SHORT COMMUNICATION

PSEUDOMONAS POPULATION CAUSING TOMATO PITH NECROSIS IN THE REPUBLIC OF MACEDONIA

S. Mitrev[1], I. Karov[1], B. Kovacevik[1] and E. Kostadinovska[1]

[1]Goce Delcev University, Faculty of Agriculture, Department of Plant and Environmental Protection, Krste Misirkov bb, 2000 Stip, Republic of Macedonia

SUMMARY

Pseudomonas species causing pith necrosis on tomato were collected from 2005 to 2012 in different regions of the Republic of Macedonia and identified as *Pseudomonas mediterranea* and *P. viridiflava*. Other *Pseudomonas* spp. were also recovered, but they are still under identification. The obtained bacterial isolates were compared with reference strains on the basis of phenotypic characteristics, pathogenicity, sensitivity to antibiotics, BIOLOG tests, DNA fingerprinting by multiplex PCR using two specific primers [type I (PC5/1 and PC 5/2) and type II (PC1/1 and PC 1/2)], and 16S rRNA–RFLP analysis. Unique DNA bands from 10 representative strains isolated from symptomatic tomato plants disclosed the presence of *P. mediterranea* by amplification of their genomic DNA. All strains produced one specific band (600 bp) compared with the representative samples IPVCT9.1 (*P. mediterranea*). Also, we used another representative sample IPVCT10.3 (*P. corrugata*), to check if we already have *P. corrugata* or no, in our samples. In order to investigate the bacterial populations causing tomato pith necrosis in Macedonia, a total of 150 isolates were collected and compared with reference strains. This is the first report on *Pseudomonas* population causing pith necrosis of tomato in the Republic of Macedonia.

Key words: tomato pith necrosis, BIOLOG, PCR, 16S rRNA–RFLP.

Symptoms of pith necrosis (TPN) in greenhouse tomato crops have been reported to be caused by different bacterial pathogens, such as *Pseudomonas corrugata* (Catara *et al*., 2000), *P. viridiflava* (Alippi *et al*., 2003), *P. fluorescent* biotype I (Malathrakis and Goumas, 1987) and *P. mediterranea* (Catara *et al*., 2002; Basim *et al*., 2005). This latter species is an ubiquitous bacterium that can be distinguished from *P. corrugata* by 16SrDNA analysis, by means of REP– and BOX–PCR profiles and random–primed PCR (Catara *et al*., 2002). Two type of primers: type I (PC5/1 and PC 5/2) and type II (PC1/1 and PC 1/2) allow PCR detection and identification of *P. corrugata* and *P. mediterranea*, respectively (Catara *et al*., 2000). Also *P. viridiflava* produces pith necrosis and other diseases in several plants (Malathrakis and Goumas, 1987; Goumas *et al*., 1999). In Macedonia, the first record of *P. mediterranea* dates to 2010 (Mitrev *et al*., 2010).

The main aim of this study was to identify *Pseudomonas* populations causing tomato pith necrosis (TPN) in the Republic of Macedonia using phenotypic and DNA techniques.

Object of this investigation were *Pseudomonas* strains isolated from greenhouse-grown tomatoes in the Strumica and Sveti Nikole region, one reference strain of *P. mediterranea* and one of *P. corrugata* strain from Italy, as well as one reference strain of *P. viridiflava* (LMG5396) from Belgium (Table 1). All strains were characterized according to Schaad (2001). Colony appearance was determined on sucrose peptone agar (SPA), nutritient agar (NA), King B medium and yeast peptone glucose agar (YPGA); pigment production and fluorescence were assessed on glucose-peptone agar and yeast glucose carbonate agar (YDCA); and their utilization of the 95 carbon sources available with the BIOLOG GN/GP MicroPlate system was also determined. Copper and streptomycin sensitivity were ascertained by the agar dilution method using SPA medium supplemented with different concentrations of copper and streptomycin in order to determine the minimal inhibitory concentration values, expressed as µg/ml (MIC). Tolerance to NaCl was assessed using yeast extract salts (YS) broth at the final concentration of 5 and 7% NaCl (Schaad, 2001).

Tomato plants of cvs Novosadski jabucar, Bele and Magnus, were grown in a growth chamber at 25°C. Inoculations were made on the stem with one point injection of 0.3 ml of a 10^7 CFU/ml bacterial suspension, 5 cm above the substrate.

For template DNA, all strains were grown on NA agar. A single colony from a 24 h NA culture was suspended in 0.5 ml of molecular grade sterile water in a micro–centrifugal tube, and was used as a DNA template. Multiplex PCR was performed with two pairs of primers, type I (PC5/1 and PC 5/2) and type II (PC1/1, PC1/2) for the

Corresponding author: S. Mitrev
Fax :+38932/390-701
E-mail: sasa.mitrev@ugd.edu.mk

Table 1. Bacterial strains used in this study with host and source

Species	Strain	Origin	Host	Cultivar	Year	Source[a]
Pseudomonas mediterranea	P.m. 1 P.m. 2 P.m. 3	Prosenikovo (Strumica)	*L. esculentum*	Bele	III. 2005–2012	1
P. mediterranea	P.m. 4 P.m. 5 P.m. 6	Monospitovo (Strumica)	*L. esculentum*	Bele	V. 2006–2012	1
P. mediterranea	P.m. 7 P.m. 8	Kuklis (Strumica)	*L. esculentum*	Magnus	V. 2006–2012	1
P. mediterranea	P.m. 9 P.m. 10	Piperovo (Strumica)	*L. esculentum*	Magnus	IV. 2005–2012	1
P. viridiflava	3.1 3.2	Prosenikovo (Strumica)	*L. esculentum*	Bele	III. 2005–2012	2
P. viridiflava	3.3	Kuklis (Strumica)	*L. esculentum*	Magnus	V. 2006–2012	2
P. viridiflava	3.4 3.5	Piperovo (Strumica)	*L. esculentum*	Magnus	IV. 2005–2012	2
Pseudomonas spp.	IIPsp.1 IIPsp.2 IIPsp.3	*Erdjelija*	*L. esculentum*	Pink Rape	IX. 2006–2012	3
Pseudomonas spp.	IIPsp.4 IIPsp.5 IIPsp.6	Prosenikovo (Strumica)	*L. esculentum*	Bele	III. 2005–2012	3
Pseudomonas spp.	IIPsp.7 IIPsp.8	Kuklis (Strumica)	*L. esculentum*	Magnus	V. 2006–2012	3
Pseudomonas spp.	IIPsp.9 IIPsp.10	Piperovo (Strumica)	*L. esculentum*	Magnus	IV. 2005–2012	3
P.mediterranea	IPVCT9.1	*Italy*	*L. esculentum*			4
P. corrugata	IPVCT 10.3	*Italy*	*L. esculentum*			5
P.viridiflava	LMG 5396	*Belgia*	*L. esculentum*			6

[a]1, 2, 3 isolates included in this study; 4, 5 IPVCT (Istituto di Pathologia Vegetale, Università degli Studi di Catania, Italy); 6, BCCMTM/LMG (Belgian Coordinated Collection of Microorganisms).

amplification of two specific PCR products (1100 or 600 bp). The PCR reaction was carried out in a final volume of 25 µl, containing 1.5 mM $MgCl_2$, 2 mM of each dNTP, 10 nmol of each primer, 1.25 U Taq polymerase, and 1 µl bacterial DNA (Catara *et al.*, 2000). The PCR program consisted of initial denaturation for 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min annealing at 62°C, elongation for 1.5 min at 72°C, followed by a final elongation step of 5 min at 72°C. PCR products were separated in 1% agarose gel in 0.5X TBE buffer. For RFLP analysis, we used three µl of PCR products, which were incubated overnight at 37°C with restriction enzymes *Alu*I and *Hae*III and performed by 3% agarose gel electrophoresis.

The results disclosed the presence of several pseudomonads as causal agents of TPN, including *P. mediterranea*, *P. viridiflava* and unidentified *Pseudomonas* spp. From 2005 till 2012, pith necrosis symptoms were observed on tomatoes grown in plastic tunnels in the Strumica region, one of the major Macedonian districts for tomato and pepper cropping. The first symptoms appeared during fructification. Large oblong spots appeared on the leaves whose blades rolled upward. The pith of the stem was brown, but the vascular system was not (Fig. 1A). The fruits did not show symptoms, but their quality and size and the yield were reduced. Infections up to 80% were observed in some polytunnels.

The colonies of the *P. mediterranea* isolates that were investigated had a rough surface and curly margins. On YDCA medium, colonies showed a dark green–blue not diffusible pigmentation in the center and creamy margins (Fig. 1B). All *Pseudomonas* strains produced a yellow diffusible pigment on NAS medium, but not on NA medium. The appearance of morphology mutants with smooth surface colonies was also observed during sub–culturing. All strains were Gram-negative and induced hypersensitive reaction in tobacco (Table 2).

The index of similarity with *P. corrugata* for isolates P.m. 1, P.m.5 and P.m. 7 was 0.593, and the index of difference was 5.84. The index of similarity for P.m. 2, P.m. 3 and P.m. 4 was 0.692 and the index of difference was 4.67. As to P.m. 6, P.m. 8, P.m. 9 and P.m. 10, they had an index of similarity of 0.600 and an index of difference of 5.67. Control IPVCT 10.3 showed an index of similarity of 0.833, and an index of difference of 0.250. For IPVCT 9.1 the index of similarity was 0.845 and the index of difference was 2.24. BIOLOG results for *P. viridiflava* yielded

Table 2. Characters used to differentiate *Pseudomonas* species

	P.m. 1	P.m. 2	P.m. 3	P.m. 4	P.m. 5	P.m. 6	P.m. 7	P.m. 8	P.m. 9	P.m. 10	3.1	3.2	IIPsp.1	IIPsp.2	IIPsp.3	IIPsp.4	IIPsp.5	IIPsp.6	IPVCT 9.1	LMG 5396	IPVCT 10.3
Gram	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Levan	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescense	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	–
Pectolitic activity	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	–
Arginine	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	+	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O/F test	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Gelatine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
5% NaCl	nd	nd	nd	+	+	+	nd	nd	nd	n d	––	–	–	–	–	–	–	–	nd	–	–
7% NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+
Aesculine	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	–
Urease	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	+
PHB	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+
4°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+
41°C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
meso–tartrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2ketogluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) – positive; (–) – negative; (nd) – not defined.

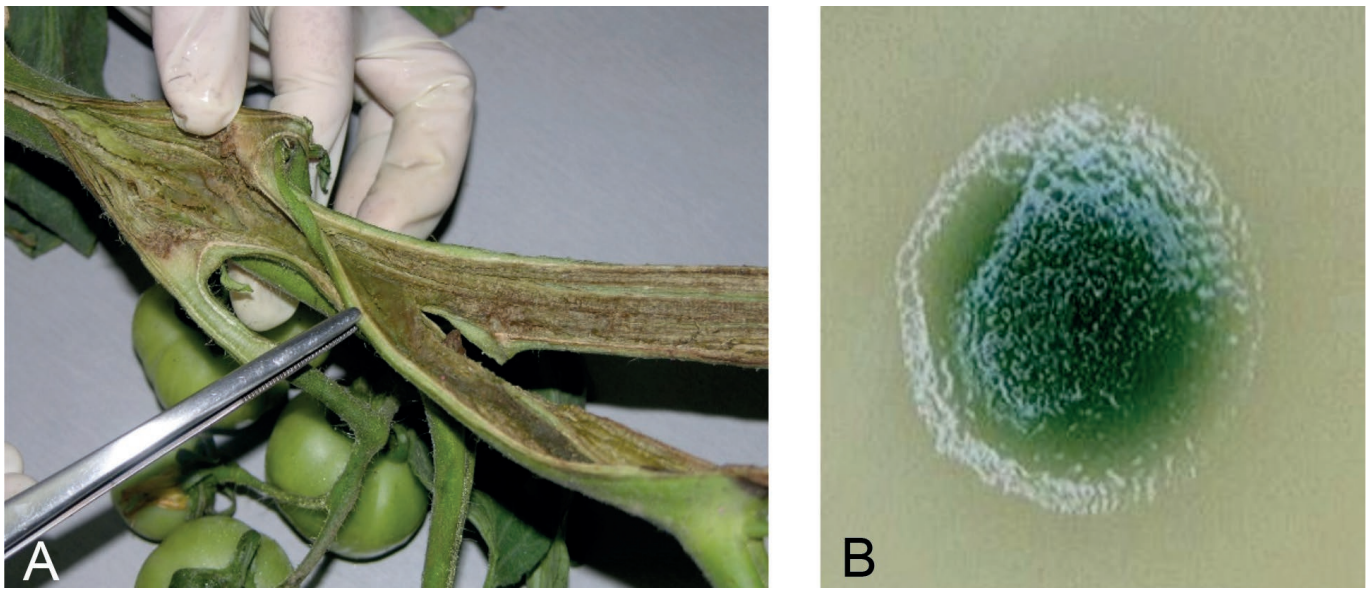

Fig. 1. A. Symptoms of tomato pith necrosis. B. Colony of *P. medditeranea* (isolate P.m. 1) on YGCA medium.

an index of similarity if 0.659, and an index of difference of 5.21. A copper concentration of 75 µg/ml suppressed the growth of Macedonian strains, while the control isolate IPVCT9.1 showed resistance at 100 µg/ml. Streptomycin was the most effective at the concentration of 20 µg/ml. All representative *P. mediterranea* strains and IPVCT9.1 showed low resistance to 5% NaCl while IPVCT 10.3 showed high resistance. None of the strains were resistant to 7% NaCl.

All *Pseudomonas* strains proved to be pathogenic to tomato plants inducing typical TPN symptoms, i.e. pith browning, brown lesions on the stem and production of adventitious roots in some inoculated plants. External and internal symptoms (pith necrosis) were recorded 45 days post inoculation. Negative control did not show any symptoms.

The results for copper and streptomycin sensitivity allow us to conclude that *P. mediterranea* is very sensitive to

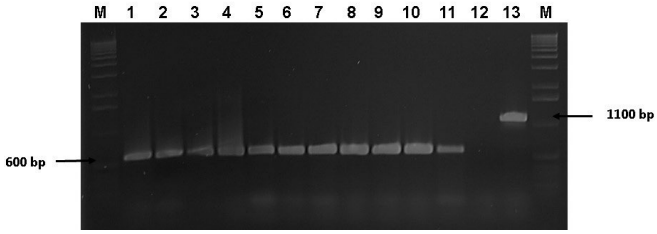

Fig. 2. PCR amplification with type I and type II primers in multiplex reaction. Lanes: 1. 1Kbp ladder; 1-10. P.m. 1 - P.m. 10 11. IPVCT 9.1 12. Blank 13. IPVCT 10.3.

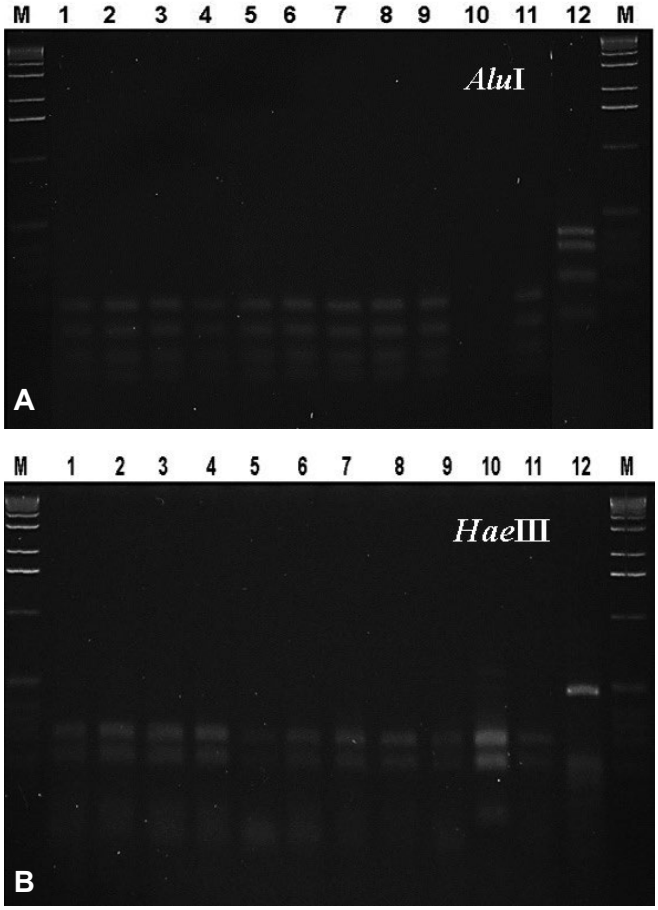

Fig. 3. RFLP patterns. A. Profiles from *Alu*I digestions of type I (PC5/1 and PC 5/2) and type II (PC1/1 and PC 1/2) PCR products. B. RFLP patterns – *Hae*III restriction enzymes, lanes 1–10. P.m. 1 – P. m. 10 11. IPVCT 9.1 12. Blank 13. IPVCT 10.3. *Pseudomonas* acronyms are listed in Table 1. Electrophoretic analyses were carried out on 3% agarose gel.

streptomycin, which can prevent the growth of bacteria *in vitro* in very low concentrations of around 10 µg/ml.

All strains from Macedonia and the control IPVCT9.1 yielded amplification products 600 bp in size. By contrast, IPVCT10.3 showed the presence of a band 1100 bp in size (Fig. 2), which confirms the absence of *P. corrugata* in our region. RFLP analysis on *P. mediterranea* samples showed identical patterns among them and the reference isolates.

In conclusion, a total of 150 bacterial isolates were recovered from tomatoes affected by pith necrosis in Macedonia and compared with reference strains of *P. corrugata*, *P. mediterranea and P. viridiflava*. All isolates were characterized by traditional phenotypic characteristics, copper and antibiotic sensitivity, BIOLOG, pathogenicity and PCR/RFLP analysis. These groups of pathogens are very virulent and cause significant losses in some plastic tunnels when spring temperatures are low and the humidity is high. These results allowed the identification of the presence of *Pseudomonas* populations causing TPN for the first time in Macedonia.

REFERENCES

Alippi M.A., Dal Bo E., Ronco L.B., Lopez M.V., Lopez A.C. Aguilar O.M., 2003. *Pseudomonas* population causing pith necrosis of tomato and pepper in Argentina are highly diverse. *Plant Pathology* **52**: 287-302.

Basim H., Basim E., Yilmaz S., Ilkucan M., 2005. First report of pith necrosis of tomato caused by *Pseudomonas mediteranea* in Turkey. *Plant Pathology* **54**: 240.

Catara V., Dawn A., Cirvilleri G., Vivian A., 2000. Specific oligonucleide primers for the rapid identification and detection of the agent of tomato pith necrosis, *Pseudomonas corrugata*, by PCR amplification: evidence for two distinct genomic groups. *European Journal of Plant Pathology* **106**: 753-762.

Catara V., Sutra L., Morineau A., Achouak W., Christen R., Gardan L., 2002. Phenotypic and genomic evidence for the revision of *Pseudomonas corrugata* and proposal of *Pseudomonas mediterranea* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **52**: 1749-1758.

Goumas D.E., Malathrakis E.N., Chatzaki A.K., 1999. Characterization of Pseudomonas viridiflava associated with a new symptom on tomato fruit. *European Journal of Plant Pathology* **105**: 927-932.

Malathrakis E.N., Goumas D.E., 1987. Bacterial soft rot of tomato in plastic greenhouse in Crete. *Annals of Applied Biology* **111**: 115-123.

Mitrev S., Spasov D., Karov I., Kostadinovska E., Kovacevik B., 2010. Identification of the causer of tomato pith necrosis in the Republic of Macedonia, Yearbook 2010, Goce Delcev University, 9-23.

Schaad N.W., 2001. Initial identification of common genera. In: Schaad N.W., Jones J.B., Chun W. (eds). Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd Ed. St Paul MN, USA, APS Press, pp. 1-16.

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