European Journal of Plant Pathology **110:** 285–292, 2004.
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Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from peppers and tomatoes in Serbia

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Accepted 7 November 2003

Key words: bacterial spot, *hrp* gene, phenotypic grouping, races, restriction analysis

Abstract

During the last two decades bacterial strains associated with necrotic leaf spots of pepper and tomato fruit spots were collected in Serbia. Twenty-eight strains isolated from pepper and six from tomato were characterized. A study of their physiological and pathological characteristics, and fatty acid composition analysis revealed that all of the strains belong to *Xanthomonas campestris* pv. *vesicatoria*. Being non-amylolytic and non-pectolytic, pathogenic on pepper but not on tomato, containing lower amounts of fatty acid 15 : 0 ante–iso, the pepper strains were designated as members of the A group of *X. campestris* pv. *vesicatoria*. However, the tomato strains hydrolyzed starch and pectate, caused compatible reactions on tomato but not on pepper, had higher percent of 15 : 0 ante–iso fatty acid, and were classified into B phenotypic group and identified as *X. vesicatoria*. PCR primers were developed which amplified conserved DNA regions related to the *hrp* genes of different strains of *X. campestris* pv. *vesicatoria* associated with pepper and tomato. Restriction analysis of the PCR product resulted in different patterns and enabled grouping of the strains into four groups. When xanthomonads isolated from pepper and tomato in Serbia were analyzed, they clustered into two groups corresponding to the grouping based on their physiological and pathological characteristics. According to the reaction of pepper and tomato differential varieties, the strains from pepper belong to races P7 and P8 and tomato strains belong to the race T2. All strains were sensitive to copper and streptomycin. Advantages and disadvantages of various bacterial spot management practices are discussed.

Introduction

Pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) are the major vegetable crops grown in Serbia. Increased market demand contributed to more intensive production of different pepper and tomato varieties in both open and protected fields. However, producers' efforts were diminished and the yield and quality of fruits were limited by the occurrence of disease problems (Mijatovic et al., 1999; Obradovic et al., 1997).

Bacterial spot of pepper caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye has become a very important disease of pepper in Serbia (Balaz, 1994; Obradovic et al., 1997; 1999; 2000; 2001a). Necrotic spots surrounded by a slight halo, appearing mostly on lower leaves, as well as scabby lesions on infected fruits were characteristic symptoms of the disease. The leaf spots tend to coalesce causing leaf blight or chlorosis and premature abscission. Fruit infection usually results in loss of marketability. Under favorable climatic conditions, the disease causes significant

production losses. However, bacterial spot of tomato has not been reported in Serbia since its original description by Sutic in 1957. In 1998, scabby lesions were observed on fruits originating from a single field in central Serbia (Obradovic et al., 2000; 2001b) and xanthomonads were routinely isolated from the lesions.

Based on pathogenicity and bacteriological determinative tests, *X. campestris* pv. *vesicatoria* was originally considered a relatively homogeneous organism (Dye et al., 1964). However, recent results indicate that this bacterium consists of two genetically and phenotypically distinct groups, A and B (Stall et al., 1994), classified as *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*, respectively (Vauterin et al., 1995). Jones et al. (1998b) identified four distinct phenotypic groups within the population of xanthomonads associated with tomato. Currently there are 11 pepper races and three tomato races of the pathogen (Jones et al., 1995; 1998b; Kousik and Ritchie, 1995; 1999; Sahin and Miller, 1998). Such diversity within this group of organisms causing bacterial spot disease of tomato and pepper, has complicated precise identification of the strains. Therefore, the first objective was to phenotypically characterize the bacterial strains associated with pepper leaf spot and tomato fruit spot in Serbia. At the same time we were interested in developing an effective diagnostic assay for rapid detection and grouping of *X. campestris* pv. *vesicatoria* strains.

Integrated disease management practices as well as routine application of bactericides have failed to provide satisfactory disease control, especially when weather conditions favored the spread of the pathogen. Breeding for resistance appears to be the most practical method for bacterial spot management. Thus, a further objective was to investigate pathogen race structure and to identify which resistance genes should be incorporated into the commercial pepper and tomato cultivars grown in Serbia.

Application of bactericides is still the most widely used control strategy for bacterial spot of pepper and tomato. However, the protection strategy could be compromised by occurrence of resistant strains (Marco and Stall, 1983; Ritchie and Dittapongpitch, 1991). Therefore, the final objective was to determine copper and streptomycin resistance of the 'vesicatoria' strains isolated in Serbia. In this paper, pepper and tomato associated strains will be referred to as *X. campestris* pv. *vesicatoria* (*Xcv*, group A) and *X. vesicatoria* (*Xv*, group B).

Materials and methods

Isolation of bacteria and growth conditions

Bacterial strains were isolated from the diseased pepper leaves and tomato fruits collected from different regions in Serbia using standard procedures (Arsenijevic, 1992; Klement et al., 1990) (Table 1). Typical yellow mucoid colonies were selected and transferred to slants of yeast dextrose carbonate medium (YDC), incubated at 28 °C for 24 h, and maintained at 10 °C as a working culture for several months. Prior to further testing, the bacterial strains were grown on plates of King's medium B (KB) at 28 °C for 24–48 h. For long-term preservation, all strains were lyophilized. The strains were compared with two strains of *Xcv* (GSPB2043, GSPB2051) isolated from tomato and pepper in Hungary and deposited in the Collection of Phytopathogenic Bacteria, University of Goettingen, Germany.

Pathogenicity tests

Plants of sweet pepper cv. Piquillo (Spain) and tomato cv. Walter were grown in a greenhouse in 10 cm pots containing a mixture of peat and sand (3 : 1) until they reached the six-leaf stage. Inoculum was prepared by growing the strains on KB plates at 28 °C for 24 h. Bacterial cells were suspended in 0.01 M magnesium sulfate (pH 7.2) and the bacterial suspension was adjusted to 10^8 CFU ml^{-1} ($OD_{660} = 0.06$). The suspension was diluted to $\sim 10^6$ CFU ml^{-1}, and introduced through stomata into leaves of pepper and tomato plants by a high pressure sprayer. Control plants were sprayed with sterile buffer. Following inoculation, the plants were placed on a greenhouse bench and observed daily for symptoms. Each strain was tested for hypersensitivity (Klement et al., 1964) by infiltrating the bacterial suspension (10^8 CFU ml^{-1}) into the intercostal tissue of tobacco leaves cv. Xanthi. Necrosis of the infiltrated area after 24 h was considered a positive hypersensitive reaction (HR).

Physiological and serological characterization of the pathogen

The strains were identified according to tests described by Dye (1980) and Sands (1990): Gram reaction, growth on YDC medium, fluorescence on

Table 1. Investigated *X. campestris* pv. *vesicatoria* strains isolated from pepper and *X. vesicatoria* strains isolated from tomato in Serbia, and their pathogenic reaction on pepper (Piquillo) and tomato (Walter)

Strain	Host plant	Year of isolation	Location isolated	Total number of strains	Pathogenicity	
					Pepper	Tomato
KFB 24[a]	Pepper	1985	Srbobran	1	+	−
KFB 25	Pepper	1986	Ruma	1	+	−
KFB 26, 27, 28	Pepper	1988	Srbobran	3	+	−
KFB 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	Pepper	1996	Becej, Karavukovo, Kikinda, Kovilj, Kruscic, Odzaci	12	+	−
KFB 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	Pepper	1997	Horgos, Kisiljevo	11	+	−
KFB 29, 30, 31, 32, 33, 34	Tomato	1998	S. Palanka	6	−	+

[a]KFB – Collection of phytopathogenic bacteria, curator A. Obradovic. All strains are deposited into Gottinger Sammlung Phytopathogener Bakterien (GSPB), curator A. Mavridis.

KB medium, growth at 37 °C, oxidative–fermentative metabolism (OF), nitrate reduction, gelatin liquefaction, aesculin hydrolysis, oxidase and catalase activity, tolerance to triphenyl tetrazolium chloride (TTC), and acid production from D-arabinose, D-glucose and mannose. An indirect enzyme-linked immunosorbent assay (ELISA) was carried out using polyclonal antibodies (Adgen Diagnostic Systems, Scotland) produced against a tomato strain *Xcv* (NCPPB 422), and alkaline phosphatase conjugated goat anti-rabbit antibodies (SIGMA, A-8025) (Hampton et al., 1990).

Pectolytic activity was determined using crystal violet pectate (CVP) medium, prepared without crystal violet (Cuppels and Kelman, 1974). Amylolytic activity was tested by growing bacteria for 72 h on nutrient agar plates containing 0.2% (w/v) soluble starch. Plates were then flooded with Lugol's iodine solution. A clear zone around the bacterial growth indicated hydrolysis of starch (Arsenijevic, 1997; Sands, 1990).

In order to analyze whole cell fatty acid profiles, the strains were grown at 28 °C on Trypticase soy broth agar (TSBA) for 24 h and assayed for fatty acids as previously described (Sasser, 1990). The data were analyzed by the Microbial Identification System software (version 3.6; MIDI, Newark, DE).

DNA amplification and restriction endonuclease analysis (REA)

In order to develop a sensitive method for rapid detection of *Xcv* we sequenced the PCR products of 10 representative A, 6 B, 20 C, and 3 D group strains (Jones et al., 2000) following amplification of the Hrp B region of the *hrp* gene cluster of *Xcv* with RST2/RST3 (840 bp) (Leite et al., 1995) and designed new primers based on conserved sequences within the regions of the four groups. The set of primers designated RST65 (5′ GTCGTCGTTACGGCAAGGTGGTCG 3′) and RST69 (5′ TCGCCCAGCGTCATCAGGCCATC 3′) amplify a 420 bp fragment of genomic DNA from all tested 'vesicatoria' strains and some other pathogenic *Xanthomonas* sp. or pathovars. In order to determine the grouping of the 'vesicatoria' strains, the PCR products were digested with restriction enzymes *Cfo*I, *Taq*I, and *Hae*III, as described by Leite et al. (1995). The following strains of *Xcv* were used as reference strains for grouping the pepper and tomato strains from Serbia: 93-1 (pepper race, group A), 75-3 (tomato race 1, group A), XV56 (tomato race 2, group B), 91118 (tomato race 3, group C), and XG101 (*X. gardneri*, group D) (Bouzar et al., 1994; Jones et al., 2000).

Race differentiation

All pepper strains were tested for elicitation of a HR on Early Calwonder (ECW) plants and the near-isogenic lines (ECW-10R, ECW-20R and ECW-30R) containing resistance genes to bacterial spot (*Bs1*, *Bs2* and *Bs3*, respectively) as well as on *Capsicum pubescens* plant introduction line PI 235047 (Jones et al., 1998b; Sahin and Miller, 1998). Tomato plants cv. Walter, Hawaii 7998 and Hawaii 7981 were used for screening for tomato races. The plants were tested at the six-leaf stage. Races of *Xcv* were identified based on the presence or absence of collapsed tissue at the infiltration site within 24–48 h after inoculation (Bouzar et al., 1994; Jones et al., 1998b; Minsavage et al., 1990; Sahin and Miller, 1998).

Copper and streptomycin sensitivity

Fresh stock solutions of copper sulfate (Sigma) and streptomycin sulfate (Sigma) were prepared in sterile distilled water. The stock solutions were filter-sterilized and added at the appropriate concentrations to sucrose peptone agar (SPA) after autoclaving. Bacterial suspensions were spotted on SPA plates amended with either $CuSO_4 \cdot 5H_2O$ (100, 200 $\mu g\,ml^{-1}$) or streptomycin sulfate (20, 50, 100 or 200 $\mu g\,ml^{-1}$) (Ritchie and Dittapongpitch, 1991) or on SPA without any copper or streptomycin. Plates were incubated for 48 h at 28 °C and observed for bacterial growth.

Results

Strains and their phenotypic characteristics

A survey of pepper and tomato fields in Serbia, conducted over a 13-year period (Table 1), resulted in the isolation of numerous bacterial strains, with 28-pepper and 6-tomato representative strains selected for further analysis.

All strains caused typical symptoms on the plant species from which they were isolated. None was pathogenic on both sweet pepper and tomato. All pepper strains induced an HR on tobacco leaves, while tomato strains were inconsistent.

All the strains were Gram negative, non-fluorescent, rod-shaped and motile. They formed yellow, convex, mucoid colonies on YDC medium, metabolized glucose oxidatively, grew at 37 °C, hydrolyzed gelatin and esculin, produced acids from D-arabinose, D-glucose and mannose, did not reduce nitrates, were oxidase negative and catalase positive, tolerant to 0.02%, but not to 0.1% of TTC. ELISA test confirmed serological similarity of the strains and *Xcv*. The pepper strains differed from the tomato strains with the former being non-amylolytic and non-pectolytic. Based on these results, both pepper and tomato strains were identified as *Xcv*, but separated into two groups according to the differentiation made by Stall et al. (1994).

Fatty acid profiles

Three fatty acids, 11 : 0 iso, 11 : 0 iso 3OH and 13 : 0 iso 3OH, characteristic of the genus *Xanthomonas* (Yang et al., 1993), were found in all the Serbian strains used in this study. The fatty acids 15 : 0 iso (23.7%) and 16 : 1 w7c (17.2%) were present in the highest concentration. These two fatty acids also were recognized as common among xanthomonads (Bouzar et al., 1994; Yang et al., 1993). However, lower amounts of 15 : 0 ante–iso (13.2%) in pepper strains compared to tomato strains (30.7%) support their differentiation into the A and B groups as was identified by Bouzar et al. (1994).

Restriction analysis

The selected primer set (RST65/69) generated a PCR product (Figure 1) from genomic DNA of all tested 'vesicatoria' strains and 9 out of 12 tested pathogenic *Xanthomonas* sp. or pathovars. No PCR product was obtained from the saprophytic bacteria and opportunistic xanthomonads. The banding patterns, obtained by cutting the PCR product of 39 '*vesicatoria*' strains with the restriction enzymes *Cfo*I, *Taq*I, and *Hae*III, enabled differentiation of four groups (A, B, C, D) (Figure 1). Eight pepper and three tomato strains isolated from different regions and in different years in Serbia, were analyzed by the same procedure (Figure 2). The amplified 420 bp product from DNA from the pepper strains, when digested with *Hae*III, had an identical pattern with the representative phenotypic group A (93-1, 75-3) and C (91118) strains, while the patterns from the tomato strains clustered with the B group representative strain (XV56) (Figure 2).

Characterization of races

The reaction of pepper and tomato differentials showed that the Serbian strains belong to at least three different races (Table 2). Based on these results we classified the pepper strains into the races P7 and P8, respectively (Table 2). However, the tomato strains were homogeneous regarding the reaction of the tomato differentials. According to the compatible reaction of the tested genotypes, the tomato strains belong to tomato race 2 (T2).

Copper and streptomycin assays

All strains grew on SPA medium amended with 100 $\mu g\,ml^{-1}$ of copper sulfate but not with 200 $\mu g\,ml^{-1}$. The lowest concentration of streptomycin sulfate (20 $\mu g\,ml^{-1}$) inhibited growth of the strains.

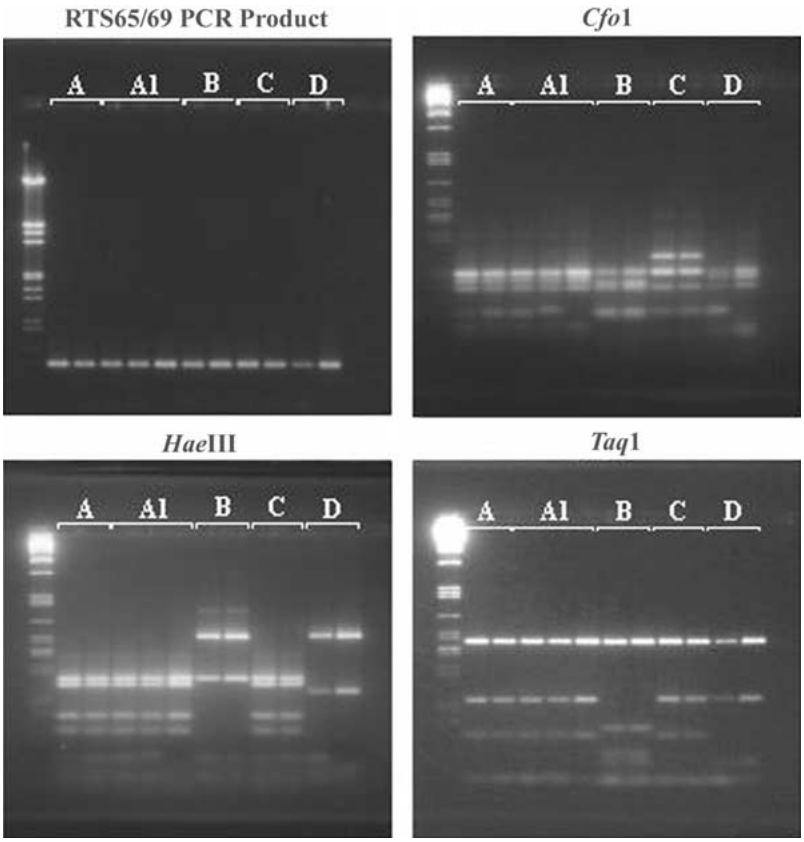


Figure 1. Amplification of 420 bp DNA fragment of the *hrp* gene cluster from *X. campestris* pv. *vesicatoria* strains with RST 65/69 primer set, and grouping of the strains according to restriction analysis of the amplified fragment restricted with the endonucleases *Cfo*I, *Hae*III, and *Taq*I.

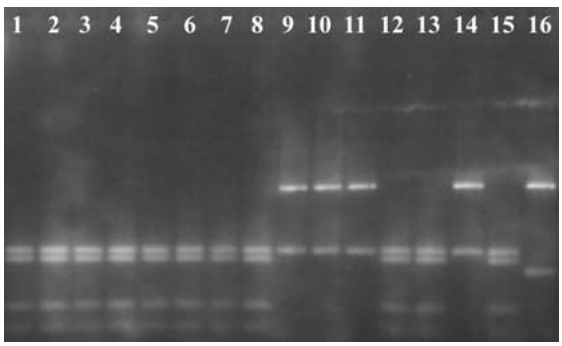


Figure 2. *Hae*III restriction endonuclease analysis of the DNA fragment of the *hrp* gene cluster amplified with RST65/69 primer set from xanthomonads associated with pepper and tomato. Lines 1–8: KFB1, 3, 5, 6, 9, 13, 24, 25 (pepper strains from Serbia); 9–11: KFB29, 30, 31 (tomato strains from Serbia); 12: 93-1 (pepper race, group A); 13: 75–3 (tomato race 1, group A); 14: XV56 (tomato race 2, group B); 15: 91118 (tomato race 3, Group C); 16: XG101 (*X. gardneri*, group D).

Discussion

Bacterial spot was detected in all major pepper growing regions in Serbia (Table 1), over a 13-year period. Although pepper and tomato were grown in the same regions, very often present in the same fields, bacterial spot of pepper prevailed (Obradovic et al., 2000). The diseased material was usually received within July and August, the warmest part of the season when most of the fields were irrigated overhead. Higher disease incidence was observed in the fields after heavy rainfall or frequent overhead irrigation. In some fields, severe leaf infection caused defoliation and complete death of the plants. However, a regeneration of the plants occurred in some cases, resulting in new foliage growth and development of fruits. Fruit size and quality were reduced compared with those from non-infected fields. Despite the high intensity of the leaf infection, fruits were not heavily affected.

Table 2. Race determination of investigated xanthomonads isolated from pepper and tomato in Serbia

Host	No. of strains	Differential pepper and tomato genotypes								Pathogen race
		ECW[1]	ECW-10R (Bs1)	ECW-20R (Bs2)	ECW-30R (Bs3)	PI[2] 235047	Walter	H[3] 7998	H7981	
Pepper	16	S	S	HR	HR	S	HR	HR	HR	P7
	12	S	S	HR	S	S	HR	HR	HR	P8
Tomato	6	HR	/	/	/	/	S	S	S	T2

S – susceptible reaction; HR – hypersensitive reaction; / – not tested.
[1]Early Calwonder.
[2]Plant introduction line 235047 (*C. pubescens*).
[3]Hawaii 7998.

The results of our study showed that two organisms were associated with bacterial spot of pepper (*Xcv*) and tomato (*Xv*) in Serbia. The pepper strains were non-amylolytic and non-pectolytic, with lower amounts of the fatty acid 15 : 0 ante–iso, uniform regarding the banding pattern following restriction enzyme *Hae*III digestion of the PCR amplification product of the *hrp* region. The pattern was identical to the pattern of the group A and C representative strains. The strains were pathogenic on pepper but not on tomato. However, tomato strains of the pathogen were isolated in 1998 (Obradovic et al., 2000; 2001b), more than 40 years since they were described for the first time in Serbia (Sutic, 1957). They hydrolyzed starch and pectate, caused a compatible reaction in tomato but not in pepper, had higher amount of 15 : 0 ante–iso fatty acid, and clustered with the B group representative strain regarding the REA of the PCR product.

Initially, 16 of the pepper strains caused an HR in ECW-30R and therefore designated into race P1 compared to the remaining 12 strains that belong to the race P3 of *Xcv* (Obradovic et al., 1999; 2000). After resistance in *C. pubescens* was reported (Sahin and Miller, 1998) the line PI 235047 was challenged with the strains. They were reclassified into P7 and P8 according to the results presented in this paper (Table 2) and a new race classification proposal (Sahin and Miller, 1998). Although a limited number of strains per year were analyzed, the data from the last two years of isolation strongly indicated that one race predominated in the season (Table 3). Shifts in races or the competitive advantage of one race over another have been already reported (Jones et al., 1998a; Kousik and Ritchie, 1996; Pohronezny et al., 1992). The pepper genotype ECW-20R, possessing the resistance gene *Bs2*, was the only one reacting hypersensitively to all investigated strains (Table 2). It was recognized as a source of resistance to races of *Xcv* present in Serbia and thus is recommended to be included in the pepper breeding program. Even though such a program results in some resistant varieties, it will probably be a temporary solution because of the high mutation rate described for the *avrBs2* gene in this pathogen (Swords et al., 1996). Pepper races 1 and 3 of *Xcv* have been found in many areas (Bouzar et al., 1999; Cook and Stall, 1982; Jones et al., 1998b). When the worldwide collection of *Xcv* has been characterized, 11 strains from pepper in Hungary and Spain were identified as races P1, T1P1, and T1P3 (Bouzar et al., 1994). Among races P1, P2 and P3 detected on pepper in Italy (Buonaurio et al., 1994), P1 and P3 predominated. However, in the region of Former Yugoslav Republic of Macedonia races P0 and P2 were described (Mitrev et al., 2001). Since pepper is intensively grown on the Balkan Peninsula and in Mediterranean countries, the seed and fresh fruit exchange between countries could contribute to the relatively uniform distribution of the pathogen races in this region. However, this presumption should be confirmed by analyzing the representative population of the pathogen coming from this area. Since there was no record of *Xv* as a tomato pathogen in Serbia during the last 40 years, the sudden appearance of this organism could be of an external origin. It could be a consequence of uncontrolled import

Table 3. Distribution of *X. campestris* pv. *vesicatoria* pepper races per year of isolation

	Year of isolation				
	1985	1986	1988	1996	1997
Number of investigated strains per year	1	1	3	12	11
Race					
P7	0	1	3	1	11
P8	1	0	0	11	0

of infected tomato seed from neighboring countries were *Xv* strains are already present. To our knowledge this is the first determination of *Xcv* and *Xv* races in Serbia and one of few related reports coming from this region.

The amplification of *hrp* sequences by PCR and REA of the amplified product is a useful tool for the detection and identification of plant pathogenic xanthomonads. By targeting the *hrp* region, the tested saprophytic bacteria and opportunistic xanthomonads were eliminated from the competition and could not produce positive results due to absence of *hrp* genes. The set of primers RST65/69 amplified a single fragment of 420 bp conserved among the 'vesicatoria' strains. As a result of the PCR product digestion with the restriction enzymes *Cfo*I, *Taq*I, and *Hae*III the strains were differentiated into four groups. Comparing banding patterns obtained by *Hae*III digestion, Serbian pepper strains, the control pepper race strain (93-1, group A), tomato race 1 (75-3, group A) and tomato race 3 (91118, group C) representative strains clustered together (Figure 2). Serbian tomato strains and T2 representative strain had identical patterns, while the third group consisted of the strain identified as *X. gardneri* (Sutic, 1957; Jones et al., 2000).

No strains of *Xcv* resistant to streptomycin were detected in Serbia. Until now antibiotic-based pesticides are not allowed to be used in plant protection in this country. Therefore the susceptibility of the strains to the lowest concentration of streptomycin was expected. The possible occurrence of resistant strains could be only of an external origin. However, copper based compounds have been used in vegetable protection in Serbia for many years. In spite of the strain susceptibility to the higher concentration of copper sulfate, chemical protection appeared to be ineffective especially when a susceptible variety was grown and when the environmental conditions favored spread of the disease. Traditional practices such as pepper production on the same commercial fields and home gardens for several years, planting of self-produced non-certified seed, and multiple use of the same substrate for the transplant production, could also play an important role in higher disease incidence. Thus, crop rotation, sowing a pathogen-free seed, disinfection or change of substrate for the transplant production, furrow or drip irrigation, growing resistant varieties, and early application of bactericides, should be a part of good agricultural practices in order to control bacterial spot of pepper and tomato in Serbia.

Acknowledgements

This research was supported by Alexander von Humboldt Foundation and Hertie Foundation ('Roman Herzog' Fellowship, granted to the first author). Thanks are due to S.A. Miller and R.E. Stall for providing the seed of pepper differential varieties.

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