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Experimental transmission by *Scaphoideus titanus* Ball of two Flavescence dorée-type phytoplasmas

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Summary

During the last 5 years Flavescence dorée (FD) has been expanding from the first loci of outbreak in the Veneto region, to the major viticultural areas of northern Italy, showing increased infection rates in 1999. The FD vector is *Scaphoideus titanus* Ball. Recently it has been demonstrated that in Veneto FD is associated with two molecularly distinguishable types of phytoplasmas, both belonging to the 16SrV group (Elm yellows and related strains). In 1997 the two phytoplasma types appeared to be geographically separated in 1997: FD-16SrV-D was found in the provinces of Verona, Vicenza, Venice and Padova, infecting mainly cvs Garganega and Chardonnay while FD-16SrV-C occurred in the Treviso province, infecting cultivars Prosecco, Chardonnay and Perera. During 1998 the two types started to merge in Treviso province, showing that 16SrV-D was transmitted more efficently and over longer distances than the other type. From the transmission trials carried out in 1998 and 1999 in order to gain more information about the epidemiology of the two types of FD agents, *S. titanus* was found to be able to transmit both 16SrV-C and 16SrV-D phytoplasmas.

K e y w o r d s : phytoplasma, transmission, PCR, RFLP, detection.

Introduction

Among grapevine yellows phytoplasma-associated diseases, Flavescence dorée (FD) is the most harmful and it is a quarantine disease; its epidemic spread is strictly conditioned by the activity of its natural vector *Scaphoideus titanus* Ball (Homoptera Auchenorryncha Cicadellidae).

This leafhopper completes the whole univoltine cycle on grapevine (*Vitis vinifera* L.) (Vidano 1966) and transmits FD from vine to vine in a selective way (Schvester *et al*. 1961, 1969). This insect is not able to transmit the "Bois noir" (BN, 16SrXII-A) (Carraro *et al.* 1994), another grapevine yellows widespread in European viticulture, although this phytoplasma was detected in the insect (Martini *et al*. 1999). This phytoplasma disease with symptoms similar to FD, is transmitted by *Hyalesthes obsoletus* Signoret (Homoptera Auchenorryncha Cixiidae) at least in Germany (Maixner 1994) and in France (Sforza and Boudon-Padieu 1998), where grapevines seem to be only a dead end host (Boudon-Padieu 1999). Recently the 16SrXII-A phytoplasma was transmitted to sugar beet by *Pentastiridius beieri* Wagner (Homoptera Auchenorryncha Cixiidae) (Gatineau *et al*. 2001)

S. titanus was shown recently to be an experimental vector of Chrysanthemum yellows phytoplasma (CY, subgroup 16SrI-B) from marguerite (*Chrysanthemum carinatum*) used as source of inoculum to grapevine seedling (Alma *et al*., 1999); phytoplasmas of this subgroup were also shown to be transovarially transmitted by the same insect (Alma *et al*. 1997).

Recently it has been demonstrated that in Veneto (North-eastern Italy) FD is associated with two molecularly distinguishable phytoplasma types both belonging to 16SrV group (Elm yellows and related strains) (Martini *et al*. 1999). The two phytoplasma types appeared to be geographically separated in FD-16SrV-D in the provinces of Verona, Vicenza, and Padova, infecting mainly cvs Garganega and Chardonnay and FD-16SrV-C in the province of Treviso infecting cvs Prosecco, Chardonnay and Perera. In 1998 the two types started to merge into the province of Treviso (Bertaccini *et al*. 1998) and in 2000 16SrV-D was identified in Emilia (South-west of the Veneto region) in Lambrusco cultivars (Bertaccini *et al*. 2000) and in the province of Pordenone (Frausin 2000), confirming that this phytoplasma is transmitted more efficiently and over longer distances than the other type and that it is able to infect many different grapevine cultivars.

In 1998-1999, trials were carried out to achieve more information about the epidemiology of these two agents and to verify their implication in transmission by *S. titanus*. Part of these results was published by Mori *et al*. 1999.

Material and Method

P h y t o p l a s m a t r a n s m i s s i o n : From June to September 1998-1999, batches of nymphs and adults of *S. titanus* were collected from 5 different vineyards in the Veneto region (Italy), where the presence of the two phytoplasma types was demonstrated by PCR (Martini *et al*., 1999).

Some insects were used directly for transmission trials, others (early instar nymphs) were reared on symptomatic grapevine plants in order to obtain variable acquisition accession times. The experimental scheme is shown in Tab. 1.

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Table 1

Experimental plan of *S. titanus* transmission trials

Location Province	Cultivars used for acquisition	Additional acquisition time (d)	Inoculation date	Insect stage during inoculation	Insects/ plant	Inoculated plants
Soave Verona	Durello	7	24/08/98	Adults	20	4
Affi	Cabernet	-	08/07/99	L_4 / L_5	30	2
Verona	Franc	22	18/07/99	Adults/L_5	30	2
Torreglia Padova	Chardonnay	-	30/06/99	L_3 / L_4	30	2
Montecchio	Cabernet	-	02/07/99	L_4 / L_5	50	6
Maggiore	Sauvignon	-	14/07/99	Adults	60	3
Vicenza		-	14/07/99	Adults	30	2
		-	07/07/99	Adults	40	2
	Trebbiano	-	21/07/99	Adults	50	2
		-	07/07/99	Adults	75	2
Farra di Soligo	Prosecco	-	28/07/99	Adults	50	6
Treviso		14	12/08/98	Adults	40	3

420 insects from the Treviso vineyards were confined to 9 grape test plants, 1130 insects from the Verona, Vicenza and Padova vineyards to 27 grape test plants; 6 plants were kept insect-free as control.

The test plants used were micropropagated, potted cv. Chardonnay vines (kindly provided by M. BORGO, MiPAF, Conegliano, TV, Italy). The plants were tested by nested PCR, as described below, before and after transmission. 3.8 % (16/420) of the insects from the Treviso vineyards and 5.3 % (60/1130) of the insects from the Verona and Vicenza vineyards were sampled from the cages and tested singularly or in batches of 2-5 individuals by nested-PCR as described below.All insects were caged for phytoplasma transmission until they died, all cages were placed in an insect-proof greenhouse.

Phytoplasma detection: The nucleic acid extraction from insects was performed as described by VEGA *et al.* (1993) but slightly modified with regard to centrifugation time and speed. Plant nucleic acid was extracted following a chloroform/phenol procedure (PRINCE *et al.* 1993). Template nucleic acids were diluted to a final concentration of 20 ng·µl^{-1}; 1µl of this dilution was used for PCR assays. Reaction mix contained 10 pmol of each primer, 200 µM of d-NTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10mM Tris-HCl (pH 9) and 0.625 U of *Taq* Polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) for a total volume of 25 µl. The amplification cycle was the one described by SCHAFF *et al.* (1992). All samples were tested by nested-PCR, using universal primers R16F1/R0 followed by R16F2/R2 (LEE *et al.* 1995) and 16R758f/16R1232r (M1/M2) (GIBB *et al.* 1995). All samples being positive after this procedure were further amplified with group 16SrV-specific primers R16(V)F1/R1 and groups 16SrI and 16SrXII-specific primers R16(I)F1/R1 (LEE *et al.* 1994, 1995) under the conditions described above.

On samples positive for EY-type phytoplasmas, another nested PCR procedure was adopted using primers 16R723f/P7 followed by primers 16R758f/M23SR1804r and by primers 16R758f/V1731r in seminested-PCR in order to amplify to the 3' end of the spacer region (MARTINI *et al.* 1999). RFLP analyses of the latter amplified products were carried out with *Taq*I (MBI, Vilnius, Lithuania) by incubation overnight at 65 °C. The samples amplified with R16(I)F1/R1primers were digested with *Mse*I (NEB, Beverly, MA, USA) for at least 16 h at 37 °C.

Control samples used in PCR/RFLP analyses for group 16SrV were grapevine FD strain B1872 (kindly provided by E. Boudon-Padieu, INRA, Dijon, France) and strain 19-TV (MARTINI *et al.* 1999) for subgroups 16SrV-D and 16SrV-C respectively; elm yellows (EY), (kindly provided by H. GRIFFITH and W. A. SINCLAIR, Cornell University, Ithaca, NY, USA) for 16SrV-A subgroup, Italian periwinkle Virescence (IPVR) for 16SrXII-A subgroup and aster yellows (AY) for 16SrI-B subgroup.

Results

The results from transmission tests are summarized in Tab. 2. To feed the vectors symptomatic grapevine plants were infected by 16SrV-D phytoplasmas if the insects were collected in the Verona and Vicenza vineyards, and by 16SrV-C phytoplasmas if they were collected in the Treviso vineyard, confirming that the two types of FD were employed for transmission tests (Tab. 2 and Figure); in the transmission trials no results were obtained with samples from the province of Padova.

Table 2

Results of FD phytoplasma transmission by *S. titanus*

Location Province	Phytoplasma detected on symptomatic grapevines used for acquisition	Phytoplasma transmitted (detected on inoculated plants)	Infected/ inoculated plants	Symptoms on infected plants
Soave Verona	FD 16SrV-D	FD 16SrV-D	1/4	+
Affi Verona	FD 16SrV-D	FD 16SrV-D AY 16SrI-B	1/4	+
Torreggia Padova	FD 16SrV-D	-	0/2	-
Montecchio Maggiore Vicenza	FD 16SrV-D	FD 16SrV-D	3/6 1/3 1/2 1/2 2/2 2/2	+ + + + + +
Farra di Soligo Treviso	FD 16SrV-C	FD 16SrV-C AY 16SrI-B	4/6 0/3	+ -

Yellowing, leaf rolling, lack of growth and of lignification, necrosis starting at the shoots and death were observed in 44.4 % of plants inoculated with *S. titanus* from Treviso and of plants inoculated with *S. titanus* from Verona and Vicenza. Symptoms of Chardonnay infected with either phytoplasma type were not different.

In three years 16/36 test plants exhibited typical symptoms, in particular 4 in the year of the inoculum and 12 in the following years; none of the 6 insect-free plants used as control was found to be positive in nested PCR analysis or showed phytoplasma-related symptoms.

Only in one case the presence of Bois noir (16SrXII-A) phytoplasmas was evident in one insect, but it was not found on test plants, confirming previous reports on the lack of transmissibility of this phytoplasma by *S. titanus*. In 1/36 test plants PCR and RFLP analyses showed the presence of 16SrI-B phytoplasmas (Tab. 2); this presence was detected on test plants only in the year of inoculation but no symptoms were associated with their presence. The tests on inoculated plants carried out twice, the year of inoculation and the following year, confirmed the presence of the two types of FD phytoplasmas (Tab. 2).

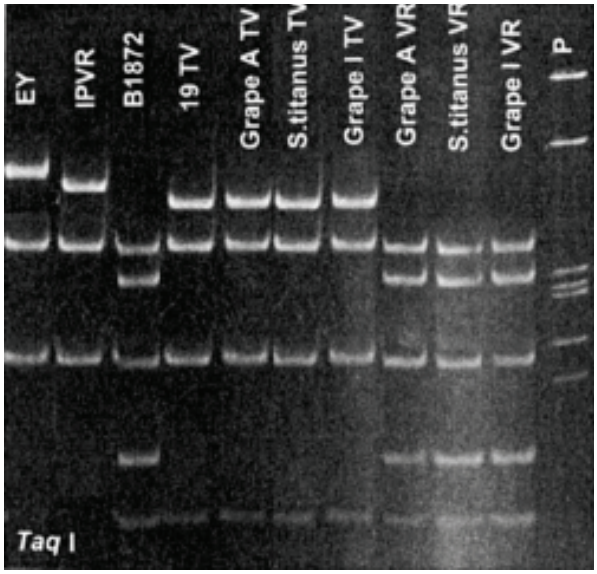

Figure: RFLP analyses of nucleic acid amplified with primers 16R758f/M23SR1804r from grapevine plants and from *S. titanus*. P, marker; EY, elm yellows (16SrV-A); IPVR, Italian periwinkle virescence (16SrXII-A), B1872 (16SrV-D) and 19TV (16SrV-C). TV, Treviso; VR, Verona. Grape A and *S. titanus* samples were collected respectively in Treviso and Verona vineyards; "Grape I" are patterns from micropropagated grapevine plants inoculated by *S. titanus*.

Discussion

The ability of *S. titanus* to transmit both 16SrV-C and 16SrV-D types of FD has been demonstrated. Carraro *et al.* (1994) used vector specificity of *S. titanus* to differentiate between the two phylogenetically widely related grapevine yellows FD and BN, showing the inability of this insect to transmit the latter phytoplasma; our data together with these of Martini *et al*. (1999) confirm this finding.

Based on the transmissibility by *S. titanus* it is possible to define both phytoplasmas 16SrV-C and 16SrV-D, as "Flavescence dorée" according to the resolutions of the

ICVG (Volos, Greece, 3-7 Sept. 1990). Vector specificity is not a valid criterion to differentiate between these closely related phytoplasmas, taking into consideration that several phytoplasma agents have a low vector specificity (BOSCO *et al*. 1997) and that is relevance to define the real biological properties of these pathogens is limited.

Some epidemiological informations indicate a different spreading speed of these two phytoplasmas in Northern Italy (MARTINI et al. 1999); it appears that 16SrV-D is transmitted more efficiently and over longer distances than the other type. More studies based on the transmission efficiency and comparative symptomatology on the same host plants are needed to elucidate the real bio-ecological differences between the two quarantine pathogen types.

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