

Leafhoppers and Cixiids in Phytoplasma-infected Carrot Fields: Species Composition and Potential Phytoplasma Vectors

Tanja Drobnjaković¹, Pantelija Perić¹, Dejan Marčić¹, Luca Picciau², Alberto Alma²,
Jelena Mitrović¹, Bojan Duduk¹ and Assunta Bertaccini³

¹*Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia*

²*DIVAPRA, Entomologia e Zoologia applicate all'Ambiente "C. Vidano", Università di Torino, Italy*

³*Dipartimento di Scienze e Tecnologie Agroambientali – Patologia vegetale, Università di Bologna, Italy*

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SUMMARY

The first molecular analysis of samples collected in southern Bačka (Serbia) confirmed the presence of aster yellows (16SrI) and stolbur phytoplasmas (16SrXII) in insects belonging to the family Cicadellidae, as well as in carrot plants where the insects were collected. A correct identification of the phytoplasmas and their vectors is essential to arrange effective control strategies to prevent diseases associated with phytoplasmas from spreading to carrots and other vegetable crops. In order to enhance knowledge about insect vectors of aster yellows and stolbur phytoplasmas in Serbia, Cicadellidae and Cixiidae (Homoptera Auchenorrhyncha), the most common vectors of these phytoplasmas, were monitored in southern Bačka during 2008. Adults leaf- and planthoppers were collected and identified at species level using standard entomological methods, and tested for phytoplasma presence by means of PCR/RFLP. A total of 13 insect species of Cicadellidae were identified, as follows: a) three species of the subfamily Agallinae: *Anaceratagallia ribauti* (Ossiannilsson), *Anaceratagallia venosa* (Fourcroy), and *Anaceratagallia laevis* (Ribaut); b) seven species of the subfamily Deltocephalinae: *Psammotettix confinis* (Dahlbom), *Psammotettix striatus* (Linnaeus) *Psammotettix alienus* (Dahlbom), *Macrosteles sexnotatus* (Fallén), *Ophiola decumana* (Kontkanen), *Errastunus ocellaris* Fallén, and *Scaphoideus titanus* Ball; c) three species of the subfamily Typhlocibinae: *Eupteryx atropunctata* (Goeze), *Eupteryx mellissae* Curtis, *Zyginidia pullula* (Boheman). Female specimens of the genus *Euscelis* (Deltocephalinae) were also collected,

as well as one species of *Reptalus quinquecostatus* (Dufour) of the family Cixiidae. Stolbur phytoplasmas were detected in *A. laevis*, *A. ribauti*, *A. venosa*, *P. striatus*, *P. confinis* and *P. alienus*. The species: *A. laevis*, *O. decumana*, and *P. confinis* were AY-infected (subgroup 16SrI-A), while subgroup 16SrI-C was found only in one specimen of *P. confinis*. Since some aster yellows- and stolbur-infected species of the genera *Psammotettix* and *Anaceratagallia* (especially *P. confinis* and *A. laevis*) were regularly and commonly found in the infected carrot fields during the whole vegetative period, they could play a significant role in transmitting and spreading these pathogens in natural environment.

Keywords: Cicadellidae; AY; STOL; Carrot

INTRODUCTION

Phytoplasmas are phloem-limited, insect-transmitted, plant pathogenic bacteria that are responsible for hundreds of diseases world-wide (Weintraub, 2007). In Serbia, plant diseases associated with phytoplasmas are detrimental to grapevine, vegetable, and fruit production (Duduk et al., 2004, 2007, 2008a). The symptoms resembling phytoplasma diseases have been recently detected for the first time in carrot plants, one of the most important vegetable crops in Serbia. Carrot is affected by phytoplasmas belonging to different subgroups of aster yellows (AY) and stolbur (STOL), 16SrI and 16SrX-II ribosomal groups respectively (Duduk et al., 2007).

Aster yellows phytoplasmas have a wide host range (Lee et al., 2004) and they are transmitted in a persistent manner by phloem-feeding leafhoppers (Homoptera: Cicadellidae) and planthoppers (Homoptera: Cixiidae) (Weintraub and Beanland, 2006). Stolbur phytoplasmas are transmitted by cixiids (Fos et al., 1992; Gatineau et al., 2001; Jović, 2009). The first molecular analysis of samples collected from carrot fields in the southern Bačka confirmed the presence of AY and stolbur phytoplasmas in specimens of the family Cicadellidae, as well as in carrot plants where the insects were collected. In spite of a low infection level in 2007 and a small number of collected insects, the results of the investigation revealed the presence of phytoplasmas and their potential vectors in the infected carrot crops. Four already known vectors of AY phytoplasmas were identified: *Anaceratagallia laevis* (Ribaut), *Macrosteles laevis* (Ribaut), *M. quadripunctulatus* (Kirschbaum), and *M. sexnotatus* (Fallén) (Duduk et al., 2008b). In order to enhance knowledge about insect vectors of phytoplasmas, which is rather limited in Serbia, the leafhopper and planthopper were continued to be monitored in the same area. The identification of phytoplasmas and their vectors is essential for setting effective control strategies to prevent phytoplasma diseases from spreading to carrot and other vegetable crops.

MATERIAL AND METHODS

Sample collection and species identification

During 2008 insects belonging to the families Cicadellidae and Cixiidae were collected and tested for phytoplasma presence. The insects were collected from the beginning of April till the end of October, in two localities in southern Bačka where outbreak of carrot diseases had been recorded: Begeč (GPS: 45°14'55"N 19°37'15"E, spring seeding) and Begeč rit (GPS: 45°14'13"N 19°34'47"E, fall seeding). In both localities the adults were collected from carrots and surrounding weeds biweekly, using standard collecting methods (yellow sticky traps and sweep nets). Yellow sticky traps (25x10cm) were set along the perimeters of the field, at carrot plants height, and simultaneously, leaf- and planthoppers were collected around the field using the entomological net. The net-captured adults were then sucked into the vial by mouth exhauster. Thus collected insects were kept in 96% ethanol and identified before extraction of nucleic acids. Identification of the Cicadellidae species was based on identification keys (Ribaut, 1952; Biedermann and Niedringhaus, 2004). For species identification of the family Cixiidae and also of some leafhopper species, the keys of Nielson (1968) and Ossiannilsson (1978, 1981, 1983) were used. Identification was based on microscopic examination of male genitalia in entomological laboratories of the Institute of Pesticides and Environmental Protection, Zemun and of the University of Turin, Italy (DIVAPRA).

Nucleic acid extraction and PCR/RFLP analyses

The insect specimens of the family Cicadellidae, belonging to subfamilies Agallinae and Deltocephalinae, were subjected to DNA extraction and PCR-RFLP analyses for detecting the presence of phytoplasmas. Total nucleic acids were extracted from dissected single specimens, dissolved in 40 µl TE buffer, and maintained

at -20 °C. Following the modified protocol described by Angelini et al. (2001), phytoplasma detection in insects was carried out by nested PCR assays with universal phytoplasma primer pair R16F2/R2 (Lee et al., 1993), followed by R16(I)F1/R1 primers (Lee et al., 1994) in nested PCR on amplicons obtained with R16F2/R2 primers diluted 1:30 with sterile distilled water. Each 25 µl PCR reaction mix contained 1 µl of template DNA diluted 1: 30 with sterile distilled water, 12.5 µl 2X PCR master mix (Fermentas, Lithuania), and 0.4 µM 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 in insects was done using restriction fragment length polymorphism (RFLP) analyses with *TruI* and *HbaI* restriction enzymes (Fermentas, Lithuania) on R16(I)F1/R1 amplicons. Separation of restriction fragments, obtained with both restriction enzymes, was accomplished by electrophoresis on 7% polyacrylamide gel. The gels were stained and visualized as described above. The reference strains were maintained in periwinkle [*Catharanthus roseus* (G.) Don.] cultured *in vitro* (Bertaccini, 2003) and were used for PCR-RFLP pattern identification. These reference strains included: *Chrysanthemum* yellows (CHRYM, 16SrI-A), American aster yellows (AY-W, 16SrI-B), Carrot yellows (CA, 16SrI-C) and stolbur from pepper from Serbia (STOL, 16SrXII-A).

RESULTS AND DISCUSSION

During 2008, 13 insect species of Cicadellidae family were identified, as follows: a) three species of the subfamily Agallinae: *Anaceratagallia ribauti* (Ossiannilsson), *A. venosa* (Fourcroy), and *A. laevis* (Ribaut); b) seven species of the subfamily Deltocephalinae: *Psammotettix confinis* (Dahlbom), *P. striatus* (Linnaeus), *P. alienus* (Dahlbom), *Macrosteles sexnotatus* (Fallén), *Ophiola decumana* (Kontkanen), *Errastunus ocellaris* Fallén, and *Scaphoideus titanus* Ball; c) three species of the subfamily Typhlocibinae: *Eupteryx atropunctata* (Goeze), *Eupteryx mellissae* Curtis, *Zyginidia pullula* (Boheman). A large number of specimens belonging to genus *Empoasca* was also collected, as well as females of the genera *Anaceratagallia* (Agallinae), *Macrosteles*, and *Euscelis* (Deltocephalinae), but they were not identified at species level due to unreliability of identification based only on female genitalia. Most of collected samples belonged to the genera *Eupteryx* (70), *Anaceratagallia* (66), and *Empoasca* (55). Besides the species belonging to the family Cicadellidae, the species *Reptalus quinquecostatus* (Dufour) of the Cixiidae family was rather abundant.

Table 1 shows the results of testing for phytoplasma presence in collected leafhoppers. Only members of Cicadellidae subfamilies already reported as phytoplasma vectors (Agallinae and Deltocephalinae) (Weintraub and Beanland, 2006; Riedle-Bauer et al., 2008) were selected for phytoplasma testing. Fourteen out of 49 samples tested positive for phytoplasma presence, which means that 28.6% of all insects were infected.

Table 1. Tested species of the family Cicadellidae collected in 2008, and detected phytoplasmas

Subfamily	Species	Co.	Te.	Inf.	Phytoplasmas ¹
Agallinae	<i>Anaceratagallia laevis</i> (Ribaut),	17	17	4	16SrI-A (2);16SrXII-A (2)
	<i>Anaceratagallia ribauti</i> (Ossiannilsson)	4	4	2	16SrXII-A (2)
	<i>Anaceratagallia venosa</i> (Fourcroy)	1	1	1	16SrXII-A (1)
Deltocephalinae	<i>Macrosteles sexnotatus</i> (Fallén)	6	6	0	–
	<i>Psammotettix striatus</i> (Linnaeus)	2	2	1	16SrXII-A (1)
	<i>Psammotettix confinis</i> (Dahlbom)	10	10	4	16SrI-A (2) 16SrI-C (1) 16SrXII-A (1)
	<i>Psammotettix alienus</i> (Dahlbom)	2	2	1	16SrXII-A (1)
	<i>Ophiola decumana</i> (Kontkanen)	2	2	1	16SrI-A (1)
	<i>Errastunus ocellaris</i> Fallén.	2	1	0	–

¹ in parenthesis is the number of infected specimens

Co. = number of collected specimens

Te. = number of tested specimens

Inf. = number of infected specimens

Direct PCR with primer pair R16F2/R2 using template DNA from insect samples yielded no amplification bands, while nested PCR reactions with primer pair R16(I)F1/R1 resulted in amplicons of the expected size (about 1,100 bp). Four out of seventeen *A. laevis* specimens, two out of four *A. ribauti*, and one out of one tested *A. venosa*, of the subfamily Agallinae, were positive; concerning the subfamily Deltocephalinae, four out of ten *P. confinis* specimens, one out of two *P. striatus*, one out of two *P. alienus*, and one out of two tested *O. decumana* were also positive.

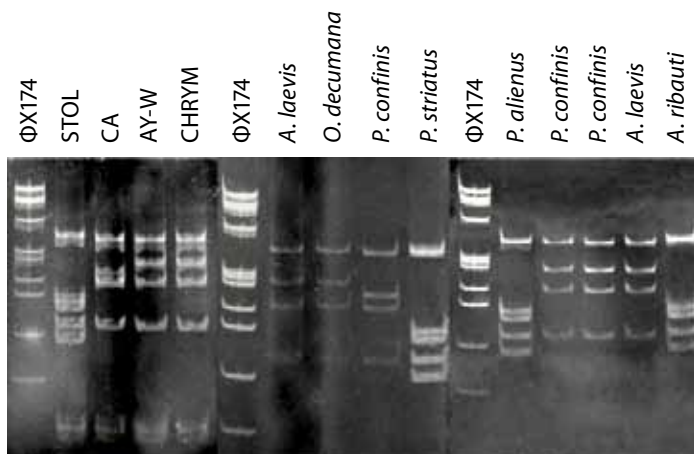


Figure 1. Polyacrylamide gels showing the restriction fragment length polymorphism profiles of reference phytoplasma strains and selected insect samples amplified with primers R16F2/R2 followed by primers R16(I)F1/R1 in nested polymerase chain reaction (PCR) and digested with restriction enzyme *TruI*. CHRYM, Chrysanthemum yellows (16SrI-A); AY-W, American aster yellows (16SrI-B); CA, Carrot yellows (16SrI-C); STOL, Stolbur from pepper (16SrXII-A); ΦX174, ΦX174 marker *HaeIII* digested, fragment sizes in base pairs from top to bottom: 1.353, 1.078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

This study of potential phytoplasma vectors in carrot fields in Serbia indicate that the number of harmful species may be larger than reported so far (Duduk et al., 2008). The following can be added to the list of potential insect vectors: *A. ribauti*, *A. venosa*, *P. striatus*, *P. confinis*, *P. alienus*, *O. decumana*, and *E. ocellaris*.

Some of the collected insects are already widely known as vectors of phytoplasmas and/or viruses: *M. sexnotatus* (Nielson, 1968; Van Slogteren, 1972), *P. striatus* (Nielson, 1968), *P. alienus* (Ossiannilsson, 1983), *S. titanus* (Boudon-Padieu, 2000), *A. laevis* (Tanne et al., 2001; Della Giustina et al., 2000), *A.*

After *TruI* digestion of R16(I)F1/R1 amplicons of all 14 positive samples, eight samples showed patterns identical to the one of reference strain STOL (16SrXII), five showed profiles identical to those of reference strains CHRYM (16SrI-A) and AY-W (16SrI-B), while one sample (*P. confinis*) showed profile identical to those of reference strains CA (16SrI-C). Digestion with *HhaI* restriction enzyme, performed on the same amplicons, yielded identical profiles for all samples which were indistinguishable from those of STOL (16SrXII), CHRYM (16SrI-A) and CA (16SrI-C).

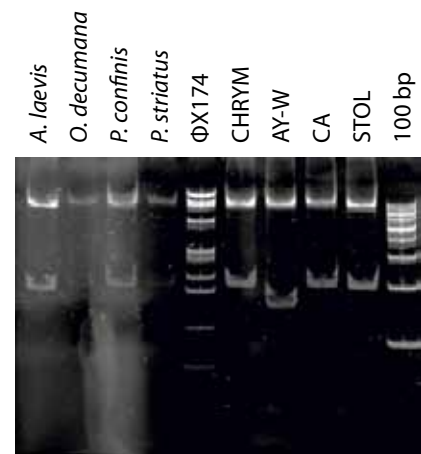


Figure 2. Polyacrylamide gels showing the restriction fragment length polymorphism profiles of reference phytoplasma strains and selected insect samples amplified with primers R16F2/R2 followed by primers R16(I)F1/R1 in nested polymerase chain reaction (PCR) and digested with restriction enzyme *HhaI*. CHRYM, Chrysanthemum yellows (16SrI-A); AY-W, American aster yellows (16SrI-B); CA, Carrot yellows (16SrI-C); STOL, Stolbur from pepper (16SrXII-A); ΦX174, ΦX174 marker *HaeIII* digested, fragment sizes in base pairs from top to bottom: 1.353, 1.078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

ribauti (Della Giustina et al., 2000; Riedle-Bauer et al., 2008), *A. venosa* (Nielson, 1968), *Empoasca* spp. (Nielson, 1968). The presence of stolbur in *R. quinquecostatus* (Trivellone et al., 2005), as well as its capability to transmit these pathogens in laboratory conditions, has already been documented by Pinzauti (2008). The other five identified leafhopper species (*E. mellissae*, *Z. pullula*, *O. decumana*, *P. confinis* and *E. ocellaris*), were not reported in literature as having a role in transmission of plant pathogens.

In our study, in the first part of vegetative period *P. confinis* was the most frequent potential vector of

phytoplasmas in carrots and nearby weeds. Its activity was strongest in July. Among the known vectors of phytoplasmas and/or viruses *A. laevis* was the most frequently encountered species, especially in the second part of vegetative period (August-October). In addition, higher numbers of positive insects were detected in samples collected during the second part of the vegetative period (collected in carrot crops in Begeč rit which were seeded later), when the infection was also present in higher number of carrot plants. Except for *E. atropunctata* and *Z. pullula*, the number of other species - so far unconfirmed phytoplasma vectors - was quite limited. The species *P. striatus* is often found in maize (Bogavac, 1968; Jović et al., 2009), while *P. alienus* is reported in red clover (Tanasijević, 1962) and in most of Serbian vineyards (Krnjajić, 2008). *P. confinis* completes at least three generations per year in Serbia; it was identified less frequently and in smaller number than *P. alienus*, but it is found very often and in abundance in red clover, along with *A. laevis* and *A. ribauti* (Tanasijević, 1962). *O. decumana* has been reported so far in Serbia only in maize and surrounding weeds (Jović et al., 2009).

In Serbia, the species of the genus *Eupteryx* (especially *E. atropunctata* and *E. mellissae*) have been detected so far in medicinal plants of the family Lamiaceae (*Melissa officinalis*, *Salvia officinalis*) (Tanasijević and Simova-Tošić, 1987). *E. atropunctata* is frequently observed in sunflower (Tanasijević, 1966) and maize (Jović et al., 2009) during the whole vegetative period. Tanasijević (1966) and Bogavac (1968) reported *Z. pullula* as the most frequent species of small cicadas in all investigated maize growing localities (appearing somewhat later in spring than other species and reproducing during the whole vegetative period). The recent findings by Jović et al., (2009) indicate that the number of *Z. pullula* in maize is higher in the second part of the vegetative period.

S. titanus is the only known vector of flavescence dorée (FD), a quarantine grapevine phytoplasma. In Europe, it feed exclusively on *Vitis* sp. (Boudon-Padieu, 2000). *S. titanus* is also the most studied cicadellid species in Serbia, and it is found in most of the vineyards (Krnjajić, 2008). Its presence in carrot fields could be explained by the vicinity of the Fruška Gora vineyards, or by the presence of wild grapevines in the surveyed carrot growing localities. *R. quinque-costatus* (fam. Cixiidae) is also a very frequent species of cicadina living in different habitats, usually dry and

warm, but most often not far from rivers. In Serbia it is often reported in maize fields. There is a possibility that this *taxon* refers to more than one species (Holzinger et al., 2003). It has been detected only in the locality of Begeč rit.

The combined RFLP analyses performed with *TruI* and *HbaI* restriction enzymes on R16(I)F1/R1 amplicons of positive insect samples, allowed to classify the phytoplasmas as members of aster yellows group (16SrI-A and 16SrI-C subgroups) and stolbur (16SrX-II-A) (Table 1). Even though positive result for phytoplasma presence in insects did not prove their transmission ability, it shed some light on potential vectors and epidemiology of the diseases.

During previous research (Duduk et al., 2008), three species of the genus *Macrostelus* were identified as potential phytoplasma vectors in carrot fields: *M. sexnotatus*, *M. laevis* and *M. quadripunctulatus*. In this study the presence of only *M. sexnotatus* is reported. Only six specimens of this species (known to be capable of transmitting AY phytoplasma and acquiring stolbur) were collected from the observed localities, but none of them tested positive for phytoplasmas.

Stolbur phytoplasma was found in one specimen of *A. venosa* and in two samples of both *A. laevis* and *A. ribauti*, while two specimens of *A. laevis* were infected with 16SrI-A phytoplasmas. Field collected *A. laevis* could transmit the yellows phytoplasma to *Catharanthus roseus* and potato plants (Tanne et al., 2001) and recently laboratory-reared *A. ribauti* adults transmit stolbur phytoplasma to *Vicia faba* seedlings (Riedle-Bauer et al., 2008).

The species *P. alienus* has never been confirmed to transmit phytoplasmas, but it is known to be capable of harbouring some of them, including AY and STOL. Though they are present in numerous localities in Serbia where the symptoms of corn reddening were recorded (Jović et al., 2009), and in grapevine yellows-infected vineyards in Serbia (Krnjajić, 2008), *P. alienus* was never found positive to phytoplasma presence. Some other studies revealed the capability of another species of the same genus, *P. striatus*, to transmit stolbur (Sabaté et al., 2003). The presence of AY phytoplasmas in *P. striatus* was recorded in southern Italy (Albanese et al., 1997). In this study, all three collected species of the genus *Psammotettix* showed to be capable of acquiring stolbur phytoplasmas. RFLP analysis also revealed that two adult insect of *P. confinis* were infected with 16SrI-A, and one was infected with 16SrI-C phytoplasmas. Although Jović et al. (2009) recorded high abundance of *O. decumana* in

corn fields infected by reddening, this species did not reveal presence of phytoplasmas. In our study, *O. decumana* tested positive for presence of AY (16SrI-A) phytoplasma.

The results of earlier research showed that AY and STOL phytoplasmas were associated with phytoplasma diseases in carrot crops in southern Bačka in Serbia (Duduk et al., 2008), which is in agreement with the results presented in this paper. Considering that AY- and STOL-infected species of the genera *Psammotettix* and *Anaceratagallia* (first especially, *P. confinis* and *A. laevis*) were regularly and most commonly present in the infected carrot fields during the whole vegetative period, they could play a significant role in transmitting and spreading of these pathogens in natural environment. Since the presence of phytoplasmas in insects does not necessarily implies their transmission ability, the role of insect not recognized yet as phytoplasma vectors in epidemiology of AY and stolbur phytoplasmas remains to be investigated.

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Cikade u šargarepi zaraženoj fitoplazmama: sastav vrsta i potencijalni vektori fitoplazmi

REZIME

Prva molekularna istraživanja uzoraka iz južne Bačke potvrdila su prisustvo aster yellows (16Srl) i stolbur fitoplazmi (16SrXII) u insektima iz familija Cicadellidae, kao i u biljkama šargarepe među kojima su insekti sakupljeni. Tačna identifikacija vrsta fitoplazmi i njihovih vektora su osnov za definisanje mera sprečavanja širenja fitoplazmoza u usevima šargarepe i drugog povrća. U cilju proširenja saznanja o insektima vektorima aster yellows i stolbur fitoplazmi, o kojima u Srbiji ima malo podataka, tokom 2008. godine nastavljeno je sa praćenjem insekata iz fam. Cicadellidae i fam. Cixiidae (kao najčešćih prenosilaca pomenutih vrsta fitoplazmi) u južnoj Bačkoj. Standardnim entomološkim postupcima sakupljana su imaga cikada, identifikovana do nivoa vrste i testirana na prisustvo fitoplazmi PCR/RFLP metodama. Identifikovano je ukupno 13 vrsta insekata iz familije Cicadellidae, od čega *a*) tri vrste iz subfamilije Agallinae: *Anaceratagallia ribauti* (Ossiannilsson), *A. venosa* (Fourcroy) i *A. laevis*; *b*) sedam vrsta iz subfamilije Deltocephalinae: *Psammotettix confinis* (Dahlbom), *P. striatus* (Linnaeus), *P. alienus* (Dahlbom), *Macrosteles sexnotatus* (Fallén), *Ophiola decumana* (Kontkanen), *Errastunus ocellaris* Fallén i *Scaphoideus titanus* Ball; *c*) tri vrste iz subfamilije Typhlocibinae: *Eupteryx atropunctata* (Goeze), *Eupteryx mellissae* Curtis, *Zyginidia pullula* (Boheman). Prikupljen je i određen broj ženki iz roda *Euscelis* (Deltocephalinae), kao i jedna vrsta iz fam. Cixiidae *Reptalus quinquecostatus* (Dufour). Prisustvo stolbur fitoplazmi zabeleženo je u *A. laevis*, *A. ribauti*, *A. venosa*, *P. striatus*, *P. confinis* i *P. alienus*. Vrste *A. laevis*, *O. decumana* i *P. confinis* bile su inficirane AY (soj 16Srl-A), dok je soja 16Srl-C detektovan samo u jednoj jedinki *P. confinis*. S obzirom da su tokom čitavog vegetacionog perioda AY i STOL zaražene vrste iz rodova *Psammotettix* i *Anaceratagallia* (pre svega *P. confinis* i *A. laevis*) bile redovno i najčešće prisutne u zaraženim poljima šargarepe, one mogu imati značajnu ulogu u prenošenju i širenju ovih patogena u prirodi.

Ključne reči: Cicadellidae; AY; STOL; šargarepa