-PCR fingerprinting is rapid, reliable and the most practical method for routine detection and identification of *X. campestris* pv. *zinniae* strains.

Introduction

Bacterial leaf spot caused by *Xanthomonas campestris* pv. *zinniae* (Hopkins and Dowson) Dye, is a serious problem on zinnia cultivars worldwide. This disease was first reported in Italy in 1929 (Nannizzi, 1930). Since then it has been observed in many other countries including Brazil, Sierra Leone, Malawi, Rhodesia, India and Australia (Bertus and Hayward 1971; Deighton, 1957; Peregrine and Siddiqi, 1972; Rangaswami and Gowda, 1963; Robbs, 1954). Occurrence of bacterial leaf spot of zinnia in the United State of America was first found in Ohio in 1972 (Sleesman et al., 1973), and a widespread outbreak of the disease was observed in 1997.

X. campestris pv. *zinniae* is not a well-studied pathogen. Zinnias (*Zinnia elegans* and *Z. haageana*) are the only known natural host of this pathogen (Jones and Strider, 1979). So far no attempts have been made to determine its host range. *X. campestris* pv. *zinniae* can attack all aboveground parts of zinnia plants and cause necrotic lesions on leaves, stems and flowers when environmental conditions are favorable (warm and wet). *X. campestris* pv. *zinniae* has been reported to survive in diseased plant residue in the field and in or on seeds (Strider, 1973; 1979a). Infested and infected seeds are also known to be the major means of long-distance dispersal and primary inoculum source of the pathogen (Strider, 1979b). Thus, the use of pathogen-free seeds and transplants, and seed treatment have been recommended for effective control of this disease (Strider, 1979a; 1980).

Detection and identification of *X. campestris* pv. *zinniae* is currently dependant on isolation of pure cultures on differential or semi-selective media, and biochemical tests, followed by pathological tests

(Strider, 1979b). Since these traditional methods are time-consuming, labor intensive, unreliable and impractical, it is necessary to develop a rapid, sensitive and reliable method for routine diagnosis of *X. campestris* pv. *zinniae* strains from diseased plant samples and contaminated seeds. Recently, a number of techniques such as fatty acid methyl ester (FAME) analysis, protein electrophoresis, serological tests, DNA–DNA hybridization, and genomic DNA fingerprinting or sequencing, have been developed and applied for characterization and classification of plant pathogenic bacteria including Xanthomonads (Gabriel et al., 1989; Gurtler and Stanisich, 1996; Sasser, 1990; Van der Mooter and Swings, 1990; Van Zyl and Steyn, 1990; Vauterin et al., 1991; Verdier et al., 1994). Yang et al. (1993) conducted a comprehensive study based on analysis of cellular fatty acids to determine the relationship among *Xanthomonas* strains representing all *Xanthomonas* species and 134 *X. campestris* pathovars including *X. campestris* pv. *zinniae*. In their study, the results showed that the strains of *X. campestris* pv. *zinniae* and many other *X. campestris* pathovars (such as *X. campestris* pv. *armoraciae*, *campestris*, *raphani*, *vesicatoria* (reclassified as *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*) and *vitians* (reclassified as *X. hortorum* pv. *vitians*)) have similar fatty acid profiles and were classified into the same FAME cluster (FAME cluster 2). More recently Vauterin et al. (1995, 1996, 2000) examined the relationships between *Xanthomonas* strains according to DNA hybridization and metabolic fingerprinting (Biolog) and FAME profiling data, and proposed a new classification. However, *X. campestris* pv. *zinniae* strains were not represented in their studies. In addition, there are many other techniques such as serology, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) fingerprinting and amplification and sequencing of the 16S–23S rDNA spacer region were found useful for analyzing phylogenetic relationships among the strains of many *X. campestris* pathovars (Alvarez et al., 1994; Bouzar et al., 1994; Jones et al., 2000; Louws et al., 1994; 1995; Opgenorth et al., 1996; Sahin, 1997; Schaad et al., 2000; Stall et al., 1994; (Swings and Civerolo, 1993) Vauterin et al., 1995; 1996; 2000). However, none of these techniques were applied to analyze the relationship within and between the strains of *X. campestris* pv. *zinniae* and other *Xanthomonas* species. Thus, application of different techniques in a comprehensive study is necessary to determine the most useful methods for differentiation and identification of *X. campestris* pv. *zinniae* strains.

The objectives of this study were: (1) to characterize strains of *X. campestris* pv. *zinniae* in terms of pathogenicity on different crops, (2) to analyze the population structure of the *X. campestris* pv. *zinniae* strains using traditional and molecular techniques and (3) to evaluate methods which could be useful for differentiation of *X. campestris* pv. *zinniae* from some other *Xanthomonas* species and *X. campestris* pathovars.

Materials and methods

Isolation and identification of X. campestris *pv.* zinniae *strains.* In 1997 and 1998, a total of 22 putative *X. campestris* pv. *zinniae* strains were isolated from contaminated seed and from naturally infected zinnia plants collected from home and commercial gardens in Ohio. Bacterial strains were isolated on CKTM semi-selective medium (Sijam et al., 1992), and identified by morphological, physiological and biochemical tests described previously (Schaad and Stall, 1988; Steel, 1961; Suslow et al., 1982). The fatty acid profiles of strains were determined (Sasser, 1990) using the Microbial Identification System (Hewlett-Packard model 5898A, Palo Alto, CA) with TSBA (Tripticase Soy Broth Agar) database in the Microbial Identification System software package (MIDI; Microbial ID, Inc., Newark, DE). For stock cultures, all strains were grown on Yeast Dextrose Carbonate (YDC) agar medium (Lelliot and Stead, 1987) at 27 °C for 48 h, and stored in sterile water at 15 °C and 15% glycerol at −80 °C. All bacterial strains used in this study are listed in Table 1. All of the tests in this study were done at least twice for all strains.

Pathogenicity and host range. All of the presumptive *X. campestris* pv. *zinniae* strains were tested for pathogenicity on zinnia (*Z. elegans* cv. Scarlet), lettuce (*Lactuca sativa* cv. Darkland), pepper (*Capsicum annuum* cv. Marengo), tomato (*Lycopersicon esculentum* cv. OH88119), cabbage (*Brassica oleracea*) and radish (*Raphanus sativum* cv. Fuego) plants. The pathogenicity test was performed as described by Sahin and Miller (1997). The inoculated plants were incubated in a mist chamber (95% relative humidity) for 3 days, and then transferred to a greenhouse at 25–28 °C. Eight days after inoculation,

Table 1. *Xanthomonas campestris* pv. *zinnia* and the reference strains used in this study

Pathogen/strains	Year isolated	Host/cultivar	Source	Location	Reference*
X. campestris pv. *zinnia*					
Xcz-1, Xcz-2, Xcz-3, Xcz-4,	1997	Zinnia/?	Leaves	Ohio	This study
Xcz-5, Xcz-6, Xcz-7, Xcz-8,	1997	Zinnia/Thumbelina mix	Leaves	Ohio	This study
Xcz-9, Xcz-10, Xcz-11, Xcz -12	1997	Zinnia/Thumbelina mix	Leaves	Ohio	This study
Xcz-13, Xcz-14, Xcz-15, Xcz-16, Xcz-17	1998	Zinnia/Canary	Seed	—	This study
Xcz-18, Xcz-19	1998	Zinnia/Scarlet	Seed	—	This study
Xcz-20, Xcz-21, Xcz-22	1998	Zinnia/Enchantress rose	Seed	—	This study
X. campestris pv. *campestris* Xcc-702a	1995	Cabbage/White	Seed	Ohio	Sahin (1997)
X. campestris pv. *armoraciae* Xca-704b	1995	Radish/Fuego	Seed	Ohio	Sahin (1997)
X. campestris pv. *raphani* DC-91-1	1991	Tomato/?	Seed	Canada	D.A. Cuppels
X. hortorum pv. *vitians* Xcvit-701a,	1995	Lettuce/Darkland	Leaves	Ohio	Sahin (1997)
X. axonopodis pv. *vesicatoria* Xcv-110c	1995	Pepper/Bell	Leaves	Ohio	Sahin (1997)
X. vesicatoria Xcv-766a	1995	Tomato/?	Leaves	Ohio	Sahin (1997)

*D.A. Cuppels, Agriculture and Agri-Food Canada, London, Ont., Canada.

plants were scored for development of characteristic bacterial leaf spot symptoms. Representative strains of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians* (causal agents of black rot of crucifers, bacterial spot of pepper and tomato, bacterial spot of tomato and bacterial leaf spot of lettuce, respectively) were used for comparisons.

Serotype determination. The serological relationship between the strains of *X. campestris* pv. *zinniae* and other test strains was determined based on their positive reaction with the 16 monoclonal antibodies (MAbs) tested in indirect enzyme-linked immunosorbent assay (ELISA) (Bouzar et al., 1994). Eight of these MAbs (Xv1, Xv5, Xv6, Xv8, Xv10, Xv15, Xv21 and Xv30) were developed using *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria* as immunogen (Bouzar et al., 1994). MAbs X9, X13, X17, X21, A11 and B35 were specific to *X. campestris* pv. *campestris* and/or *X. campestris* pv. *armoraciae*, and MAbs X1 and X11 were *Xanthomonas* genus-specific (Alvarez et al., 1994). All these MAbs were provided by J.B. Jones (University of Florida) and A.M. Alvarez (University of Hawaii).

Rep-PCR genomic fingerprinting. Total bacterial genomic DNA was isolated and purified (Louws et al., 1994). The DNA was amplified in the PCR assay using the primers REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′). These primers were derived from a rep sequence common to eubacteria. PCR amplifications were performed in an automated thermocycler (Amplitron II, Thermolyne, Dubuque, Iowa) (Louws et al., 1994). The amplified DNA product was detected using the Nighthawk™ Image Analysis System with Diversity database™ software v1.0 (*phi*, Huntington Station, NY) after electrophoresis in 1% agarose gels and staining with ethidium bromide.

Amplification and sequencing of the 16S–23S rDNA spacer region. The 16S–23S rDNA spacer region of the bacterial strains was amplified by PCR using the oligonucleotide primers 4F (5′-GGCTTGGATCA CCTCCTT-3′) and 7R (5′-GGTTACCTTAGATGTTT CAGTTTC-3′) (Gurtler and Stanisich, 1996). The 4F and 7R primer sequences correspond to positions 1525–1541 in 16S rDNA and 188–208 in 23S rDNA of *Escherichia coli*, respectively (Brosius et al., 1980; Gurtler and Stanisich, 1996). PCR amplification of the target sequences was carried out according to the protocol described by Laguerre et al. (1994). The amplified DNA products were detected as described above and analyzed for length polymorphism. The amplified PCR products from five bacterial strains including *X. campestris* pv. *zinniae* (Xcz-1 and Xcz-21), *X. campestris* pv. *campestris* (Xcc-702a), *X. axonopodis* pv. *vesicatoria* (Xcv-110c) and *X. hortorum* pv. *vitians* (Xcvit-701a) were purified using QIAquick PCR purification kits (Qiagen Inc, Chatsworth, CA) according to the manufacturer's instructions. Direct sequencing of purified amplifi-

cation products was performed in the Biopolymer Facilities, The Ohio State University, Columbus, OH.

Sequence analysis. Sequence alignments were performed and similarity values were calculated using the Clustal method of the MAGALIGN program in the DNASTAR software package (DNA Star, Madison, WI). GeneBank, EMBL, DDBJ and PDB databases were searched for sequence similarities using Gapped BLAST and PSI-BLAST programs (Altschul et al., 1997).

Nucleotide sequence accession numbers. The 16S–23S rDNA sequences of the following strains, *X. campestris* pv. *zinniae* Xcz-1 and Xcz-21, *X. campestris* pv. *campestris* 702a, *X. axonopodis* pv. *vesicatoria* 110c and *X. hortorum* pv. *vitians* 701a, have been deposited in the GeneBank nucleic acid sequence database under the accession numbers AF064517, AF064518, AF064519, AF064520 and AF060177, respectively.

Results

Identification of X. campestris *pv.* zinniae *strains.* A total of 22 bacterial strains were isolated and purified from contaminated seed and diseased plant samples. All of the strains grew on CKTM medium and produced large round, mucoid pale yellow colonies that are characteristic for *Xanthomonas*. Morphological, physiological and biochemical test results showed that all strains were Gram-negative, rod-shaped, motile, aerobic, catalase-positive, oxidase-negative, amylolytic, nonpectolytic and grew at 35 °C on YDC plates. All of these strains isolated from zinnias in this study were identified as *X. campestris* on the basis of FAME profiles which matched the strains to different *X. campestris* pathovars (such as *armoraciae*, *campestris*, *raphani*), *X. hortorum* pv. *vitians* or *X. axonopodis* pv. *vesicatoria* with similarity indices (SI) ranged from 0.175 to 0.806. No significant (qualitative or quantitative) differences in cellular fatty acid contents of the strains were observed.

Pathogenicity and host range. Pathovar identity of all 22 strains isolated from zinnias in Ohio were determined as *X. campestris* pv. *zinniae* based on pathogenicity tests. All strains produced characteristic leaf spot symptoms on inoculated zinnia (cv. Scarlet) plants in greenhouse conditions. Moreover, tomato (cv. OH88119) plants inoculated with *X. campestris* pv. *zinniae* strains were observed with necrotic leaf spots, whereas other crops including cabbage, radish, pepper and lettuce, show no diagnostic disease symptoms. However, none of the representative strains of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians* tested in this study were pathogenic on zinnia plants.

Serotype determination. None of the 22 *X. campestris* pv. *zinniae* strains reacted with MAbs (Xv1, Xv5, Xv6, Xv8, Xv10, Xv15, Xv21 and Xv30 (specific to *X. axonopodis* pv. *vesicatoria* and/or *X. vesicatoria* strains) or MAbs X9, X13, X17, X21, A11 and B35 (specific to *X. campestris* pv. *campestris*/pv. *armoraciae* strains). However, all the *X. campestris* pv. *zinniae* strains reacted with *Xanthomonas* genus-specific MAbs X1 and X11.

Rep-PCR genomic fingerprinting. Analysis of the rep-PCR profiles data showed that strains of *X. campestris* pv. *zinniae* were homogenous, displaying a unique banding pattern consisting of eight distinct bands ranged approximately from 0.4 to 3 kb (Figure 1). The representative strains of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria* and *X. hortorum* pv. *vitianas* had different banding patterns, which were readily distinguished from those of *X. campestris* pv. *zinniae* strains (Figure 1).

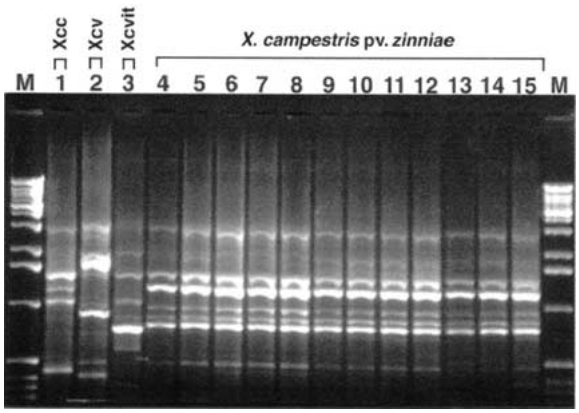

Figure 1. Rep-PCR genomic fingerprint patterns of *X. campestris* strains; Lanes: (1) *X. campestris* pv. *campestris* Xcc-702a; (2) *X. axonopodis* pv. *vesicatoria* Xcv-110c; (3) *X. hortorum* pv. *vitians* Xcvit-701a; (4–15) *X. campestris* pv. *zinniae* strains (Xcz-1, Xcz-2, Xcz-4, Xcz-6, Xcz-7, Xcz-9, Xcz-11, Xcz-14, Xcz-16, Xcz-18, Xcz-19, Xcz-20); (M) DNA ladder (1 kb).

Amplification and sequencing of the 16S–23S rDNA spacer region. Amplification of the 16S–23S rDNA spacer regions from the strains of *X. campestris* pv. *zinniae* and other *Xanthomonas* species, listed in Table 1, yielded a single amplicons in size of approximately 680 bp. Direct sequencing of the amplicons from strains of *X. campestris* pv. *zinniae* Xcz-1 and Xcz-21, *X. campestris* pv. *campestris* 702a, *X. axonopodis* pv. *vesicatoria* 110c and *X. hortorum* pv. *vitians* 701a, revealed significant sequence differences in the 16S–23S rDNA spacer regions of the strains (Figure 2). The degree of sequence similarity between the strains of *X. campestris* pv. *zinniae* Xcz-1 and Xcz-21 was relatively high (96.9%)

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Xcz-21      CAGGTCGGTATGCGAA- GTCCCTTT- TGGGGCCTTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGG
Xcv-110c    CAGGCCGATATGCGAAAGTCCCATCATGGGGCCTTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGG
Xcvit-701   CAGGTCGGTATGCGAA- GTCCCTTT- TGGGGCCTTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGG
Xcc-702     CAGGTCGGTATGCGAA- GTCCCTTT- TGGGGCCTTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGG

Xcz-1       GGTCGTCGGTTCGATCCCGACAGGCTCCACCATATTGAGTGAAAAGACTTCGGGTCTGTAGCTCAGGTGGT-214
Xcz-21      GGTCGTCGGTTCGATCCCGACAGGCTCCACCATATTGAGTGAAAAGACTTCGGGTCTGTAGCTCAGGTGGT
Xcv-110c    GGTCGTCGGTTCGATCCCGACAGGCTCCACCAT- TTGAGTGAAACGACTTTGGGTCTGTAGCTCAGGTGGT
Xcvit-701a  GGTCGTCGGTTCGATCCCGACAGGCTCCACCATATTGAGTGAAAAGACTTCGGGTCTGTAGCTCAGGTGGT
Xcc-702a    GGTCGTCGGTTCGATCCCGACAGGCTCCACCATATTGAGTGAAAAGACTTCGGGTCTGTAGCTCAGGTGGT

Xcz-1       TAGAGCGCACCCCTGATAAGGGTGAGGTCGGTAGTTCGAGTCTACCCAGACCCACCACTCTGAATGTAGTG-288
Xcz-21      TAGAGCGCACCCCTGATAAGGGTGAGGTCGGTAGTTCGAGTCTACCCAGACCCACCACTCTGAATGTAGTG
Xcv-110c    T- GAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCACTCTGAATGTAGTG
Xcvit-701a  TAGAGCGCACCCCTGATAAGGGTGAGGTCGGTAGTTCGAGTCTACCCAGACCCACCACTCTGAATGTAGTG
Xcc-702     TAGAGCGCACCCCTGATAAGGGTGAGGTCGGTAGTTCGAGTCTACCCAGACCCACCACTCTGAATGTAGTG

Xcz-1       CACACTTAAGAATTTATATGGATCAGCGTTGAGGCTGATACATGTTCTTTTATAACTTGTGA- CGTAGCGAGC-358
Xcz-21      CACACTTAAGAATTTATATGGATCAGCGTTGAGGCTGATACATGTTCTTTTATAACTTGTGA- CGTAGCGAGC
Xcv-110c    CACACTTAAGAATTTATATGGCTCAGCGTTGAGGCTGAGACATGTTCTTTTATAACTTGTGAACGTAGCGAGC
Xcvit-701a  CACACTTAAGAATTTATATGGATCAGCGTTGAGGCTGAGACATGTTCTTTTATAACTTGTGA- CGTAGCGAGC
Xcc-702a    CACACTTAAGAATTTATATGGATCAGCGTTGAGGCTGAGACATGTTCTTTTATAACTTGTGA- CGTAGCGAGC

Xcz-1       GTTTGAGATATCTATCTAAACGTGTCGTTGAAGCTAAGGCGGGGACTTCGAGTCCCTAAA- TAATTGAGTCG-430
Xcz-21      GTTTGAGATATCTATCTAAACGTGTCGTTGAAGCTAAGGCGGGGACTTCGAGTCCCTAAAATAATTGAGTCG
Xcv-110c    GTTTGAGATATCTATCTAAACGTGTCGTTGAGGCTAAGGCGGGGACTTCGAGTCCCTAAA- TAATTGAGTCG
Xcvit-701a  GTTTGAGATATCTATCTAATCGTGTCGTTGAGGCTAAGGCGGGGACTTCGAGTCCCTAAA- TAATTGAGTCG
Xcc-702a    GTTTGAGATATCTATCTAAACGTGTCGTTGAAGCTAAGGCGGGGACTTCGAGTCCCTAAA- TAATTGAGTCG

Xcz-1       TATGTTCGCGTTTGGTGGCTTTGTACCCCACACAACACG- GCATAT- AGCTCCGAGGCAACTTGGGGTTATAT-494
Xcz-21      TATGTTCGCGTTTGGTGGCTTTGTACCCCACACAACACG- GCATAT- AGCTCCAAGGCAACTTGGGGTTATAT
Xcv-110c    TATGTTCGCGTT- GGTGGCTTTGTACCCCACACAACACG- GCATG - - - ACCCTGAGGCAACTTGGGGTTATAT
Xcvit-701a  TATGTTCGCGTT- GGTGGCTTTGTACCCCACACAACACGTACATGT- AGCTCCAAGGCAACTTGGGGTTATAT
Xcc-702a    TATGTTCGCGTTGGGTGGCTTTGTTACCCACACAACACGTACATGTTAGCTCCGAGGCAACTTGGGGTTATAT

Xcz-1       GGTCAAGCGAATAAGCGCACACGGTGGATGCCTAGGCGGTCAGAGGCGATGAAGGACGTGGTAGCCTGCG-564
Xcz-21      GGTCAAGCGAATAAGCGCACACGGTGGATGCCTAGGCGGTCAGAGGCGATGAAGGACGTGGTAGCCTGCG
Xcv-110c    GGTCAAGCGAATAAGCGCACACGGTGGATGCCTAGGCGGTCAGAGGCGATGAAGGACGTGGTAGCCTGCG
Xcvit-701a  GGTCAAGCGAATAAGCGCACACGGTGGATGCCTAGGCGGTCAGAGGCGATGAAGGACGTGGTAGCCTGCG
Xcc-702a    GGTCAAGCGAATAAGCGCACACGGTGGATGCCTAGGCGGTCAGTGGCGATGTAGGACGTGGTAGCCTGCG

Xcz-1       AAAAGTGTCGGGGAGCTGGCAACAAGCTTTGATCCGGCAATATCCGAATGGGGAAACCCACTGCTTCGG-633
Xcz-21      AAAAGTGTCGGGGAGCTGGCAACAAGCTTTGATCCGGCAATATCCGAATGGGGAAACCCACTGCTTCGG
Xcv-110c    AAAAGTGTCGGGGAGTTGGCAACAAGCTTTGATCCGGCAATATCCGAATGGGGAAACCCACTGCTTCGG
Xcvit-701a  AAAAGTGTCGGGGAGCTGGCAACAAGCTTTGATCCGGCAATATCCGAATGGGGAAACCCACTGCTTCGG
Xcc-702a    AAAAGTGTCGGGGAGCTGGCAACAAGCTTTGATCCGGCAATATCCGAATGGGGAAACCCACTGCTTCGG

Xcz-1       CAGTATCCTGCAGTGAATTCATAGCTGCTGGAAGCGAACCCCGT-677
Xcz-21      CAGTATCCTGCAGTGAATTCATAGCTGCTGGAAGCGAACCCCGT
Xcv-110c    CAGTATCCTGCAGTGAATTCATAGCTGCTGGAAGCGAACCCGGT
Xcvit-701a  CAGTATCCTGCAGTGAATTCATAGCTGCTGGAAGCGAACCCCGA
Xcc-702a    CAGTATCTTGCAGTGAATTCATAGCTGCTTGAAGCGAACCCCGT
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Figure 2. Sequences alignment of the 16S–23S rDNA spacer region of *X. campestris* pv. *campestris* 702a, *X. axonopodis* pv. *vesicatoria* Xcv-110c, *X. hortorum* pv. *vitians* Xcvit-701a and *X. campestris* pv. *zinniae* strains Xcz-1 and Xcz-21. Nucleotide deletions are indicated by dashes. Two transfer RNA genes $tRNA^{Ala}$ and $tRNA^{Ile}$ (underlined) are located within the spacer region between nucleotides 98–174 and 193–269, respectively.

compare to the similarity between *X. campestris* pv. *zinniae* strains and other *Xanthomonas* species tested was in the range of 87.7–94.8% (Figure 3). Comparisons of these sequences with GeneBank, EMBL, DDBJ and PDB databases (Altschul et al., 1997) showed that the 16S–23S rDNA spacer regions of all *X. campestris* pathovars contain two conserved regions corresponding to transfer RNA (tRNA) genes $tRNA^{Ala}$ and $tRNA^{Ile}$ (Figure 2).

Discussion

This is the first study showing that *X. campestris* pv. *zinniae* can cause bacterial spot disease on tomato and zinnias, but not on cabbage, lettuce, pepper and radish. These results suggest that tomato may have potential to be an alternative host of this pathogen in field conditions. Since none of the *X. campestris* pv. *zinniae* type strains were included in this study, it is difficult to speculate that tomato is an alternative host for all known *X. campestris* pv. *zinniae* strains from different locations. Therefore, it would be useful to conduct a further and more comprehensive study to determine the relationship among *X. campestris* pv. *zinniae* strains in terms of pathogenicity and host range. Furthermore, this study also demonstrated that the other *Xanthomonas* species tested (*X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians*) were not pathogenic on zinnia. Thus, *X. campestris* pv. *zinniae* strains can be distinguished from *X. campestris* pv.*campestris*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians* on the basis of pathogenicity on zinnia.

The phenotypic relationships among the strains of *X. campestris* pv. *zinniae* isolated from zinnias in Ohio in 1997 and 1998, and other *Xanthomonas* species were examined based on FAME analyses and indirect ELISA. FAME profiles of *X. campestris* pv. *zinniae* strains appeared to be closely homologous. FAME analysis data in this study also confirmed the previous report (Yang et al., 1993) which showed that *X. campestris* pv. *zinniae* and some other *X. campestris* pathovars (such as *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians*) have similar FAME profiles. Thus, FAME analysis is not a useful method for identification of *X. campestris* pv. *zinniae* strains at the species or pathovar level.

Serological reaction patterns of the strains with the 16 MAbs in indirect ELISA showed that only two *Xanthomonas* genus-specific MAbs (X1 and X11) weakly reacted with all of the *X. campestris* pv. *zinniae* strains whereas none of the remaining 14 MAbs (including eight *X. axonopodis* pv. *vesicatoria* and/or *X. vesicatoria*-specific MAbs Xv1, Xv5, Xv6, Xv8, Xv10, Xv15, Xv21, Xv30 or six *X. campestris* pv. *campestris*/*armoraciae*-specific MAbs X9, X13, X17, X21, A11 and B35) (Alvarez et al., 1994; Bouzar et al., 1994), reacted with any of the *X. campestris* pv. *zinniae* strains tested. Serological reaction patterns of the strains indicated that there were no serological differences among *X. campestris* pv. *zinniae* strains. In addition, screening of the pathovar-specific MAbs against a collection of the strains representing different pathovars of *X. campestris* demonstrated that seven of these MAbs (Xv5, Xv6, Xv8, Xv10, X21, A11 and B35) were reacted with the strains of other *X. campestris* pathovars such as *X. campestris* pv. *campestris*, *X. campestris* pv. *armoraciae* and *X. campestris* pv. *raphani* and/or *X. hortorum* pv. *vitians* (Sahin, 1997; Sahin et al., 2002). Consequently, ELISA with a panel of the 16 MAbs may be useful for discrimination of *X. campestris* pv. *zinniae* strains from *X. campestris* pv. *raphani*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria* and *X. hortorum* pv. *vitians* strains, which are known as causal agents of bacterial spot disease on tomato (Sahin and Miller, 1998; Sahin et al., 2002). However, in the absence of specific MAbs, this method could not be used for detection and identification of *X. campestris* pv. *zinniae* strains.

The genotypic relationships between *X. campestris* strains were determined based on rep-PCR fingerprints and sequence analysis of the 16S–23S rDNA spacer regions. The genomic fingerprint data generated with rep-PCR demonstrated that all *X. campestris* pv. *zinniae* strains had an identical banding pattern. However, rep-PCR profiles of *X. campestris* pv. *zinniae* strains were different than those of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria* and *X. hortorum* pv. *vitians*. This finding indicates that *X. campestris* pv. *zinniae* strains can be distinguished by rep-PCR profiles.

Amplification of a single PCR product (approximately 680 bp) from the 16S–23S rDNA spacer regions of the strains representing six different pathovars of *X. campestris* including *X. campestris* pv. *zinniae* suggested that *X. campestris* pathovars are too closely related to be discriminated on the basis of size variations of spacer region. Sequence analysis of the

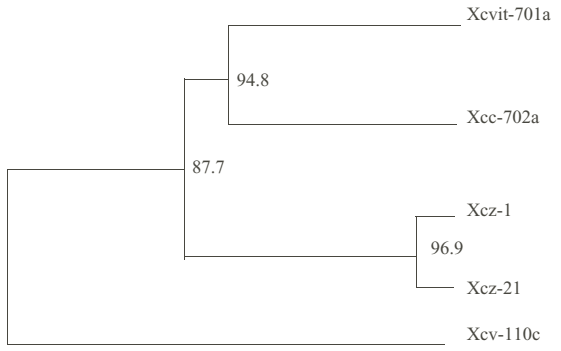

Figure 3. Dendrogram estimated phylogenetic relationships based on sequences similarity in the 16S–23S rDNA spacer regions of *X. campestris* pv. *zinniae* strains Xcz-1 and Zcz-21, *X. axonopodis* pv. *vesicatoria* Xcv-110c, *X. hortorum* pv. *vitians* Xcvit-701a and *X. campestris* pv. *campestris* Xcc-702a.

PCR-amplified DNA fragment from the 16S–23S rDNA spacer regions revealed relatively a high level of sequence similarity (approximately 89–97%) among the strains representing *X. axonopodis* pv. *vesicatoria*, *X. hortorum* pv. *vitians*, *X. campestris* pv. *campestris* and *X. campestris* pv. *zinniae* (Figures 2 and 3). However, the sequence differences between strains was significant enough to separate *X. campestris* pv. *zinniae* strains from the others tested. These results confirmed the previous studies suggesting that rep-PCR fingerprinting and sequence analysis of 16S–23S rDNA spacer regions are powerful molecular techniques not only for estimating genetic relatedness, but also for detection and identification of bacterial strains such as *X. campestris* pv. *zinniae* (Brosius et al., 1980; Gurtler and Stanisich 1996; Louws et al., 1994; 1995; Opgenorth et al., 1996; Vauterin et al., 2000). Since sequencing of 16S–23S rDNA spacer regions for routine diagnosis would be costly and time-consuming. It is necessary to conduct a further study for designation of specific PCR primers from 16S–23S rDNA spacer region sequences that could be used for rapid detection and identification of *X. campestris* pv. *zinniae* and other *Xanthomonas* species pathogenic on tomato.

This is the first study to show that there is a high level of phenotypic and genotypic relationships among *X. campestris* pv. *zinniae* strains which can easily be diagnosed by pathogenicity test on zinnia, rep-PCR fingerprinting and sequence analysis of the 16S–23S rDNA spacer regions. However, rep-PCR appears to be the most practical method for detection and identification of this pathogen.

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