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Hyalesthes obsoletus in Serbia and its role in the epidemiology of corn reddening

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Abstract

Surveys were carried out in order to verify and monitor the presence of *Hyalesthes obsoletus* Signoret (Homoptera Cixiidae) populations in Serbia under different environmental conditions. *H. obsoletus* was present in all the localities investigated and different population dynamics were observed in relationship to the host plant. The development cycle of the vector and population density collected on *Convolvulus arvensis* L. was earlier and lower than those on the population collected on *Urtica dioica* L. In Udovice region, both nymphs and adults were observed on *Artemisia vulgaris* L. Since many larvae were found on the root apparatus of *A. vulgaris*, *H. obsoletus* appears to be adapted to the species and able to reproduce as well. Moreover, transmission trials were carried out with *H. obsoletus* population collected on nettle near corn field, allow verifying the ability of the cixiid to transmit the corn reddening disease. Amplification of phytoplasma DNA was obtained after nested PCR assays from corn samples tested after 40 days from insect caging and from batches of *H. obsoletus* collected on nettle from the same population used for cage-transmission. RFLP analyses allow identification of the detected phytoplasmas as "stolbur" in both, corn experimentally infected and *H. obsoletus* specimens tested.

Key words: "stolbur" phytoplasma, PCR/RFLP analyses, maize redness, vector.

Introduction

Corn reddening was first observed in 1957 in middle and south Banat region (Serbia) and, after this sporadic appearance, the disease entered into an epidemic phase (Marić and Savić, 1965). Another epidemic phase in the same region was reported during the late 1990s and early 2000s (Šutić *et al*., 2003). Between these epidemics the disease was always present erratically in the region, although it appeared to be more widespread in dry years, and it was reported as the most important disease of corn in the region due to its impact on yield (Blaženčić, 1982; Šutić, 2003). Symptoms of reddening begin in the second half of July on the main leaf midrib; then they spread to the stalk and eventually affect the whole plant. The most obvious symptoms are present in August and September (Šutić *et al*., 1983). Corn reddening was also reported in other regions of Serbia, in Romania, Bulgaria (Šutić *et al*., 2002), and recently in Italy (Calari *et al*., 2010) and Hungary (Acs *et al*., 2011).

In 2006 in Serbia, corn reddening was associated with "stolbur" phytoplasma (subgroup 16SrXII-A) (Duduk and Bertaccini, 2006) that is a widespread phytoplasma in Serbia, historically reported since 1949 (Martinović and Bjegović, 1950) and recently identified on grapevine (Duduk *et al*., 2004) and several other plant species (Duduk *et al*., 2006; Mitrović *et al*., 2013; Ivanović *et al*., 2011; Jović *et al*., 2011; Trukulja *et al*., 2011; Pavlović *et al*., 2011; Mitrović and Duduk, 2011).

Among the insect species, some of those belonging to Cixiidae family are reported as vectors of "stolbur" phytoplasma to different crops: *Pentastiridius leporinus* (L.) on herbaceous crops (Bogoutdinov, 2003) and on sugarbeet (Gatineau *et al*., 2001, Bressan *et al*., 2009) and recently the leafhopper *Anaceratagallia ribauti* (Ossiannilsson) (Riedle-Bauer *et al*., 2008). *Reptalus panzeri* (Low), after having been found infected by "stolbur" phytoplasma in Hungary (Palermo *et al*., 2004), was demonstrated to be involved in the epidemiology of corn reddening in Serbia (Jović *et al*., 2007; 2009).

However the cixiid, *Hyalesthes obsoletus* Signoret, was reported as the most important vector of "stolbur" phytoplasmas on solanaceus plants (Suhov and Vovk, 1946; Musil, 1956; Aleksić *et al*., 1967; Brčak, 1979) and grapevine (Maixner, 1994; Sforza *et al*., 1998; Alma *et al*., 2002; Bressan *et al*., 2007). The species is circummediterranean originating in the middle Asia region, and its most northern occurrence has been found in Germany and Poland. In Europe it is univoltine on wild plants, that are mainly field bindweed, *Convolvulus arvensis* L. (Maixner and Reinert, 2000; Langer *et al.,* 2003), stinging nettle, *Urtica dioica* L. (Alma *et al.*, 2002; Lessio *et al*., 2007), *Artemisia vulgaris* L. (Alma *et al*., 1988) and *Lavandula angustifolia* Mill. (Leclant and Lacote, 1969; Sforza *et al*., 1999). In Serbia, *H. obsoletus* was first reported by Aleksić *et al*. (1967) as abundant both on wild plants (i.e. *C. arvensis* and *Amaranthus retroflexus* L.), and cultivated corn (*Zea mays* L. var. *saccharata*). However, *H. obsoletus* was reported as absent or rare in and around the corn-field areas affected by corn reddening (Jović *et al*., 2007; 2009) and in Serbian vineyards (Cvrković *et al*., 2013).

Aim of this work was to investigate the role of *H. obsoletus* in the epidemiology of corn reddening in Serbia under field conditions.

Materials and methods

H. obsoletus sampling

In order to study the presence and ecology of *H. obsoletus* in Serbia, three localities were chosen (table 1), where "stolbur" phytoplasma was previously detected in symptomatic cultivated or wild plants (Duduk *et al*., 2004; 2006; Mitrović *et al*., 2013). In particular "stolbur" phytoplasma was detected in Smederevo in grapevine and bindweed, in Kovačica in corn and in Udovice in peach.

In each locality, from June to August 2012, the populations of *H. obsoletus* were surveyed every 10-15 days. The nymph instars were sampled by digging the roots of the reported hosts plants (*A. vulgaris*, *C. arvensis* and *U. dioica*) found in the investigated area. At least ten holes were made for each plant species. The adults were monitored using sweep nets on herbaceous vegetation. For each locality, at least 10 sampling of 100 square meters size, were chosen. For each sampling site the herbaceous vegetation was swept 6 times, collecting the adults captured in the net. Identification of collected insects was carried out by visual inspection, selected specimens for each locality were identified under dissection microscope using the morphological identification reported in Musil (1956), and Cargnus *et al*. (2012) for nymph instars and in Holzinger *et al*. (2003) and Bertin *et al*. (2010) for the adults. On July 19th-20th, during the flight period, specimens for each region were collected and stored in 96% ethanol for phytoplasma detection and identification.

Transmission trials

Transmission trials were set up using wild *H. obsoletus* insect population from Kovačica locality in the Banat Region, collected from nettle at the borders of corn fields, in which the corn reddening was reported (Duduk and Bertaccini, 2006). At the end of May 2012, asymptomatic corn seedlings, in a corn field, were isolated in cages (1.0 × 1.0 × 1.8 m) and tested for "stolbur" phytoplasma presence. Four cages were made each containing 7-9 corn hybrid AS72 seedlings, of which two cages were maintained as negative control without insects, while into the other two, 100 specimens each of *H. obsoletus*, collected on *U. dioica* from the field borders were released on July 25th 2012. From the same population a batch of 35 specimens was also collected and maintained in 96% ethanol in order to verify "stolbur" phytoplasma presence. The plants in the cages were examined for the symptoms presence on August 11th and 26th and on September 8th, when the cages were disassembled and plants sampled for the molecular analyses to verify phytoplasma presence.

"Stolbur" phytoplasma detection and identification

Insect specimens (table 2) were grouped in 30 batches of 3 insects each, and subjected to nucleic acid extraction with CTAB following a published protocol (Angelini *et al*., 2001). After the final isopropanol precipitation, nucleic acid extracts were re-suspended in TE buffer.

Corn samples were tested twice i.e. before the release of insects and after (May 28 and September 8, 2012 respectively) using the same nucleic acid extraction protocol.

Direct PCR assays with the universal phytoplasma primer pair R16F2/R2 (Lee *et al.*, 1995) and nested PCR on obtained amplicons, diluted 1:30, with primer pair R16(I)F1/R1 (Lee *et al*., 1994) were carried out. Each 25 µL PCR reaction mix contained 20 ng template DNA, 1 × PCR Master Mix (Fermentas, Vilnius, Lithuania) and 0.4 µM of each primer. Thirty-five PCR cycles were performed for both primer pairs, 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 analyzed in 1% agarose gel, stained with ethidium bromide

Table 1. Characteristic of the investigated localities.

Locality	GPS coordinates	Altitude (m a.s.l.)	Species reported to be infected by "stolbur"phytoplasma
Smederevo central Serbia	44°35'24.54"N 20°57'20.01"E	89	grapevine
Udovice central Serbia	44°37'51.95"N 20°51'25.17"E	187	peach
Kovačica south Banat	45°11'31.32"N 20°37'05.22"E	72	corn, tobacco

Table 2. "Stolbur" phytoplasma presence in the three *H. obsoletus* populations.

Locality	Host plant	Number of collected and analyzed individuals	Number of tested batches (3 insects)	Number of infected batches	Tuf-type b batches
Smederevo	*C. arvensis*	30	10	9	5
Udovice	*U. dioica* *A. vulgaris*	12	4	4	1
Kovačica	*U. dioica*	49	16*	3	2

*One batch of this population was made out of four specimens.

and visualized with UV transilluminator. Samples devoid of nucleic acid were used as negative control in all direct and nested PCR assays.

The amplicons obtained with R16(I)F1/R1 primers (1.1 kb) were analyzed by RFLP analysis with *Tru1*I restriction enzyme (Fermentas, Vilnius, Lithuania) following the instructions of the manufacturer. Separation of bands generated from restriction digests was performed in 6.7% polyacrylamide gels, staining and visualization of DNA was as described above for agarose gels. The nucleic acid of the positive insect and corn plant samples were also tested on *tuf* gene using in direct and nested-PCR assays respectively, primers Tuf1f/r (Schneider *et al*., 1997) and Tuf AYf/r (Langer and Maixner, 2004) under published conditions. Amplicons were digested with *Hpa*II (Fermentas, Vilnius, Lithuania) at 37 °C for 16 h following the instruction of the manufacturer.

Results

H. obsoletus presence

H. obsoletus was present in all the localities investigated. In Smederevo locality *H. obsoletus* nymphs and adults were collected on *C. arvensis*. In Udovice region, both nymphs and adults were observed feeding on *A. vulgaris* and *U. dioica,* but scattered distribution of the two herbaceous plants did not allow distinguishing the adults catches on each plant. In Kovačica locality nymphs and adults were collected on *U. dioica*.

The population density of *H. obsoletus* collected in Smederevo on *C. arvensis* was ten times lower than the one of the Kovačica population collected on *U. dioica*, and the flights were earlier on *C. arvensis* than on *U. dioica*: late May to mid July and late June to mid August, respectively.

All tested insect populations were infected by "stolbur" phytoplasmas. The infection rates, using the maximum likelihood estimation reported in Walter *et al*. (1980) was 53% for the Smederevo population, 13% for the Kovačica one and not estimable (4/4) for Udovice region. Tuf-type b was the only one detected in the three insect populations however only 40 to 60% of the batch positive on ribosomal gene were also amplified and therefore characterized on this gene (table 2).

Transmission trials

On August 11th no symptomatic plants were observed in any of the cages, while on August 26th appearance of the typical corn reddening symptoms have been observed only in the cages with *H. obsoletus*. On September 8th, when the cages were disassembled, the conditions of the plants in cages with and without insects were very different. Since the summer 2012 was extremely hot and dry in Serbia, the symptomatic plants suffered from sudden dry, however the symptoms were still visible and green areas were present in some of the leaves (figure 1), therefore samples were collected for phytoplasma presence analyses and corn cobs were harvested. In the cages without insects the corn plants were completely asymptomatic, the leaves were green and no dry or red areas were observed, on the other hand also the difference between corn cobs collected in cages with and without insect inoculation was very evident (figure 1).

"Stolbur" phytoplasma detection and identification

"Stolbur" phytoplasma was not detected in the corn samples tested before insect caging and from cages not containing insects. Amplification of phytoplasma DNA was obtained after nested PCR assays with primers R16(I)F1/R1 from 2 out of 7 corn samples tested after 40 days from insect caging and from 3 out of 16 batches of *H. obsoletus* collected on nettle from the same population used for cage-transmission.

RFLP analyses allow identification of the detected phytoplasmas as "stolbur" (16SrXII-A), tuf-type b in both, corn experimentally infected and *H. obsoletus* batches tested (figure 2).

Figure 1. Corn plants under cages and cobs collected; on the left after inoculation with *H. obsoletus*, and on the right without insects.
(In colour at www.bulletinofinsectology.org)

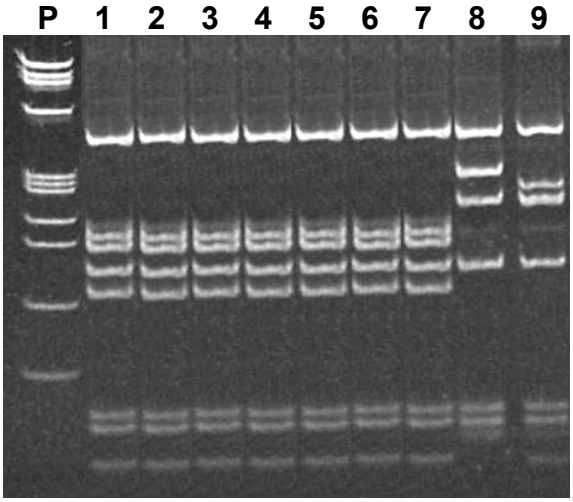

Figure 2. RFLP profiles of 1.1 kb products amplified by nested-PCR using primer pair R16(I)F1/R1, respectively from batches of *H. obsoletus* collected on bindweed in Smederevo (samples #1 and #2), on *A. vulgaris* in Udovice (samples #3 and #4) and on nettle in Kovačica (samples #5 and #6); reference strains in periwinkle are #7, STOL, "stolbur" from pepper from Serbia (16SrXII-A); #8, AY, aster yellows from Germany (16SrI-B) and #9, KVG, clover phyllody from Germany (16SrI-C). PCR products were digested separately with restriction enzyme *Tru1*I and separated by electrophoresis through 6.7% polyacrylamide gel. P, marker phiX174 *Hae*III digest, fragment sizes (bp) from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; 72.

Discussion

H. obsoletus is widespread in Serbian regions where its most important host plant is *U. dioica*, beside *C. arvensis* which is already well known (Aleksić *et al*., 1967). This situation is reported for the other European countries as well, and areas with similar climatic conditions (Maixner, 2007; Mori *et al*., 2008a; 2008b; Kessler *et al*., 2011; Johannesen *et al*., 2012). It is however the first time that in Serbia population of the insect are found connected with *A. vulgaris* plants, and considering that nymphs were found on the root apparatus, this population appears to be adapted to the species and able to reproduce as well.

H. obsoletus is able to transmit "stolbur" phytoplasma to herbaceous and woody plants (Aleksić *et al*., 1967; Brcak, 1979; Lee *et al*., 1998). We demonstrated here the ability of *H. obsoletus* to harbour and transmit "stolbur" phytoplasma also to corn seedlings inducing corn reddening in two out of the seven plants tested. The real transmission rate may be higher, but we were unable to detect phytoplasma presence in the other symptomatic plant tested probably due to extreme drought and extremely hot weather during summer 2012 (Smailagić *et al*., 2013) which induced fast drying of symptomatic plants in the cages with *H. obsoletus*.

On vineyards *H. obsoletus* transmits "stolbur" to grape from bindweed (Maixner 1994; Sforza *et al*., 1998) and nettle (Alma *et al*., 2002; Bressan *et al*., 2007) as phytoplasma inoculum source. Considering that *U. dioica* is mainly located in the vineyard surrounding areas and edges, effects on spatial distribution of symptomatic grapevines and vector density were described, showing that nettles are an important source of "stolbur" phytoplasma for grapevines (Bressan *et al*., 2007; Mori *et al*., 2008; Maixner, 2010). Although only the presence of tuf type-b of "stolbur" phytoplasmas was demonstrated in Serbia so far, recent findings indicate that tuf-type b was detected also in nettle in central and southeastern Europe (Riedle-Bauer *et al*., 2008), therefore this aspect should be further evaluated in Serbian environments.

In this study we demonstrate that *H. obsoletus* from wild nettle at the borders of corn fields is able to transmit "stolbur"/16SrXII-A phytoplasmas to healthy corn seedlings and that it is present in significant numbers on the borders of corn cultivated area. As the Banat region is well known as one of the windiest regions of Serbia, the infected population of *H. obsoletus* can be blown to the neighbouring corn fields. Moreover, since nettle areas are present in drenches near to the corn fields where humidity stays longer and may dry only during the extremely dry years, when the epidemic phase of corn reddening are present, it may represent the source of stolbur infected *H. obsoletus*, specially during the epidemic phases.

As *R. panzeri* that is involved in the epidemiology of "stolbur" phytoplasmas on corn (Jović *et al*., 2007) and grapevine (Cvrković *et al*., 2013), also *H. obsoletus* could play a role in the epidemiology of "stolbur" not only of grapevine and solanaceous crops (Maixner, 1994), but also of corn as shown in this work. Study is in progress to further investigate the importance of *H. obsoletus* role in the corn reddening epidemiology.

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