Eur J Plant Pathol
DOI 10.1007/s10658-014-0554-y

Monitoring the occurrence of bacterial spot of tomato and pepper in the Czech Republic and development of new PCR primers for detection of *Xanthomonas vesicatoria*

Pavel Beran · Ivan Mraz · Blanka Kokoskova · Andrea Bohata

Accepted: 16 October 2014
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2014

Abstract We tested 212 bacterial isolates isolated from tomato and pepper plants suspected of being infected by *Xanthomonas vesicatoria* (causing bacterial spot). Samples from plants were collected in the years 2007–2010 from fields located in central Bohemia and central and south Moravia. For detection of the pathogens we used PCR with two previously published primer pairs (RST65, RST69 and XCVF, XCVR) and one primer pair (Xv1, Xv2) designed for specific detection of *Xanthomonas vesicatoria*. In total, 121 of 212 (57 %) samples were positive in PCR. In this article we provide data for additional verification of the newly designed (Xv1, Xv2) primers. Our results confirm the need of precise detection methods, because several tomato and pepper pathogens can share similar visual symptoms. We also suggest, that new PCR primers for detection of bacterial spot of tomato and pepper pathogens should not be overlooked, because they can improve currently used PCR assays for detection of these pathogens.

Keywords *atpD* · Oligonucleotides · *Xanthomonas euvesicatoria* · *Lycopersicon esculentum* · *Capsicum annuum*

P. Beran (✉) · I. Mraz · A. Bohata
Faculty of Agriculture, University of South Bohemia, Studentska 13, , 370 05 Ceske Budejovice, Czech Republic
e-mail: pavel.beran@centrum.cz

I. Mraz
Department of Plant Virology, Institute of Plant Molecular Biology, Biology Centre of the Academy of Sciences of the Czech Republic,
Branisovska 31, 370 05 Ceske Budejovice, Czech Republic

B. Kokoskova
Laboratory of Diagnostics and Epidemiology of Microorganisms, Plant Medicine Division, Crop Research Institute,
Drnovska 507, 161 06 Prague 6, Czech Republic

Bacterial spot of tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum* L.) can cause considerable losses (up to 30 % depending on climate and year) in productivity and fruit quality especially in areas of warmer and humid climate (Jones et al. 1998; Al-Dahmani et al. 2003). There are four phenotypically and genotypically distinct *Xanthomonas* species causing the bacterial spot of tomato and pepper–*X. euvesicatoria* Jones et al. 2006 (group A, which contains most of the strains previously known as *X. campestris* pv. *vesicatoria* or *X. axonopodis* pv. *vesicatoria*), *X. vesicatoria* (ex Doidge 1920) Vauterin et al. 1995 (group B), *X. perforans* Jones et al. 2006 (group C), and *X. gardneri* (ex Sutic 1957) Jones et al. 2006 (group D). While strains belonging to groups A, B and D are pathogenic on tomato and pepper, group C strains are pathogenic on tomato only. Also geographic distribution varies among the groups. Groups A and B are distributed worldwide, whereas distribution (and resulting economical importance) of groups C and D seems to be limited to U.S., Mexico, Brazil, Korea, regions bordering the Indian Ocean (group C), and former Yugoslavia, Canada, Costa Rica, U.S, Brazil and regions of the

Published online: 23 October 2014

Indian Ocean (group D) so far (Jones et al. 2004; Potnis et al. 2011).

In this study we present results of a monitoring for the occurrence of *X. euvesicatoria* (group A) and *X. vesicatoria* (group B) in central Bohemia and central and south Moravia conducted in the years 2007–2010. For detection of the mentioned pathogens we used PCR. Many PCR primer pairs for detection of the bacterial spot related pathogens have been published (Araújo et al. 2013; Koenraadt et al. 2009; Leite et al. 1994; Obradovic et al. 2004; Moretti et al. 2009; Park et al. 2009). In this study we used two PCR primer pairs for detection of *X. euvesicatoria* designed by Obradovic et al. (2004) - RST65, RST69 and Park et al. (2009) - XCVF, XCVR. Third primer pair (Xv1, Xv2) was newly designed for specific detection of *X. vesicatoria* (group B). This group of bacteria was chosen because it is distributed worldwide but number of published PCR primers for its detection is small (Araújo et al. 2013; Beran and Mráz 2013; Koenraadt et al. 2009) and the publications are relatively recent compared to those for detection of *X. euvesicatoria*. Therefore, there is not enough evidence whether *X. vesicatoria* is rarely found in host plants, or it has not been detected due to lack of detection primers.

A total of 212 bacterial isolates (Table 1) were collected from tomato and pepper plants (one isolate per plant) showing visual symptoms of bacterial spot pathogen. Plant tissue was mechanically crushed in 5 ml of PBS buffer (pH=7.4) and 100 ul of the resulting extract was cultured on MPAg agar medium (meat-peptone agar with glucose: 40 g of nutrient agar no. 2, 5.2 g of yeast extract, 10 g of glucose, 20 g of agar–ensuring solid surface, added to 1 l of distilled H_2O, pH adjusted to 7.2 with 1 M NaOH) at 27 °C for 24–48 h. When yellow bacterial colonies showed up, they were re-cultured under the same conditions to obtain pure cultures.

Another 49 various bacterial strains (Table 2) for testing the Xv1, Xv2 primers specificity were obtained from the Belgian Co-ordinated Collections of Micro-organisms–Bacteria Collection, Gent (BCCM/LMG), French Collection of Plant associated Bacteria, Beaucouze Cedex (CFBP), Czech Collection of Microorganisms, Brno (CCM), Crop Research Institute Collection, Czech Republic, Prague - Ruzyne (CRI) and National Collection of Plant Pathogenic Bacteria, UK, York (NCPPB). All strains were cultured on MPAg agar at 27 °C for 24–48 h. Total DNA of all Gram-positive and Gram-negative bacteria (Tables 1 and 2) was

Table 1 Number of isolates examined by corresponding primer pairs in individual years

Location	Plant	Number of isolates collected				Detected by Xv1, Xv2 primers				Detected by RST65, RST69 primers				Detected by XCVF, XCVR primers			
		2007	2008	2009	2010	2007	2008	2009	2010	2007	2008	2009	2010	2007	2008	2009	2010
South Moravia, Breclav	Tomato	5	3	4	11	0	0	0	0	2	3	2	9	2	3	2	9
South Moravia, Breclav	Pepper	3	3	2	13	0	0	0	0	1	3	0	8	1	3	0	8
South Moravia, Mikulov	Pepper	6	5	8	12	0	0	0	0	4	5	2	10	**5**	5	2	10
South Moravia, Znojmo	Pepper	5	6	3	13	0	0	0	0	3	3	1	10	3	3	**2**	10
South Moravia, Znojmo	Tomato	2	5	8	9	0	0	0	0	0	4	4	2	0	4	4	2
Central Moravia, Prostejov	Tomato	8	3	4	8	**6**	0	0	0	2	3	1	5	2	3	1	5
Central Moravia, Kromeriz	Pepper	3	6	5	8	0	0	0	0	3	5	2	0	3	5	2	0
Central Moravia, Olomouc	Tomato	5	4	6	4	0	0	0	0	0	3	2	1	0	3	2	1
Central Bohemia, Nymburk	Pepper	2	7	8	5	0	0	0	0	2	2	3	2	2	2	3	**3**
Total		39	42	48	83	6	0	0	0	17	31	17	47	18	31	18	48
		212				6				112				115			

Table 2 List of bacterial strains used to test specificity of Xv1 and Xv2 primers

Name of bacteria as listed in collection	Collection and number	Detected by Xv1, Xv2 primers	Detected by XCVF, XCVR primers	Detected by RST65, RST69 primers
Xanthomonas vesicatoria	BCCM/LMG 911	+	–	–
Xanthomonas vesicatoria	BCCM/LMG 916	+	–	–
Xanthomonas vesicatoria	BCCM/LMG 919	+	–	–
Xanthomonas vesicatoria	BCCM/LMG 920	+	–	–
Xanthomonas vesicatoria	BCCM/LMG 923	+	–	–
Xanthomonas vesicatoria	BCCM/LMG 925	+	–	–
Xanthomonas vesicatoria	BCCM/LMG 934	+	–	–
Agrobacterium tumefaciens	CCM 2835	–	–	–
Clavibacter michiganensis subsp. *insidiosus*	CRI 12/5/98	–	–	–
Clavibacter michiganensis subsp. *michiganensis*	BCCM/LMG 5727	–	–	–
Clavibacter michiganensis subsp. *michiganensis*	BCCM/LMG 7333	–	–	–
Clavibacter michiganensis subsp. *nebraskensis*	BCCM/LMG 3697	–	–	–
Clavibacter michiganensis subsp. *sepedonicus*	NCPPB 3467	–	–	–
Curtobacterium albidum	CCM 2296	–	–	–
Curtobacterium flaccumfaciens	BCCM/LMG 3651	–	–	–
Dickeya spp.	CCM 989	–	–	–
Erwinia amylovora	NCPPB 696	–	–	–
Pantoea agglomerans	CCM 2406	–	–	–
Pantoea dispersa	CCM 4414	–	–	–
Pectobacterium atrosepticum	BCCM/LMG 2396	–	–	–
Pectobacterium carotovorum subsp. *carotovorum*	BCCM/LMG 2404	–	–	–
Pseudomonas corrugata	CRI 8892-85Re	–	–	–
Pseudomonas fluorescens	CCM 2115	–	–	–
Pseudomonas marginalis pv. *marginalis*	BCCM/LMG 2210	–	–	–
Pseudomonas syringae pv. *phaseolicola*	CRI 186/2	–	–	–
Pseudomonas syringae pv. *pisi*	NCPPB 3496	–	–	–
Pseudomonas syringae pv. *syringae*	BCCM/LMG 1247	–	–	–
Pseudomonas syringae pv. *tomato*	CRI 2/1/99Re	–	–	–
Pseudomonas viridiflava	BCCM/LMG 2352	–	–	–
Rahnella aquatilis	CCM 4086	–	–	–
Ralstonia solanacearum	NCPPB 2505	–	–	–
Xanthomonas arboricola pv. pruni	BCCM/LMG 852	–	–	–
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 667	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 668	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 909	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 922	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 926	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 931	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	BCCM/LMG 933	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	CFBP 1604	–	+	+
Xanthomonas axonopodis pv. *vesicatoria*	CFBP 3274	–	+	+
Xanthomonas campestris pv. *vesicatoria*	BCCM/LMG 918	–	+	+
Xanthomonas campestris pv. *vesicatoria*	BCCM/LMG 921	–	+	+
Xanthomonas campestris pv. *vesicatoria*	BCCM/LMG 927	–	+	+

Table 2 (continued)

Name of bacteria as listed in collection	Collection and number	Detected by Xv1, Xv2 primers	Detected by XCVF, XCVR primers	Detected by RST65, RST69 primers
Xanthomonas campestris pv. *vesicatoria*	BCCM/LMG 928	–	+	+
Xanthomonas fragariae	BCCM/LMG 708	–	–	–
Xanthomonas gardneri	BCCM/LMG 962	–	–	–
Xanthomonas hortorum pv. *pelargonii*	BCCM/LMG 7192	–	–	–
Xanthomonas perforans	NCPPB 4321	–	–	–

BCCM/LMG Belgian Co-ordinated Collections of Micro-organisms–Bacteria Collection; *CFBP* French Collection of Plant Pathogenic Bacteria; *CCM* Czech Collection of Microorganisms; *CRI* Crop Research Institute Collection; *NCPPB* National Collection of Plant Pathogenic Bacteria

extracted from an amount of approximately 10^9 bacterial cells (adjusted by spectrophotometer according to Dominguez et al. 2001) using the Wizard SV Genomic DNA Purification System (Promega, USA), using the manufacturer's protocol.

The PCR was performed using the following mixture (for all primer pairs): 10 μl of PPP MasterMix Combi (Top-Bio, Czech Republic) composed of 150 mM Tris–HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02 % Tween 20, 5 mM $MgCl_2$, 400 μM of each nucleotide, 100 U ml^{-1} Taq DNA polymerase, 38 nM anti-Taq monoclonal antibody, stain, stabilizer and additives, 7 μl of PCR grade water (Top-Bio, Czech Republic), 1 μl of each primer (10 pmol) and 1 μl of template DNA. PCR conditions for the XCVF, XCVR primers were taken from the corresponding publication. Annealing temperature of RST65, RST69 primers was set to 67 °C to avoid non-specific amplification. The PCR primers Xv1 (5'–TGGGAAATCCATCGCACT–3') and Xv2 (5' –TTGCCCTTGCCGTTCTCG–3') were designed for detection of *X. vesicatoria* in the same manner as in Beran and Mráz (2013). Annealing temperature for Xv1, Xv2 primers was optimized using gradient PCR. Adequate number of cycles was determined manually. All reactions were performed as previously described in Beran and Mráz (2013)

The primers Xv1, Xv2 were tested for specificity by PCR with 49 bacterial strains obtained from various collections of microorganisms (Table 2). All seven *X. vesicatoria* samples were positive and sharp band for the 404 bp product was observed (data not shown) while no other amplified DNA was visible in any of the 42 other (even closely related) bacterial strains analysed. The PCR assays for each reference sample (Table 2) were repeated at least three times with identical results. Best specificity of the Xv1, Xv2 primers was achieved with the following PCR program: 25 cycles, each consisting of 45 s at 94 °C, 45 s at 69 °C and 40 s at 72 °C, with an initial 5 min denaturation at 94 °C and final extension of 10 min at 72 °C. To determine reproducibility of the PCR (Park et al. 2009), three different PCR cyclers from three different manufacturers were used and they produced consistent results. Sensitivity of the Xv1, Xv2 primers was tested together with previously published XV1F, XV1R primers (Beran and Mráz 2013) according to Moretti et al. (2009). The sensitivity of both primer pairs was approximately 5 ng ml^{-1}, which is similar to the levels obtained in other studies (Moretti et al. 2009; Leite et al. 1994). Although there is not much difference between the two primer pairs, the newly designed primer pair (Xv1, Xv2) is external to the previously published XV1F, XV1R (Beran and Mráz 2013), so a nested PCR assay can be designed easily.

Altogether 212 bacterial isolates taken from the field grown pepper and tomato plants were tested (Table 1). All the samples were tested by PCR with three primer pairs (RST65, RST69; XCVF, XCVR; Xv1, Xv2). A total of 112 samples were positive in PCR with RST65, RST69 primers. In addition to these samples, three more were positive in PCR with XCVF, XCVR primers with overall 115 positive samples. Only six samples were positive in PCR with Xv1, Xv2 primers. None of the six samples was, however, positive in PCR with RST65, RST69 or XCVF, XCVR primers. Such results were expected, because Xv1, Xv2 primers are designed for detection of *X. vesicatoria* (group B) while XCVF, XCVR primers are designed primarily for detection of *X. euvesicatoria* (group A). The primers RST65, RST69 were designed for detection of all tomato and pepper related xanthomonads (Obradovic et al. 2004). According to BLAST (GenBank), however, the only

tomato or pepper related *Xanthomonas* with 100 % homology to the RST65, RST69 primers is *X. euvesicatoria*. These data suggest that the primers RST65, RST69 and XCVF, XCVR can be used almost interchangeably or to verify the results obtained by the other primer pair.

DNA sequences of 16S rDNA (Accession numbers: KM198913, KM198914, KM198915, KM198916, KM198917, KM198918) and the *atpD* gene (Accession numbers: KM198919, KM198920, KM198921, KM198922, KM198923, KM198924) were obtained from the six samples positive in PCR with Xv1 and Xv2 primers to confirm its belonging to *X. vesicatoria* (group B) species using BLAST (GenBank). Although the number of the positive samples obtained with Xv1, Xv2 primers is not high, it should not be omitted. Multiplexing Xv1, Xv2 primers with other primers for detection of bacterial spot pathogens should not significantly increase the price of PCR and would contribute to avoid false negatives during PCR detection of these quarantine bacteria. This approach would, however, require additional testing. Our intention was also to provide data which can be useful for further verification of new primers Xv1, Xv2 according to EPPO standards.

Acknowledgments This study received financial support from Grant Agency of the Czech Republic (GA CR) grant 521/08/H042, from the Ministry of Agriculture of the Czech Republic, grant QH71229, from the Grant Agency of the Academy of Sciences of the Czech Republic, grant AV0Z50510513 and from Czech Ministry of Education, Youth and Sports, grant No. CZ.1.07/2.3.00/30.0049

References

Al-Dahmani, J. H., Abbasi, P. A., Miller, S. A., & Hoitink, H. A. J. (2003). Suppression of bacterial spot of tomato with foliar sprays of compost extracts under greenhouse conditions. *Plant Disease, 87*, 913–919.

Araújo, E. R., Ferreira, M. A. S. V., & Quezado-Duval, A. M. (2013). Specific primers for *Xanthomonas vesicatoria*, a tomato bacterial spot causal agent. *European Journal of Plant Pathology, 137*, 5–9.

Beran, P., & Mráz, I. (2013). Species-specific PCR primers for detection of *Xanthomonas vesicatoria. Crop Protection, 43*, 213–215.

Doidge, E. M. (1920). A tomato cancer. *Journal of the Department of Agriculture, Union of South Africa., 1*, 718–721.

Dominguez, M. C., de La Rosa, M., & Borobio, M. V. (2001). Application of a spectrophotometric method for the determination of post-antibiotic effect and comparison with viable counts in agar. *Journal of Antimicrobial Chemotherapy, 47*, 391–398.

Jones, J. B., Stall, R. E., & Bouzar, H. (1998). Diversity among Xanthomonads pathogenic on pepper and tomato. *Annual Review of Phytopathology, 36*, 41–58.

Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., & Schaad, N. W. (2004). Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Systematic and Applied Microbiology, 27*, 755–762.

Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., & Schaad, N. W. (2006). Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Systematic and Applied Microbiology. Erratum: Systematic and Applied Microbiology, 29*, 85–86.

Koenraadt, H., van Betteray, B., Germain, R., Hiddink, G., Jones, J. B., & Oosterhof, J. (2009). Development of specific primers for the molecular detection of bacterial spot of pepper and tomato. *ISHS Acta Horticulturae, 808*, 99–102.

Leite, R. P., Jr., Minsavage, G. V., Bonas, U., & Stall, R. E. (1994). Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria. Applied and Environmental Microbiology, 60*, 1068–1077.

Moretti, C., Amatulli, M. T., & Buonaurio, R. (2009). PCR-based assay for the detection of *Xanthomonas euvesicatoria* causing pepper and tomato bacterial spot. *Letters in Applied Microbiology, 49*, 466–471.

Obradovic, A., Mavridis, A., Rudolph, K., Janse, J., Arsenijevic, M., Jones, J., Minsavage, G., & Wang, J. (2004). Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from peppers and tomatoes in Serbia. *European Journal of Plant Pathology, 110*, 285–292.

Park, D. S., Shim, J. K., Kim, J. S., Lim, C. K., Shrestha, R., Hahn, J. H., & Kim, H. G. (2009). Sensitive and specific detection of *Xanthomonas campestris* pv. *vesicatoria* by PCR using pathovar-specific primers based on *rhs* family gene sequences. *Microbiological Research, 164*, 36–42.

Potnis, N., Krasileva, K., Chow, V., Almeida, N. F., Patil, P. B., Ryan, R. P., Sharlach, M., Behlau, F., Dow, J. M., Momol, M., White, F. F., Preston, J. F., Vinatzer, B. A., Koebnik, R., Setubal, J. C., Norman, D. J., Staskawicz, B. J., & Jones, J. B. (2011). Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper. *BMC Genomics, 12*, 146.

Sutic, D. (1957). Bakterioze crvenog patlidzana (Tomato bacteriosis). Posebna Izdanja Institut za zastitu bilja Beograd (Special Edition Institute for Plant Protection Belgrade) 6, 1–65. *English Summary Review of Applied Mycology, 36*, 734–735.

Vauterin, L., Hoste, B., Kersters, K., & Swings, J. (1995). Reclassification of *Xanthomonas. International Journal of Systematic Bacteriology, 45*, 472–489.