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NEW EVIDENCE FOR THE STOLBUR PHYTOPLASMA DEVELOPMENT IN PEPPER IN REPUBLIC OF MACEDONIA

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Abstract

Sweet red pepper and *Capsicum annuum* var. cerasiforme plants collected from the fields in Eastern Macedonia in the summer period of 2015/16, were proven stolbur positive by performing multilocus genetic analysis, and a comparison with related changes of pigment plastids content in healthy and symptomatic tissue was carried out. PCR amplification on specific *tuf*, *vmp*1 and *stamp* gene, showed that the identified phytoplasma strains 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 *tuf* gene, it was possible to identify the *Hpa*II RFLP profiles associated with *tuf*-type *b* genes. The concentration of chlorophyll *a*, chlorophyll *b*, chlorophyll $(a + b)$ and carotenoids of non-symptomatic and 100% stolbur-affected pepper leaves were estimated. There was a general trend of decrease of chlorophyll *a*, chlorophyll *b* and chlorophyll $(a + b)$ concentration, while there was an increase of carotenoids concentration in stolbur-affected pepper plants compared to their non-symptomatic counterparts.

This is the first report of the presence of stolbur phytoplasma on pepper in the Republic of Macedonia.

Key words: pepper, stolbur phytoplasma, multilocus genetic analysis, pigment plastids

Introduction. Phytoplasmas are obligate, phloem-limited phytopathogens. They are pleomorphic prokaryotes without cell walls. Phytoplasmas belong to the

class *Mollicutes* and are the putative causal agents of yellows diseases that affect more than a thousand plant species worldwide. Phytoplasmas are transmitted to plants in the process of feeding of their vectors, sap-sucking hemipteran insects, mainly leafhoppers, planthoppers, and psyllids.

Since the identification of phytoplasmas until recently was not possible to be performed by standard microbiological methods in routinely grown laboratory cultures, they are classified in a system of groups and subgroups based on DNA fingerprints (RFLP patterns) of 16S rRNA genes (16S rDNA) [1]. Their genome is unusually small – from 530 to 1350 kbp [2]. 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 16SrXII-B subgroup.

16SrXII-A phytoplasma strains are associated with stolbur disease in numerous cultivated and wild plants, hence they are commonly known as stolbur phytoplasmas. Their major host is *Vitis vinifera* (grape) [3], and their minor hosts include corn, wheat, strawberry, stone fruits including apricot and peach, hosts from *Solanaceae* family like potato, tomato and pepper [4].

Symptoms of stolbur infection in solanaceous hosts vary greatly and may be absent or hardly distinguishable. Various wild (weedy) hosts act as pathogen reservoirs – for example *Convolvulus arvensis* (bindweed), *Urtica dioica* (stinging nettle), etc. Phytoplasmas may also be transmitted from infected to healthy plants through the parasitic plant dodder (*Cuscuta* spp.). Experimental transmission of phytoplasmas from infected to healthy dodder of the same or different species, is one of the main ways by which experimental phytoplasma transmission is achieved [5].

In Macedonia the agriculture is with well-established traditions in the production of vegetables, more specifically various pepper cultivars [6,7]. Strumica and Kocani are the best developed regions in Eastern Macedonia for the cultivation of different varieties of pepper (*Capsicum annuum* L.) in the open field and under greenhouse or glasshouse conditions. A great agrobiological diversity of 129 domestic and 2205 imported varieties of *Capsicum spp.* [8] have been documented in Macedonia and throughout the last 30 years most of them have been successfully introduced for fresh consumption as well as for industrial processing.

In this study we report for the first time the molecular detection and characterization of stolbur phytoplasma in sweet red pepper grown in Strumica as well as in *Capsicum annuum* L. var. cerasiforme (common name Hungarian cherry pepper) collected from the sunny valley of Kocani – the only one in Macedonia that lays on a bed of warm thermal waters coupled with excellent climatic factors for pepper cultivation.

Since the yellows diseases caused by stolbur phytoplasmas change the concentration of plastids in the plant cells, we followed that change by estimating

the concentration of chlorophyll a, chlorophyll b, chlorophyll $(a + b)$ and the carotenoids in stolbur-affected and healthy pepper plants.

Materials and methods. ***Collection of stolbur symptomatic pepper plants.*** During our field surveys carried out from the beginning of August till the end of October 2015/16, pepper leaf samples were collected from forty symptomatic plants from eleven localities in Eastern Macedonia, in Strumica and Kocani regions. Out of all analyzed samples, four sweet red pepper plants from Borievo (Strumica region, latitude 41°25′24″ N, longitude 22°45′54″ E) and one *Capsicum annuum* L. var. cerasiforme (Mill.) Irish plant from Cesinovo (Kocani region, latitude 41°52′18″ N, longitude 22°17′24″ E) have been identified and characterized for stolbur disease with the molecular methods described below.

Total DNA extraction. Leaf veins, separated from laminas by a sterile razor, and all parts of the *Cuscuta* and *Convolvulus* spp. plants, were stored at −80 °C. Total nucleic acids were extracted from 1 g of frozen plant tissues by cetyltrimethylammonium bromide (CTAB) extraction procedure [9]. The concentration of the total DNA was measured by spectrophotometer NanoDrop (Jenova Nano Spectrophotometer).

Molecular identification of stolbur phytoplasmas. Phytoplasma detection was carried out by means of amplification of 16S rDNA in nested PCR assays primed by P1/P7 [10,11] followed by primer pair R16F1/R16R0 [12], and subsequent *Alu*I-, *Bfa*I-, *Bst*UI-, and *Mse*I-RFLP assays on the amplicons obtained. PCR and RFLP reaction conditions were as previously described [1,13]. 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 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. Reaction mixtures and PCR-RFLP conditions used for amplifying and digesting the genomic segments of *tuf* [14,15] and *stamp* [16] genes were as previously described.

Estimation of chlorophyll a, chlorophyll b and carotenoids content in stolbur symptomatic and healthy pepper leaves. For extraction and estimation of chlorophyll a, chlorophyll b, chlorophyll $(a + b)$ and carotenoids, the method of Arnon [17] has been followed. Fresh healthy and stolbur-affected leaves of 0.5 gm were ground with 10 ml of 80% acetone using mortar and pestle. The homogenate was centrifuged at 10 000 rpm for 10 min. The supernatant was collected and the pellet was reextracted with 5 ml of 80% acetone each time until it became colourless. All aliquots of supernatant were collected and utilized for pigment plastid estimation. The absorbance was read at 452.5 nm, 645 nm and 663 nm in spectrophotometer using 80% acetone as blank. The content of

chlorophyll a, chlorophyll b, chlorophyll $(a + b)$ and carotenoids was calculated using the formula of Arnon [17]:

$$\text{Chlorophyll } a \text{ (mg/ml)} = 12.7\Delta A_{663} - 2.69\Delta A_{645},$$
$$\text{Chlorophyll } b \text{ (mg/ml)} = 22.9\Delta A_{645} - 4.68\Delta A_{663},$$
$$\text{Chlorophyll } (a + b) \text{ (mg/ml)} = 8.02\Delta A_{663} + 20.2\Delta A_{645},$$
$$\text{Carotenoids (mg/ml)} = 4.75\Delta A_{452.5} - 0.226C(a + b),$$

where, ΔA is the absorbance at the respective wavelength.

Results and discussion. ***Symptomology of the pepper plants.*** During our field surveys carried out from the beginning of August to the end of October 2015/16, leaf samples were collected from 40 symptomatic plants of pepper and tomato, from eleven localities in Eastern Macedonia. Three *Cuscuta* spp. and two *Convolvulus* spp. plants coiled around the above-mentioned symptomatic cultivated plants were also collected from the field. Among all plants, analyzed for stolbur disease, four sweet red pepper plants and one *Capsicum annuum* var. cerasiforme were proven stolbur positive with the molecular methods used in this study. In pepper plants the typical assessed symptoms on the leaves in the course of the disease progress were yellowing, stunting, and wilting (Fig. 1A, B). The fruits were smaller and without taste. The anthers and the filaments of the flowers were distorted and grown into one whole entity. The roots of the symptomatic plants were dry and/or not well developed.

Molecular identification of stolbur phytoplasmas. Due to variations of the typical symptoms of stolbur phytoplasmas on the field, PCR-based amplification of 16S rRNA gene was performed to prove that some of the examined samples were affected by stolbur phytoplasmas. PCR amplification on specific *tuf*, *vmp*1 and *stamp* gene, showed that the identified phytoplasma strains 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. 2A).

For *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 [15]. Our samples were positive for *tuf* type *b*, VK-II (Fig. 2B).

Estimation of pigment plastids (chlorophyll a, chlorophyll b, chlorophyll (a + b) and carotenoids) content in stolbur symptomatic and healthy leaves of pepper plants. The concentration of chlorophyll a, chlorophyll b, chlorophyll $(a + b)$ and carotenoids may vary depending on the variety of the pepper plants and the cultivation conditions [18]. In the present study the plastid pigments' content of non-symptomatic and 100% stolbur-affected leaves were estimated. There was a decrease of chlorophyll a concentration in both analyzed pepper varieties. The decrease of chlorophyll a concentration of

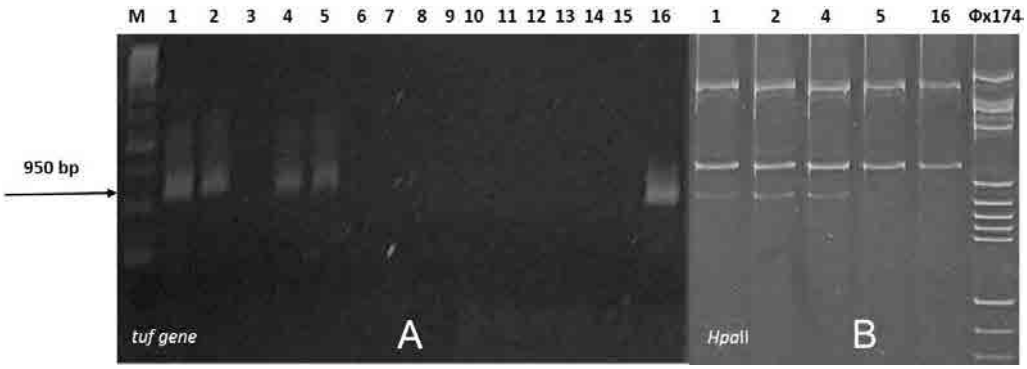

Fig. 2. A) PCR pattern on specific *tuf* gene for stolbur phytoplasma including tomato and pepper plants (M – marker 1 kb DNA ladder). 1–7 – pepper, Strumica, Borievo, 8 – healthy plant of pepper as negative control, 9 – healthy plant of tomato as negative control, 10–13 – pepper, Strunmica, Dobrejci, 14–16 – pepper, Kochani, Cesinovo. B) RFLP profiles from *Hpa*II digestions of positive fTufAY/rTufAY PCR products (*tuf* gene) using 3% agarose gel, Φx174 – marker

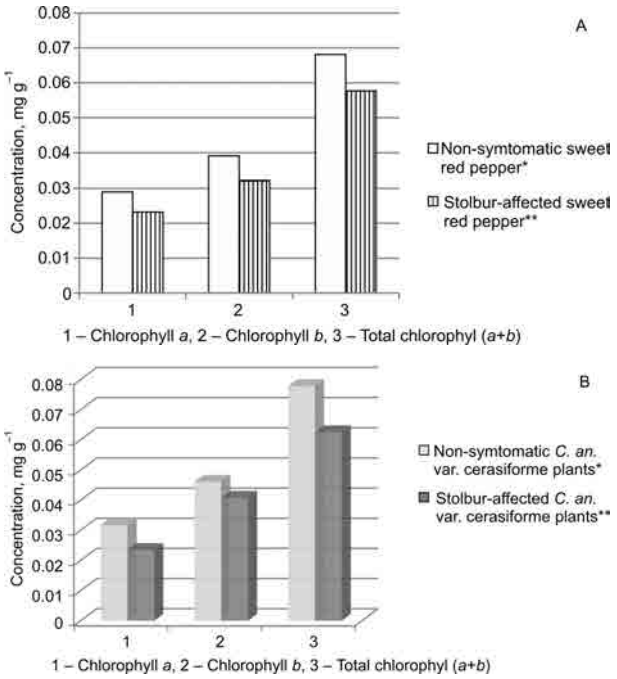

Fig. 3. A) Chlorophyll a, chlorophyll b and chlorophyll $(a+b)$ concentrations in non-symptomatic and stolbur-affected *Capsicum annuum* var. cerasiforme plants. *Values are the average of five different non-symptomatic plant samples, **Values are the average of five different stolbur-affected plant samples. B) Chlorophyll a, chlorophyll b and chlorophyll $(a+b)$ concentrations in non-symptomatic and stolbur-affected sweet red pepper plants. *Values are the average of five different non-symptomatic plant samples, **Values are the average of five different stolbur-affected plant samples

stolbur-affected *Capsicum annuum* var. cerasiforme plants was 33% on the average compared with control non-symptomatic plants, and in comparison with the decrease of chlorophyll *a* concentration in sweet pepper plants it was by 8% higher (Fig. 3A, B). There was a reverse trend in the decrease of chlorophyll *b* – it was by 7% higher in stolbur-affected sweet red pepper plants than in *Capsicum annuum* var. cerasiforme plants (Fig. 3A, B). The overall decrease of the concentration of chlorophyll $(a + b)$ was higher in stolbur-affected *Capsicum annuum* var. cerasiforme plants than in stolbur-affected sweet red pepper plants (Fig. 3A, B). On the contrary, the content of carotenoids was shown to be higher in both varieties of stolbur-affected pepper plants than in their non-symptomatic counterparts. The increase of carotenoids was 23% higher in stolbur-affected *Capsicum annuum* var. cerasiforme plants than in their non-symptomatic counterparts, while that increase was 18% in stolbur-affected sweet red pepper compared to their non-symptomatic counterparts (Fig. 4).

Conclusion. Stolbur disease is an old and well-known disease mainly in Europe but in other continents as well. The symptoms in pepper vary depending on the pepper variety, the geographical region and the cultivation conditions. Although it has been noticed in Macedonia multiple times in the last forty years, it has not been laboratory tested and proven until this present study. The aim of this study was to check the distribution of this disease in Eastern Macedonia, in Strumica and in Kocani as the main pepper cultivation regions, and to iden-

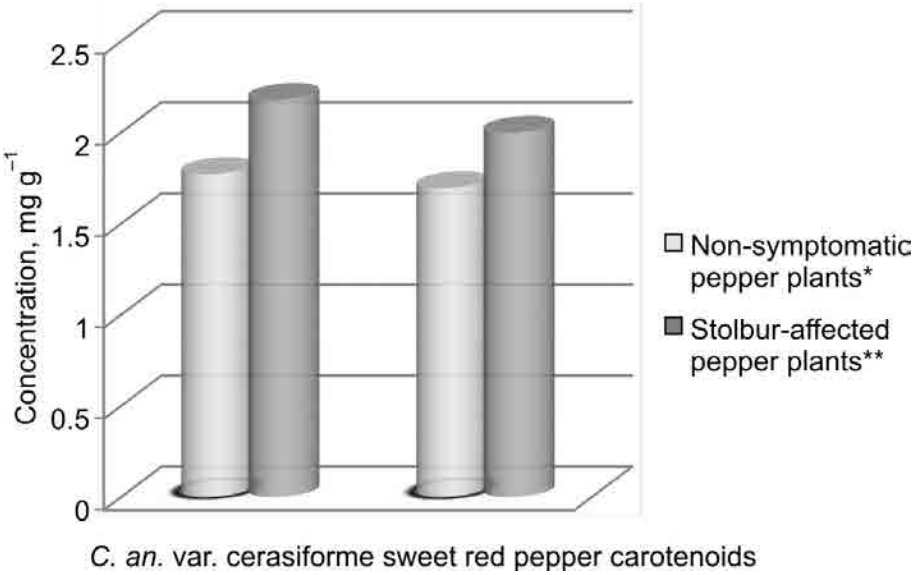

Fig. 4. Carotenoids concentration in non-symptomatic and stolbur-affected *Capsicum annuum* var. cerasiforme and in sweet red pepper plants. *Values are the average of five different non-symptomatic plant samples, ** Values are the average of five different stolbur-affected plant samples

tify and characterize the disease with modern and reliable molecular methods, including multilocus genetic analysis of the 16S rRNA gene.

Five out of all forty analyzed pepper plants were molecularly identified and characterized with stolbur symptoms. The other three tomato plants, three *Cuscuta* spp. and two *Convolvulus* spp. plants, coiled around pepper plants, failed to be positive for stolbur disease. Therefore, it can be concluded that the disease incidence is still sporadic in Eastern Macedonia, and the crop damages are not substantial yet. Nevertheless, it is very important to emphasize the presence of stolbur disease in pepper in the country and to keep the alarm turned on for eventual future local or countrywide outbreaks.

REFERENCES

[1] Lee I.-M., D. E. Gundersen-Rindal, R. E. Davis, I. M. Bartoszik (1998) Revised classification scheme of phytoplasmas based on RFLP analyses of 16SrRNA and ribosomal protein gene sequences, Int. J. Systematic Bacteriology, **48**, 1153–1169.

[2] Rédei G. P. (2008) Encyclopedia of Genetics, Genomics, Proteomics, and Informatics, Springer.

[3] Kostadinovska E., F. Quaglino, S. Mitrev, P. Casati, D. Bulgari, P. A. Bianco (2014) Multiple gene analyses identify distinct "bois noir" phytoplasma genotypes in the Republic of Macedonia, Phytopathologia Mediterranea, **53**(3), 491–501.

[4] Mitrovic J., S. Pavlovic, B. Duduk (2013) Survey and multigene characterization of stolbur phytoplasmas on various plant species in Serbia, Phytopathologia Mediterranea, **52**(3), 434–441.

[5] Pribylova J., J. Spak (2013) Dodder transmission of phytoplasmas, Methods in Molecular Biology, **938**, 41–6.

[6] Gudeva L. K., M. Spasenoski, F. Trajkova (2007) Somatic embryogenesis in pepper anther culture: The effect of incubation treatments and different media, Scientia Horticulturae, **111**, 114–119.

[7] Mitrev S., D. Spasov (1999) Health status of pepper in Strumica region in 1998, J. Agriculture Plant Sci., **10**, 163–173.

[8] Gudeva L. K., F. Trajkova (2008) *Capsicum* spp. genetic resources in the genebank of Faculty of agriculture, "Goce Delchev" University. In: Proc. of the 3rd Cong. Ecologists of the Republic of Macedonia with Int. Participation, 06–09.10.2007, Struga. Special issues of Macedonian Ecological Society, Skopje, **8**, 303–309.

[9] Angelini E., D. Clair, M. Borgo, A. Bertaccini, E. Boudon-Padieu (2001) Flavescence dorée in France and Italy – occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder phytoplasma, Vitis, **40**, 79–86.

[10] Deng S., C. Hiruki (1991) Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction, Phytopathology, **81**, 1475–1479.

[11] Schneider B., E. Seemüller, C. D. Smart, B. C. Kirkpatrick (1995) Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: Molecular and Diagnostic Procedures in Mycoplasmology (eds S. Razin, J. G. Tully), San Diego, California, USA, Academic Press, 369–380.
[12] Lee I.-M., A. Bertaccini, M. Vibio, D. E. Gundersen (1995) Detection of multiple phytoplasma in perennial fruit trees with decline symptoms in Italy, Phytopathology, **85**, 728–735.
[13] Quaglino F., Y. Zhao, P. A. Bianco, W. Wei, P. Casati, G. Durante, R. E. Davis (2009) New 16Sr subgroups and distinct single nucleotide polymorphism lineages among grapevine Bois noir phytoplasma populations, Ann. Appl. Biol., **154**, 279–289.
[14] Schneider B., K. S. Gibb, E. Seemüller (1997) Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas, Microbiology, **143**, 3381–3389.
[15] Langer M., M. Maixner (2004) Molecular characterisation of Grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA, Vitis, **43**, 191–200.
[16] Fabre A., J.-L. Danet, X. Foissac (2011) The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection, Gene, **472**, 37–41.
[17] Arnon D. (1949) Copper enzymes isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*, Plant Physiology, **24**, 1–15.
[18] Gayathri N., M. Gopalakrishnan, T. Sekar (2016) Phytochemical screening and antimicrobial activity of *Capsicum chinense* Jacq., Int. J. Adv. Pharmaceutics, **5**(1), 12–21.

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Fig. 1. A) Symptoms of stolbur disease on the whole sweet red pepper plant

Fig. 1. B) Symptoms of stolbur disease on the whole *Capsicum annuum* var. cerasiforme plant