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MULTIGENE CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' IN PEPPER AND TOMATO PLANTS IN THE REPUBLIC OF MACEDONIA

Emilija Arsov[1], Galina Ivanova[1], Sasa Mitrev[1]

[1]*Department for Plant and Environmental Protection, Faculty of Agriculture, Goce Delcev University, Krste Misirkov, 10-A, 2000 Stip, Republic of Macedonia*

emilija.arsov@ugd.edu.mk; givanova1@gmail.com (M.Sc. student); sasa.mitrev@ugd.edu.mk

Abstract

'*Candidatus* Phytoplasma solani' is a phytoplasma distributed worldwide, associated in the Euro-Mediterranean area with various diseases in most spreader and cultivated plants including grapevine, maize, potato, pepper, celery and tobacco.

Molecular multigene characterization was performed for the first time on phytoplasma strains collected from the Eastern part of Macedonia from symptomatic and asymptomatic pepper and tomato plants, during the summer period of the 2015/16 season. PCR amplification on specific *tuf*, *vmp*1 and *stamp* genes showed that the phytoplasma strains identified belong to subgroup 16SrXII-A, since their restriction patterns were indistinguishable from one another and from the patterns characteristic of the STOL (16SrXII-A) reference strain.

For samples positive for the *tuf* gene, it was possible to identify the *Hpa*II RFLP profiles associated with *tuf* type b genes.

This is the first field observation and molecular laboratory testing for the presence of stolbur phytoplasma in pepper and tomato in the Republic of Macedonia.

Key words: *pepper, tomato, stolbur phytoplasma, multilocus genetic analysis*

INTRODUCTION

Phytoplasmas are obligate, phloem-limited phytopathogens. They are pleomorphic prokaryotes without cell walls. Phytoplasmas are transmitted to plants in the process of feeding of their vectors, sap-sucking hemipteran insects, mainly leafhoppers, planthoppers, and psyllids (Bertaccini et al., 2014).

Since the identification of phytoplasmas by standard microbiological methods in routinely grown laboratory cultures was not possible until recently, they are classified in a system of groups and subgroups based on DNA fingerprints (RFLP patterns) of 16S rRNA genes (16S rDNA) (Lee et al., 1998). Based on 16S rRNA gene sequence identity and biological properties, group 16SrXII encompasses several species, including STOL – '*Candidatus* Phytoplasma solani' in subgroup 16SrXII-A, and '*Candidatus* Phytoplasma australiense', '*Candidatus* Phytoplasma japonicum' and '*Candidatus* Phytoplasma fragariae' within subgroup 16SrXII-B. 16SrXII-A phytoplasma strains are associated with stolbur disease in numerous cultivated and wild plants, hence they are commonly known as stolbur phytoplasmas (Bertaccini et al., 2014).

Bois noir phytoplasma was shown to be transmitted by the planthopper *Hyalesthes obsoletus*, which normally feeds on herbaceous weeds, and occasionally also on grapevines (Berger et al. 2009).

In Macedonia, agriculture uses well-established traditions in the production of vegetables, more specifically various pepper cultivars (Gudeva et al., 2007; Mitrev and Spasov, 1999). Till now, only grapevines, the most spreader cultivars in the Tikves region, are known as host plants for stolbur phytoplasma in Macedonia (Kostadinovska et al., 2014a, 2014b; Mitrev et al., 2008, 2012).

Strumica and Kocani are the best-developed regions in Eastern Macedonia for the cultivation of different varieties of pepper

(*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.) in the open field and most often under greenhouse or glasshouse conditions. Great agrobiological diversity, of 129 domestic and 2205 imported varieties of *Capsicum* spp. (Gudeva and Trajkova, 2008), has been documented in the country; throughout the last 30 years, most of them have been successfully introduced for fresh consumption as well as for industrial processing.

The aim of this study was to check the distribution of this disease in Eastern Macedonia, in Strumica and in Kochani as the main pepper cultivation regions, and to identify and characterize the disease with modern and reliable molecular methods, including multilocus genetic analysis of the 16S rRNA gene.

MATERIAL AND METHODS

Collection of stolbur-symptomatic pepper and tomato plants

During our field surveys carried out from the beginning of August till the end of October 2015/16, pepper and tomato leaf samples were collected from 24 symptomatic and asymptomatic plants from 11 localities in Eastern Macedonia, in Strumica and Kocani regions (Figs. 1 and 2). Of all samples analysed, four sweet red pepper plants from Burievo (Strumica region, latitude 41° 25′ 24″ N, longitude 22° 45′ 54″ E) and one *C. annuum* L. var. *cerasiforme* (Mill.) Irish plant from Cesinovo (Kocani region, latitude 41° 52′ 18″ N, longitude 22° 17′ 24″ E) were identified and characterized for stolbur disease with the molecular methods described below.

Tomato plants were collected randomly without symptoms or with atypical symptoms, because we had difficulty finding tomato plants in the open field, as most tomato production in Macedonia is in controlled conditions (green- or glasshouses).

Figure 1. (a) Pepper field in the Strumica locality; (b) symptoms of stolbur phytoplasmas on a whole *Capsicum annuum* var. *cerasiforme* plant.

Table 1 shows the number of plants collected from the field with the presence of visual symptoms, and included characteristics of location, region and plant host.

Table 1. Field observation during the summer period 2015/16.

Number	Strain	Location	Region	Plant host	Symptoms*
1	1–5/15	Strumica	Burievo	Pepper plant	+++
2	8/16	Strumica	Prosenikovo	Healthy pepper plant	–
3	9/16	Strumica	Piperovo	Healthy tomato plant	–
4	10–13/16	Strumica	Dobrejci	Pepper plant	+?
5	14–18/16	Kocani	Cesinovo	Sweet pepper plant	++
6	25–30/16	Kocani	Ciflik	Pepper plant	++++
7	37–43/16	Kocani	Gorni Podlog	Pepper plant	++++

*Plants were chosen randomly, and symptoms were not clear phytoplasma symptoms: – negative; +? symptoms not clear; ++ good symptoms; +++ very good symptoms; ++++ obvious phytoplasma symptoms.

Total DNA extraction

Leaf veins, separated from laminas by a sterile razor, and all parts of the tomato and pepper plants were stored at –80 °C. Total nucleic acids were extracted from 0.5 g of frozen plant tissues by a cetyltrimethylammonium bromide (CTAB) extraction procedure (Angelini et al., 2001). The concentration of total DNA was measured with a NanoDrop spectrophotometer (Jenova Nano Spectrophotometer).

Molecular identification of stolbur phytoplasmas

'*Ca.* P. solani' strains from symptomatic and asymptomatic pepper and tomato plants were employed for further molecular characterization carried out on the *tuf* (encoding translation elongation factor Tu), *vmp*1 (encoding a putative membrane protein) and *stamp* genes (encoding an antigenic membrane protein).

The *tuf* gene was amplified in a nested PCR procedure using primer pairs tuf1f/r/ tufAYf/r (Langer and Maixner, 2004), and amplicons were subjected to RFLP analysis with *Hpa*II enzyme.

The nested PCR products of the *vmp*1 gene were obtained with StolH10F1/R1 (Cimerman et al., 2006) and TYPH10F/R (Fialova et al., 2009) primers.

Nested PCR for the *stamp* gene was done with StampF/R0 followed by StampF1/R1 primers (Fabre et al., 2011).

PCR and RFLP reaction conditions were as previously described (Lee et al., 1998; Quaglino et al., 2009). PCRs were performed by using *Taq* polymerase (Promega) in an automated thermal cycler (MasterCycler Gradient, Eppendorf). PCR and enzymatic digestion products were electrophoresed through 1% and 3% agarose gel, respectively, in TBE buffer, stained with ethidium bromide and visualized under a UV transilluminator.

Characterization of stolbur phytoplasmas through multilocus genetic analysis

Molecular characterization of phytoplasma strains was performed by nested PCR/RFLP-based assays of two phytoplasma genomic portions, including *tuf*, *vmp*1 and *stamp* genes.

RESULTS AND DISCUSSION

Symptomology of the pepper and tomato plants

During our field surveys carried out from the beginning of August to the end of October 2015/ 16, leaf samples were collected from 24 symptomatic/asymptomatic pepper and tomato plants from 11 localities in Eastern Macedonia. Among all plants analysed for stolbur disease, four sweet red pepper plants and one *C. annuum* var. *cerasiforme* were proven to be stolbur-positive with the molecular methods used in this study. In pepper plants, typical symptoms assessed in the leaves in the course of disease progress were yellowing, stunting and wilting. The fruits were smaller and without taste. The anthers and the filaments of the flowers were distorted and grown into one whole entity (Fig. 2). The roots of symptomatic plants were dry and/or not well developed (Fig 3.).

Figure 2. Pepper plants with distorted anthers and filaments of the flowers, and grown into one whole entity.

Figure 3. The roots of symptomatic plants were dry and/or not well developed.

Molecular identification of stolbur phytoplasmas

Due to variations in typical symptoms of stolbur phytoplasmas in the field, PCR-based amplification of 16S rRNA genes was performed to prove that some of the samples examined were affected by stolbur phytoplasmas. PCR amplification of specific *tuf*, *vmp*1 and *stamp* genes showed that the phytoplasma strains identified belong to subgroup 16SrXII-A, since their restriction patterns were indistinguishable from one another and from the patterns characteristic of the STOL (16SrXII-A) reference strain (*tuf* profiles are presented and *stamp* profiles are not presented in this study) (Fig. 4).

For the *tuf* gene, it was possible to identify *Hpa*II RFLP profiles associated with *tuf* type a (two strains) and *tuf* type b (16 strains), formerly named VK-I and VK-II (Langer and Maixner, 2004). Our samples were positive for *tuf* type b, VK-II (Fig. 4a and b).

Also, only PCR amplification was done to confirm *vmp*1 and *stamp* gene-positive profiles (Fig. 5).

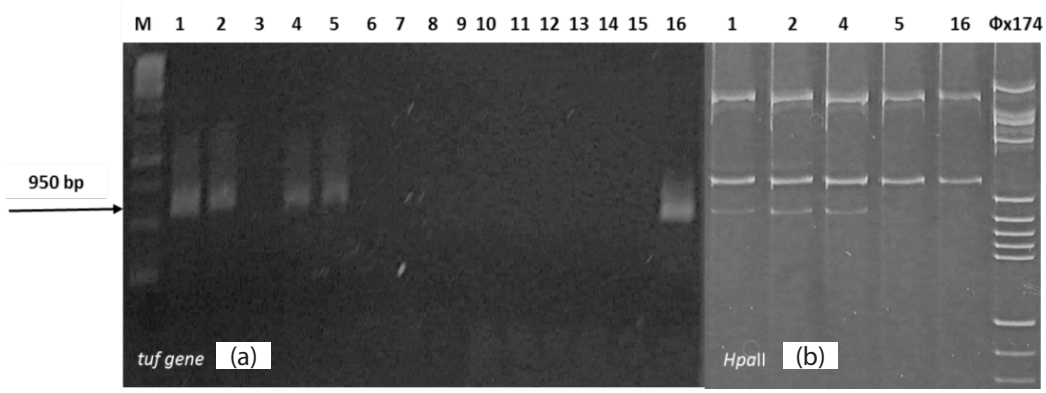

Figure 4. (a) PCR pattern of specific *tuf* gene for stolbur phytoplasma, including tomato and pepper plants: M – 1 kb DNA ladder marker; (1–7) pepper, Strumica, Burievo; (8) healthy pepper plant as negative control; (9) healthy tomato plant as negative control; (10–13) pepper, Strumica, Dobrejci; (14–16) pepper, Kocani, Cesinovo; (b) RFLP profiles from HpaII digestions of positive fTufAY/rTufAY PCR products (*tuf* gene) using 3% agarose gel: Φx174 – marker. b) RFLP profiles from *Hpa*II digestions of positive fTufAY/rTufAY PCR products (*tuf* gene) using 3% agarose gel: Φx174 – marker.

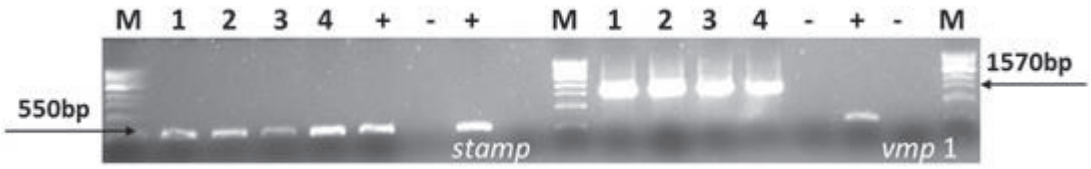

Figure 5. PCR pattern of specific *vmp*1 gene (1570 bp) and *stamp* gene (550 bp) for stolbur phytoplasma, including only positive pepper plants: M – 1 kb DNA ladder marker; (1–4) pepper, Strumica, Burievo; (–) healthy pepper plant as negative control.

CONCLUDING REMARKS

Stolbur disease is an old and well-known disease, mainly in Europe but in other continents as well. Symptoms in pepper vary depending on the pepper variety, geographical region and cultivation conditions. Although it has been noticed in Macedonia multiple times in the last 40 years, it has not been laboratory-tested and proven until this present study for pepper and tomato.

The present molecular multigene characterization is the first detection of stolbur phytoplasmas on garden crops (pepper and tomato) and corresponded to stolbur phytoplasma (16SrXII-A), the same one that we've already had in the vineyards (*tuf* type b).

Four of the 24 pepper and tomato plants analysed were molecularly identified and characterized with stolbur symptoms. Only samples collected from peppers showed positive PCR/RFLP profiles. In this study, we did not find tomato plants with positive profiles, so future field observations and laboratory analysis are required.

Therefore, it can be concluded that incidence of the disease is still sporadic in Eastern Macedonia, and crop damage is not yet substantial. Nevertheless, it is very important to emphasize the presence of stolbur disease in pepper plants in the country, and to keep the alarm turned on for eventual future local or countrywide outbreaks.

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МУЛТИГЕНЕТСКА КАРАКТЕРИЗАЦИЈА НА *CANDIDATUS PHYTOPLASMA SOLANI* КАЈ ПИПЕРКА И ДОМАТ ВО РЕПУБЛИКА МАКЕДОНИЈА

Емилија Арсов[1], Галина Иванова[1], Саша Митрев[1]

[1]Катедра за заштита на растенијата и животната средина, Земјоделски факултет, Универзитет „Гоце Делчев" - Штип

emilija.arsov@ugd.edu.mk; givanova1@gmail.com *(магистранд/студент);* sasa.mitrev@ugd.edu.mk

Резиме

Candidatus phytoplasma solani е фитоплазма дистрибуирана во светски рамки во евромедитеранската област поврзана со разни болести кај најраспространетите и култивирани растенија, вклучувајќи ги виновата лоза, пченката, компирот, пиперката, целерот и тутунот.

Во ова истражување молекуларната мултигена карактеризација за првпат беше изведена на соеви на фитоплазмите собрани во источниот дел на Република Македонија од симптоматски и асимптоматски растенија од пипер и домати во текот на летниот период во сезоната 2015/2016. PCR амплификацијата на специфичен ген за трите специфични гени *tuf*, *vmp*1 и *stamp* покажа дека идентификуваните соеви на фитоплазмата ѝ припаѓаат на подгрупата 16SrXII-A, бидејќи нивните ограничувачки шеми не се разликуваат една од друга и од шаблоните кои се карактеристични за референтниот вид на STOL C фитоплазмата (16SrXII-A).

За позитивни примероци на генот *tuf* беа направени типизација и идентификација на *Hpa*II RFLP профилите поврзани со гените од типот *tuf - type* b.

Ова е прво молекуларно лабораториско тестирање за присуство на столбур фитоплазма кај пипер и домат во Република Македонија.

Клучни зборови: *пипер, домат, столбур фитоплазма, мултилокусна генетска анализа*.