

Phytoplasmas in carrots: disease and potential vectors in Serbia

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Abstract

The prevalent presence of aster yellows phytoplasmas belonging to the subgroup 16SrI-A together with the presence of samples infected with phytoplasmas belonging to the subgroups 16SrI-B and 16SrXII-A is described in Serbian carrot fields where a low level of epidemic was present. Among nine leafhopper and treehopper species captured and identified in the carrot fields, three species, *Macrostelus quadripunctulatus* (Kirschbaum), *Macrostelus sexnotatus* (Fallén), and *Macrostelus laevis* (Ribaut) were tested positive for the same phytoplasmas identified in the infected plant samples, therefore are potential vector species of these phytoplasmas. In particular *M. quadripunctulatus* and *M. sexnotatus* specimens were positive for aster yellows phytoplasmas, while a *M. laevis* specimen was positive for stolbur phytoplasmas.

Key words: carrot, aster yellows, insect vector, *Macrostelus quadripunctulatus*, *Macrostelus sexnotatus*, *Macrostelus laevis*.

Introduction

Phytoplasma diseases are known to affect vegetables in the major production areas worldwide (Lee *et al.*, 2003; 2004). Carrot is one of the most important vegetable crops in Serbia, covering 15,000 ha, with marketable production of about 120,000 t (Thorogood *et al.*, 2003), and is susceptible to phytoplasma infections. Recently, phytoplasmas affiliated with different subgroups of the aster yellows (AY) phytoplasma group (16SrI) were found to be associated with low incidence of diseases in carrots showing redness of leaves, shoot proliferation and reduced tap roots quality (figure 1) (Duduk *et al.*, 2007). AY phytoplasmas have a wide host range (Lee *et al.*, 2004) and their spreading among vegetable crops are carried out by vector species belonging to the order Homoptera (Weintraub and Orenstein, 2004; Weintraub and Beanland, 2006; Battle *et al.*, 2008).

Considering the economic importance of the crop and the possibility of rapid spreading of phytoplasmas in carrot fields as well as in other crops, it is necessary to prevent large epidemics. The present study was aimed at determination of phytoplasma populations and identification of potential vector species.

Materials and methods

Sample collection and nucleic acid extraction

In order to identify potential Auchenorrhyncha vector species, insects were collected from carrot fields at Begeč in the South Bačka region of Serbia, covering approximately 40 ha (GPS: 45°14'55''N 19°37'15''E), where phytoplasma diseases incidence was reported (Duduk *et al.*, 2007).

The insects were collected at two-week intervals, from June through October 2007, by sweep netting and by

using yellow sticky traps (25 x 10 cm) placed at plant level and regularly distributed in the field. Collected insects were stored in 95% ethanol until species identification and nucleic acids extraction. Total nucleic acids were extracted from single specimens of all collected species following the protocol described by Angelini *et al.* (2001), dissolved in TE buffer, and maintained at -20 °C.



Figure 1. Symptomatic carrot showing leaf reddening, formation of chlorotic adventitious shoots, and reduction in taproot size and quality.
(In colour at www.bulletinofinsectology.org)

During August 2007, leaf samples were also collected from 40 symptomatic carrot plants (figure 1), and total nucleic acids were extracted from 0.5 g of fresh leaf tissues following the same protocol described for insects.

Nucleic acids were diluted with sterile distilled water 1:100 for plants, and 1:30 for insects before performing polymerase chain reaction (PCR) assays.

PCR-RFLP analyses

Phytoplasma detection in carrot was carried out by direct PCR assays with universal phytoplasma primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), and in insect by nested PCR with F1/B6 primers (Duduk *et al.*, 2004) on amplicons obtained with P1/P7 primers diluted 1:30 with sterile distilled water. Each 25 µl PCR reaction mix contained 20 ng template DNA, 2.5 µl 10X PCR buffer, 0.8 U *Taq* polymerase (Polymed, Italy), 0.2 mM dNTPs, 1.5 mM MgCl₂ and 0.4 mM each primer. Samples lacking DNA were employed as negative controls. Thirty-five PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94 °C, 2 min for annealing at 50 °C, and 3 min (10 min for the last cycle) for primer extension at 72 °C. Six µl of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized under UV transilluminator.

Identification of phytoplasmas was done using restriction fragment length polymorphism (RFLP) analyses with *Hha*I, *Taq*I, and *Tsp*509I (New England Biolabs, Beverly, MA, USA) restriction enzymes on P1/P7 amplified fragments obtained from plant samples, and with *Tru*I (New England Biolabs, Beverly, MA, USA) on F1/B6 amplicons obtained from insects. Separation of restriction fragments was accomplished by electrophoresis on a 5% polyacrylamide gel, DNA bands were stained with ethidium bromide and visualized under UV transilluminator.

The reference strains were maintained in *in vitro* culturedperiwinkle [*Catharanthus roseus* (G.) Don.] (Bertaccini, 2003) and were used for PCR-RFLP pattern identification. These reference strains included: *Chrysanthemum* yellows (CHRY, 16S ribosomal subgroup I-A), European aster yellows (EAY, 16S ribosomal subgroup I-B), *Catharanthus* virescence (CVB, 16S ribosomal subgroup I-F), carrot yellows (CA, 16S ribosomal group 16SrI-C), primula yellows (PRIVA, 16S ribosomal subgroup I-L), clover phyllody from France (KVF, 16S ribosomal subgroup I-C), ranunculus phyllody from Udine (RA, 16S ribosomal subgroup III-B), and stolbur from pepper from Serbia (STOL, 16S ribosomal subgroup XII-A).

Results

Insects

Insects were caught in relatively small numbers, however Auchenorrhyncha specimens belonging to the families Cicadellidae (leafhoppers) and Membracidae (treehoppers) were identified. The leafhoppers belonged to four subfamilies: Deltocephalinae, *Macrosteles quadripunctulatus* (Kirschbaum), *Macrosteles sexnotatus* (Fallén), *Macrosteles laevis* (Ribaut), *Psammotettix notatus* (Melichar); Agalliinae, *Anaceratagallia laevis* (Ribaut); Typhlocybiniae, *Empoasca* sp., *Eupteryx* sp., and Cicadellinae, *Cicadella viridis* (L.). Besides the leafhoppers, the treehopper *Stictocephala bisonia* (Kopp et Yonke) was also identified. The species that were tested positive for phytoplasmas are listed in table 1.

Direct PCR using template DNA from insect samples yielded no or, in a few cases faint amplification bands, while nested PCR reactions with primer pair F1/B6 resulted in amplicons of expected size (about 1,700 bp) from eight out of 43 insects tested. *A. laevis* (one specimen), *Empoasca* sp. and *Eupteryx* sp. (10 specimens each), and *C. viridis* (seven specimens) resulted all negative. Molecular identification of F1/B6 positive samples with *Tru*I restriction enzyme showed the presence of phytoplasmas belonging to different 16S ribosomal groups and/or subgroups (table 1 and figure 2).

Restriction profile of the amplicon from a single *M. laevis* specimen was identical to the one from reference strain STOL, belonging to subgroup 16SrXII-A; restriction profiles of two amplicons from *M. sexnotatus* were identical to the one from reference strain EAY, belonging to subgroup 16SrI-B; while one amplicon was identical to the one from reference strain CHRY, belonging to subgroup 16SrI-A. In one specimen of *P. notatus* restriction profile identical to the one from reference strain RA, belonging to subgroup 16SrIII-B was obtained, while from *M. quadripunctulatus* different restriction profiles were obtained in the two positive specimens: one was identical to reference strain EAY, belonging to 16SrI-B (table 1), and the other to the profile of reference strain CHRY, belonging to 16SrI-A ribosomal subgroup (figure 2 and table 1). One of the two tested specimens of *S. bisonia* was positive for 16SrX-A phytoplasmas (apple proliferation) (table 1).

Plants

Direct PCR with P1/P7 primer pairs resulted in amplicons with expected size (about 1,800 bp) from 36 out of the 40 symptomatic carrot samples tested; no amplification was obtained from four symptomatic as well as

Table 1. Auchenorrhyncha collected in carrot field at Begeč: results of phytoplasma identification.

| Family | Species | No infected/No tested | Phytoplasmas * |
|-----------------------------------|-----------------------------|-----------------------|--------------------------|
| Cicadellidae (Deltocephalinae) | <i>M. quadripunctulatus</i> | 2/2 | 16SrI-A (1); 16SrI-B (1) |
| | <i>M. sexnotatus</i> | 3/6 | 16SrI-A (1); 16SrI-B (2) |
| | <i>M. laevis</i> | 1/3 | 16SrXII-A |
| | <i>P. notatus</i> | 1/2 | 16SrIII-B |
| Membracidae | <i>S. bisonia</i> | 1/2 | 16SrX-A |

* in parenthesis number of infected specimens.

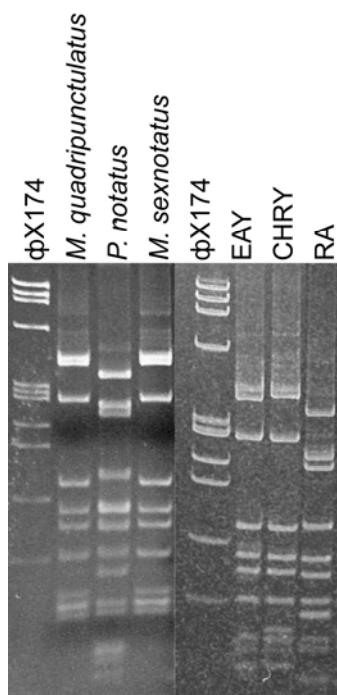


Figure 2. RFLP picture of polyacrylamide 5% gel showing restriction profiles of F1/B6 amplicons from insect samples digested with *TruI*; abbreviations of phytoplasma control strains are given in materials and methods. ϕ X174: marker phiX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

from negative control (figure 3). RFLP analyses were performed on P1/P7 amplicons using the three restriction enzymes listed above. The results showed that restriction profiles from 31 carrot samples were identical to the reference strain CHRY, belonging to subgroup 16SrI-A, the restriction profiles from 4 samples were identical to reference strain EAY, belonging to subgroup 16SrI-B, while one sample showed restriction profile identical to the reference strain STOL, belonging to subgroup 16SrXII-A (figure 4).

Discussion and conclusions

The results obtained in this work revealed the presence, in symptomatic carrot, of phytoplasmas belonging to three different subgroups: 16SrI-A, 16SrI-B and 16SrXII-A. Such results are consistent with the findings from previous study conducted in the same area in that AY phytoplasmas are mainly responsible for the carrot phytoplasma disease in Serbia (Duduk *et al.*, 2007). In addition, the results from present study indicated for the first time that a stolbur phytoplasma infection also contributed to the carrot disease. The diverse phytoplasmas detected can not be differentiated by specific symptomatology as often reported for carrot, other vegetables, and woody hosts such as grapevine (Bertaccini *et al.*, 1995; 1999; Lee *et al.*, 2006). For example, carrot plants exhibiting similar symptoms from commercial and experimental fields in Israel were found to be infected by phytoplasmas belonging to three phylogenetically di-

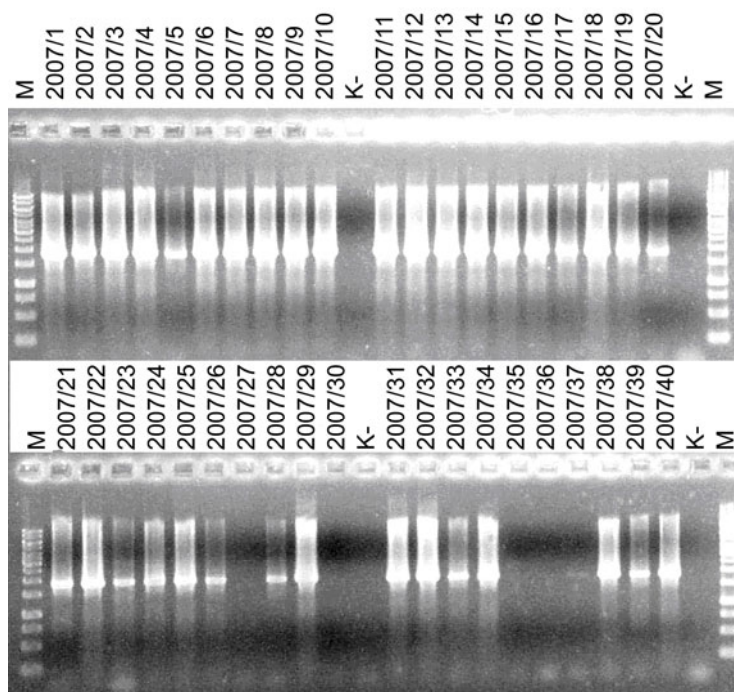


Figure 3. Agarose gels showing results of PCR assays with P1/P7 primers on carrot samples. 2007/1-2007/40: carrot samples; K-: negative control; M: Marker DNA 1 kb; fragment sizes in base pairs from top to bottom: 10,000; 8,000; 6,000; 5,000; 4,000; 3,500; 3,000; 2,500; 2,000; 1,500; 1,000; 750; 500; and 250.

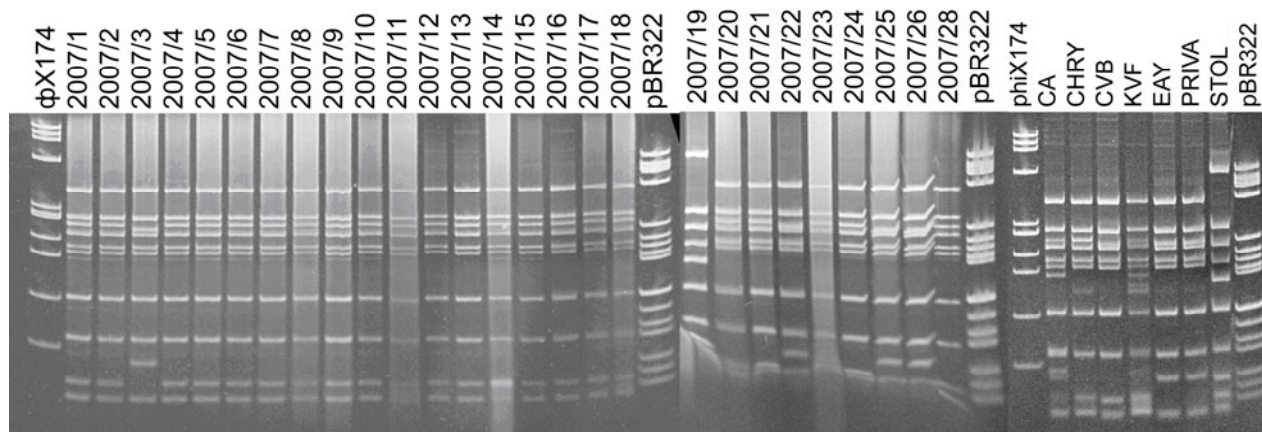


Figure 4. RFLP picture of polyacrylamide 5% gel of P1/P7 amplicons from carrot samples digested with *Tsp509I*. 2007/1-2007/28: carrot samples; abbreviations of phytoplasma control strains are given in materials and methods. ϕ X174: marker phiX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72; pBR322: marker pBR322 *Hae*III digested; fragment sizes in base pairs from top to bottom: 587; 540; 502; 458; 434; 267; 234; 213; 192; 184; 124; 123; 104; 89; 80; 64; 57 and 51.

vergent groups, 16SrI, 16SrIII and 16SrV (Orenstein *et al.*, 1999; Weintraub and Orenstein, 2004).

In an epidemiological study conducted on commercial agricultural plots affected by stolbur phytoplasma in Spain, Battle *et al.* (2008) evaluated the potential of *M. quadripunctulatus* as a vector of stolbur in carrots. The results indicated that this species acquired and was capable to transmit the phytoplasma after feeding during a single day on infected plants followed by a 19-day latent period on healthy plants.

In this study, among the leafhopper species considered as potential vectors of phytoplasmas (Weintraub and Orenstein, 2004; Weintraub and Beanland, 2006), *M. quadripunctulatus*, *M. sexnotatus* and *M. laevis* were identified and collected. Although a limited number of specimens was tested, aster yellows phytoplasmas were detected in *M. quadripunctulatus* and *M. sexnotatus*, while stolbur phytoplasma was detected in *M. laevis*. Such results provided evidence to suggest that the three insect species may truly play a role in transmitting phytoplasmas to carrot plants in the fields.

M. laevis was reported as a vector of “stolbur virus” from infected to healthy clover (Valenta *et al.*, 1961); the report should be confirmed with molecular identification of phytoplasmas, but it represents an indication of stolbur transmissibility by this species. Controlled transmission studies are in progress to prove the capability of specific phytoplasma transmission to carrot by the three insect species harbouring aster yellows and stolbur phytoplasmas.

Two more insect species among those collected in carrot fields were found to be infected with phytoplasmas that were not detected in carrot, but were already reported in Serbia in other plant species. In particular the 16SrIII-B subgroup phytoplasmas reported here for the first time in *P. notatus*, were previously described in Serbia infecting wild *Cirsium arvense*, and pear plants (Rancic *et al.*, 2005; Duduk *et al.*, 2008); and apple proliferation phytoplasmas detected in *S. bisonia* were already reported infecting apple, even if in other Serbian

areas (Duduk *et al.*, 2008). The detection of these two phytoplasmas in previously unreported insect species is confirming the widespread diffusion of diverse phytoplasmas in important agricultural districts; therefore further research is necessary to verify their transmissibility to plants and their host range.

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