

GENETIC SIMILARITY OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* STRAINS SHOWING VARIOUS VIRULENCE

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Abstract: Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* appeared to be recently the most important disease on tomato in Poland. The genetic relationships among four Polish strains of race 0 *P. syringae* pv. *tomato* of different origin, isolated from tomato plants, were examined by RAPD and PCR-RFLP techniques. Amplification of bacterial DNA using 33 primers with RAPD technique showed, that similarity of strains expressed by the Nei-Li coefficient was very high (above 0.8). Next, the restriction analysis of amplified region ITS with the use of 5 endonucleases revealed, that profiles obtained from electrophoretic separation of DNA fragments were also very similar. On the basis of those analyses it was concluded that all strains tested constituted a closely related group. However, they showed various level of virulence as was demonstrated on the inoculated leaves of tomato plants growing in the greenhouse.

Key words: bacterial speck, RAPD, PCR-RFLP

INTRODUCTION

Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young, Dye & Wilkie is of great economic significance for greenhouse and field tomato throughout the world (Bashan et al. 1978; Devash et al. 1980; Smitley and McCarter 1982). In Poland, severe outbreaks of bacterial speck on tomato fields have been reported during the past decade, causing lower productivity and quality of varieties. The problem is mainly related to the susceptible tomato cultivars grown in Poland (Macias 1999). Therefore, research on different aspects on breeding for resistance to bacterial speck of tomato was undertaken at the Research Institute of Vegetable Crops, Skierniewice to enable the selection of a number of resistant cultivars (Kozik 2004).

Knowledge of the possible variations in pathogenic ability of *P. syringae* pv. *tomato* is very important for planning of breeding programs designed to develop disease-

resistant cultivars. Our earlier studies (Kozik and Sobiczewski 2000) revealed that variation found in virulence of pathogen strains (Mitchell et al. 1983; Bashan et al. 1978) affects symptom expression and creates difficulties in determination of resistant genotypes during breeding programs. To study the genetic relatedness of bacterial strains the random amplified polymorphic DNA (RAPD) and the restriction fragment length polymorphism (RFLP) techniques have been used widely (Denny 1988; Denny et al. 1988; Cook et al. 1989; Henderson et al. 1992; Louws et al. 1994).

The purpose of the present study is to report on relationship of *P. syringae* pv. *tomato* strains isolated in Poland in terms of their virulence and genetic characteristics.

MATERIALS AND METHODS

Bacterial strains

Four strains of race 0 *P. syringae* pv. *tomato* of different Polish origin were used: Pst 1 and Pst 4 – from the Department of Plant Protection, Institute of Pomology and Floriculture, Skierniewice; Pst 3 – from the Department of Plant Protection, Research Institute of Vegetable Crops, Skierniewice; Pst 5 – from The Collection of Plant Pathogens, Institute of Plant Protection, Poznan. Stock cultures of all bacterial strains were maintained on 2.3% nutrient agar (Difco) with 1.6% glycerol under paraffin oil at 5°C.

Preparation of inoculum

For the preparation of inoculum, bacteria were incubated at 24°C on nutrient agar medium supplemented with 1% glucose. The inoculum was prepared by washing 24 hours-old cultures of bacteria with sterile distilled water. Concentration of bacteria in the suspension was determined spectrophotometrically and adjusted to 10^8 and 10^7 cfu/ml by serial dilution plating method. Before inoculation of tomato plants the strains were checked with hypersensitivity tests on tobacco cv. Samsun according to Klement (1963).

Evaluation of bacterial virulence

The test was performed on tomato seedlings of A100 and Ontario 7710 cultivars grown in a greenhouse. A100 is a highly inbred line, commonly used as a susceptible standard in our tomato breeding program for resistance to various diseases of biotic origin, including bacterial speck. Ontario 7710 is widely recognized to be a highly resistant cultivar to this disease. When the tomato seedlings of both cultivars reached four leaf stage they were sprayed with inoculum of each strain separately. The plants were kept under plastic cover for 4–5 days after inoculation to maintain a relative humidity of 100%. The temperature was set up at 21–27°C day/night. 45 plants of each cultivar were inoculated with inoculum concentration of 10^7 or 10^8 cfu/ml.

Virulence of tested bacteria was determined on the basis of bacterial speck severity. The lesions of bacterial speck on leaves were counted about 14 days after inoculation, then an overall rating for the plot using the scale of Chambers and Merriman (1975) was calculated as follows: 0 = no lesions; 1 = 1–10 lesions per plant; 2 = 11–20 lesions; 3 = 21–40 lesions; and 4 = more than 40 lesions per plant. All data were subjected to analysis of variance and separation of means was performed using t-Student's test at 5% of probability.

Bacterial DNA isolation

Bacteria were grown on King B medium for 48h at 26°C. DNA of tested bacteria

was isolated using the method of Aljanabi and Martinez (1997). DNA concentration and quality was estimated using spectrophotometer BioPhotometer (Eppendorf, Germany) and by electrophoresis in 0.8% agarose gel.

RAPD analysis

DNA of four tested strains (Pst 1, Pst 3, Pst 4, Pst 5) was amplified with application of 31 random 10-mer oligonucleotide primers (Operon Technologies Inc., USA) and 2 primers labelled CUGEA1 and CUGEA5 (Momol et al. 1997) (Table 1).

Table 1. Sequences of RAPD primers used for *Pseudomonas syringae* pv. *tomato* strains analysis

Primer	Sequence 5' → 3'	Primer	Sequence 5' → 3'
OPA01	CAGGCCCTTC	OPB10	CTGCTGGGAC
OPA02	TGCCGAGCTG	OPB12	CCTTGACGCA
OPA03*	AGTCAGCCAC	OPB18*	CCACAGCAGT
OPA05	AGGGGTCTTG	OPC02*	GTGAGGCGTC
OPA07*	GAAACGGGTG	OPC06	GAACGGACTC
OPA08	GTGACGTAGG	OPC07*	GTCCCGACGA
OPA09*	GGGTAACGCC	OPC08*	TGGACCGGTG
OPA10*	GTGATCGCAG	OPD04	TCTGGTGAGG
OPA11*	CAATCGCCGT	OPD16	AGGGCGTAAG
OPA15*	TTCCGAACCC	OPE06*	AAGACCCCTC
OPA16*	AGCCAGCGAA	OPE14	TGCGGCTGAG
OPA20*	GTTGCGATCC	OPU19*	GTCAGTGCGG
OPAR03	GTGAGGCGCA	OPW06*	AGGCCCGATG
OPB05*	TGCGCCCTTC	OPW20	TGTGGCAGCA
OPB06	TGCTCTGCCC	CUGEA1	TCGCCAGCGA
OPB07	GGTGACGCAG	CUGEA5	CGATCGATGC
OPB08	GTCCACACGG		

*primers which gave polymorphic products differentiating tested strains

The reaction mixture (15 µl) contained 30 ng of template DNA, 0.8 U of *Taq* DNA polymerase (Gibco BRL/Invitrogen Sand Diego, USA), 10 pmol of a single primer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 10 × PCR reaction buffer. PCR reaction conditions: initial denaturation at 94°C for 3 min, 40 cycles of amplification: 94°C for 45 s, 36°C for 45 s, 72°C for 1 min and final elongation step was completed at 72°C for 10 min. Amplifications were done in thermocycler TRIO – Thermoblock (BIOMETRA, Göttingen, Germany) and products were separated in 1.5% agarose gels. All analyses were done at least twice. Each amplification band was scored as 1 (present) and 0 (absent) for all strains. Genetic distances were calculated using Nei and Li formula (1979). The strains were clustered by the unweighted average pair group method (UPGMA).

PCR-RFLP of ITS region

On the basis of computer analysis of 16S–23S rDNA ITS region sequence of strain Pst DC3000 (GenBank AE 0116875), five endonucleases: *Taq* I, *Hind* III, *Pvu* II, *Hae* III, *Hpa* II (Fermentas, Lithuania) were selected as the most appropriate for RFLP analysis. ITS region

of *P. s. pv. tomato* strains tested was amplified with primers 1406f and 23S (Fisher and Triplett 1999) in 50 μ l of reaction mixture. Amplification was performed in PCR buffer (10 mM TRIS-HCl pH 8.8; 50 mM KCl; 0.08% Nonidet P40) with 0.2 mM dNTPs, 50 pmol of each primer, 2.5 mM MgCl₂ and 2 U of *Taq* DNA polymerase (Fermentas, Lithuania). Initial denaturation was performed at 94°C for 2 min., followed by 30 cycles of denaturation at 94°C for 15s, annealing at 55°C for 15s, extension at 72°C for 45s and a final extension step for 2 min. Amplification products were precipitated with 2.5 volume of 96% ethanol and 0.1 volume of 3M NaAc pH 3.2. Precipitated DNA was dissolved in 11 μ l of TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0). One μ l of obtained DNA solution was analysed for estimation of DNA concentration by electrophoresis in 1.5% agarose gel. Two μ l of DNA was used for each restriction analysis with chosen enzymes. RFLP products were separated in 2% agarose gel.

Because the general opinion that *P. s. pv. tomato* host range is limited to closely related plant species (Denny 1988; Denny et al. 1988), we have examined the diversity of the pathogen on strains that were isolated only from one host that was tomato.

RESULTS

Virulence of bacteria

All tested strains caused necrosis of tobacco leaf tissue after 24 hours from its infiltration, which indicates typical HR reaction. All strains also produced typical symptoms of bacterial speck on leaves characterized by dark specks of 2–3 mm in diameter surrounded by chlorotic halo observed 3–4 days after inoculation. Disease severity on inoculated tomato seedlings depended on tested strain and cultivar used. Rating performed 2 weeks after inoculation indicated that A 100 showed significantly higher disease severity than Ontario 7710 at both concentrations (10⁷ and 10⁸ cfu/ml) (Table 2).

Table 2. The severity of bacterial speck caused by four *Pseudomonas syringae* pv. *tomato* strains on two tomato genotypes

Genotype	Strain			
	Pst 1	Pst 3	Pst 4	Pst 5
Inoculum concentration 10 ⁸ cfu/ml				
A 100	3.3 aB	3.9 aC	3.7 aC	2.0 aA
Ontario 7710	0.4 bA	1.1 bB	0.4 bA	0.3 bA
Inoculum concentration 10 ⁷ cfu/ml				
A 100	1.1 aA	2.1 aC	2.4 aC	1.8 aB
Ontario 7710	0.0 bA	0.2 bA	0.3 bA	0.1 bA

Means followed by the same capital letter(s) indicate no significant difference between strains for each genotype within each test (inoculum concentration); means followed by the same small letter indicate no difference between cultivars for each strain within one test (inoculum concentration)

The strain Pst 3 applied at concentration of 10⁸ cfu/ml appeared to be the most virulent on both cultivars. The lowest average number of lesions was caused by Pst 5. However, at concentration of 10⁷ cfu/ml the highest virulence showed strain Pst 4 and in case of Pst 3 it was slightly but not significantly lower. It should be pointed out that both tested cultivars significantly differed in reaction to infection.

RAPD analysis

Number of obtained products in reaction with each out of 33 primers ranged from 2 for primer OPB06 to 13 for primer OPC02. All used primers generated 230 informative PCR bands (amplification products). From among all used primers (Table 1) as a result of amplification with 16 primers polymorphic products differentiating tested strains were obtained (Fig. 1). The dendrogram constructed on the basis of combined data of all 33 primers used shows mutual similarity of studied strains and reveals their low diversity (coefficient of Nei and Li > 0.76) (Fig. 2). The most divergent was strain Pst 4 but its similarity to other strains was still high.

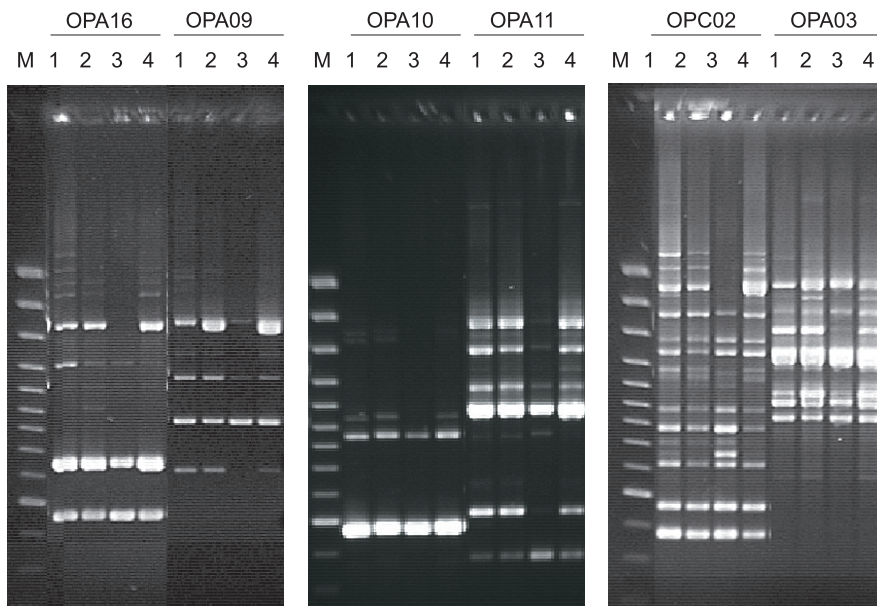


Fig. 1. Gel electrophoresis of DNA fragments of four *Pseudomonas syringae* pv. *tomato* strains from amplification with six chosen RAPD primers. M – 100 bp DNA Ladder (Fermentas, Lithuania), 1 – Pst 1, 2 – Pst 3, 3 – Pst 4, 4 – Pst 5

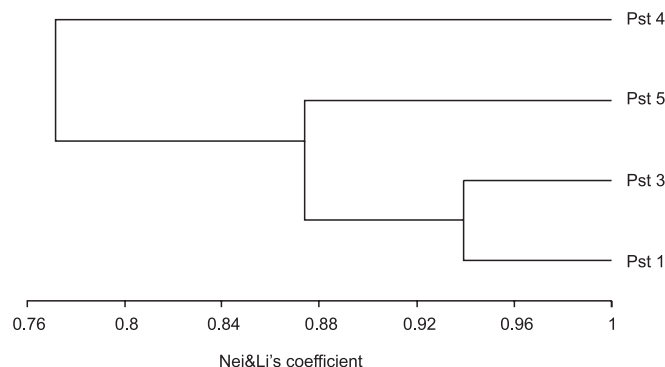


Fig. 2. Dendrogram representing computed identity and similarity values among *Pseudomonas syringae* pv. *tomato* strains based on RAPD data

PCR-RFLP analysis

As a result of DNA amplification with the primers 1406f and 23S, the two bands of approximately 800 and 900 bp were obtained. However, the 900 bp product was very weak. RFLP analysis of products of amplified region ITS at 800 bp with five restriction enzymes (*Taq* I, *Hind* III, *Pvu* II, *Hae* III, *Hpa* II) used showed no distinctive profiles that clearly differentiate strains of *P. s. pv. tomato* strains (Fig. 3).

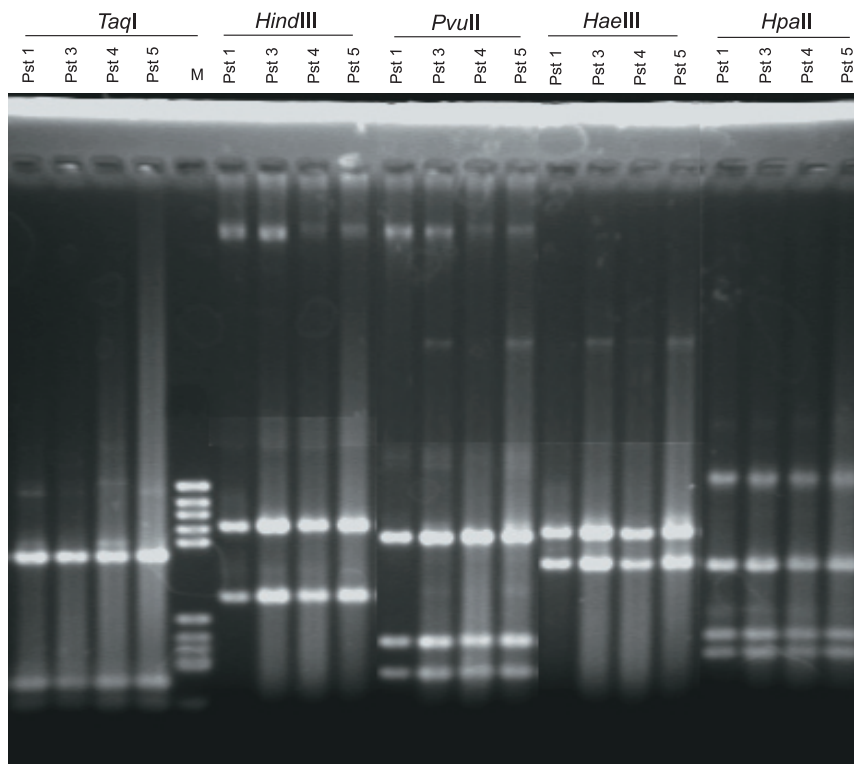


Fig. 3. Gel electrophoresis of PCR-amplified ITS fragments of *Pseudomonas syringae* *pv. tomato* strains digested with five endonucleases: *Taq* I, *Hind* III, *Pvu* II, *Hae* III, *Hpa* II (Fermentas, Lithuania). M – DNA ladder 100 bp

DISCUSSION

Before starting a breeding program for resistance to bacterial speck of tomato one of the primary objective was to provide its causal agent inducing the symptoms that will allow genotypes conferring adequate resistance to be distinguished from susceptible genotypes. Our study revealed the general thesis about high divergence of bacteria. It was found that tested strains presented diversified virulence. At higher concentration of inoculum applied Pst3 strain caused the highest disease severity. This strain in subsequent tests was found to be the least variable and the most virulent (Kozik 2004; data unpublished). Pathogenically different strains of *P. syringae* *pv. tomato* were reported also by other authors (Henderson et al. 1992; Jones et al. 1983; Wiebe and Cambell 1993). A varying degree of virulence towards tomato could

be explained by different expression of genes: *agress*, *dsp*, *ice*, *cor*, *hrp*, responsible for bacterial pathogenicity (Kazempour 2002). Variability of virulence/pathogenicity within tested strains may also reflect some environmental differences prevailing in the greenhouse chamber during the test (Kozik and Sobiczewski 2000). Lawson and Summers (1984) noted that even small environmental differences made consistent identification and qualification of susceptible genotypes impossible.

In contrast to earlier pathogenicity tests (Kozik and Sobiczewski 2000) where significant differences in virulence were found among examined strains our analysis of DNA of bacterial strains showed their high homogeneity. Amplification of bacterial DNA using 33 primers with RAPD technique showed, that similarity of strains expressed by Nei-Li coefficient was very high (above 0.8). Next, the restrictive analysis of amplified region ITS with the use of 5 endonucleases revealed, that profiles obtained from electrophoretic separation of DNA fragments were also very similar. Similar results were obtained by Denny et al. (1988), where close relationship among *P. s. pv. tomato* strains was shown by multilocus electrophoresis and RFLP analysis. In the work similar to ours, Manceau and Horvais (1997) reported using ITS region analysis to distinguish 29 strains of different *P. syringae* pathovars by 14 endonucleases. They found high heterogeneity of studied *P. s. pv. tomato* strains. Close phylogenetic relationship not only within tomato pathovar but also between *P. s. pv. tomato*, *P. s. pv. syringae*, and for the most of *P. s. pv. maculicola* strains was observed by Peters et al. (2004). RFLP analysis proved no significance variation in genetic structure among *maculicola* and *tomato* pathovars and both of them have been regarded as closely related pathovars that have more than 75% DNA homology (Wiebe and Campbell 1993). Therefore, we can conclude that strains used in our studies possess even more DNA homology what was shown by their high similarity based on RAPD and RFLP analysis. The results of our study support also the view of other authors (Gardan et al. 1995; Denny et al. 1988; Shafik 1994) that tomato pathovar is even more homogenous than others within *Pseudomonas syringae* species.

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POLISH SUMMARY**GENETYCZNE PODOBIEŃSTWO SZCZEPÓW *PSEUDOMONAS SYRINGAE* PV. *TOMATO* O ZRÓŻNICOWANEJ WIRULENCJI**

Bakteryjna cętkowość pomidora powodowana przez *Pseudomonas syringae* pv. *tomato* wyrządza straty o znaczeniu ekonomicznym na plantacjach pomidora w Polsce, zwłaszcza w uprawie polowej. Genetyczne relacje między 4 szczepami *P. syringae* pv. *tomato* wyizolowanymi z roślin pomidora w Polsce i zaklasyfikowanymi do rasy 0, badano metodami RAPD i PCR-RFLP. Amplifikacja bakteryjnego DNA metodą RAPD z wykorzystaniem 33 starterów wykazała, że podobieństwo między szczepami wyrażone współczynnikiem Nei-Li było bardzo wysokie (powyżej 0,8). Również analiza restrykcyjna amplifikowanego regionu ITS z użyciem 5 endonukleaz ujawniła, że profile po elektroforetycznym rozdziale fragmentów DNA były bardzo podobne. Na podstawie tych analiz stwierdzono, że badane szczepy tworzyły bardzo spokrewnioną grupę. Szczepy te wykazały jednak zróżnicowaną wirulencję na zainokulowanych liściach roślin pomidora rosnących w szklarni.