Vitis **43** (4), 201–203 (2004)

Presence of stolbur phytoplasma in Cixiidae in Hungarian vineyards

S. Palermo[1)], M. Elekes[2)], S. Botti[3)], I. Ember[2)], A. Alma[1)], A. Orosz[4)], A. Bertaccini[3)] and M. Kölber[2)]

[1)] Di.Va.P.R.A., Entomology and Zoology applied to the Environment "Carlo Vidano", University of Torino, Italia
[2)] Central Service for Plant Protection and Soil Conservation, Budapest, Hungary
[3)] DiSTA, Patologia Vegetale, Alma Mater Studiorum, University of Bologna, Italia
[4)] Hungarian Natural Scientific Museum, Budapest, Hungary

Summary

Bois Noir (16SrXII-A) phytoplasmas were identified in three viticultural areas of Hungary in 18 % of *Hyalesthes obsoletus*, and in an asymptomatic nettle sample. The cixiid *Reptalus panzeri* was found to be infected with 16SrXII-A and with 16SrIII (X-disease) phytoplasmas. The latter pathogen was also detected in symptomatic wild *Cirsium* spp. and *Convolvolus* collected inside Bois Noir-infected and *R. panzeri*-infested vineyards.

K e y w o r d s : Bois Noir, grapevine, *Reptalus panzeri*, phytoplasmas, PCR/RFLP.

Introduction

Among the yellows diseases of grapevine, Flavescence dorée (FD) is the most dangerous. Its agent belongs to ribosomal subgroups 16SrV-C and 16SrV-D in Italy and in France (Angelini *et al*. 2001) and is transmitted by the leafhopper *Scaphoideus titanus* Ball (Auchenorrhyncha: Cicadellidae). Originally described in France, FD is currently spread in several European countries and was recently found also in Serbia (Duduk *et al*. 2004). However the FD causal agent and its insect vector have never been reported from Hungary, where symptoms related to phytoplasma diseases were first observed in grapevine in the 1970s (Kölber *et al*. 1997 a). A nation-wide survey for grapevine yellows began in 1993 and typical symptoms of phytoplasma diseases were found in County of Tolna in 1994, then in other Counties (Kölber *et al*. 1997 a). Molecular analyses of the pathogens detected in Hungary allowed the identification of BN phytoplasmas (Kölber *et al*. 1997 b; Viczián *et al*. 1998). Between 1997 and 2002, visual inspections of 29,800 grapevine plants (33 cultivars) in the main Hungarian wine-growing areas indicated the presence of phytoplasma symptoms in 7.9 % of the inspected plants. Besides BN, other phytoplasmas associated with yellows diseases such as Aster Yellows (AY), Elm yellows (EY), Clover phyllody (CPh) and European stone fruit yellows (ESFY) as well as mixed infections were identified (Kölber *et al*. 2003). Studies on vector populations carried out between 1996 and 1998 in the vineyards, where BN phytoplasmas were firstly identified, showed that almost 70 Auchenorrhyncha species occurred in the vineyards, including *H. obsoletus* (Orosz *et al.* 1996).

Considering the detection and the spreading of BN in grapevines, further studies focused on monitoring *H. obsoletus*, and other potential vectors and weed host species were studied in 2002-2003 in the frame of an Italian-Hungarian cooperation.

Material and Methods

L o c a t i o n s : Potential phytoplasma-vector Auchenorrhyncha species and plant samples were collected in 2002 and 2003: (1) in the Eger region in a vineyard planted with Chardonnay in 1978, (2) in the Villány region, in two vineyards planted with Zweigelt in 1987 and 1991, (3) in the Szekszárd region in a vineyard planted with Chardonnay and Zweigelt in 1981, and (4) in an abandoned vineyard.

Along the borders of these vineyards many wild plants were identified and among them *Artemisia vulgaris* L., *Cirsium arvense* (L.) Scopoli (Canada thistle), *Convolvolus arvensis* L. (bindweed), *Conyza* spp., *Prunus spinosa* L. (blackthorn), *Rubus* spp., *Sonchus* spp., *Taraxacum* spp. and *Urtica dioica* L. (nettle) were the most abundant.

C o l l e c t i o n o f i n s e c t s : Insects were collected with D-vac, gathering specimens along 80-100 m of the grapevine canopy and about 150 m in the weed layer, spending 10 min for each sampling. Fifteen and 11 samplings were carried out in the canopy of grapevines and in the weed layer, respectively, while 6 and 2 samplings were made in nettle and blackthorn, respectively, in July 2002 and 2003. The trapped material was cleaned using a Vortis insect selector. Other samplings of Cixiidae specimens were performed using a sweep net on symptomatic grapevines and their undergrowth, as well as on nettles, growing in and around the vineyard, and on blackthorn. Collected insects were killed with chloroform and the Auchenorrhyncha species were sorted out within 24 h after collection. Specimens of the insects known as potential phytoplasma vectors were stored for molecular testing.

C o l l e c t i o n o f p l a n t s a m p l e s : Samples of grapes and herbaceous plants were collected in the same experimental sites in both years of the survey. Asymptomatic bindweed, showing yellows and stunting, Canada thistle with multiple inflorescence and stunting, and other wild plants were sampled.

M o l e c u l a r a n a l y s e s i n i n s e c t s a m p l e s : Nucleic acids were isolated from 220 individual cixiids using

Correspondence to: Dr. A. Bertaccini, DiSTA, Plant Pathology, via Filippo Re 8, 40126 Bologna, Italy. Fax: +39-051-2091451. E-mail: Bertaccini_A@biblio.cib.unibo.it

a modified nucleic acid preparation method (MARZACHÍ *et al.* 1998) and the final products were re-suspended in 100 µl TE buffer [10mM Tris-HCl, 1mM EDTA (pH 8.0)]; 1.5 µl of the suspension were employed in direct PCR. Phytoplasma universal primers P1/P7 were applied to amplify phytoplasma 16SrDNA, or 16SrDNA plus spacer region, followed by R16F2/R16R2 or by phytoplasma group-specific primers R16(I)F1/R16(I)R1 and R16(III)F1/R16(III)R1. All nested-PCR reactions were performed on P1/P7 amplicons diluted 1: 30 (see in DUDUK *et al*. 2004).

Molecular detection in plant samples: One g of fresh, frozen or dry leaf midribs and phloem tissue of branch samples was used to extract nucleic acids by means of a chloroform/phenol procedure. Final products were re-suspended in 50 µl TE buffer and diluted to adjust the final DNA concentration to 20 ng·µl^{-1}; 1 µl of this dilution was used for PCR assays. Direct PCR was performed with the same primers described above followed in first nested-PCR by R16F1/B6 (=M23SR$_{1804}$) (DUDUK *et al*. 2004) and by R16F2/R16R2 in second nested-PCR on amplicons obtained from R16F1/B6 diluted again 1: 30. Group-specific primers described above were used in second nested-PCR on R16F2/R16R2 amplicons.

Reaction and cycling conditions for plant and insect material were conducted in an automated thermal cycler following the procedure described in DUDUK *et al*. (2004). Nested-PCR products were analysed in a 1 % agarose gel, stained with ethidium bromide, and visualised under UV light. Purified DNAs of control samples described in the Figure were used as positive controls. DNA from an asymptomatic grapevine plants and a sample containing the reaction mixture without DNA were included as negative controls.

Nested-PCR products were digested with the restriction enzymes *Tru9*I and *Hpa*II (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. RFLP profiles were resolved in 2.5 % agarose or in 5 % polyacrylamide gels, stained with ethidium bromide and visualised by UV light.

Results

Samplings carried out in 6 viticultural areas in July 2002 and 2003 allowed to collect 2,037 Auchenorrhyncha specimens belonging to 40 species, among these 16.5 % were cixiids. The two species of ciixids identified, *H. obsoletus* and *Reptalus panzeri* (Löw), were selected for molecular testing to verify the presence of phytoplasmas. *H. obsoletus* specimens were mostly collected on *U. dioica* (171) and weeds (41), while 13 and 9 samples were collected on grapevine and *P. spinosa*, respectively. The majority of *R. panzeri* samples (24) were collected on grapevine, 12 were collected on *P. spinosa* and 5 on weeds. In total, 18 % of the specimens of *H. obsoletus* resulted to be infected by 16SrXII-A phytoplasmas: 9 % of the individuals collected on grapevine and 24.2 % of the insects caught on nettles (Table). The higher percentages of *H. obsoletus* positive to stolbur phytoplasmas were detected in samples collected in Pécs (29.9 %) and in Nagytotfalu (28 %). Among *R. panzeri*, 9.2 % of the tested individuals were infected by 16SrXII-A

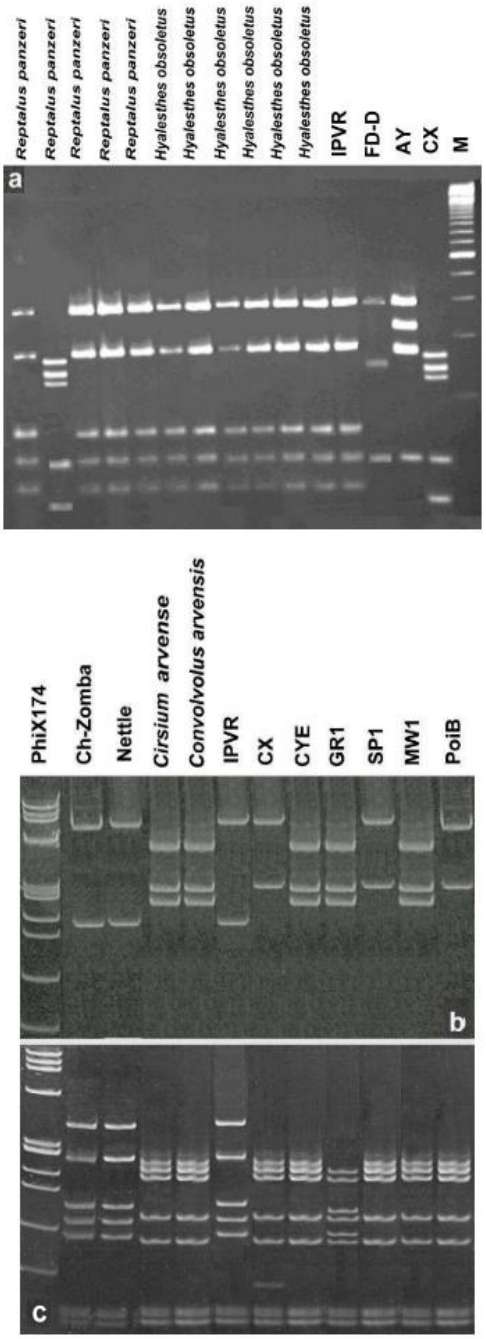

Figure: **a**) Agarose gel (2.5 %) showing the RFLP patterns of selected insect samples and phytoplasma control strains of DNA fragments amplified with R16F2/R16R2 primers analysed with *Tru* 9I restriction enzyme. Acronyms of samples: IPVR, Italian periwinkle virescence (16SrXII-A, stolbur group); FD-D, flavescence dorée from Italy (16SrV-D, elm yellows group); AY, aster yellows (16SrI-B, aster yellows group); CX, X-disease (16SrIII-A, X disease group). Marker, 100 bp DNA marker. **b**) Polyacrylamide gel (5 %) showing the RFLP patterns of selected plant samples and phytoplasma control strains of DNA fragments amplified with R16F2/R16R2 primers analysed with *Hpa*II and **c**) *Tru* 9I restriction enzymes. Acronyms of samples: Ch-Zomba, grapevine Chardonnay from Zomba; CYE, clover yellow edge (16SrIII-B, X disease group); GR1, Goldenrod yellows from US (16SrIII-D, X disease group), SPI, spirea stunt (16SrIII-E, X disease group); MW1, Milkweed yellows (16SrIII-F, X disease group); PoiB, poinsettia branch inducing (16SrIII-H, X disease group). Marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72.

T a b l e

Phytoplasmas detected by PCR-RFLP in single *Hyalesthes obsoletus* and *Reptalus panzeri* samples collected by D-vac or sweep net in BN-infected vineyards in 2003

host plant	*H. obsoletus* samples			*R. panzeri* samples		
	positive/tested	%	Phytoplasma	positive/tested	%	phytoplasma
Grapevine	6/67	9	16SrXII-A	1/32	3.1	16SrXII-A
U. dioica	24/99	24.2	16SrXII-A	2/6	33.3	16SrXII-A + 16SrIII
P. spinosa	-			1/12	8.3	16SrXII-A
weeds	-			0/4	0	
Total	30/166	18		5/54	9.2	

phytoplasmas, and one of them resulted positive in PCR also to 16SrIII phytoplasmas, in mixed infection with 16SrXII-A (Table, Figure).

In vineyards where the Auchenorrhyncha specimens were collected BN phytoplasmas were detected by PCR-RFLP assays in both symptomatic grapevines and rootstock varieties (13 samples out of 24), and in asymptomatic nettle plants (1 out of 4). Moreover 16SrIII-B phytoplasmas (Clover yellow edge, CYE) were identified in *C. arvense* and *C. arvensis* symptomatic samples (Figure).

Discussion

A relatively high number of *H. obsoletus* shown to be infected by BN phytoplasmas, confirms the major role of this vector species in BN disease occurrence in Hungary, as has already been demonstrated for other countries (Maixner *et al.* 1995; Alma *et al.* 2002).

In the present survey, relatively high populations of the cixiid *R. panzeri* were recorded on grapes and blackthorn in one location (Andornaktálya) and some specimens were positive for BN phytoplasma presence. We plan to assess the *R. panzeri* ability in phytoplasma transmission on different host plant species and its role in spreading of grapevine yellows. In particular, molecular analyses on *P. spinosa* samples could clarify the role of this plant as a potential Stolbur phytoplasma reservoir.

BN phytoplasmas were detected in diverse weed species in Europe (Maixner *et al.* 1995; Alma *et al.* 2002); and in Hungary, in *Datura stramonium* L. (thorn apple), *Silene vulgaris* L., *Taraxacum officinale* L. (Viczián *et al*. 1998). In this study BN phytoplasmas were found, besides grapevine, only in one asymptomatic nettle plant. This might suggest an important role of this plant species in spreading BN, also considering the high number of *H. obsoletus* collected on nettles in vineyards. Further analyses would be needed to assess the epidemiological role of this plant in the epidemiology of BN disease in Hungary.

Acknowledgement

The work was performed in the frame of the Hungarian-Italian Intergovernmental S&T cooperation (I-43/1999) as a result of the joint research supported by the Research-Development Sub-Secretariat of the Ministry of Education and Ministero degli Affari Esteri Direzione Generale delle Relazione Culturali, Rome, Italy.

References

Alma, A.; Soldi, G.; Tedeschi, R. M.; Marzachí, C; 2002: Ruolo di *Hyalesthes obsoletus* Signoret (*Homoptera: Cixiidae*) nella transmissione del Legno nero della vite in Italia. Petria **12**, 411-412.

Angelini, E.; Clair, D.; Borgo, M.; Bertaccini, A.; Boudon-Padieu, E.; 2001: Flavescence dorée in France and Italy - Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. Vitis **40**, 79-86.

Duduk, B.; Botti, S.; Ivanović, M.; Krstić, B.; Dukić, N.; Bertaccini, A.; 2004: Identification of phytoplasmas associated with grapevine yellows in Serbia. J. Phytopathol. **152**, 575-579.

Kölber, M.; Davis R. E.; Dally, E. L.; Tőkés, G.; Lázár, J.; Szendrey, G.; Krizbai, L.; Mikulás, J.; 1997 b: Stolbur alcsoportba tartozó fitplazma kimutatása és azonosítása, mint a szőlő sárgaság (grapevine yellows) lehetséges kórokozója Magyarországon. Növényvédelmi Tudományos Napok, 1997.

Kölber, M.; Ember, I.; Varga, K.; Botti, S.; Martini, M.; Lázár, J.; Bertaccini, A.; 2003: Six-year survey of grapevine yellows distribution in Hungary. 14th Meeting of ICVG, 12-17th September, 2003. Locorotondo, 99-100.

Kölber, M.; Lázár, J.; Davis, R. E.; Dally, E. L.; Tőkés, G.; Szendrey, G.; Mikulás, J.; Krizbai, L.; Papp, E.; 1997 a: Occurrence of Grapevine Yellows Disease in Grapevine Growing Regions of Hungary, 73-74. 12th Meeting of ICVG, 29 September-2 October 1997, Lisboa.

Maixner, M.; Ahrens, U.; Seemüller E.; 1995: Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. Eur. J. Plant Pathol. **101**, 241-250.

Marzachí, C.; Veratti, F.; Bosco, D.; 1998: Direct PCR detection of phytoplasmas in experimentally infected insects. Ann. Appl. Biol. **133**, 45-54.

Orosz, A.; Elekes, M.; Cziklin, M.; Dulinafka, Gy.; Gál, Sz.; Győrffy-Molnár, J.; Gyulai, P.; Havasréti, B.; Szendrey, G.; Tóth, B.; Vörös, G.; 1996: Detection of leafhopper vectors of phytoplasmas causing grapevine yellows in Hungary. 17. Workshop on Integrated Crop Management in Horticulture, Budapest, 26 November 1996.

Viczián, O.; Süle S.; És Gáborjányi, R.; 1998: A sztolbur fitoplazma természetes gazdanövényei Magyarországon. Növényvédelem **34**, 617-620.

Received July 23, 2004