SHORT COMMUNICATION

CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS* PV. *VESICATORIA* ISOLATED FROM PEPPERS IN MACEDONIA

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SUMMARY

The bacteria were isolated from the spots of pepper plants, surveys in open-field during 1996-2001 in Macedonia. Symptoms similar to natural symptoms were reproduced following inoculation on pepper leaves. The strains were negative for amylolytic activity and ability to utilize *cis*-aconitate and belong to type A of *Xanthomonas axonopodis* pv. *vesicatoria*, and were identified to race P0 and race P2. All strains were sensitive to copper sulfate and streptomycin sulfate and were homogeneous according to their Polymerase chain reaction (PCR). Polyclonal antibodies produced clear positive reaction in I-ELISA with all strains from Macedonia and reference strains from abroad.

Key words: *Xanthomonas axonopodis*, identification, phenotypic characteristics, serology, PCR, pathogenicity.

Xanthomonas axonopodis pv. vesicatoria, the causal agent of bacterial leaf spot of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum*), occurs worldwide in regions of pepper and tomato production (Bouzar, 1994). This bacterium was first diagnosed in the early 1920s (Hayward, 1964). The disease reduces plant growth, fruit yield, quality and it is characterized by necrotic lesions on leaves, steams, and fruits. In warm and rainy weather, bacterial spot may cause severe defoliation of the plants that results in reduced yield, and diseased fruits may not be suitable for fresh-market sale (Leite, 1995).

Macedonian pepper (*Capsicum annuum* L.) is traditional crop, cultivated on about 9.000 ha of open field and plastic covered greenhouses. Bacterial spot is an important pepper disease in many production areas in the country and cause significant losses in the field. The losses caused by this bacterium in Macedonia were different every year. They were estimated on about 10-20%, but some years, the damages were extremely higher as a result of favorable climatic conditions, warm and rainy summer (Mitrev *et al*., 1999).

The objective of this research, was to characterize the strains of *Xanthomonas axonopodis pv. vesicatoria* isolated from pepper plants (*Capsicum annuum* L.) in Republic of Macedonia, to confirm their pathogenical, morphological, biochemical, physiological, genetic and serological characteristics, sensitivity to copper sulfate and streptomycin sulfate, and presence of races.

The bacteria were isolated from the spots of pepper plants, surveys in open-field, performed during June - September of 1998-2003 from different production areas in Macedonia. Collected leaves were surface-disinfected, cut small pieces of leaf tissue from margins of spots with sterilized razor blade and comminuted in sterile deionized water (SDW). Suspension was streaked on plate's surface of nutrient agar (NA) medium or yeast dextrose carbonate (YDC) medium (Schaad *et al.*, 2001). Plates were incubated at 26°C/48h. Representative round, convex, mucoid, yellow colonies on yeast dextrose carbonate medium (YDC), or small, yellow colonies on nutrient agar (NA) were selected and purified by repeated restreaking on yeast dextrose carbonate (YDC) medium. These pure cultures were preserved in tube on yeast dextrose carbonate (YDC) slope medium on 4ºC for short-term storage, or at -20ºC in 15% glycerin for long-term storage (Schaad *et al.*, 2001). Identification and determination of *X.c.* pv. *vesicatoria* strains were performed on few hundred bacterial strains isolated from pepper in Macedonia. There were performed plenty of different biochemical and nutritional tests (Lelliot, 1987; Klement *et al.*, 1990; Bouzar, 1994; Schaad *et al*., 2001).

Biochemical tests presented that all investigated bacterial strains were Gram-negative, aerobic, catalase- and aminopeptidase-positive, oxidase- and urease-negative.

They hydrolyzed aesculin, gelatin, tween 80 but did not hydrolyzed starch. Grew at 35°C, produce hydrogen sulfide from cysteine but did not reduce nitrates and did not show tolerance of 1% NaCl, 0,1 and 0,02% triphenyl-tetrazolium chloride (TTC).

Bacteria were grown and tested for utilization of 95 carbon sources available on the Bilog GN MicroPlate and samples were analyzed according to the manufacture protocol of Biolog Identification System. (Biolog

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Table 1. Bacterial strains used in this study.

Strain	Place of isolation	Host	Species
P-01/1; P-01/4; P-01/6; P-01/9	Kuklis, MK	pepper	*X. a.* pv. *vesicatoria*
P-02/3; P-02/4; P-02/5; P-02/7	Prosenikovo, MK	pepper	*X. a.* pv. *vesicatoria*
P-03/1; P-03/3; P-03/7	Dabile, MK	pepper	*X. a.* pv. *vesicatoria*
P-04/3; P-04/4; P-04/5	Radovis, MK	pepper	*X. a.* pv. *vesicatoria*
P-05/5; P-05/7; P-05/8	Kumanovo, MK	pepper	*X. a.* pv. *vesicatoria*
P-06/3; P-06/5; P-06/7	Aracinovo, MK	pepper	*X. a.* pv. *vesicatoria*
P-07/1; P-07/3; P-07/7; P-07/8	Stip, MK	pepper	*X. a.* pv. *vesicatoria*
P-08/1; P-08/2; P-08/5; P-08/6	Valandovo, MK	pepper	*X. a.* pv. *vesicatoria*
P-09/2; P-09/3; P-09/5; P-09/9	Rosoman, MK	pepper	*X. a.* pv. *vesicatoria*
2524	Sacramento, USA	pepper	*X. a.* pv. *vesicatoria*
1840	Sacramento, USA	pepper	*X. a.* pv. *vesicatoria*
93-1 (race 3)	Florida, USA	pepper	*X. a.* pv. *vesicatoria*
E-3 (race 2)	Florida, USA	pepper	*X. a.* pv. *vesicatoria*
71-21 (race 1)	Florida, USA	pepper	*X. a.* pv. *vesicatoria*
P-2026	Novi Sad, SCG	pepper	*X. a.* pv. *vesicatoria*
P-150	Strumica, MK	pepper	*P. s. pv. syringae*
T122	Sacramento, USA	cotton	*X. c.* pv. *malvacearum*

Inc., Hayward, CA USA). Bacteria were grown on trypticase soy agar (BBL Microbiology Systems) for 24 h at 25ºC. Bacterial cells were removed from plates with sterilized cotton swabs and transferred to a sterile saline solution (0,85% (w/v) NaCl). The density of the cell suspension was then adjusted to match the Biolog GN MicroPlate system's turbidity standards. Biolog GN Microplates were immediately inoculated with 150 µl of cell suspension per well and incubated for 24 h at 28ºC. Plates were read visually and results were analyzed with Biolog GN database version 3.01. Positive or negative growth reactions were recorded after 28 hours. After analyzing the results with Biolog GN Database all investigated strains from Macedonia were similar to phytopathogenic bacteria *Xanthomonas axonopodis pv. vesicatoria*, group A.

The morphological, pathological, biochemical and physiological tests confirmed that the characteristics of our strains were similar with the characteristics of control strains of bacteria *X.a.* pv. *vesicatoria* 71-21, E-3 and 93-1. Additional characterization of strains showed that all strains from Macedonia were negative for amylolytic activity and able to utilize dextrin and *cis*-aconitate. The only substrate that discriminate strains between A and B group of *X.a.* pv. *vesicatoria* is cis-aconitate. Our strains utilized this component and belong to strains of type A of *X.a.* pv. *vesicatoria* and none of them belong to type B (Jones *et al.* 1987; Sahin *et al.*, 1996; Schaad *et al.*, 2001).

Sensitivity of strains to copper and streptomycin was assayed in sucrose peptone agar (SPA) medium amended with either $CuSO_4 \cdot 5H_2O$ (200 µg/ml) or streptomycin sulfate (100 µg/ml) (Buonaurio, 1994). Plates were incubated at 26°C for 48 h and the presence or absence of growth was recorded. All investigated bacterial strains were not presented growth on SPA medium and therefore were considered as unresisting to copper sulfate and antibiotics.

Streptomycin sulfate and copper sulfate resistant strains were not detected in this study. Two plausible explanations for streptomycin sensitive strains are that the use of them is not permitted in Macedonia, or that the pathogen was introduced on seed, which had been produced in countries where antibiotics are not used or they are not permitted.

Bacterial suspensions ($1x10^7$cfu·ml^{-1}) prepared by dispersing bacteria from 24-48 h nutrient agar (NA) cultures in sterile deionized water (SDW), were used to inoculate leaves of the young paprika plants cv "*kurtovska kapija*" by injection into six inoculation points using 26 swg needle. There inoculations were made by spraying and plants were covered with plastic bags for 24 h at room temperature. Control plants were inoculated with sterile deionized water (SDW). The strains: 71-21, E-3, 93-1 and P-2026 of *X.a.* pv. *vesicatoria* isolated from pepper were used as a positive control. Pepper plants cv "*kurtovska kapija"* reacted intensely after 2-4 days with characteristic necrotic spots appeared around the place

of inoculation. Necroses progressively spread and whole leaves were destroyed. Spraying of bacterial suspension on the leaf surface of pepper plants showed similar symptoms.

X.a. pv. *vesicatoria* races were differentiated using the pepper cultivar ECW and a set of near-isogeneic pepper lines derived from and including ECW: ECW-10R, ECW-20R and ECW-30R containing the resistance genes bs1, Bs1, Bs2 and Bs3, in consequence (Minsavage, 1990). Pepper race 1 (strain 71-21), race 2 (strain E-3), and race 3 (strain 93-1) of *X.a.* pv. *vesicatoria* were used as reference cultures. Investigated strains were suspended in sterile, distilled water, adjusted to a concentration approximately 10^8cfu◊ml^{-1}, and infiltrated into fully expanded leaves of the pepper lines. Plants were incubated for 48 h under greenhouse conditions and hypersensitive response (HR) was recorded within 24 to 48 h. Each strain was tested three times (Sahin, 1996). Seven strains (22% of strains) were identified as race P0, nineteen strains (59% of strains) were identified as race P2, and the remaining strains (19% of strains) could not be identified to race using these differential pepper lines.

The bacterial strains of *X.a.* pv. *vesicatoria* obtained from diseased pepper plants showed that pepper races P0 and P2 were dominant in Republic of Macedonia. The strains were identified as race P2, less P0, and the remaining strains could not be identified to race using these differential pepper lines. The predominant pepper races in the world were: P0, P1, and P3. Races P0, P2 and P4 are unusual pepper races, which have been previously found only in USA (North Carolina and Florida), Mexico, Australia, Caribbean and Central America (Bouzar, 1994). In Europe the pepper races were determined in Italy (central and southern part of Italy), and were found races 1 (39%), 2 (16%) and 3(45%), (Buonario, 1994). Previously, there was not any indication for proving the pepper races of *X.a.* pv. *vesicatoria* in Macedonia. The use and deployment of resistant cultivars to the races of *X.c.* pv. *vesicatoria* may provide the best disease-management strategy.

All assays utilized the indirect double antibody sandwich technique in which flat bottom 96-well microtiter plates were coated with a polyclonal antibody (Agdia Inc., Elkhart, Indiana, USA), developed in white rabbits against *X.a.* pv. *vesicatoria* strain. The results were interpretated visually. Wells in which color developed indicated positive results and wells in which there were not significant color developments indicate negative reaction. Polyclonal antibodies produced clear positive reaction in I-ELISA with all eight strains from Macedonia and two reference strains of *X.a.* pv. *vesicatoria* (2524 and 1840) from California, but strain TT122 of *X.c.* pv. *malvacearum* did not show any reaction. The reaction between antibodies and antigens of investigated bacteria on the plates was clearlier and faster when the bacterial suspension before use was heated at 60ºC for 30 minutes.

The procedure described by Ausubel (1987) with minor modification was used to extract total genomic DNA. Bacterial cells were briefly centrifuged in an Eppendorf micro centrifuge for 6 minutes at 15,000 x g. The pellet was washed in 1 ml of distilled water, pelleted again, and resuspended in 200 µl 1X PBS buffer and 750 µl CTAB buffer and vortexed. After incubation at 60ºC for 20 minutes and vortexed every 5 minutes, DNA was extracted with chloroform/octanol (24:1) and vortexed vigorously, centrifuged for 10 minutes and remove 1ml supernatant to a clean 2,0 ml microfuge tube, than added an equal volume of ice cold isopropanol, and incubated on -20ºC over night. Next day the tubes were centrifuged for 10 minutes at 15,000 x g and discard the supernatant, washed the pellet with 2 drops of ice cold ethanol (80%) and dry inverted for 1 hour. The dried pellet resuspended in 50 µl of PCR grade water.

One set of oligonucleotide primers: RST2 (5'AGGCCCTGGAAGGTGCCCTGGA3') and RST3 (5'ATCGCACTGCGTACCGCG CGCGA3') was used in this research. The set of primers which was delineated an

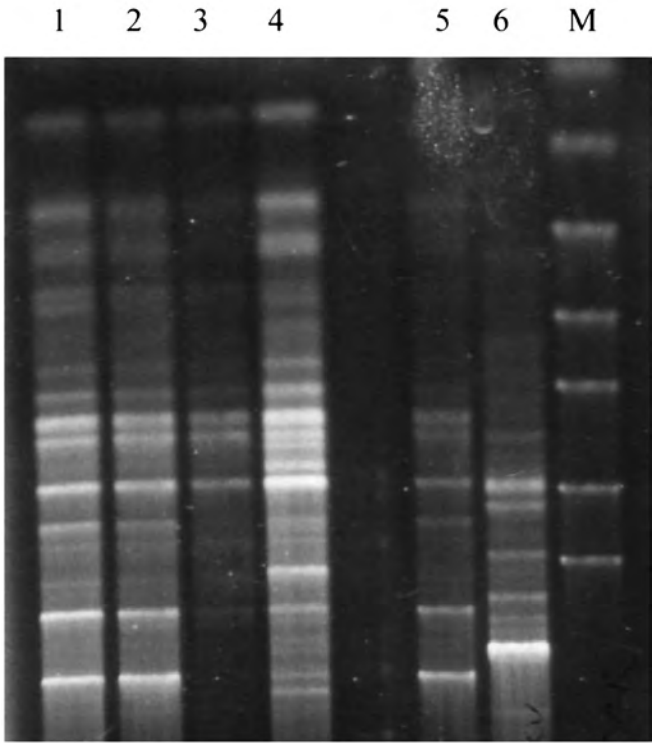

Fig. 1. Amplification of the 840 bp *hrp* fragment was done using one set of oligonucleotide primers: RST2 (5'AGGCCCTGGAAGGTGCCCTGGA3') and RST3 (5'ATCGCACTG CGTACCGCG CGCGA3'). 1: *X.c. pv. vesicatoria* strain P-1; 2: *X.c. pv. vesicatoria* strain P-2; 3: *X.c. pv. vesicatoria* strain P-3; 4: *X.c. pv. vesicatoria* strain P-4; 5: *X.c. pv. vesicatoria* strain 2524; 6: *X.c. pv. vesicatoria* strain 1840; M: 100 bp DNA ladder (Sigma, PCR Marker, P9577).

840-bp fragment (Leite, 1994) was selected from the nucleotide sequence of the *hrp* region of *X.c.* pv. *vesicatoria.* To each of the 0.5 ml READY•TO•GO beads (Pharmacia Biotech, Piscataway, NJ, USA) added the 23 µl of prepared suspension (diluted RST2 and RST3 primers and Polymerase chain reaction (PCR) grade water) and 2µl of DNA sample. Polymerase chain reaction (PCR) mixtures were overlain with one drop of mineral oil (Sigma, Saint Louis, USA). PCR amplification was performed in thermocycler using following cycles: 1 initial cycle at 95ºC for 1 min; 30 cycles at 94ºC for 1 min, annealing at 44ºC for 1 min, extension at 65ºC for 8 min, single final extension cycle at 65ºC for 15 min and a final soak at 4ºC. Amplified PCR products in 5µl were separated by gel electrophoresis on 2% agarose gel in 0.75% running buffer, TAE buffer (40mM Tris acetate, 1 mM EDTA, pH 8.2), for 2 hours at 150 W; 151 mA and 90 V (Ephortec 3,000). Stain was done with ethidium bromide and photographed on a UV transilluminator using Polaroid type 665, positive/negative land film, ISA 3,000/36º. The reprints generated from different strains were compared visually. Variation in the intensity of DNA bands was not considered as a true difference between strains if bands had the same apparent migration distance.

DNA reprints were generated from total chromosomal DNA extracted from eight strains of *X.a. pv. vesicatoria*, seven originated from various parts of Macedonia, one from California and one negative control of other *Xanthomonads*. A unique genomic reprint of *X.a.* pv. *vesicatoria* was generated by Polymerase Chain Reaction (PCR) and amplified fragments of the expected 840 bp in length from total genomic DNA of strains by using RST2 and RST3. All investigated strains from Macedonia, which were positive in pathogenicity tests, produced very similar Polymerase chain reaction (PCR) banding patterns. These patterns were similar to the patterns from *X.a.* pv. *vesicatoria* reference strains from California and they were distinct from reference strains of other X*anthomonads* examined in this work (Fig. 1).

The use of oligonucleotide primers provides a sensitive and specific tool for detection of DNA by amplification. The results presented here indicate that strains of *X.a.* pv. *vesicatoria* can be detected and identified by analysis of DNA fragments amplified with *hrp* gene-specific primers.

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