

Molecular characterization of stolbur phytoplasmas in pepper and tomato from Bulgaria

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Abstract. Tomato and pepper are the main vegetable crops cultivated in Bulgaria. Phytoplasma diseases, mainly stolbur, are important plant diseases for these crops in Bulgaria. The goal of the present paper was to verify association of phytoplasmas with the observed disease symptoms in tomato and pepper and to identify the phytoplasmas detected using RFLP analysis of conserved genes and other uncharacterised phytoplasma chromosomal regions. The presence of phytoplasmas was confirmed in all the samples of tomato and pepper showing typical stolbur symptoms. A phytoplasm sample, which caused severe symptoms, showed the same pattern as the reference strain Mol, while all other phytoplasmic reference strains showed different polymorphisms. RFLP profiles were found useful in distinguishing phytoplasmas in stolbur subgroup (16SrXII-A) in natural plant hosts.

1 Introduction

Tomato and pepper are the main vegetable crops cultivated in open field in Bulgaria. Phytoplasma diseases, primarily stolbur, are important plant diseases for these crops in Bulgaria. During the period 2014-2017 years we analyzed the spreading of stolbur disease on tomato and pepper in vegetable growing areas in Southern Bulgaria. In some regions, located to the north of Maritza river, the losses of pepper yield caused by stolbur reached up to 75-100%, tomato losses - 65-75%. On the territory of Experimental field of Agricultural University in Plovdiv the symptoms of classical stolbur on tomato and pepper were also observed.

The goal of the present paper was to verify association of phytoplasmas with the observed disease symptoms in tomato and pepper and to identify the phytoplasmas detected using RFLP analysis of conserved genes and other uncharacterised phytoplasma chromosomal regions.

2 Materials and methods

Plants with typical disease symptoms were collected from the experimental field of Agricultural University of Plovdiv, Southern Bulgaria, in the Maritza river valley. Standard area plots covered by each plant species and cultivar were about 1 ha each. Samples collected from petioles, midribs, young shoots and flowers of plants (Table 1) were exploited for molecular analysis.

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Table 1. Number of samples of the diseased plants in the analysis

Plant species	Cultivar	Plant organs	Number of samples
<i>Capsicum annuum</i> L.	Kurtovska kapia ser. var. grossum type kapia	midribs	15
		young shoots	10
<i>Lycopersicon esculentum</i> L.	Konserven	petiols	10
		midribs	25
		flowers	20
	Trapesitza	petiols	15
		midribs	25
		flowers	25
	Konserven UC-134	petiols	15
		midribs	25
		flowers	20

Genomic DNA was extracted using standard chloroform/phenol method [1] from 2 g/sample of fresh plant tissue. The DNA samples were resuspended in TE buffer and diluted to concentration of 20 ng/μl. DNA samples of identified phytoplasma strains Stolbur (Stol), Deperissement du lavandin (Dep) and Molière disease (Mol) belonging to subgroup 16SrXII-A and strain elm yellows (EY) belonging to 16SrV-A group were used as a reference [2-4]. Two strains from grapevine infected with Bois Noir disease from Italy (Gr1 and Gr2) were also included in the study [2]. DNA samples from asymptomatic pepper and tomato were used as a negative control.

DNA samples were tested with different PCR systems. PCR reaction mixtures (25 μl) contained: 0.8 μl template DNA (20 ng/μl), 200 μM each of dNTP, 0.625 U Taq polymerase (Perkin Elmer, Norwalk, Conn., USA); 0.4 μM each of primer, 10 mM TRIS buffer (pH 8.3), 50 mM HCl, 1.5 mM MgCl₂ and 0.001% (w/v) gelatine as PCR buffer. For the nested PCR, two primer set were used: 1) Phytoplasma ribosomal general P1/P7 outer primers [4, 5] were used to amplify a DNA fragment between positions 18 and 1832 in the 16SrDNA and spacer region and 2) inner primers 16R758f/M23SR1804r (=M1/B6) or R16F2/R2 [6-8]. Chromosomal primers G35p/m [10] and Stol4f/r [5] amplifying phytoplasma DNA regions with unknown functions were also exploited in direct PCR.

The protocol of amplification described by Schaff et al. [10] was used for all the primers except Stol4f/r for which the PCR conditions were described by Daire et al. [5]. PCR products (6 μl) were separated in 1% agarose gel stained with ethidium bromide.

RFLP analysis of PCR products was performed to identify the phytoplasmas and to verify genomic variability. The restriction enzymes were chosen among those being most significant in phytoplasma genomic diversity studies [5]. MseI was used to verify ribosomal subgroup identity [8] against the product amplified with P1/P7, R16F2/R2 and M1/B6 primers; the presence of genomic differences among phytoplasma isolates from pepper and tomato was verified using Tsp509I, SspI and DraI against the DNA regions with unknown functions. All digestions with restriction enzymes were performed at least for 16 hours according to the manufacturer's instructions.

3 Results

The first symptoms on tomato cv. Trapesitza were observed on 15-25 July. The symptoms in the whole plant were strong decrease in leaf dimensions, chlorosis, reduction of top leaves number, purple coloration of the stem. Flowers showed the typical malformations: the calyces were 3-4 times longer than normal, narrow, freely or accrete; the flowers were green with

stamen reduction and phyllody. The fruits of diseased tomato were of smaller size, woody and unfit for processing, possessed irregular coloring and bad taste.

First disease symptoms in pepper cv. Kurtovska kapia were observed on 15-25 July. The chlorosis, leaf deformation, yellowing, crinkling of midribs, purple colour of leaf ribs were observed on leaves on the top of the plant. Most of the flowers were sterile, they dried and fell down. The fruits of diseased pepper were small with prematurely coloration, with very thin and dry pericarp. Several weeks after first detection of symptoms the leaves of the whole plants totally dried and the roots decayed, starting from the tip. The bark was soft and necrotic.

All the tested pepper and tomato samples were positive for phytoplasma and have been found to be infected with phytoplasmas belonging to the stolbur group (16SrXII-A) except for the tomato sample cv UC-134. The DNA of all the phytoplasma strains from pepper and strains from tomato varieties Konserven and Trapesitza were successfully amplified using direct PCR with primers P1/P7. The amplification was further confirmed by the primers R16F2/R2 and M1/B6 used in nested PCR. In particular, primers M1/B6 showed higher sensitivity as they amplified the samples from tomato Konserven which were not amplified with the other nested PCR primers. PCR with chromosomal primers G35p/m and stol4f/r was positive only for two experimental samples from pepper (P2 and P3).

RFLP analysis of DNA fragment of 16S rRNA region has also shown that all phytoplasmas from infected pepper and tomato belong to the stolbur group (16SrXII-A). Electrophoretic profiles obtained for paper samples with stol4f/r primers did not show variability using the two restriction enzymes, though some variation was observed when primers G35p/m were exploited. The strain Dep from France showed polymorphisms in DNA fragments amplified with Stol4f/m and G35p/m primers when digested by *SspI* and *Tsp509I*, respectively. The control strain Mol expressed the same pattern as P3 when DNA was amplified with primers G35p/m and digested with *Tsp509I*.

4 Discussion

The presence of phytoplasmas was confirmed in all the samples of tomato and pepper showing typical stolbur symptoms, however the tomato UC-134 showed symptoms that could also be associated with other pathogens or stress factors not determined in this study.

Different intensity of symptoms was observed between the two pepper samples amplified with primers G35p/m and this could be associated with the RFLP patterns observed after digestion with *Tsp509I* but further research is necessary to confirm this association. It is interesting to note that P3 phytoplasm sample, which caused severe symptoms, showed the same pattern as the reference strain Mol, while all other phytoplasmic reference strains showed different polymorphisms. Only two samples from Italian grapevines caused “Bois Noir” symptoms showed identical RFLP profiles, indicating that this phytoplasma chromosomal fragment could be useful in distinguishing phytoplasmas in stolbur subgroup (16SrXII-A) in natural plant hosts.

References

1. J.P. Prince, R.E. Davis, T.K. Wolf., I.-M. Lee, B.D. Mogen, E.L. Dally, A. Bertaccini, R. Credi, M. Barba, *Phytopathol.* **83**, 1130 (1993).
2. A. Bertaccini, M. Borgo, M. Martini, N. Mori, E. Murari, G. Posenato, P. Sancassani, S. Sartori, M. Vibio, *L'Informatore Agrario* **54**, 85 (1998).
3. A. Bertaccini, L. Carraio, D. Davies, M. Laimer da Camara Machado, M. Martini, S. Paltrinieri, E. Seemüller, EFPP 2000 Taormina-Giardini Naxos, 19-22 September 2000, **122**, 38 (2000).
4. S. Deng, C. Hiruki, *Phytopathol.* **81**, 1475 (1991).
5. K.S. Gibb, A.C. Padovan, B.A. Mogen, *Phytopatol.* **85**, 169 (1995).
6. I.-M. Lee, A. Bertaccini, M. Vibio, D.E. Gundersen, *Phytopathol.* **85**, 728 (1995).
7. I.-M. Lee, D.E. Gundersen, R.E. Davis, I.M. Bartoszyk, *Int. J. Syst. Bacteriol.* **48**, 1153 (1998).
8. M. Martini, E. Murari, N. Mori, A. Bertaccini, *Plant Dis.* **83**, 925 (1999).
9. A.C. Padovan, K.S. Gibb, A. Bertaccini, M. Vibio, R.E. Bonfiglioli, P.A. Magarey, B.B. Sears, *Aust. J. Grape Wine Res.* **1**, 25 (1995).
10. D.A. Schaff, I.-M. Lee, R.E. Davis *Biochem. Biophys. Res. Comm.* **186**, 1503 (1992).
11. B. Schneider, E. Seemüller, C. Smart, B.C. Kirkpatrick, *Molecular and diagnostic procedures in mycoplasmaology* (Academic Press, New York, 1995) 369.
12. E. Seemüller, C. Marcone, U. Lauer, A. Ragozzino, M. Groschl, *J. Plant Pathol.* **80**, 3 (1998).