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Screening for phytoplasma presence in leafhoppers and planthoppers collected in Bulgarian vineyards

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Abstract

In the beginning of 2003, the Bulgarian Food Safety Agency started the monitoring program for quarantine pests on grapevine. The object of surveys was to verify the possible presence of 'flavescence dorée' phytoplasmas. At the same period entomological surveys were carried out with yellow sticky traps in the vineyards surveyed. Identification of *Scaphoideus titanus* Ball. was done in the summer of 2006 in Varna and Veliko Turnovo regions. For the control of natural spreading of phytoplasmas in Bulgarian vineyards individual testing by PCR of the collected planthoppers and leafhoppers in order to define phytoplasma presence and increase knowledge on the grapevine phytoplasma vectors in Bulgaria was carried out.

Key words: *Scaphoideus titanus* Ball., nested PCR, grapevine phytoplasmas, insect vectors.

Introduction

'Flavescence dorée' (FD) and 'bois noir' (BN), are serious diseases of grapevine (*Vitis vinifera* L.) in temperate European areas. FD, quarantine organisms in Bulgaria and in EU, is associated with phytoplasmas that belongs to the elm yellows (EY) group (16SrV group) (Bertaccini *et al*., 1995). This phytoplasma is naturally transmitted by the leafhopper *Scaphoideus titanus* Ball (Hemiptera, Cicadomorpha, Cicadellidae). BN diseases is associated with stolbur (STOL, 16SrXII) phytoplasmas, it is the second economically important yellows of grapevine in Europe and is transmitted by the planthopper *Hyalestes obsoletus* Signoret. The disease was reported in Bulgaria since 2006 (EPPO, 2006; Sakalieva *et al*., 2007; Avramov *et al*., 2008).

In the beginning of 2003, the National Plant Protection Service in Bulgaria (now Bulgarian Food Safety Agency) started the monitoring program for quarantine pests on grapevine. The object of surveys was to verify the presence of grapevine yellows diseases, especially FD. The samples were checked for phytoplasmas in the Central Laboratory for Plant Quarantine. At the same period entomological observations were done with yellow sticky traps. For the control of natural phytoplasma spreading in vineyards individual testing of the collected planthoppers and leafhoppers in order to define phytoplasma infection and increase knowledge on the grapevine phytoplasma vectors.

Materials and methods

Insect collection. Since 2008 yellow sticky traps sampling have been conducted from July until September in vineyards and 230 specimens in total between adult insects and nymphs were captured and examined. The leafhoppers and planthoppers were collected in all regions of Bulgaria, where vineyards are present except *Scaphoideus titanus* Ball witch is spread only in a North part of Bulgaria (table 1).

DNA extraction. Total nucleic acids were extracted from single leafhoppers by the method of Doyle and Doyle (1990) as adapted by Marzachì *et al*. (1998). Each individual was ground in a 1.5 ml Eppendorf tube along with 500 µl of pre-heated (60°C) CTAB buffer [2% w/v cetyl-trymethyl-ammonium-bromide, 1.4 M NaCl, 20mM EDTA pH 8.0, 100 mM Tris-Cl, pH 8.0, 02.% v/v ß-mercaptoethanol]. After incubation for 30 min at 60°C, nucleic acid was extracted with 1 vol of 24:1 chloroform:isoamyl alcohol, precipitated with 1 vol of cold isopropyl alcohol and freed from salts by 70% (v/v) ethanol washings. Final products are suspended in 200 µl of sterile double distilled water or TA buffer [10 mM Tris and 1 mM EDTA, pH 8].

PCR assays. DNA extracted from insect specimens was tested by nested PCR (Lee *et al*., 1993), using two pairs of generic primers (P1/P7, R16F2/R16R2) that amplify phytoplasma 16S rDNA and followed by RFLP analyses for phytoplasma identification using restriction enzymes *Rsa*I *(Afa*I*)* and *Alu*I (Amersham Biosciences, USA). PCR and RFLP products were analysed by electrophoresis on 1.5% agarose gel followed by staining with ethidium bromide and visualization of DNA bands with a UV transilluminator.

Results and discussion

After entomological identification the specimens were distributed as follows: *S. titanus* - 48 units, *H. obsoletus* – 57 units, *Reptalus* spp. – 52 units, *Empoasca vitis* Goethe – 23 units, *Neoaliturus fenestratus* Her. Schaffer – 16 units, *Zigina rhamni* F. – 12 units, *Eupteryx* spp. – 11 units and *Philaenus* spp. – 11 units.

H. obsoletus known as vector of stolbur phytoplasma in Bulgaria and BN disease of grapevine was identified in the regions of Bourgas, Varna, Vratsa, Rousse, Veliko Tarnovo, Stara Zagora and Pleven. For the first two years of the monitoring programme *S. titanus* was not detected; it was first identified in the summer of 2006 in Veliko Turnovo region.

Table 1. Results of molecular tests on collected leafhopper and planthopper with yellow sticky traps in vineyards in Bulgaria*.

Species	Regions (number of captured specimens)	PCR tests carried out	Positive results
S. titanus	Vratsa (14), Montana (9), Russe (6), Veliko Turnovo (6), Vidin (6), Pleven (4), Lovech (3)	48	none
H. obsoletus	Veliko Turnovo (10), Varna (12), St Zagora (6), Russe (6), Lovech (4), Vratsa (3), Vidin (4), Plovdiv (5), Pleven (4), Bourgas (3)	57	12 (In all 10 regions)
Reptalus spp.	St. Zagora (10), Plovdiv (10), Pleven (8), Veliko Turnovo (7), Lovech (3), Vratsa (3), Russe (2), Varna (2), Bourgas (2), Vidin (2), Blagoevgrad (2), Haskovo (1)	52	10 (In 9 out of 12 regions)
Empoasca spp.	Plovdiv (8), Pleven (5), Vratsa (4), Bourgas (3), St. Zagora (2), Russe (1)	23	none
N. fenestratus	Lovech (3), Vratsa (3), St Zagora (2), Vidin (2), Varna (2), Plovdiv (1), Veliko Turnovo (1), Bourgas (1), Blagoevgrad (1)	16	none
Zigina spp.	Plovdiv (4), Bourgas (3), Vratsa (2), St Zagora (2), Varna (1)	12	none
Eupteryx spp.	Pleven (3), VelikoTurnovo (3), Bourgas (2), Plovdiv (1), Vidin (1), Haskovo (1)	11	none
Philaenus spp.	Plovdiv (4), Pleven (3), Varna (3), Bourgas (1)	11	none

* Not all identified insects were tested: approximately equal numbers from different regions were selected.

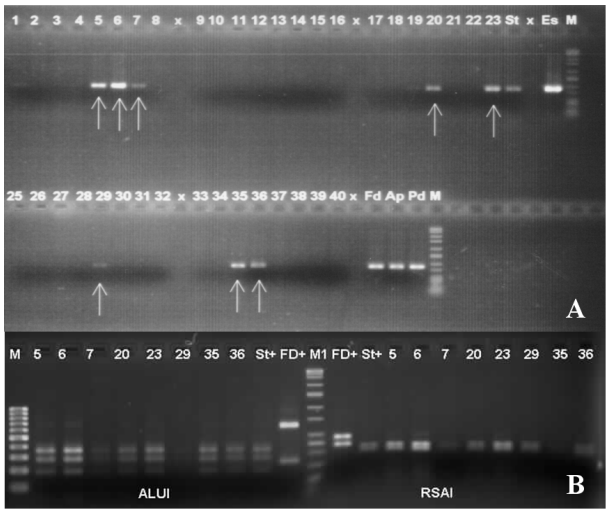

Figure 1. Results of nested PCR. A) P1/P7 follwed by R16F2n/R16R2. Line 1 - 40 insect samples, St, Fd, Es, Ap, Pd – positive controls for stolbur, FD, ESFY, PD. M – wide range marker, Sigma, 10 kbp, lines 5, 6, 7, 20, 23, 29, 35, 36 are samples with positive results for phytoplasma presence. B) RFLP results in agarose gel.

After the performing nested PCR analysis the phytoplasma presence was detected in 22 insects. *S. titanus* was never found to carry phytoplasmas. All positive samples produced showed 16SrXII profiles (figure 1) suggesting the presence of stolbur phytoplasmas associated with BN presence in vineyards. Infection of BN phytoplasma presence was confirmed only in the cixiids *H. obsoletus* - 12 and *Reptalus* species – 10 positive results. Phytoplasma infection was not detected into the others species (table 1). Based on the results of RFLP analyses, tested phytoplasma isolates were classified as 'bois noir'.

No FD infections were found in vineyards in Bulgaria inspite of the presence of the FD vector *S. titanus*. There is good evidence that *H. obsoletus* and *Reptalus* spp. are the insect vector of grapevine phytoplasma infections detected in Bulgaria so far.

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