

## Prevalence of aster yellows (AY) and elm yellows (EY) group phytoplasmas in symptomatic grapevines in three areas of northern Italy

by

P. A. BIANCO<sup>1)</sup>, R. E. DAVIS<sup>2)</sup>, P. CASATI<sup>1)</sup> and A. FORTUSINI<sup>1)</sup>

<sup>1)</sup> Centro C.N.R. per il Miglioramento Sanitario delle Colture Agrarie, c/o Istituto di Patologia Vegetale, Università di Milano, Italia

<sup>2)</sup> Molecular Plant Pathology Laboratory-ARS USDA, Beltsville, USA

**S u m m a r y :** Flavescence dorée (FD) and others important grapevine yellows (GY) diseases are known to occur in northern Italy. In this communication we report the results of an extended survey of naturally yellows diseased grapevines growing in three provinces of northern Italy, Vicenza, Brescia and Pavia. The plants were sampled at the end of June and the beginning of August. Direct PCR assay using group-specific primers for universal phytoplasma 16S rDNA amplification or for amplification of 16S rDNA from 16SrI (aster yellows and related phytoplasmas), 16SrIII (X-disease and related phytoplasmas) and 16SrV (elm yellows and related phytoplasmas) phytoplasma groups, and RFLP analysis of amplified DNA, were conducted. Phytoplasmas belonging to group 16SrV were detected only in grapevines in the Vicenza province; whereas, phytoplasmas belonging to group 16SrI subgroup G were found in grapevines in all three provinces.

**K e y w o r d s :** phytoplasma, mycoplasma, flavescence dorée, PCR.

### Introduction

The grapevine yellows disease known as flavescence dorée (FD) was first reported in France (CAUDWELL 1957) and in northern Italy (BELLI *et al.* 1973). Subsequently, similar diseases were reported in several countries including Germany (GÄRTEL 1965), USA (PEARSON *et al.* 1985) and Australia (MAGAREY *et al.* 1986). Where the diseases have been studied by electron microscopy, mycoplasma-like organisms (MLOs), now called phytoplasmas (SEARS and KIRKPATRICK 1994), have been found in symptomatic plants (CAUDWELL *et al.* 1971; MAGAREY *et al.* 1988; QUARONI *et al.* 1988; GRANATA and GRIMALDI 1991). So far, the inability to isolate and cultivate phytoplasmas in pure culture has hindered efforts to critically assess their presumed causal role in the disease, but the knowledge of grapevine yellows has progressed nevertheless. Using serology, phytoplasmas have been detected in the reported FD insect vector, *Scaphoideus titanus*, and in diseased grapevines, although the identity of the detected phytoplasmas was not determined (BOUDON-PADIEU *et al.* 1989; MAIXNER *et al.* 1993; CHEN *et al.* 1993). Currently, DNA-based techniques are being used extensively for both, detection and identification of phytoplasmas associated with grapevine yellows diseases. E.g., DAIRE *et al.* (1992) found that cloned FD phytoplasma DNA probes shared homology with DNA of several phytoplasmas including those associated with X-disease of stone fruits, ash yellows, and elm yellows diseases in the U.S.A..

Restriction fragment length polymorphism (RFLP) analyses using cloned DNA fragments from Italian periwinkle virescence (IPVR) phytoplasma led DAVIS *et al.*

(1992 a) to propose that no less than two distinct phytoplasmas may be involved in grapevine yellows in Italy. An extensive study by PRINCE *et al.* (1993) revealed that strains in the aster yellows (AY), elm yellows (EY), and X-disease phytoplasma groups were associated with grapevine yellows syndromes in different countries and identified a presumed agent of FD *sensu stricto* (as defined by ICSVG, ANONYMOUS 1990) as a member strain of the elm yellows phytoplasma 16S rRNA group (group 16SrV). Results by DAIRE *et al.* (1993 a, b) were consistent with these findings. Additional reports confirmed that diverse phytoplasmas are associated with grapevine yellows diseases (ALBANESE *et al.* 1996, PADOVAN *et al.* 1995, MAIXNER *et al.* 1996).

While enlarging our understanding of grapevine yellows, this new knowledge underscores the complexity of disease diagnosis, as data suggest that taxonomically diverse phytoplasmas may be involved. E.g., studies have indicated that a number of grapevine yellows-associated phytoplasma strains belong to the aster yellows strain cluster of 16S rRNA gene group 16SrI (aster yellows and related phytoplasmas, DAVIS *et al.* 1992 a) and that some of these strains are present in grapevines in regions where group 16SrV phytoplasmas also infect grapevines. To the group 16SrI strains belongs Italian periwinkle virescence phytoplasma strain G and other closely related strains that belong to a subgroup designated 16SrI-G by VIBIO *et al.* (1994) which also contains European stolbur phytoplasma (DAVIS and DALLY, unpublished). Previously, we reported that phytoplasmas in group and strains belonging to group 16SrV (elm yellows and related phytoplasmas) are present in grapevines with typical symptoms of FD in regions of

northern Italy. Some plants in Vicenza, for example, were doubly infected by strains belonging to both phytoplasma groups (BIANCO *et al.* 1993).

In 1993, a severe outbreak of the grapevine yellows disease exhibiting typical symptoms of FD was observed in several areas of northern Italy, particularly in the province of Vicenza. Since phytoplasmas belonging to at least two distinct taxonomic groups have previously been found in diseased grapevines in this province (BIANCO *et al.* 1993), we investigated the prevalence of various phytoplasma groups in the current epidemic. In the same year, we investigated phytoplasmas associated with grapevine yellows in two other provinces, Brescia and Pavia, and compared these findings with those in Vicenza. A preliminary report has been published (BIANCO *et al.* 1994).

### Materials and methods

Samples from 42 naturally diseased grapevines of different varieties (Barbera, Chardonnay, Pinot noir, Pinot gris, Garganega) exhibiting symptoms of grapevine yellows and from symptomless grapevines were collected in the provinces of Vicenza, Brescia and Pavia at the end of June 1993; at the beginning of August 1993, 24 of these grapevines were inspected again and samples were collected.

Reference strains of aster yellows (strain AY1), elm yellows (strain EY1), and X-disease phytoplasmas have been previously described (PRINCE *et al.* 1993). The reference strains were maintained by grafting on a white-flowered clone of periwinkle (*Catharanthus roseus* (L) Don) plants. Healthy grapevine seedlings were grown as control plants in a greenhouse in Beltsville, MD (USA).

DNA extractions from plant tissues and polymerase chain reactions (PCR) were carried out as previously described (BIANCO *et al.* 1993). Each reaction was primed by: (1) one of several oligonucleotide pairs r16SF2/r16SR2 (abbreviated F2/R2), which primes amplification of a 1.2 kb ribosomal (r) DNA fragment from all known phytoplasmas tested (LEE *et al.* 1993); (2) primer pair G35pm (DAVIS *et al.* 1992 b), which primes amplification of DNA from stolbur and various other strains in the same group; (3) ribosomal RNA gene group-specific primer pairs designed (LEE *et al.* 1994) for amplification of 16S rDNA from phytoplasmas affiliated with groups containing aster yellows (primer pair R16 [I] F1/R1), peach X-disease (primer pair R16 [III] F2/R1), or elm yellows (primer pair R16 [V] F1/R1) related phytoplasmas.

Digoxigenin labeled probes were used for Southern hybridizations of PCR products. Two probes, termed probe G35 (DAVIS *et al.* 1992), and probe 758 were used. Probe 758 was generated from 16S rDNA using primer pair 16R758/16R1232R (GIBB *et al.* 1995) in PCR. Both labeled probes were prepared as described earlier (BIANCO *et al.* 1993). Hybridization was conducted overnight at 42 °C after a pre-hybridization step of 1 h at the same temperature. Two washes at 52 °C were performed with 2 x SSC, 0.1 % SDS followed by successive two washes in 0.2 x SSC and 0.1 % SDS. Chemiluminescent visualization (Gen-

ius™ Boehringer Mannheim Corporation, Indianapolis, IN, USA) of hybridized probe was performed according to the manufacturer's instructions.

RFLP analyses using *Mse*I, *Kpn*I and *Alu*I were conducted by single enzyme digestions of products from PCR primed by R16[I]F1/R1 or R16[V]F1/R1; the fragments obtained were separated by electrophoresis through 5 % polyacrylamide gel, and visualized by staining with silver nitrate.

### Results

No evidence was obtained for the presence of phytoplasmas in all the tests conducted on samples from asymptomatic plants. However, nearly all samples collected from grapevines in June showing symptoms typical of those described as flavescence dorée and grapevine yellows diseases (BELLI *et al.* 1983) were found to be infected by phytoplasmas (Tab. 1). While no evidence was obtained for infection of grapevines by strains related to X-disease phytoplasmas (group 16SrIII), phytoplasmas related to elm yellows (group 16SrV), and aster yellows (group 16SrI) were detected and identified in symptomatic grapevines.

Analyses of samples collected in the field at the end of June 1993 showed that symptomatic grapevines largely contained phytoplasmas belonging to group 16SrI. Phytoplasmas belonging to the elm yellows group 16SrV were found only in grapevines from Vicenza province, where a considerable number of grapevines was infected by both, group 16SrI and group 16SrV strains (Tab. 1).

Table 1

Results from a survey of 42 symptomatic grapevines for the presence of phytoplasmas in three areas of northern Italy during June 1993

Area	Total number of plants	Number of plants			
		containing phytoplasma group indicated <sup>1)</sup>		negative for presence of phytoplasma	
		I	V	I + V	
Vicenza	21	3	11	5	2
Pavia	11	8	0	0	3
Brescia	10	9	0	0	1
Totals	42	20	11	5	6

<sup>1)</sup> I = group 16SrI (aster yellow and related phytoplasmas); V = group 16SrV (elm yellows and related phytoplasmas).

Of the 42 grapevine plants (Tab. 1), 24 were initially in June 1993 and again sampled at the beginning of August. Results from analyses of these 24 plants are summarized in Tab. 2. Group 16SrI strains were found in diseased grapevines in all three provinces, whereas group 16SrV strains were found only in grapevines in Vicenza (Tab. 2). Three grapevines apparently singly infected by group 16SrV strains at the end of June were infected by both group 16SrV and group 16SrI strains at the onset of August (Tab. 2).

Tab. 3 presents detailed data concerning the detection of different phytoplasmas in the grapevine samples noted in Tab. 2. Of all the oligonucleotide primer pairs used in PCR with template derived from samples in August, only G35pm primed sufficient amplification for detection of amplicons by gel electrophoresis and staining alone. In PCR using all other primer pairs, it was necessary to carry out DNA hybridizations after Southern blotting of PCR

products to detect amplicons (Tab. 3). In most cases, phytoplasmas were detected in grapevines exhibiting symptoms in June. Plants 20 and 21 were exceptions, since, no phytoplasmas were detected in June, in spite of the fact that these plants were symptomatic. In August, phytoplasmas were detected in all symptomatic plants sampled. Although phytoplasmas detected by PCR using primer pair G35pm belong to group 16SrI (DAVIS *et al.* 1992), it is in-

Table 2

Comparison of results from PCR tests with detected phytoplasmas in 24 symptomatic grapevine plants sampled in June and August 1993

Area	Number of plants sampled	Number of plants containing group I and/or group V phytoplasmas*)						Total of plants with symptoms of phytoplasmas	
		group I		group V		group I + V		June	August
		June	August	June	August	June	August		
Vicenza	7	2	2	5	2	0	3	7	7
Brescia	9	8	9	0	0	0	0	8	9
Pavia	8	5	8	0	0	0	0	5	8

\*) Group I: 16SrI; group V: 16rV.

Table 3

Location and cultivars where phytoplasmas were detected by use of PCR primed by different oligonucleotide pairs in symptomatic plants sampled in June and August 1993

Area	Plant number	Grapevine cultivars	Detection of phytoplasmas							
			F2/R2		G35pm		R16[I]		R16[V]	
			June	August	June	August	June	August	June	August
Vicenza	1	Pinot gris	+	+	-	+	-	-	+	+
	2	"	+	+	-	-	-	-	+	+
	3	Chardonnay	+	+	-	-	-	-	+	+
	4	"	+	-	+	+	+	+	-	-
	5	"	-	+	-	+	+	-	-	-
	6	"	+	+	-	+	-	-	+	+
	7	"	+	+	-	+	-	-	+	+
Brescia	8	Chardonnay	+	+	+	+	+	+	-	-
	9	"	-	+	-	+	+	+	-	-
	10	"	+	+	+	+	-	-	-	-
	11	"	-	+	-	+	+	-	-	-
	12	"	+	+	+	+	+	-	-	-
	13	"	+	+	+	+	+	-	-	-
	14	"	-	+	-	+	+	+	-	-
	15	"	+	+	+	+	+	+	-	-
	16	"	+	+	+	+	+	+	-	-
Pavia	17	Chardonnay	+	+	-	+	+	+	-	-
	18	"	+	+	+	+	+	+	-	-
	19	"	+	+	-	+	-	-	-	-
	20	"	-	+	-	+	-	+	-	-
	21	"	-	+	-	+	-	+	-	-
	22	"	+	+	+	+	+	+	-	-
	23	"	+	+	+	+	-	+	-	-
	24	"	+	+	+	+	-	+	-	-

teresting that in several cases phytoplasmas detected using G35pm were not detected in PCR using group 16SrI-specific primer pair R16F1/R1. This may be explained by a different efficiency of priming of DNA amplification by these two oligonucleotide pairs. In plant samples 5, 9, 14, and 17, however, phytoplasmas were detected in June using primer pair R16F1/R1 but not using G35pm; this result is attributed to the presence of group 16SrI phytoplasmas that were different from those detectable using primer pair G35pm.

Moreover, it was possible to detect phytoplasmas in 85 % of symptomatic plants in June, while in August we detected phytoplasmas in 100 % of symptomatic plants.

### Discussion

We report the detection and identification of phytoplasmas infecting grapevines with symptoms characteristic of flavescente dorée (FD) and grapevine yellows in the provinces of Vicenza, Pavia, and Brescia in northern Italy. These results agree with a previous report of phytoplasmas similarly infecting diseased grapevines in Vicenza by BIANCO *et al.* (1993); they also reported such infections to occur in Lombardia (northern Italy). In these investigations, we have detected phytoplasma infections in several symptomatic grapevine cultivars, including Chardonnay, Barbera, Garganega, Pinot noir, and Pinot gris.

It is interesting that the symptoms observed in all diseased grapevine cultivars in our present and earlier study (BIANCO *et al.* 1993) were those characteristic of the grapevine yellows disease known as FD. Although PRINCE *et al.* (1993) have shown that a reference strain of the presumed causal agent of so called FD *sensu stricto* (CAUDWELL 1993) is a member of group 16SrV (elm yellows and related phytoplasmas), our work has confirmed that the grapevines with typical FD symptoms may not be infected by phytoplasmas in that group. Instead, our results indicate a broad prevalence of symptomatic grapevine infections by strains belonging to group 16SrI phytoplasma (aster yellows and related phytoplasmas).

It is not possible to diagnose FD on the basis of symptoms alone, because this disease is defined on the basis of transmission of its causal agent by an insect, specifically *S. titanus* (ANONYMOUS 1990). Since the pathogen of FD *sensu stricto* has been identified as a member of group 16SrV (elm yellows and related phytoplasmas) (PRINCE *et al.* 1993), it appears that symptomatic grapevines studied during 1993 in the provinces of Brescia and Pavia did not represent cases of FD *sensu stricto*. We suggest that these grapevines were affected by grapevine yellows, probably induced by strains in group 16SrI, subgroup 16SrI-G, because the RFLP patterns of DNA amplified from the phytoplasmas detected in these plants were indistinguishable from those observed for Italian periwinkle virescence (Figure) and Stolbur phytoplasmas, which are members of subgroup 16SrI-G (DAVIS and DALLY, unpubl.). It may be important to investigate possible transmission of subgroup

16SrI-G phytoplasmas between grapevine and known hosts of Stolbur phytoplasma in Italy in the light of related work in Germany (MAIXNER *et al.* 1995).

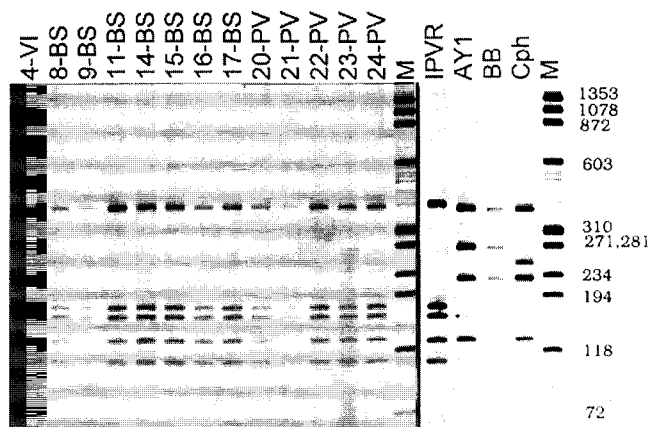


Figure: RFLP patterns obtained after *Mse*I digestions of amplified 16S rDNA amplified in PCR primed by oligonucleotide primer pairs specific for amplification of 16SrI phytoplasma DNA. Amplified DNA was from 13 grapevine samples collected in the field in August 1993. Numbers on top indicate grapevine samples reported in Tab. 3. Lanes IPVR, AY1, BB and Cph correspond to 16SrI digested fragments amplified from the following phytoplasma subgroup reference strains: Italian periwinkle (IPVR), aster yellows 1 (AY1), tomato big bud (BB) and clover phyllody (Cph). Lanes M: molecular weight marker ( $\rightarrow$ X RF 174 DNA digested by *Hae* III).

The finding of grapevines doubly infected with group 16SrI and group 16SrV strains is consistent with a previous report of such mixed infection (BIANCO *et al.* 1993). It is conceivable that symptoms in doubly infected grapevines may be due to strains belonging to both phytoplasma groups, without evident synergism between the strains.

The high percentage of group 16SrI phytoplasma detection would be consistent with a significant role of phytoplasmas in this group in the etiology of the FD-like symptoms. In Vicenza, however, group 16SrV may be responsible for early symptoms in some plants, since group 16SrV and not group 16SrI strains was found in such symptomatic plants in June. Group 16SrI might have played a role in disease development in these plants later, because by the beginning of August, most symptomatic plants (Tab. 3: sample nos. 1, 6 and 7) containing group 16SrV were also infected by group 16SrI strains. It is interesting that data from PCR using primer pair G35pm showed a higher frequency of phytoplasma detection in grapevine plants assayed in August than in June, possibly reflecting an increase in phytoplasma titers in infected plants or spread of phytoplasma group 16SrI strains in the vineyards.

In June, a phytoplasma was found in plant 19 but group 16SrI, 16SrIII or 16SrV phytoplasmas were not detected (Tab. 3).

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