THE SITUATION OF GRAPEVINE YELLOWS AND CURRENT RESEARCH DIRECTIONS: DISTRIBUTION, DIVERSITY, VECTORS, DIFFUSION AND CONTROL

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Grapevine yellows (GY) are known now for 50 years. After the first appearance of Flavescence dorée (FD) in West-South France in the 1950's, similar diseases have been observed in vineyards of other regions or countries (22) in Europe, North-America, Asia Minor and Australia. Typical symptoms are leaf rolling and discoloration of veins and laminae, uneven or total lack of lignification of canes, flower abortion or berry withering. Eventually, severe decline and death occur with sensitive varieties or with particular GY diseases. All these diseases have been associated with phytoplasmas.

Phytoplasmas, discovered in 1967, are wall-less intracellular bacterias restricted to phloem sieve tubes and transmitted only by vector insects in which they multiply and circulate. Recently, comparisons of conserved regions in their genomic DNA, have permitted to classify all known phytoplasmas into about 20 groups and subgroups within a monophyletic clade in the Class Mollicutes, closest to the *Acholeplasma* clade (57, 78). Numerous DNA probes have been designed that permit diagnosis and identification of phytoplasmas in plant tissues and in insects. This, together with transmission assays, has also permitted the recent identification of new phytoplasma vectors.

Though Koch's postulate cannot be fully satisfied with non-culturable pathogen agents, it is now considered that phytoplasmas are responsible for typical GY symptoms. These conclusions have been reached because of transmission experiments with natural vectors in the case of Flavescence dorée (FD) and Bois noir (BN), of the similarity of symptoms caused world wide by GY diseases on numerous grapevine cultivars and of consistent detection of phytoplasmas in affected grapevines and in infective insect vectors. Other symptoms have been associated to phytoplasmas, such as Restricted growth (RG) or Late season leaf curl (LSLC) in Australia, and symptoms resembling GY in Tunisia, but the role of phytoplasmas is these diseases is not clear (28, 33, 49).

When the taxonomic position of insect and plant hosts are compared to phylogenetic trees of phytoplasmas, it is obvious that the latter are more related to their vectors than to their host plants. For example, phytoplasmas belonging to very different groups can be responsible for similar diseases in a given plant species. By contrast, phytoplasmas transmitted to different host plants by insects of the same family, are often classified in the same group or subgroup. This has been confirmed for GY phytoplasmas. Table 1 shows the present knowledge on etiology and vectors of GY diseases world wide.

The evolutive relationships between phytoplasma and their vector has very important consequences on the epidemiology of diseases. Phytoplasmas transmitted by polyphage insects may infect numerous plants representing important, wide spread and possibly discrete reservoirs. Instead, a mono- or oligophage insect will transmit phytoplasmas that are restricted to a few plant genera or families. It can also be assumed that the incidence and severity of symptoms also depend not only on the importance and infestation of vector populations but also on the number of inoculative events, i.e., of attractiveness of the plant. Hence, incidence and severity of diseases should be higher on natural host-plants of vectors than on plants which are only probed by occasional visiting insects.

The study of phytoplasma genome is difficult because these organism are not cultured. One of the main hitch in this field is the preparation of enough phytoplasma cells from natural hosts. Tanne *et al.* (83) have been using the approach of purification of phytoplasma DNA from the insect saliva of vectors fed through Parafilm on artificial diet (82). Important fragments of DNA could be cloned to build a library into which structural genes have been identified. Purification of whole size chromosomes with pulse field gel electrophoresis (PFGE) from high titre infected hosts is another approach (31).

Detection of phytoplasma in grapevine and vectors

Numerous improvements have been developed in molecular methods to insure a more reliable diagnosis and an easy characterization of GY phytoplasmas (6, 10, 14, 30, 33, 45, 47, 54, 65, 70, 73). Main hitches are the low titre of phytoplasma, the distribution in the plant depending on season and year (33), the presence in grapevine tissues of inhibitors of the Polymerase chain reaction (PCR) (10, 54), and also the use of poly-specific tools to detect one or the other of the main GY phytoplasma occurring in the same region. As one of the important point is quick identification of FD or BN phytoplasma, efforts have been devoted to the simultaneous detection of phytoplasma belonging to the EY group (FD and PGY in grapevine) and to the stolbur group (Bois noir / Vergilbungskrankheit / Legno nero) (30). Preliminary results have been obtained with microarrays of DNA probes (45) or TaqMan® assays (14).

Transmission to feeding medium (82) instead of plant inoculation is an alternative to transmission trials to demonstrate the role in diffusion of the disease, of species showing wild specimen testing phytoplasma-positive. Its efficiency was assessed for several vectors or potential vectors of grapevine phytoplasma in Germany (48).

Flavescence dorée (FD)

Diffusion. Flavescence dorée is a quarantine organism in the European Community. It has spread actively during the last few years in France and Italy in spite of mandatory regulations for control, eradication and protection of mother plants. Disease declaration and detailed control measures are mandatory in France since 1987 and in Italy since 2000. An important outbreak has occurred in France in Savoie in 2000 and is still developing (20). In Italy, most of Northern provinces are now affected (11, 15, 17, 25, 27, 36, 37). The disease is clearly linked to the presence of its only known vector, *S. titanus*,

which occupies a large climatic area in Europe (1, 18, 75, 77, 79, 81). Reported recently in the north of Portugal (75), it has been found also at a similar southern latitude in Umbria (Italy) (77). To the north, it has now settled in North Burgundy (France) (20). Great concern is given to the possible outbreak of new foci (80), especially where Bois noir is present, because symptoms of the two diseases can be mistaken.

Genomic and biological diversity of FD phytoplasma strains. The diversity reported earlier among strains of FD (13, 39), has been explored (6, 7, 64). It has been shown that all strains of FD were more similar to one another than to other phytoplasma strains belonging to the same group (Elm yellows related, EY-group or 16SrV). Within FD strains, one strain was identical in France and Italy (FD92 = FD-D) for all characters observed and this strain appeared to be more widespread that the others. It was also found in Spain (39). Another strain, FD-C, not found in France, was the more diverging among FD strains. It was detected in several Italian regions (17, 37, 63, 64) and transmission by *S. titanus* was successful (68). It was detected in grapevine in Serbia but not *S.titanus* (41). Moreover, FD-C was identified in wild Clematis in the vicinity of an affected vineyard in Treviso (Italy) (8). More work is necessary to verify if *S. titanus* might be involved in its transmission from *Clematis* to grapevine. It is also very interesting that an Italian alder phytoplasma, ALY, was shown to be very close to FD *sensu stricto* (6, 7, 64), and that several closely related strains of alder phytoplasma have been shown to infect grapevine in Germany and to be transmitted to grapevine by the alder leafhopper *Oncopsis alni* (Macropsinae) (61). The genomic diversity of FD strains is also under investigation through comparison of whole size chromosomes (31).

Table 1. GY diseases, associated phytoplasmas, known vectors and host plants

GY disease	phytoplasma name	ribosomal group (and subgroup)	known insect vector to grapevine	preferred host plants of vector	Occurrence	Other natural vectors of phytoplasma in the same group
Flavescence dorée	FD	EY or 16SrV (-C, -D)	*Scaphoideus titanus* Ball	*Vitis* sp	France, Italy, Spain	
Palatinate Grapevine Yellows	PGY	EY or 16SrV	*Oncopsis alni* Schrank	*Alnus glutinosa* L.	Germany	*S. luteolus* (A) *Macropsis* sp (E).
Bois noir, Legno nero, Vergilbungs-krankheit	stolbur	stolbur or 16SrXII-A	*Hyalesthes obsoletus* Sign	*Convolvulus arvensis* L. *Urtica dioica* L. *Ranunculus*, *Solanum Lavandula*	Europe, Israel, Lebanon	*Pentastiridius* sp. (Cixiidae) (E) (*Macrosteles quadripunc-tulatus*)
Australian grapevine yellows	*Candidatus* Phytoplasma australiense	stolbur or 16SrXII-B	ND	ND	Australia	*Oliarius arkinsoni* (Cixiidae) (NZ)
Australian grapevine yellows	Tomato big bud	FBP or 16SrII	ND	ND	Australia	*Oriosus argentatus* (Aus)
Buckland valley grapevine yellows	BVGY	AY or 16SrI-related	ND	ND	Australia	
Grapevine yellows	aster yellows	AY or 16SrI-A	ND	ND	Italy	*Euscelis*, *Dalbulus Macrosteles*
Nth-American grapevine yellows	Western X	W-X or 16SrIII-I	ND	ND	New York, Virginia	*Colladonus*

Bois noir (BN) / Vergilbungskrankheit (VK) / Legno nero (LN)

Etiology and epidemiology. GY diseases associated to a stolbur (16SrXII) phytoplasma are distributed in all countries of Europe and in Asia Minor (21). They have been called by different names in France, Germany and Italy, but no or minor differences have been demonstrated up to now between stolbur phytoplasma strains, especially in grapevine (39, 70, 78). However, Langer *et al.* (54) have shown differentiation of stolbur strains in Germany, where the incidence of VK can be very high and new foci appear in some vineyards on the slope of the valley of rivers, due to the favorable temperature conditions for the vector *Hyalesthes obsoletus* (40). Interestingly, the authors could relate three strains detected in grapevine and in specimen of the vector *Hyalesthes obsoletus*, to three different host plants of the vector, i.e. *Convolvulus arvensis*, *Urtica dioica* and *Calystegia sepium*. Another Cixiid species has been shown to be a vector in France of stolbur phytoplasma to sugar beet (46) and *Urtica dioica* was also a host plant for Stolbur phytoplasma in the vicinity.

Numerous reports have confirmed the presence of the LN disease in all regions of Italy (4, 15, 25, 29, 36, 62), but also in Switzerland, Slovenia, Croatia, Spain, Israel (38, 51, 70, 80) and Lebanon (Boudon-Padieu, unpublished). In Piemonte (Italy), the role of *H. obsoletus* in transmission of LN has been confirmed (3) and *U. dioica* was also characterized as the main host plant of phytoplasma and vector. In Spain, *H. obsoletus* was eventually found, but populations of the species were

scarce in some vineyards affected with BN (9). *Macrosteles quadrinotatus* was shown to be an efficient vector of stolbur phytoplasma to several plant species, but experimental transmission to grapevine was not achieved (5).

Symptom expression. The expression of symptoms of BN fluctuates on consecutive years (56, 59, 71). A measure of its incidence and of the frequency of potential vectors suggest a relationship to rootstock varieties in Israel (76).

Australian grapevine yellows

Three different phytoplasmas have been associated to GYs in Australia (Table 1) (32, 34, 49, 52). Australian grapevine yellows (AGY) phytoplasma, related to stolbur phytoplasma and Tomato Big Bud (TBB) phytoplasma occurred in the same regions. The Buckland valley grapevine yellows phytoplasma (BVGY), related to AY but possibly forming a new group, was restricted to a limited region and could be clearly associated with GY symptoms. Epidemiological observations suggested that infection with BGVY resulted from aerial transmission rather than from infected planting material (35), however no potential vector has been identified. A cixiid planthopper, *Oliarus atkinsoni*, is a vector of Phormium yellow leaf phytoplasma (similar or identical to AGY phytoplasma) in New Zealand.

Other grapevine yellows

Phytoplasma of the W-X (16SrIII) group have been reported the USA and in Israel.

Phytoplasma in the 16SrI group are consistently detected in grapevine in Italy and Israel and sometimes in dual infections with another phytoplasma, but no evidence of epidemic spraying of such phytoplasma in grapevine has been reported. Recently, grapevines with symptoms of GY in several regions of Chile have been shown to contain phytoplasmas belonging to diverse groups. AY group (16SrI - subgroup C) was the more frequent (44). It must be reminded that phytoplasmas in the AY group are the more numerous (57, 78) and very ubiquitous, with many reported vectors. Erratic transmission of such phytoplasmas to grapevine might occur with no significant pathological or epidemiological consequences. Such diseases should nevertheless be surveyed in case of important change in their epidemiological factors.

It was reported that four leafhopper species can be experimental vectors of an AY phytoplasma to grapevine (2). *S. titanus* was one of the latter species, confirming previous French experimental data. However, AY phytoplasma have never been found naturally infecting *S. titanus*, which remains the more important phytoplasma vector leafhopper species living on grapevine. Search for potential phytoplasma vectors in vineyards have been reported (23, 50, 53)

Control and prophylaxis

Control of vectors

Numerous insecticides have been registered to control *S. titanus* (18, 74). Insecticide treatments against *S. titanus* are designed according to the biology of the insect and the biology of phytoplasma transmission. Since a latency of one month is necessary for the movement of phytoplasma from the gut after acquisition to the salivary glands, a first treatment is applied 30 days at latest after the first day of hatching (beginning of May) in the vineyard. Other treatments are applied according to the remanence of specialties and the importance of populations (18).

The natural enemies fauna of *S. titanus* was searched in New York in 2001 and 2002. Three different kinds of parasitoids for larvae and eggs were identified, belonging to several species of Hymenoptera and Diptera. Specimen have been introduced in France (INRA, Antibes) to be reared in a quarantine laboratory and to check their efficiency on reared *S. titanus.* First results have been published (69).

Prognosis and integrated management are another alternative to chemical control (24, 60). Control of *H. obsoletus* is not possible with insecticides because the species is ubiquitous. Langer *et al* (55) have compared the incidence of the species in organic and conventional vineyards. A control strategy is focusing on cultural practices in the vineyard and soil coverage by competitive plants to suppress *C. arvensis*.

Plant-phytoplasma interactions.

The suppression of symptom expression on stolbur-infected tobaccos with elicitins has been reported (58). It provide a way to study defense reactions of plants towards phytoplasma and eventually to control phytoplasma diseases.

Recovery is a complex phenomenon (26, 71, 72) that has been used in France for the sanitation of FD-affected vineyards. This is no longer used because FD-diseased grapevines are uprooted to suppress important reservoirs (20, 21, 42). It is not known if recovered plants still contain infective phytoplasma, though acquisition by *S. titanus* was not possible (26).

The effect of winter pruning in the reduction of FD has been checked (85). Observations should be conducted for several years, since phytoplasma are present in the roots of grapevine, even in the absence of symptoms (33).

Sanitation of dormant planting material with hot water treatment. Caudwell *et al* (1990) have proposed curing of phytoplasma infected plant material with soaking into hot water (19). Numerous assays have been done in France and Italy, using a variety of cultivars of *V. vinifera* and rootstock (12, 16, 43, 66, 67, 84) to check the efficient conditions of hot water treatment for curing FD- and BN-infected material, and the effect of treatment on survival and growth of the material. Numerous data confirmed that a 50 °C - 45 mn treatment was efficient and that a careful application on standard quality planting material is not detrimental (84).

Conclusion

Numerous questions on GY diseases remain unsolved, because biology, genomic and speciation of phytoplasmas remain largely unknown. FD remains the more important and dangerous GY. The incidence of BN (VK / LN) seems to be increasing in several regions. Generally speaking, phytoplasma diseases are emerging diseases that should be watched and studied carefully because of epidemiological risks due new farming practice or to introduction of exotic phytoplasma or vectors. The diversity and variability of phytoplasma genome and the basis for specific transmission by vectors should be

studied thoroughly. Molecular epidemiology should bring new information on the natural emergence and diffusion of diseases. Hot water treatment should be applied to exchanged material. International procedures for diagnosis and a common certification scheme to prevent propagation of infected material, should be established.

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CONSTRUCTION OF PHYTOPLASMAL GENOMIC LIBRARIES AND CHARACTERIZATION OF PHYTOPLASMAL GENES

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Phytoplasmas are unculturable, insect-transmissible, plant pathogens belonging to the class Mollicutes. To be transmitted, the phytoplasmas replicate in the insect body and are delivered to the insect's salivary glands, from where they are injected into the recipient plant (2). Because phytoplasma cannot be cultured, any attempt to recover phytoplasmal DNA from infected plants or insects has resulted in preparations with a large background of host DNA (1). Thus, studies of the phytoplasmal genome have been greatly hampered, and aside from the rRNA genes, only a few genes have hitherto been isolated and characterized.

In order to get rid of the host background in phytoplasma preparations, we devised a method of collecting insect saliva, and showed, by PCR analysis of rRNA genes, that phytoplasmal DNA can be easily detected in that saliva (4). Based on this method, we collected DNA from insect feeding medium, cloned and analyzed it. For translation of the sequences we used two codon usage tables: the general codon usage table and the mycoplasmas-mitochondril codon usage table in which the stop codon UGA is read as tryptophan (3). Applying the criteria for ORF definition as described below, we concluded that phytoplasma read UGA as tryptophan and best ORF designation was obtained through the mycoplasmas-mitochondria codon usage table. Our study presents 32 Clones in total length of 32,753 bases, 27,407 bases out of them are ORFs which show high gene density (83.7%). Most of the clones have low G:C content (under 35%). Clones were also tested in order to ascertain that the aforedescribed clones were of phytoplasmal origin and did not represent host sequences of plants or insects. Genomic DNAs or RNAs of non-infected, and infected plants, were subjected to Southern or northern blot analysis using several inserts of selected clones as probes. In both analyses, the probes reacted with DNA from infected plants and not with non-infected plants. To further assess the specificity of the phytoplasmal DNA inserts, primers were generated from different clones and used for PCR analysis of genomic phytoplasma-infested insects and phytoplasma-free insects. In all tested cases, PCR products were obtained only with DNA extracted from phytoplasma-infested insects. Most Blastx analysis of our clones showed homology to ribosomal proteins, enzymes and factors involved in protein synthesis, rRNA processing, tRNA acylation, enzymes involved in energy metabolism, components of RNA and DNA polymerases, and enzymes involved in DNA modification and integration. Our method for isolating background-free phytoplasmal DNA enabled the construction of phytoplasmal genomic libraries and demonstrated the ability to systematically analyze phytoplasmal genes. The usefulness of the method is demonstrated by its almost tripling the volume of phytoplasmal gene information in a short time.

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IDENTIFICATION OF PHYTOPLASMAS INFECTING GRAPEVINE BY LIGASE DETECTION REACTION AND UNIVERSAL ARRAY

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Grapevine yellows is a complex of diseases caused by phytoplasmas and transmitted by insect vectors: the most dangerous form of GY, Flavescence dorée (FD), is so far reported only in some regions of France, Italy and Spain. FD is efficiently transmitted by *Scaphoideus titanus* and is caused by phytoplasmas belonging to 16SrV group. For this reason, FD has been included in several quarantine and sanitary selection programs in order to exclude its presence from grapevine certified propagating material. Other yellows, as Bois noir (BN), are known to affect grapevine but genetically distinct phytoplasmas are associated with the diseases (2, 4) and their spread in field is very slow. Nevertheless, symptoms are identical to those observed on FD affected vines. Therefore, the need of suitable tests for faster, specific and reliable detection of phytoplasmas in grapevine is becoming urgent.

The present work aims to evaluate the potential for Microarray technology as phytoplasma detectors and to develop a fast and reliable diagnostic tool for the identification of phytoplasmas infecting grapevine.

The procedure we used is based on the Universal DNA Microarray approach(5). This relies on the discriminative properties of the DNA ligation reaction and requires the design of two probes, specific for each target sequence.

Samples of leaves were collected in September 2002 from 6 different grapevine plants showing typical symptoms of yellows. Total DNA was extract from grapevine and from periwinkle plants (grown in greenhouse) infected by phytoplasmas belonging to genetically distinct phytoplasmas (2, 3, 4).

Six probes have been designed: two specific sequences for phytoplasma group 16SrXII (Stol1 and Stol2) while three probes were specific for group 16SrV. A universal probe, matching with all the known phytoplasmas in the 16SrDNA sequences, has been designed and named PhytoUniv894.

All the samples from infected plants reacted positively with the probe designed for detection of all the phytoplasma known. Specific detection was obtained with probes Stol1 and Stol2 with grapevine affected by Bois noir (BN) disease Also, among the probes designed for detection of phytoplasmas belonging to 16SrV group, ULW1 and FD1 gave poorer values with EY reference. On the other hand, the probe FD2, designed for the detection of phytoplasmas associated with FD (1, 3, 7, 8) showed higher specificity since this probe reacted positively only with grapevine samples infected by phytoplasma of the subgroup 16SrV-C and 16SrV-D.

The present work shows the feasibility of the specific detection and identification, using Microarray technology, of phytoplasmas in grapevine affected by two different diseases of crucial importance for the viticulture. In particular we obtained the detection of single base mutation in the 16SrDNA sequence: the importance of such SNPs (single nucleotide polymorphisms) is related to the possibility to identify specific phytoplasmas associated with different diseases and, in this case, allow to discriminate the agents of FD from the phytoplasma associated with Bois noir.

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REAL-TIME PCR DETECTION OF BOIS NOIR AND FLAVESCENCE DORÉE FROM FIELD COLLECTED SYMPTOMATIC GRAPEVINES

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Phytoplasma-induced grapevine yellows diseases (GYs) can be etiologically different but symptomatically similar. Phytoplasmas of diverse 16SrDNA RFLP subgroup affiliations have been associated, in single or mixed infection, with GY-affected grapevines worldwide. Bois noir (BN) in France and the equivalent disorders in central Europe and the whole Mediterranean Basin are consistently associated with XII-A subgroup members (stolbur) (1). BN phytoplasmas are transmitted to grapevine in nature by the cixiid planthopper *Hyalesthes obsoletus* Signoret (2). Flavescence dorée (FD) was originally described in southern France, and is now also reported from northern Spain and Italy. It is caused by phytoplasmas of subgroups V-C and –D. The natural vector is the monophagous grapevine-limited leafhopper *Scaphoideus titanus* Ball (3). FD represents perhaps the biggest threat to viticulture and is one of the most dreaded diseases for quarantine in Europe. Phytoplasmas are generally present in low concentration and randomly distributed in the phloem of the host plant and their detection is often difficult. Diagnosis of FD and BN is routinely performed by PCR. Total DNA is extracted from leaf veins of symptomatic plants and then amplified with universal phytoplasma-specific primer pairs. Phytoplasma titer in the plant host is often below the detection limit of the PCR technique. False negative results from symptomatic grapevines can be reduced following different protocols: nested PCR and serological or molecular detection of PCR products. These techniques, when applied to the screening of high number of grapevines, although very sensitive are also highly time-consuming. Quicker and reliable diagnostic methods are therefore needed for mass screening. To this purpose, we have designed specific primers to detect BN and FD in real-time PCR. Developed in the mid 1990s for the analysis and quantification of nucleic acids, real-time PCR is a technique gaining rapidly in popularity.

For the diagnosis of BN, primers (StolFw1/ StolRev1) have been designed on a stolbur-specific non-ribosomal sequence (4). The specificity of these primers was evaluated in traditional PCRs using periwinkle-maintained DNA of phytoplasmas belonging to different 16SrDNA groups. Real-time PCR conditions were then designed for optimal detection of BN. Total DNA from grapevines collected in 2001 and 2002 was used as template DNA in both standard and real-time PCRs. BN infection was first evaluated in each sample by nested amplification with phytoplasma-specific universal primers and then group-specific ribosomal ones, followed by RFLP analyses of the amplicons. BN was then detected by real-time PCR with primers StolFw1/ StolRev1. PCR products were detected by staining with SYBR Green I (BioRad). Specificity of the PCR was confirmed by gel electrophoresis of the amplicon and by melting curve analyses of each amplicon together with a plasmid control.

For the diagnosis of FD a group V-specific primer pair, directed to 16S rDNA sequences (fAY/rEY; 5), was used. These primers detect FD in traditional PCR approaches with high sensitivity and specificity using total DNA extracts from field-collected grapevines and insect vectors (6). Real-time PCR conditions were designed for optimal detection of FD. Total DNA from grapevines collected in 2001 and 2002 was used as template DNA in both standard and real-time PCRs. FD infection was first evaluated in each sample by nested amplification with phytoplasma-specific universal primers followed by group-specific ribosomal ones. FD was then detected by real-time PCR with primers fAY/rEY. PCR products were detected by staining with SYBR Green I (BioRad). Specificity of the PCR was confirmed by gel electrophoresis of the amplicon and by melting curve analysis of each amplicon together with a plasmid control.

The specificity of PCR products was documented with gel electrophoresis and resulted in a single products with the desired length for each system. Melting curve analysis of the obtained amplicons also resulted in single product specific melting temperatures for each system. Preliminary results have shown that StolFw1/StolRev1 could specifically detect stolbur phytoplasma among other periwinkle-maintained 16SrDNA phytoplasma groups in traditional PCR. StolFw1/StolRev1 could be used in real-time PCR for the detection of BN using as templates DNA from symptomatic field-collected grapevines with a good degree of sensitivity. Preliminary results have indicated that fAY/rEY primer pair could be used for the specific detection of FD in real-time approach, although further work is needed to improve the sensitivity of the system.

Both primer pairs have also been tested for the diagnosis of BN and FD in real-time PCRs from field-collected *H. obsoletus* and *S. titanus* with high efficiency and specificity (7).

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BIOLOGY AND EPIDEMIOLOGY OF AUSTRALIAN GRAPEVINE PHYTOPLASMAS

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Grapevine yellows symptoms were first reported in 1976 in Australia and the disease referred to as Australian Grapevine yellows (AGY) (10). Although Koch's postulates have not been fulfilled, phytoplasmas are considered to be the most likely cause of AGY. Various techniques have confirmed an association between phytoplasmas and AGY (4, 6, 9, 13, 14, 16). The disease and associated phytoplasmas are found in many viticultural regions of Australia (3; 13). AGY occurs more often in the warmer inland districts of Sunraysia in New South Wales and Victoria, Riverina in New South Wales and the Riverland in South Australia compared to other viticultural regions. Chardonnay and Riesling appear to be affected more often than other varieties (12). Three different phytoplasmas have been associated with AGY and include the AGY phytoplasma, tomato big bud (TBB) phytoplasma and the Buckland Valley grapevine yellows (BVGY) phytoplasma (7, 9). AGY phytoplasma is more frequently detected in grapevines compared to TBB phytoplasma (6; 9). BVGY phytoplasma has only been found in two vineyards in the Buckland Valley grape growing region of Victoria (8).

Polymerase chain reaction (PCR) to detect phytoplasmas in AGY affected shoots was most reliable in summer, when the symptom expression is also highest (9). Recently, research has shown that AGY and TBB phytoplasmas could be detected using the polymerase chain reaction (PCR) from shoots, cordons, trunks and roots throughout the year and that phytoplasmas appear to infect Australian grapevines persistently from year to year (6). However, phytoplasmas were not always detected in samples from the same part of the grapevine from one sampling period to the next. PCR detection of phytoplasmas was improved by sampling from shoots, cordons and trunks and spring was the best time to test for phytoplasmas when these three tissue types were sampled.

Alternative hosts for the AGY phytoplasma have not been consistently identified within or around AGY disease affected vineyards. No insect vector has been reported for the AGY phytoplasma. TBB phytoplasma has many plant hosts. In other crops, *Orosius argentatus* (Evans) is a vector of TBB phytoplasma (15). Studies have shown that TBB phytoplasma can be acquired from grapevine by *O. argentatus* and subsequently transmitted to *Vicia faba*, but the ability of the leafhopper to transmit TBB phytoplasma back to grapevines has not been confirmed (1). Surveys, using PCR for detection of TBB phytoplasma, have shown that the incidence of inoculative *O. argentatus* is low within Australian vineyards (1) Evidence suggests aerial transmission of the BVGY phytoplasma but no insect vector has been identified (8).

Other diseases thought to have a possible association with phytoplasmas include restricted growth (RG) and late season leaf curl (2; 9, 16). Phytoplasmas and diseases with a real or putative association with phytoplasmas, especially RG, are of considerable concern to the Australian viticulture industry because of reduced grapevine health and potential yield loss. No significant association between phytoplasmas and shoots of grapevines affected by RG alone has been shown (4; 9, 16). However in another study, phytoplasmas were more frequently detected in grapevines that had displayed both AGY and RG symptoms compared to grapevines displaying AGY alone (6). The association between phytoplasmas and LSLC is unclear. In one study phytoplasmas were detected in 48/59 shoot samples affected by LSLC (late AGY; 4) but in another study phytoplasmas were detected in 12/126 LSLC affected shoot samples (9). An additional study showed that phytoplasmas were detected more frequently in grapevines affected by LSLC and AGY compared to grapevines affected by either disease alone (6).

Surveys have also been conducted in several Australian vineyards to determine the incidence and possible association between AGY, RG and LSLC. From observations in one vineyard over three years it was proposed that AGY, RG and LSLC diseases were related and that LSLC was followed by the presence of AGY in the same grapevines in following years (2). It was also proposed that AGY often led to the presence of RG in subsequent years. A recent analysis of surveys conducted in four Chardonnay vineyards for three to six years indicated that while some grapevines exhibited a combination of AGY and RG or AGY and LSLC, both RG and LSLC can occur independently of AGY (5). Statistical analyses using Log-linear models also indicated that RG and LSLC were not always associated with AGY. Thus, it is possible that phytoplasmas are not the cause of RG or LSLC and their association is coincidental. All three diseases were characterised by remission of disease in some grapevines, recurrence of disease in other grapevines and new observations of disease in previously unaffected grapevines in each of the Chardonnay vineyards in each year. The pattern of temporal incidence of each disease was different between each of the Chardonnay vineyards for the survey period. A survey for AGY, RG and LSLC was also conducted in one Shiraz vineyard for three years (5). Expression of AGY in Shiraz grapevines occurred later in the season compared to Chardonnay. Very little recurrence of AGY was observed in the Shiraz grapevines indicating that the variety Shiraz responds differently to phytoplasma infections, assuming that AGY in Shiraz is a phytoplasma caused disease. RG and LSLC were not observed on any grapevine in the Shiraz vineyard.

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IDENTIFICATION OF A GRAPEVINE FLAVESCENCE DORÉE-C PHYTOPLASMA AND TWO DELETION MUTANTS IN CLEMATIS

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Introduction

Phytoplasmas responsible for the most serious damage to European viticulture are associated with Flavescence dorée (FD), which has been declared a quarantine disease in Europe. They are transmitted from one vine to another by the ampelophagus leafhopper *Scaphoideus titanus*.

FD-associated phytoplasmas belong to ribosomal groups 16SrV-C and 16SrV-D. The following phytoplasma isolates have been identified in grapevine up to the present time: FD-D, belonging to subgroup 16SrV-D and indistinguishable from FD88 and FD92; FD70, FD-C, FD-Lomb/Piem and FD2000, belonging to subgroup 16SrV-C. None of these isolates has ever been detected in plant species other than *Vitis* spp. Research into and identification of FD phytoplasma reservoirs could be very important for vineyard protection with regard to FD disease.

The aim of this work was to identify woody or herbaceous plant species that in nature harbour phytoplasmas which cause FD disease and their possible insect vectors. Visual sanitary controls carried out in the Veneto region (north-east Italy) over several years showed that in some vineyards the FD epidemics began from the border of the vineyard and spread out to the remaining vines only later (3). We therefore started a study on plants and insects living in underbrush close to a vineyard where Flavescence dorée epidemics had previously shown this characteristic trend.

Materials and Methods

During 2001-2002 about 40 different species of wild herbaceous and woody plants and 20 different species of insects were collected in underbrush close to a vineyard located in the province of Treviso. None of the plants showed any clear symptoms of yellows disease.

PCR assays were carried out using DNA extracted from the leaves of each plant sample and from each insect in order to detect if phytoplasmas were present. DNA extraction was performed using the CTAB method (1). Nested-PCR was performed on the 16S-23S rRNA gene and on a fragment of SecY protein gene with the following primer pairs: P1/P7, followed by R16F2n/R2, 16r758f/M23Sr, R16(V)F1/R1 or R16(I)F1/R1; FD9f/r, followed by FD9f3/r2 or FD9f/r2.

RFLP analyses were performed on R16F2n/R2, 16r758f/M23Sr and FD9f3/r2 DNA fragments using *Taq*I and *Mse*I restriction enzymes. FD9f/r2 (about 1200 bp) and 16r758f/M23Sr (about 1050 bp) nested-PCR products obtained from infected samples were double-strand-sequenced.

Results and Conclusion

Of the 40 different species of wild herbaceous and woody plants tested, the only species infected with phytoplasmas was *Clematis vitalba*, which had never previously been reported to host phytoplasmas. About 30 clematis samples were examined in two years, of which approximately half tested positive with primer pairs specifically used for 16SrV-group phytoplasma detection.

The RFLP and nucleotide sequencing data for the 16S ribosomal RNA gene and the SecY translocase gene fragment revealed that most of the infected clematis harboured the same FD-C phytoplasma isolate, which had caused Flavescence dorée in the nearby vineyard and which is widespread in some geographical areas in the Veneto region (Fig. 1, lanes 2,3,5 and 8-10). The nucleotide identity was 100% in both the DNA regions examined (about 2000 nt).

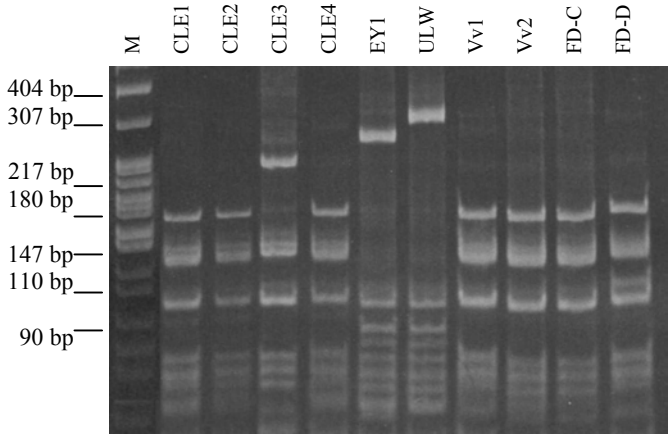

Fig. 1. Polyacrylamide gel (10%) showing RFLP patterns of non ribosomal nested-PCR products for some clematis and grapevine samples from the vineyard and underbrush which are the subject of this study. The FD9f3/r2 fragment was digested using *Taq*I restriction endonucleases. Phytoplasmas: CLE1, CLE2, CLE3, CLE4, samples from clematis; Vv1, Vv2, samples from grapevine. Reference phytoplasmas: FD-C, FD-D, Flavescence Dorée isolates; EY1, American elm yellows; ULW, elm witches'-broom. M: marker of molecular weight, pBR322/*Msp*I digested (New England Biolabs).

Three clematis samples showed particular RFLP patterns in the non ribosomal fragment digested with *Tru*I (Fig. 1, lane 4). Two samples were similar to each other, while the third exhibited a unique restriction profile. Nucleotide sequencing of the two DNA regions revealed the presence of two new phytoplasma isolates, both of which were identical to FD-C in the 16S-23S rDNA fragment and different from FD-C in the *secY* gene fragment only because of? a wide deletion of 51 and 57 nucleotides respectively. The deletion was located in the same position in the translocase gene and entailed the lack of 27 and 29 aminoacids respectively in the codified protein.

This is, in effect, the first report of FD phytoplasma in a plant host other than *Vitis* spp. Furthermore, it is also the first report of deletion mutants among the FD *sensu stricto* phytoplasma isolates. The findings demonstrate that FD phytoplasma reservoirs other than grapevine do exist and raise questions regarding FD epidemics. As matters stand, the presence of FD reservoirs should not be undervalued and the possibility that *C. vitalba* could serve as a reservoir for FD or other phytoplasmas should be taken into account.

Indeed the discovery in clematis of the FD-C isolate and of two deletion mutants leads us to argue a strict relationship between vineyard and underbrush involving an exchange of pathogens, and raising the question of where this phytoplasma originates from: grapevine or clematis? In fact FD-C phytoplasma, which is found only in restricted geographical areas of the Veneto region, appeared to be the most divergent among FD strains identified in grapevine (1,2,4). The finding of deletion mutants of the FD-C phytoplasma in clematis could help us to understand FD phytoplasma evolution and selection in different environmental conditions: the evolutive constraints in the two plant hosts are certainly quite different. Moreover, this lends weight to the theory that the FD-C phytoplasma has been present in clematis for a long time.

In order to understand the relationship between grapevine FD disease and FD phytoplasmas identified in clematis it is necessary to study the potential vectors. The presence of *S. titanus* individuals on clematis in the underbrush was observed, and some samples of *S. titanus* and several other species of *Homoptera* were collected from the clematis. PCR-RFLP assays to detect the presence of phytoplasmas in the insects are currently being carried out.

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MOLECULAR VARIABILITY IN FLAVESCENCE DORÉE PHYTOPLASMAS AS MARKER FOR THE DISEASE OUTBREAKS IN VINEYARDS

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Objectives

Flavescence dorée is a devastating disease of grapevine widespread in several EU countries such as France, Italy and Spain. Genetic variability among Italian and French FD strains was reported (1, 2, 6, 7) after RFLP analyses on 16S DNA plus spacer region, on a fragment of the ribosomal protein operon and on the non-ribosomal DNA fragment FD9. Sequencing and phylogenetic analysis validated the delineation of 4 distinct FD strain types. There were reports of FD presence both in areas where the disease is epidemic and in other where no epidemic spreading was detected; a study to verify both the possibility to use these phytoplasma DNA fragments as markers for FD population variability and the possible relationships between genetic variability and FD epidemiology was undertaken.

Material and Methods

Phytoplasmas in affected grapevine samples used in this study were from 15 different plants of various cultivars, collected in diverse fields in several Italian regions where FD disease was reported: Veneto, Lombardy, Piemonte (7), Liguria, Emilia, and Tuscany. As controls were employed the French strains FD70, FD88 and B1872 kindly provided by E. Boudon-Padieu (INRA, Dijon, France), a Spanish strain (10), and a Serbian strain identified as subgroups 16SrV-C (4) in grapevine. Alder yellows (ALY) and rubus stunt (RuS) (from C. Marcone, University of Basilicata, Italy); elm yellows (EY1) (from H. Griffiths and W. A. Sinclair, Cornell University, Ithaca, NY); elm witches'-broom ULW (from E. Seemüller, BBA, Dossenheim, Germany); jujube witches'-broom from China (JWB) in jujube shoots grown *in vitro* were also employed. Total nucleic acids were extracted from 1 g of grapevine, jujube and periwinkle mid-vein tissues, according to the DNA extraction protocol described by Prince *et al.* (8). The nucleic acids were precipitated in ethanol and the pellet, suspended in 100 µl of TE buffer and diluted to 20 ng/µl final concentration in sterile deionized water. One to 3 µl of this dilution were used in PCR with the universal primer pair P1/P7 (3, 9) in a total volume of 25 µl PCR mixture containing 2.5 µl of 10X PCR buffer, 200 µM of each dNTP, 0.4 µM of primers pair and 0.625 U of Taq polymerase (Polymed, Florence, Italy, EU). For PCR amplification 35 cycles were conducted under the following conditions: 1 min (2 min for the first cycle) denaturation step at 94°C, 2 min for annealing at 50°C and 3 min (10 min for the last cycle) at 72° C for primer extension. PCR products were analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide, and then visualized with a UV transilluminator. 100 to 200 ng of P1/P7 amplicons were digested with *Tru*I and *Taq*I at 65°C and separated by electrophoresis in a 5% polyacrylamide gel in 1X TBE buffer, stained with ethidium bromide, and visualized as above. To further characterize the 15 strains the ribosomal protein operon, containing the 3' end of *rpl22* gene and *rps3* gene was amplified in semi-nested PCR with primer pair rpVF1/rpR1 followed by rpVF2/rpR1 (5). RFLP analysis was performed with the enzymes *Tru*I and *Tsp509*I. A chromosomal DNA fragment amplified using the primer pair FD9f3/r2 in nested PCR (1) was also employed and digested with *Tru*I, *Taq*I, *Tsp*509I, and *Alu*I. The PCR reagents and the reaction conditions employed were as previously reported (1, 7).

Results

PCR using universal primers P1/P7 detected the presence of phytoplasmas in all the grapevine samples as well as in the positive controls. RFLP analyses of the P1/P7 products (about 1.8 kb) with *Tru*I and *Taq*I resolved five distinct RFLP patterns, four of them were detected in sample from grapevine (Table 1). RFLP analyses with 4 enzymes on the chromosomal fragment amplified with primers FD9f3/r2 delineated 12 different groups 7 of them in the samples from symptomatic grapevine (Table 1). Semi-nested PCR using the two primer pairs rpVF1/rpR1 and rpVF2/rpR1 (5) generated one DNA fragment (about 950 bp) from all the strains employed and their RFLP analyses defined 13 different patterns 8 of which from grapevine samples (Table 1). Comprehensive RFLP analyses showed that these conserved phytoplasma DNA sequences allowed to differentiate 16 different phytoplasma variants inside the ribosomal group 16SrV: 5 of them belong to the controls employed that were not from grapevine, three can not be reported as FD agents since RE and MO31 samples belong to subgroup 16SrV-A that was not reported to be transmitted by *Scaphoideus titanus* Ball., and the Serbian sample could not be associated with the presence of the vector. Among the other samples it was possible to distinguish 6 different variants of FD agents belonging to ribosomal group 16SrV-C and 2 belonging to ribosomal group 16SrV-D. These molecular data allow to confirm that only some of the FD identified variants can be assumed as epidemic such as the two 16SrV-D variants identified in Italy and in Spain. Among 16SrV-C strains only those identified in Piemonte, Lombardy (7), and Liguria are identical and appear to have epidemic ability, while all the others detected in Veneto TV54 (7) and VR32, in Western Emilia (PC4), and in Tuscany (MS) appear to be not in epidemic at the moment. Interestingly it was detected in grapevine the presence of elm yellows phytoplasmas in two variants both distinguishable from the strains isolated from elm in US and in EU. These DNA fragments employed as molecular markers seem to be a useful tool to follow FD agent variants and therefore their epidemical spreading.

Table 1. Results of FD-related phytoplasma differentiation based on RFLP of diverse chromosomal fragments.

Strain	Country/year	16S-23S rDNA P1/P7	FD9 non-ribosomal fragment FD9f/r					**Ribosomal protein fragment** rpVF2/rpR1			
		TruI/TaqI	*Taq*I	*Tru*I	*Tsp509*I	*Alu*I		*Tru*I	*Tsp509*I		
FD70	France, EU/1970	16SrV-C	B	C	A	B	III	A	B	III	**3**
AL202	Piemonte, Italy/2000	16SrV-C	A	A	A	A	I	A	A	I	**1**
PV259	Lombardy, Italy/2000	16SrV-C	A	A	A	A	I	A	A	I	**1**
LIG	Liguria, Italy/2001	16SrV-C	A	A	A	A	I	A	A	I	**1**
TV54	Veneto, Italy/2000	16SrV-C	A	A	A	A	I	B	E	II	**2**
SERBIA	Serbia/2002	16SrV-C	A	A	A	A	I	B	B	XI	**13**
VR32	Veneto, Italy/2001	16SrV-C	B	D	F	B	X	C	F	XII	**14**
PC4	Emilia, Italy/2002	16SrV-C	B	D	A	B	II	A	E	XIII	**15**
MS	Tuscany, Italy/2002	16SrV-C	C	B	C	D	IX	B	E	II	**12**
FD88	France, EU/1988	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
B1872	France/EU	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
PCc	Emilia, Italy/2002	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
PR	Emilia, Italy/2000	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
MO30	Emilia, Italy/2002	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
PD26	Veneto, Italy/2000	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
TV29	Veneto, Italy/2000	16SrV-D	B	D	E	E	IV	I	B	IV	**4**
BO13	Emilia, Italy/2002	16SrV-D	B	D	E	E	IV	I	E	X	**11**
Catalogna	Spain, EU/2001	16SrV-D	B	D	E	E	IV	I	E	X	**11**
RE	Emilia, Italy/2001	16SrV-A	C	E	H	F	XI	D	C	V	**5**
MO31	Emilia, Italy/2001	16SrV-A	C	E	H	F	XI	E	D	VI	**16**
EY1	NY. State, USA	16SrV-A	C	F	D	F	VI	E	D	VI	**7**
ULW	France, EU	16SrV-A	C	E	I	F	V	E	D	VI	**6**
JWB	China	16SrV-B	D	G	G	C	VII	F	H	VII	**8**
ALY	Campania, Italy	16SrV-C	B	D	F	B	X	G	B	VIII	**9**
RuS	Campania, Italy	16SrV-E	B	H	B	G	VIII	H	I	IX	**10**

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POSSIBLE INSECT VECTORS OF NORTH AMERICAN GRAPEVINE YELLOWS PHYTOPLASMA IN VIRGINIA

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North American Grapevine Yellows is a lethal disease of winegrapes causing yellowing of leaves, die back of shoot tips and abortion of developing fruit. Infected grapevines often die within months of the onset of symptoms and significant losses of vines have been observed. The disease is particularly destructive to Chardonnay vines, but has been observed in other varieties including Riesling, Sauvignon blanc, and Cabernet franc (1). In Virginia two phytoplasmas, aster yellows (group 16SrI, subgroup A) and X disease (group 16SrIII, subgroup I) have been found in symptomatic winegrapes and the aster yellows phytoplasma has been detected in asymptomatic native grape species, as well. (2, 3). Phloem-feeding leafhoppers, planthoppers or, occasionally, psyllids (4) have been identified as the vectors of many phytoplasma-caused plant diseases, including grapevine yellows in Europe (5). Hence, one or more insect vectors are suspected of transmitting the grapevine yellows phytoplasmas to winegrapes in Virginia.

Using sweep net sampling and yellow sticky traps, surveys of the incidence and abundance of potential insect vector species in and around infected vineyards were made in the 2002 growing season and starting in April 2003. Whole DNA was extracted from captured insects and assayed for the presence of grapevine yellows phytoplasmas by nested polymerase chain reaction (PCR) using universal phytoplasma primers R16F2n/ R16R2 (6) followed by 16R758F/ 16R1232R (7). To determine the group specificity of the phytoplasma detected in PCR-positive samples, aster yellows and X disease specific primers (R16(I)F1/ R1 and R16(III)F2/ R1, respectively) (8) were used in PCR assays on the product of the first universal reaction. In 2002 insect samples, twelve leafhopper species and one planthopper species tested positive for aster yellows phytoplasma; two leafhopper species tested positive for X disease phytoplasma.

Because phytoplasmas have been detected in non-vector insect species (9), transmission experiments using commonly observed candidate vector species were initiated to determine which could acquire and transmit grapevine yellows phytoplasma. Propagated yellows-infected Chardonnay cuttings and infected Chardonnay in test vineyards were used as phytoplasma acquisition hosts and faba bean plants, *Vicia faba*, and rooted Chardonnay cuttings were used as inoculation-access or indicator plants. Three leafhopper species, *Agallia constricta*, *Macrosteles quadrilineatus* and *Tylozygus bifidus*, a sharpshooter, transmitted phytoplasma to indicator faba bean plants. *Agallia constricta* also transmitted phytoplasma to test Chardonnay.

Membrane feeding trials also were performed to determine which candidate species released North American grapevine yellows phytoplasmas along with saliva into Tris-EDTA-sucrose (5%) solutions during feeding (10). *Agallia constricta*, *Paraphlepsius irroratus*, *Deltocephalus flavicosta*, *Exitianus exitiosus*, and *Endria inimica* introduced aster yellows phytoplasma into test solutions.

On three dates in 2002 and on one date in 2003 leaf and stem samples were collected from a variety of grasses, forbs, shrubs and trees found in and around test vineyards. Using PCR analysis, aster yellows phytoplasma was detected in *Trifolium pratense*, *T. repens*, *Lespedeza virginica*, *Prunus serotina* and *ulmus* sp.; X disease phytoplasma has been found in native *Vitis* sp. and in *Platanus occidentalis*.

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CHARACTERIZATION OF ISOLATES OF VERGILBUNGSKRANKHEIT-PHYTOPLASMA BY RFLP-ANALYSIS AND THEIR ASSOCIATION WITH GRAPEVINE, HERBACEOUS HOST PLANTS AND VECTORS

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Vergilbungskrankheit (Bois noir) is the most widespread Grapevine Yellows in Germany. It occurs in nine of the 13 viticultural areas in Germany, although significant damage is limited to vineyards on the slopes of the valleys of the rivers Mosel, middle Rhine, and Nahe. A phytoplasma of the stolbur group (16SrXII-A) is associated with Vergilbungskrankheit (VK) (3). Although the genetic diversity within this group seems to be low, differences based on RFLP- or sequence-analyses have been described between stolbur isolates from weeds (5) and from grapevine (6, 8), and genome sizes vary considerably between stolbur phytoplasmas (4).

The epidemiology of VK is principally determined by the presence of its vector, the Cixiid planthopper *Hyalesthes obsoletus* Signoret, and the occurrence of herbaceous alternative host plants of the phytoplasma and its vector. In order to understand the recent spread and dissemination of VK in German viticulture we studied the distribution of VK in Germany as well as the infestation of natural host plants by the VK phytoplasma and the association of *H. obsoletus* with those plants. The suitability of different amplified DNA-fragments for a further differentiation of VK phytoplasma isolates was investigated and samples of grapevine, herbaceous plants and vector insects were analyzed. Additional experiments were carried out to study a possible adaptation of *H. obsoletus* populations to particular host plant species.

Samples were taken from symptomatic grapevine from the different viticultural regions of Germany. Host plants of *H. obsoletus* and other plants with suspicious symptoms growing in vineyards or adjacent areas were also sampled. Periwinkle (*Catharanthus roseus*) isolates of stolbur phytoplasma obtained from grapevine (SA-1, SA-2, CA-1, CH-1, [1]; GGY, VK1925 [Maixner, unpublished]) or other hosts (DEP, STOLF [M-T. Cousin, Versailles, France]; MOL [M. Lansac, Bordeaux, France]; STOL [D. Sutic, Serbia]) were included in this study. Specimens of *H. obsoletus* were collected directly from different herbaceous plants. In addition, roots of herbaceous plants were checked for the presence of larval instars of *H. obsoletus* in order to identify its genuine host plants. All plant and insect samples were subjected to PCR tests with the stolbur-specific primers f/rStol (3). Positive samples were further used to amplify other ribosomal and non-ribosomal DNA fragments using primers P1/P7 or f/rStol11, f/rStol4 (2), and f/rTufAY (7) respectively. Amplification products were analyzed by digestion with various restriction enzymes and separation of the restriction fragments on polyacrylamide gels. The survival of adult *H. obsoletus* that were caught either from bindweed (*Convolvulus arvensis*) or stinging nettle (*Urtica dioica*) on their homologous and heterologous host plants was studied under controlled conditions in a growth chamber in order to identify a possible adaptation of the vector populations to the respective host plants.

C. arvensis was the only host plant that was consistently exploited by *H. obsoletus* in all viticultural areas. Patches of this plant but also of different perennial species of *Ranunculus* yielded very high population densities. *U. dioica*, on the other hand, was utilized only locally. Hedge bindweed (*Calystegia sepium*) was newly identified as a host of *H. obsoletus*. Stolbur phytoplasma was detected beside grapevine only in *C. arvensis* and *C. sepium* as well as in *Prunus spinosa*, a woody plant that grows frequently on the borders of the vineyards. However, *H. obsoletus* carrying stolbur phytoplasma was collected not only from *C. arvensis* and *C. sepium* but also from *U. dioica* and *Ranunculus* spp. Inoculation experiments revealed that the latter plant dies rapidly after infection with stolbur and is therefore a bad source of infection for *H. obsoletus* in the field. Levels of infestation of *H. obsoletus* populations varied between 30 % and 66 % on bindweed, between 1% and 5% on *Ranunculus*, and from 1% to 10% on stinging nettle.

Restriction profiles achieved from the ribosomal fragment P1/P7 and the non-ribosomal fragment Stol11 showed no polymorphism. Analysis of the Stol4 fragment with *Dra*I (6) allowed to distinguish three restriction profiles. Two of them where detected in field samples and one in the periwinkle isolate DEP (Table 1). Type A was only known from the Palatinate area previously. It was now also detected at the Mosel. Type B is widespread and present in all of the seven viticultural regions that were analyzed so far, while type C was slightly different from A and unique for DEP. Since the amplification of the Stol4 fragment is often difficult we tried to find another non-ribosomal fragment for a further characterization of the VK phytoplasma. Primers f/rTufAY allow the specific amplification of a 940 bp fragment from phytoplasmas of the aster yellows and stolbur groups (7). Digestion of the amplified fragments with *Hpa*II allowed the differentiation of phytoplasmas from the two groups and led to three different restriction profiles of stolbur from grapevine, herbaceous plants and vectors. The combination of the results achieved from the analysis of Stol4 and tufAY made it possible to define three types of VK-phytoplasma from the field samples. Type I was detected in grapevine, *H. obsoletus*, and several periwinkle isolates. It corresponds to the previously described Stol4-Type A. This type is more widely distributed than we thought before. Positive samples of grapevine or *H. obsoletus* were found not only in the Palatinate region, but also in vineyards in Baden, Bergstrasse, Mosel, and Nahe. Type II (Stol4-B) is the most widespread. It was found in grapevine, *H. obsoletus*, and various herbaceous host plants, while type III was identified in *C. sepium* and *H. obsoletus* from the Mosel only. More diseased vines need to be tested to examine the presence of this type in *Vitis vinifera*. It is remarkable that the data indicate a certain "herbaceous host preference" of the different types of VK-phytoplasma. Type I was the only one that could be found in *H. obsoletus* collected from nettle in different viticultural areas and it was never detected in other host plants. We were not able to detect phytoplasma in nettle itself, but an *U. dioica* sample from France (provided by E. Boudon-Padieu, Dijon) proved to

Table 1: RFLP-profiles of stolbur phytoplasma obtained from periwinkle isolates or natural host plants and vectors sampled in and around vineyards.

VK-Type	RFLP profiles		Source of phytoplasma	Geographic distribution in Germany
	Stol4 / *Dra*I	tufAY / *Hpa*II		
I	A	a	*Vitis vinifera* *Urtica dioica* (France)[1] *Hyalesthes obsoletus* from *Urtica dioica* (Periwinkle: SA-1, SA-2, VK1925)	Baden, Bergstrasse, Mosel, Nahe, Palatinate
II	B	b	*Vitis vinifera* *Convolvulus arvensis* *Hyalesthes obsoletus* from *C. arvensis* *Calystegia sepium, Prunus spinosa, Solanum nigrum* (Periwinkle: CH-1, CA-1, GGY, Stol, StolF)	All viticultural areas affected by VK
III	B	c	*Calystegia sepium* *Hyalesthes obsoletus* from *C. sepium*	Mosel
-*	C	b	(Periwinkle: DEP)	

*not present in grapevine [1]sample provided by E. Boudon-Padieu, Dijon

be infected by Type I. Type III, on the other hand seems to be restricted to *C. sepium* and the vectors feeding on this plant. Only type II occurred in all plants except of nettle.

Field and laboratory observations indicate an adaptation of *H. obsoletus* to particular host plant species. For example, the flight of adult planthoppers that developed on nettle is delayed by approximately three weeks compared to the insects that live on bindweed. Furthermore, laboratory experiments with bindweed and nettle revealed that insects that were kept in the laboratory on their original host species survived significantly longer than those that were kept on the heterologous host (Insects from nettle: 8.4 vs. 2.5 days; bindweed: 3.8 vs. 2.9 days).

It can be concluded that a genetic diversity exists within VK-isolates in Germany. The different types can be readily distinguished by a RFLP analysis of the tufAY fragment with *Hpa*II. The most widespread VK type II occurs in all viticultural regions and is common in herbaceous plants, particularly *Convolvulus arvensis*, which is the main source of inoculum in Germany. Type I, on the other hand, appears to be restricted to areas where *H. obsoletus* feeds on *Urtica dioica*. Type III seems to be limited to the newly identified host *Calystegia sepium* and the vectors feeding from this plant. Its presence in grapevine has still to be proved. The restriction of the different types of VK-phytoplasma to particular herbaceous hosts could be explained by a preference of different *H. obsoletus* populations to particular plant species. Although there is some evidence for this assumption, a detailed analysis of the genetic variability between vector populations would be necessary to prove this hypothesis.

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ROGUING: A CONTROVERSIAL PRACTICE TO ERADICATE GRAPE YELLOWS CAUSED BY PHYTOPLASMAS

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The grapevine yellows known as "Flavescence doreé" (FD) and "Bois noire" (BN) are both epidemic and economically important diseases in North-eastern Italy. Even if only the first is a quarantine disease, both are largely subjected to rogue as a preventive control measure. Evidences gained during over 10 years of observations strongly indicate that this costly practice could be either avoided or at least reduced.

In fact it was repeatedly ascertained that both FD - and BN - infected grapevines do recover from the symptoms as a rule (2, 3). Recovery – considered as the disappearance of symptoms from infected plants - is influenced by the cultivar, the environmental conditions and probably also by different strains of the pathogen. It can be permanent, transient or recurrent. It has been reported that BN epidemics with a consequent large recovery occurred in some viticultural areas, followed by a second wave of natural infections and a second important recovery. In North-eastern Italy the most cultivated varieties do recover from both FD and BN. In an important area of Veneto called Valdobbiadene, famous for the Prosecco wine, a complete and permanent recovery was achieved just avoiding to rogue and by applying insecticides against the FD vector *Scaphoideus titanus*. Perera is the only one cultivar that does not recover, because it is extremely sensitive to FD. For FD, the mean annual rate of recovery ranges from 20 to 45%; for BN from 12 to 28% (4). In general the probability of recovery increases during the four years after the symptom appearance. Moreover the percentage of recovery is bigger in grapes showing mild symptoms of GY than in severely infected grapes (2).

Recently it has been verified that the phytoplasma infection tends to disappear from the leaves of recovered grapevines (Table 1). In fact nested-PCR analyses performed on leaves of grapevines, that have been recovered for three or four years, gave negative results. Data from a thesis done at the Department of Plant Protection of Udine, have permitted to assume that the disappearance of the BN phytoplasma from the grapevine crown is progressive, reaching its completion the third year after recovery (1).

According to the reported observations it seems right to avoid indiscriminate roguing. This practice is recommended when recovery is absent or low, when attempts are carried out to eradicate recent epidemics and when valuable nursery areas need protection from fastidious infections. It is also suggested to rogue the severely infected plants. Anyway in all cases it is necessary to know the real level of recovery before roguing. Finally it seems that even for FD - where grapes are not "dead end hosts" - the recovered plants do not act as active potential sources of inoculum for natural transmission.

As already stated the reasons of recovery are still unknown; while its practical importance has been recently better understood.

Table 1. Positive results of nested -PCR analyses performed in North-eastern Italy on leaves of grapevines 3 - 4 years after recovery from BN or FD. Similar results are reported for apple and apricot after recovery from AP and ESFY respectively.

Phytoplasma	Plant host cultivar	Years after recovery	PCR results of recoverd plants (%)	PCR results of symptomatic plants (%)
BN	Chardonnay and Pinot noire	3 - 4	0	100
FD	Prosecco	3 - 4	0	96
AP	Florina	2 - 3	0	100
ESFY	Several cvs. of apricot	3 - 4	85	100

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HOT WATER TREATMENT: CURING EFFICIENCY FOR PHYTOPLASMA INFECTION AND EFFECT ON PLANT MULTIPLICATION MATERIAL

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Hot water treatment (HWT) has been proposed to cure dormant woody plant material from intracellular pathogenic microorganisms and to suppress surface parasites and pests. This is particularly useful in the case of phytoplasmas (1, 2) which are difficult to detect reliably in woody material and grapevine. Symptom expression may be delayed in sensitive varieties. Phytoplasma may also be hosted by tolerant plants such as *Vitis* rootstock varieties (3). Circulation of phytoplasma-infected material is forbidden for Flavescence dorée (FD) which is a quarantine disease, and it might be dangerous for other phytoplasmas, since potential vectors may exist in the area of introduction.

HWT conditions recommended for curing of grapevine planting material from phytoplasmas, have first been devised for infected scion material (1, 2). They consist in soaking fully dormant material for 45 mn in agitated water at 50°C with a procedure ensuring efficiency and security (4). In the last decade we have conducted numerous assays on a variety of material from scion and rootstock cultivars, with several dates of treatment and we checked the possibility to use lower temperature and shorter duration of treatment. We have also assessed the effect of efficient HWT conditions on different varieties and type of material by checking mortality, bud-bursting and growth of treated plants compared to untreated control. The present paper gives a summary of data obtained. In all assays, phytoplasma infection was confirmed with PCR diagnosis.

Efficiency of HWT to cure phytoplasma-infected vine material.

Rootstocks. It has been reported that rootstock mother plants can be highly FD-infected but that the distribution of FD in plants, expressed as presence of infected sensitive index, is uneven (3). Table 1 shows that when healthy Chardonnay scions were grafted on FD-infected 3309C rootstock, only the plant material treated with the [50°C- 45 mn] conditions remained symptomless for 2 years after grafting. When treatments with milder temperature or shorter duration were used, FD symptoms could be observed on the first and/or the second year (i.e. in the nursery or in an insecticide-treated plot.).

Material winter 1999		Nursery 2000						Plantation 2001			
Treatment	Batch	Nr grafts.	dead	% dead	alive FD	% FD	% healthy	Nr plants	dead	FD	% FD
Untreated	1	52	24	46	**6**	**12**	42	22	0	**1**	**4,5**
	2	204	60	29	**3**	**1**	69	141	0	**0**	/
45° - 60'	1	54	12	22	**3**	**6**	72	39	0	**0**	/
	2	203	51	25	**0**	/	75	152	0	**0**	/
48° - 30'	1	53	14	26	**3**	**6**	68	36	0	**1**	**2,8**
	2	202	64	32	**0**	/	68	138	0	**0**	/
50° - 20'	1	53	20	38	**0**	/	62	33	0	**1**	**3**
	2	204	60	29	**0**	/	71	144	0	**0**	/
50° - 45'	1	51	9	18	**0**	/	82	42	0	**0**	/
	2	205	65	32	**0**	/	68	140	0	**0**	/

Table 1. Compared curing efficiency of different HWT conditions on immediate and delayed symptom expression. Cuttings from FD-infected rootstock material (3309C) of two orignins (batch 1 or 2) were graft-indexed with healthy scions of Chardonnay and checked over two years for FD expression.

Assay	treatment	Nr grafts	Nr dead	alive with BN	% growth
1994 BN on Chardonnay	untreated	258	168	10	31
	50° - 45'	258	182	0	29
2001 BN on Chardonnay	untreated	137	70	5	45
	50° - 30'	134	69	0	49
	50° - 45'	136	67	0	51

Table 2. Curing efficiency of HWT on BN-infected scions.

Bois noir (BN) infected scions. In 1994 and 2001, normal lignified canes were taken on BN-affected plants of Chardonnay. Cuttings were HW treated (30 or 45 mn at 50 °C) or not, then planted in a greenhouse for symptom expression. Table 2 shows that BN-infected individual buds could produce symptomatic plants and that HWT suppressed the expression of symptoms. The low percentage of growth was similar in untreated and treated batches. Treated plants from the 1994 trial were grafted on HW-treated rootstock, planted in an experimental plot and have been symptomless since.

Permanent curing. The durable efficiency of HWT was checked since 1999 on Pinot N and Chardonnay grafted on two batches of FD-infected 3309C rootstocks (3). After nursery, symptomless plants were planted in two plots protected with insecticides. HWT suppressed FD symptoms consistently (Table 3) but symptoms were observed on untreated plants on the 2nd and 3rd year. The growth percentage of healthy plants to total (column 7) was similar or higher in treated batches.

Scion index variety	Treatment	Nursery 1999					Plantation 2000				2001	2002
		Nr grafts	% dead	Alive FD	% alive FD	% alive healthy	Nr plants	% dead	alive FD	% alive FD	Observations	
Pinot N	50 °C - 45'	1000	17.8	/	/	82.2	763	**0.5**	**0**	/	None	None
	Untreated	1000	19.4	**10**	**1**	79.6	673	**0.7**	**0**	/	None	None
Chardonnay	50° - 45'	1000	18	/	/	82	820	**0**	**0**	/	None	None
	Untreated	1000	18.5	**6**	**0.6**	80.9	805	**1**	**0**	/	None	None
Class A motherplants (3)												
Pinot N	Untreated	40	32.5	**7**	**17.5**	50	20	**5**	**1**	**5**	None	**1 FD**
Chardonnay	Untreated	45	26.7	**9**	**20**	53.3	24	**8**	**0**	/	None	None

Table 3. Behaviour of HW-treated and untreated Chardonnay and Pinot N grafted on FD-infected 3309C rootstocks.

Conditions for HWT applied to material before grafting or on grafted rootlings

Before grafting. Trials conducted every year since 1991 on cultivars Marselan, Merlot, Syrah, Macabeu, Caladoc, Chardonnay and Pinot N, associated to rootstock cultivars such as 3309C, R110, SO4 and Fercal (total rootlings : 16,822 over 12 years), have shown that the percentage of growth of HW-treated to non treated was 85 % to 100 %. In the case of Merlot, Macabeu and Marselan, a medium to strong delay in bud-bursting of treated batches was observed, especially when HWT was given late in the cold conservation period. However vegetation was not affected and homogeneity between treated and untreated plants was recovered in Summer. Of these, 2,444 Chardonnay and 6,138 Pinot N (HWT in February or April) have been planted (beginning of June) in 1995, 1996, 1997 and 1999. Growth, vigor and yield have been observed since and were excellent (data not shown).

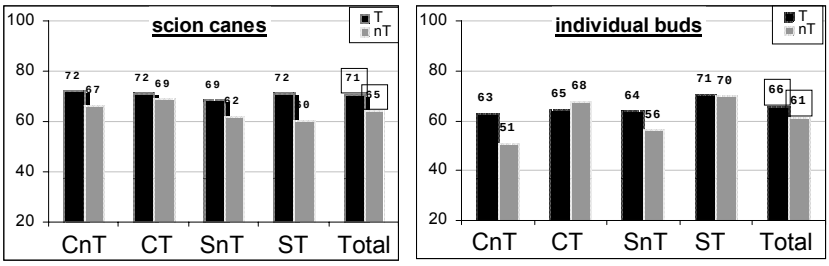

Figure 1. Percentage of growth of 16 batches (600 individuals per batch) of cv. Merlot grafted on SO4

HWT was applied separately on scion and rootstock material before grafting. Scion material was treated as 1-m-long canes or individual buds. Rootstock material was treated or untreated as 1.20-m-long canes (CT, CnT) or 30-cm-long sections (ST, SnT). ■: treated scion material; ▒: untreated scion material

Figure 1 is part of a trial that also included cv. Grenache grafted on rootstock R110. Batches using short wood pieces (individual buds or rootstock sections) was the less favorable. However, treated and untreated material gave similar data.

Treatment	Mortality (%)	
	clone 174	clone 470
Untreated	4	10
50 °C-45'	2	1
50 °C-1h	4	4
50 °C-2h	10	26
50 °C-3h	50	43
52 °C-45'	13	35
55 °C-15'	5	34

Table 4: Effect of HWT conditions on grafted rootlings from 2 clones of Syrah

Grafted rootlings. HWT was also applied before plantation (after nursery) to grafted rootlings of two clones of cv. Syrah on SO4. Table 4 shows that mortality was not increased with a 1-hour treatment.

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AUSTRALIAN ADVANCES IN HOT WATER TREATMENT RESEARCH

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Hot water treatment of *V. vinifera* cuttings at 500C for 30 minutes (HWT) is widely used in Australian vine nurseries as a simple, cost-effective commercial process for the control of a range of specific pests and diseases (1). HWT will not eliminate virus infection or improve poor quality propagating material. It is generally a safe, reliable process, however, some Australian vine nurseries have occasionally experienced significant levels of cutting mortality following HWT. This paper summarises two sections of a collaborative research project funded by the Australian Grape and Wine Research and Development Corporation responsible for the identification of key factors influencing cutting performance following HWT.

Factors influencing cutting development during propagation ('The Jacob's Creek Trials') - A series of 3 large scale commercial trials were conducted annually between 2000 and 2001 in association with the Orlando-Wyndham Group at the Jacob's Creek Vine Nursery, Barossa Valley, South Australia. Each trial utilised up to 54,000 *V. vinifera* cuttings in factorial designs with up to 95 treatment combinations. The trails assessed the effect of cutting variety, cutting harvest date, post harvest handling time, cutting hydration time, presence or absence of HWT and presence or absence of autumn/early winter irrigation. Assessments were made on sub-samples to rate callus formation, root initiation and root growth 21 days after the commencement of callusing (ACC) and shoot development 28 days after potting (AP). This assessment enabled the initial relative sensitivity of cutting varieties to different treatments to be determined. Good nursery management generally allowed cuttings to recover and mask any adverse growth responses.

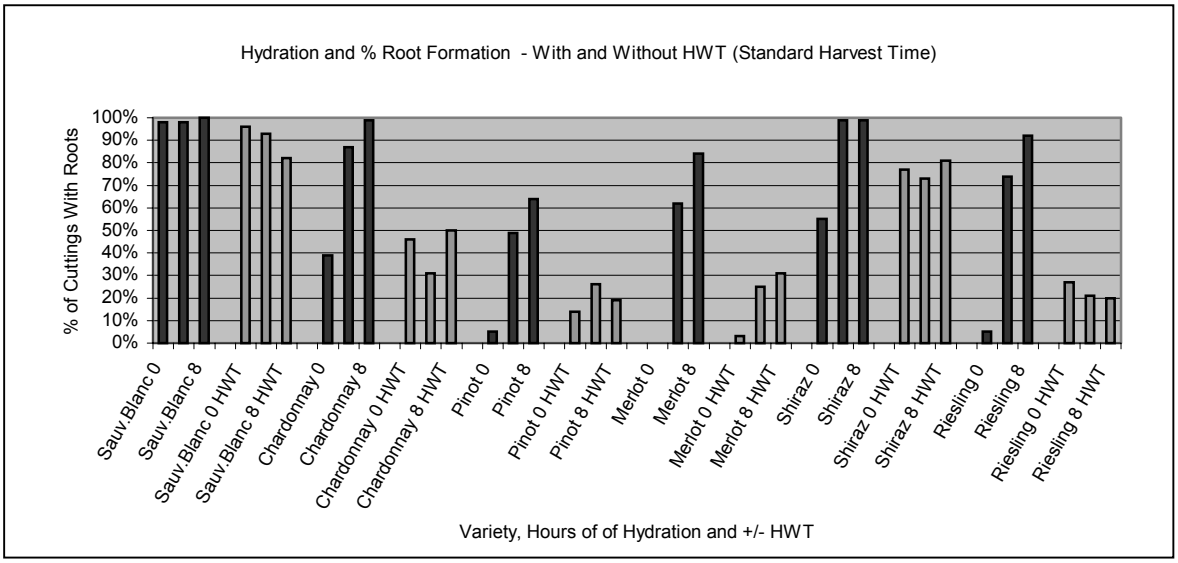


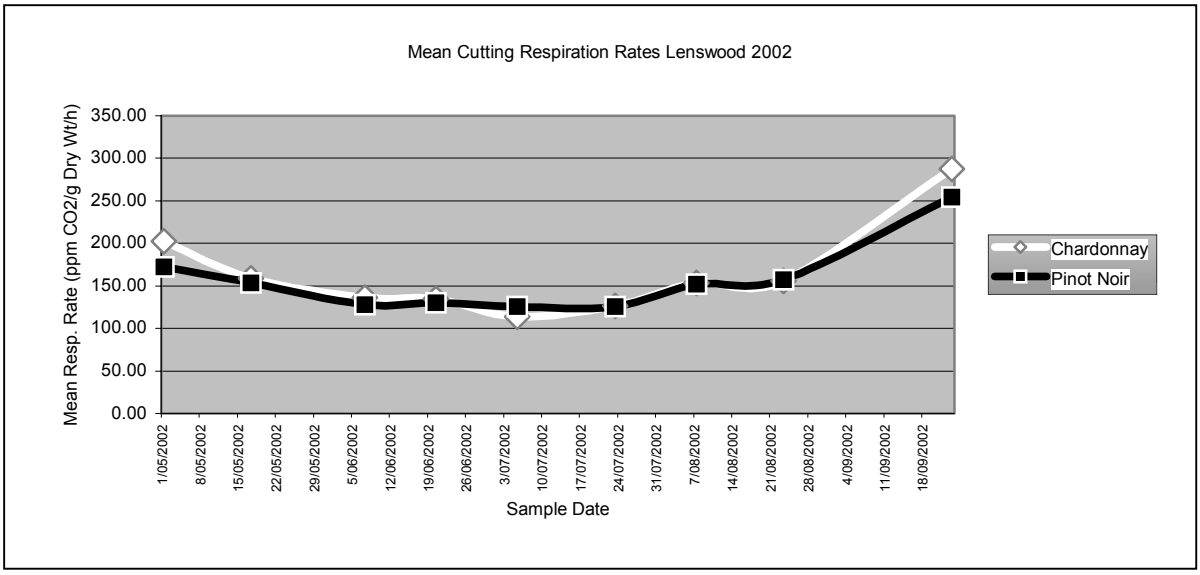
Figure 1. Response of *V. vinifera* cutting varieties to different pre-HWT hydration durations (0, 1 and 8 hours), % of cuttings demonstrating root development at 21 days ACC, 2001 data. (No HWT = black, + HWT = grey).

Analysis of results indicated that *V. vinifera* cuttings harvested when fully dormant, with short handling and cold storage times after cutting harvest and a cutting hydration duration not exceeding 8 hours generally resulted in cuttings being less sensitive to HWT at 21 days ACC and 28 days AP. Cuttings of Cabernet Sauvignon and Sauvignon Blanc were consistently least sensitive to HWT treatment regimes, (Fig 1.), while Pinot Noir, Merlot and Riesling were most sensitive. The response of Chardonnay between seasons was variable. Good nursery hygiene and good nursery management procedures enabled most cuttings to recover from treatments. Early responses to treatments were eventually masked to a point where no significant differences between treatments were observed at the time of harvest as rootlings from the field nursery (3). Initial analysis of results from the 2002 trial suggest that the application of irrigation water to cutting source vines during autumn and early winter, particularly in a drought year, has a greater impact on *V. vinifera* cutting sensitivity to HWT than any other single factor so far assessed.

The Jacob's Creek trials illustrate that cuttings of *V. vinifera* varieties respond differentially to HWT and associated propagation regimes. Where varieties are sensitive, nurseries must pay specific attention to good nursery practice, nursery hygiene and ensure that shadehouse and field nursery irrigation is well managed to reduce the incidence of cutting mortality.

Results of this research program will be used to amend Australian Vine Improvement Association (AVIA) and Vine Industry Nursery Association (VINA) propagation protocols for use under their respective Australian Nursery Accreditation schemes. The Jacob's creek trials also provided a platform for the development of a bar code driven nursery QA system that allows individual cuttings to be tracked through every stage of the propagation process. It allows data to be collected against cutting batches on all propagation treatments and has facility to trace back from field plantings to cutting source areas. This system will become available commercially in Australia during 2003.

Measurement of Cutting Dormancy. Opinion within Australian vine nurseries suggests that fully endodormant vine cuttings tolerate exposure to HWT more readily than cuttings harvested prior to endodormancy or following the commencement of ecodormancy (2). Respirative rate (rate of CO2 production/g dry weight/hour) was used for the determination of dormancy state in source area vines. The measurement of fluctuating cane moisture content, µm variations in cane diameter and fluctuations in starch/sugar ratio were eliminated as commercially adaptable techniques for dormancy measurement (4). Preliminary respirative measurements during 2000 indicated that basal respirative rates in Chardonnay increased significantly up to 5 weeks before bud swell and well before external signs of broken dormancy could be observed. Studies during 2001 confirmed this observation (3).



Observations during 2002 demonstrated that basal respiratory rate in Chardonnay and Pinot Noir progressively falls from the onset of senescence in late April to late June (winter in southern hemisphere) where it plateaus before beginning to rise in late July approximately 5 weeks prior to bud swell. This data suggests that Pinot Noir and Chardonnay cuttings harvested at Lenswood between late June and late July (2002) were metabolically quiescent. VINA members indicate that cuttings harvested during the interval between late June and late July are traditionally preferred by the nursery industry and appear to be least sensitive to HWT exposure. These observations were confirmed during the Jacob's Creek trials. Future studies will refine the use of respiration rate as an indicator of dormancy state and will seek to correlate flowering in non-related deciduous species with the measured increases in cutting respiration rates to identify suitable bio-indicators for the onset of increased metabolic activity in vine source areas.

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EFFECT OF ROOTSTOCK ON GRAPEVINE YELLOWS – FACTS AND EXPLANATIONS.

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Grapevine yellows are a serious problem in some areas in Israel. Stolbur phytoplasma was found to be the dominant type (82% of the infected vines), followed by AY (12.6%) and Western X (5.4%) (1). Disease incidence is highly dependant on the geographical location of the vineyard and, as known from all phytoplasma infected regions, on the grape cultivar. Richter 110 is the dominant rootstock used in the Golan area, but a sub-plot in one of the highly infested vineyards is grafted to Castel 216-3 rootstock and this subplot is consistently less symptomatic then the rest of the vineyard (2). The goal of the work presented here is to determine whether these differences are due to different insect populations (vectors) in vines grafted on different rootstocks.

The work took place in three Cabernet sauvignon vineyards, located in the south (Gshur), center (Yonatan) and north (Ortal) of the Golan. In each vineyard the majority of the vines are grafted on Richter 110 rootstock, and a small plot (ca. 0.5 hectare) is grafted on Castel 216-3. From the summer of 2002 through 2003 we surveyed the vineyards for disease symptoms (red-orange hue of leaves, dried or shriveled clusters, non-lignified shoots). From some symptomatic vines, leaves were analyzed by nested PCR (P1/P7 and U3/U5) followed by RFLP with Alu I.

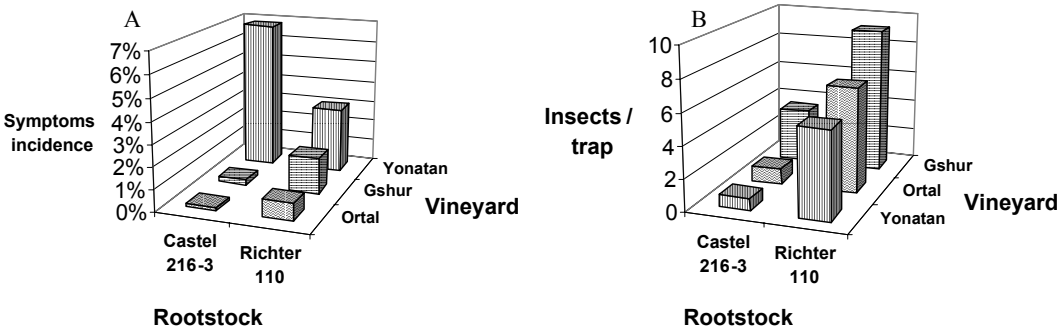
Four methods were used to monitor insect populations in April, July, September and December: sticky traps, pitfall traps, light traps and soil samples, with five repetitions in each vineyard and plot. Yellow sticky traps were placed for 1 week on the trunk of the vines 30 cm. above the ground, pitfall traps were placed in the row under the vines, soil samples were collected under the vines using a soil corer (500 cc), and insects were separated using Berlese funnels. One light trap was put in each sub-plot (each with a different rootstock) at twilight for three hours for three consecutive evenings (repetitions) in July and September.

Results

Phytoplasma survey

Five to eight times more symptomatic vines were found grafted on Richter 110 than on Castel 216-3 in Ortal and Gshur vineyards respectively, but twice more were found on Castel 216-3 in Yonatan vineyard (Fig. 1 A). The differences in incidence in Gshur and Yonatan were found to be statistically significant using GLM procedure and Duncan test after arcsin transformation.

Fig 1: Effect of rootstocks on A: symptom expresion, B: Total potential vector population.



Three symptomatic vines grafted on Castel 216-3 and seven on Richter 110 were tested by molecular means. A healthy *Vinca rosea* served as a negative control and two infected *V. rosea* – one with Stolbur and one with FD were used as positive controls. All symptomatic vines were found positive for Stolbur phytoplasma.

Potential vectors population

Ten species of leaf- and plant-hoppers, known as vectors of different types of phytoplasmas in different crops, were found during the survey (table 1). Of those, *Hyalesthes obselutus* is the only proved vector to grapevines. Two species (*D. diabolai* and *A. sinuata*) and larval stages were only detected in light or pitfall traps, but in very low numbers; therefore, in 2003 the work concentrated on data from sticky traps.

Significantly more potential vectors were caught in the "Richter" plots compared to the "Castel" plots in the three vineyards (Fig 1 B). In 2003 the total numbers of potential vectors was significantly higher (t-test $p<0.0001$) in "Richter" plots (6.6 per trap compared to the "Castel" plots (1.9/ trap), this consisted in all three locations: in Gshur 9.25 and 3.5 ($p< 0.01$), in Yonatan 5.5 and 0.75 ($p<0.005$), in Ortal 6.67 and 1.0 ($p<0.05$) respectively per trap.

Table 1. Potential vectors trapped in each vineyard with yellow sticky traps.

Vineyard	Gshur		Yonatan		Ortal	
Species	Richter	Castel	Richter	Castel	Richter	Castel
*Anaceratagallia laevis**	5.25	2.5	0	1	1	1
Circulifera spp.*	1	1	4.2	1	3	1
*Megopthalmus scabripennis**	4	3.3	1	1	1	1
*Neoaliturus fenestratus**	0	0	0	0	2	0
*Macrosteles quadripunctulatus**	1	0	4	0	2	0
*Orosius orientalis***	0.13	0.07	0	0	0.1	0
*Hyalesthes obsoletus***	0	0.13	0.8	0.53	0	0.2
Psammotettix sp.**	0.2	0.07	0.07	0.13	0.2	0
*Dryodurgades diabolai****						
Austroagallia sinuate ***						

* insects/trap during April 2003
** insects/trap during 2002
*** found only in light or pitfall traps

Expression of phytoplasma symptoms in grapevines is a result of complex interactions on different levels. The fact that the rootstock affects the disease incidence can either be a result of specific physiological interaction between the rootstock and the phytoplasma (2) through the effect of the stock on the vine vigor, or, as shown in this work, due to a different insect population around vines grafted on the two rootstocks. We are continuing to monitor and learn the dynamics of the different potential vectors in the vineyards, trying at the same time to learn whether certain properties of the grafted vines themselves (rooting, growing habits, mineral composition) makes them more or less susceptible to phytoplasma infection.

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PRESENCE OF GRAPEVINE YELLOWS PHYTOPLASMAS VECTORS (*HOMOPTERA, AUCHENORRHYNCHA*) IN NORTHWESTERN ITALY

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Grapevine yellows (GY) are economically important diseases caused by phytoplasmas and transmitted by leafhoppers and planthoppers (4, 9). In Italy, two species are known to be GY vectors: Flavescence dorée (FD), which belongs to 16Sr-V or Elm yellows group (EY), is transmitted by *Scaphoideus titanus* Ball (Cicadellidae), a monophagous species introduced in Europe from the US in 1950s (1); Bois noir (BN), that belongs to 16Sr-XIIA or Stolbur group, is transmitted by *Hyalesthes obsoletus* Signoret (Cixiidae), that lives on nettle but can occasionally feed on vine (2). Therefore, knowledge of vectors presence is important in order to proceed with IPM measures (10).

Aim of this work was to determine the presence and the flight activity of *S. titanus* and *H. obsoletus* and to identify the peak of affected individuals within the season, in order to proceed with IPM measures.

Studies on vectors and phytoplasmas presence have been conducted in piedmontese vineyards during 2001 and 2002. In five vineyards, yellow sticky traps were set up on vine rows to detect the presence and the flight activity of both species. *S. titanus* adults were also sampled on vine canopy by means of sweep net. *H. obsoletus* individuals were collected with D-Vac device in other three vineyards, both on vine canopy and within nearby nettle-beds. *S. titanus* individuals were also divided into males and females. DNAs were extracted from single adults of both species (7) and subjected to amplification driven by the primer pairs fAY/rEY (6) and M1/P8 (8) to detect FD and BN phytoplasmas, respectively.

S. titanus flight activity occurred from mid-July to end-September, and showed a peak at mid-August. 864 individuals were trapped, 502 males and 362 females (sex ratio 1.4). Males occurred mainly in the first part of the monitoring period, while later on much more females were found (fig. 1). On the other hand, captures by means of sweep net showed a sex ratio of 0.6 (83 males and 138 females). Presence of FD phytoplasma within insects was maximum (35.7%) at the end of August (fig. 2). No *H. obsoletus* individuals were found on sticky traps and only 2 were collected on vine rows by means of D-Vac, while many specimens were sampled from nettle-beds from the beginning to the end of July. Individuals affected by BN occurred mainly in the second half of July (fig. 3).

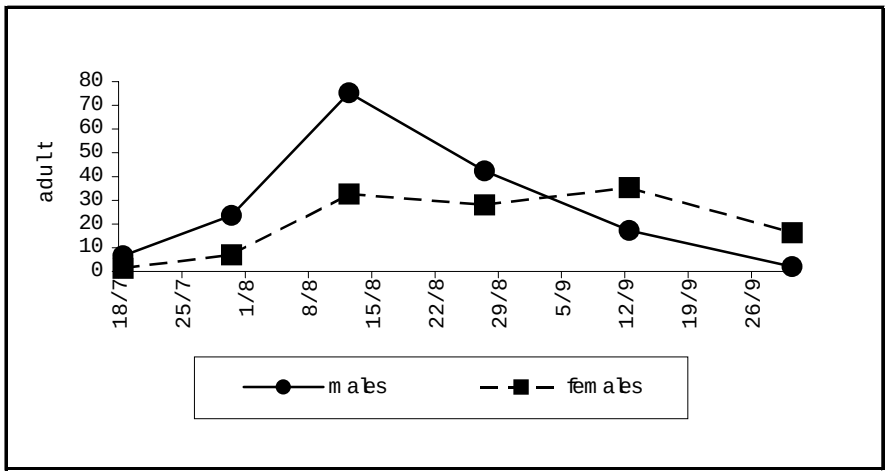

Fig. 1. Flight activity of *Scaphoideus titanus* Ball detected by mean of yellow sticky traps.

Yellow sticky traps could help in *S. titanus* chemical management, by detecting the flight peak. However, presence of a high number of positive individuals in the later part of the season must be taken into account. It has also to be demonstrated if gender matters in the FD transmission process: differences in dispersal between males and females (which have been pointed out from differences in sex ratios between sweep net and traps sampling) could influence disease's outbreaks if differences in transmission, as how happens for other leafhoppers (3), were proved. Presence of *H. obsoletus* has not been detected by mean of sticky traps in vineyards, in contrast to results of other experiments (5, 11). If compared to *S. titanus*, adults occur for a shorter lack of time but seem more likely to acquire phytoplasmas. More information is needed on the dispersal patterns of this species and especially on when and how much does it move from nettle or other weeds to grapevine, in order to apply to IPM at the right time, and only if necessary.

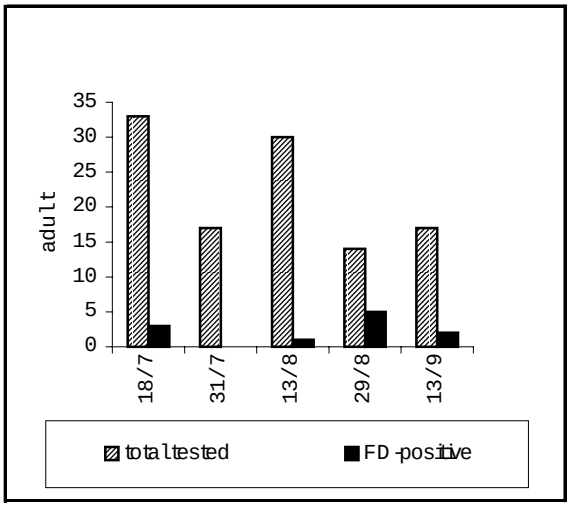

Fig. 2. Rate of FD-positive *Scaphoideus titanus* Ball adults collected from vine canopy with sweep-net.

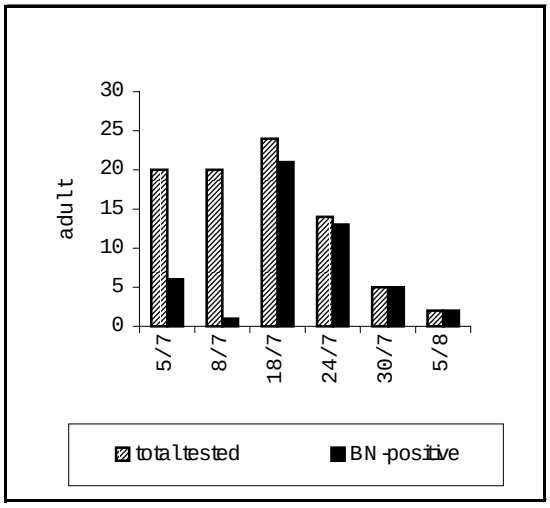

Fig. 3. Rate of BN-positive *Hyalesthes obsoletus* Signoret adults collected from nettle-beds with D-vac.

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AN INTERNAL POSITIVE CONTROL IN PCR-TESTS FOR THE DETECTION OF PHYTOPLASMA IN PLANTS AND INSECTS

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PCR is the most widely used technique to detect phytoplasma in grapevine. Grapevine tissue, however, contains considerable concentrations of enzyme inhibiting polyphenolic compounds or polysaccharides that could inhibit the PCR detection. Thus, it is often difficult to decide whether a negative PCR result is due to a negative sample or to a blocked PCR reaction. This is a problem particularly for critical applications such as quarantine tests. We observed an additional fragment in a nested PCR procedure that could be used as an internal positive control in PCR tests.

When P1/P7 (2, 3) nested fU5/rU3 (1) primers were used for detection of stolbur or elm yellows group phytoplasma we realized that the expected band of approximately 850 bp was usually accompanied by an unexpected band of 360bp. The same phenomenon was observed in a fU5/P7 half nested fU5/rU3 reaction, while no such fragment was amplified if only primer fU5 or rU3 were added in the second PCR. The additional band was separated on low melting agarose, excised and purified from the gel and diluted 1:100 in water. The fragment could be re-amplified with primers fU5/rU3. If both the fU5/P7 fragment and the unexpected fragment are mixed in a PCR reaction the latter is preferably amplified because of its considerably smaller size. To avoid inhibition of the amplification of the target fragment, the small fragment was diluted in 10fold series to the lowest concentration that could be steadily detected in a single round PCR with primers fU5/rU3 (fig. 1).

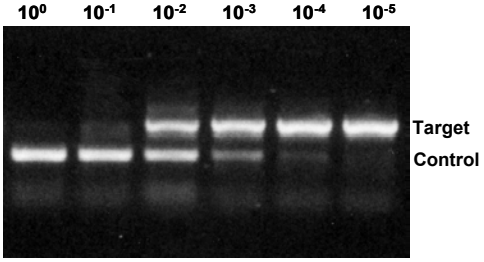

Figure 1. Single round PCR with primers fU5/rU3. The gel shows PCR products achieved when template DNA of a periwinkle isolate of Grapevine palatinate yellows (elm yellows group) phytoplasma was mixed with the control fragment in serial dilution from 100 to 10-5.

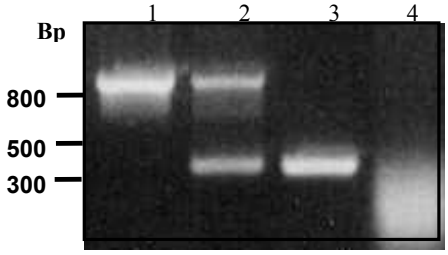

Figure 2. Use of the extra fragment fU5'/rU3 as an internal standard; 1: Positive result with high concentration of template DNA; 2: Positive result with a low concentration of template DNA; 3: Negative result but successful PCR; 4: PCR failed.

The analysis of the sequence obtained from the phytoplasma associated with Vergilbungskrankheit (Bois noir) (GenBank X76428; 4) revealed a partial complementary site for primer fU5. Six continuous bases of the 3'-end of this primer are complementary to the sequence and three more bases are matching. This is obviously sufficient to initialize the amplification of the observed extra band. The calculated distance of this site to the binding site of primer rU3 is 364 bp and corresponds exactly to the observed fragment size. Due to the only partial homology of the fU5 primer to the alternative binding site the extra fragment fU5'/rU3 disappears with increasing annealing temperature while the target sequence is still amplified.

We tested the suitability of the small fragment as an internal standard for PCR. Tests were carried out with grapevine and insect samples infected by stolbur or elm yellows group phytoplasma as templates. The diluted fU5'/rU3 fragment was added to the reaction mixture. An example of the results is shown in figure 2. The target sequence was consistently amplified from positive samples. High template concentration led to the exclusive amplification of the target while both the target and the control fragment were amplified when the template concentration was low. Only the control fragment was amplified from negative samples. Inhibition of the reaction was indicated by a missing fragment.

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DETECTION AND IDENTIFICATION OF PHYTOPLASMAS BELONGING TO 16SrV-D IN *SCAPHOIDEUS TITANUS* ADULTS IN PORTUGAL

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Flavescence dorée (FD), the epidemic form of grapevine yellows (GY) is an harmful disease caused by wall-less bacteria, now named phytoplasmas, and transmitted by *Scaphoideus titanus* Ball. So far, this phloem-feeding leafhopper of neartic origin is present in several countries of the new and the old world. Recently, *S. titanus* was identified in the North of Portugal (7): for this reason, an extensive survey was conducted by the Direcção General da Protecção das Cultares (DGPC) and Regional Services (DRAs): several vineyards were examined all over the country in the years 2001 and 2002 with the aim to acquire information about the possible presence of Flavescence dorée. Preliminary results shows that *S. titanus* in present only in the North of Portugal (Trás-os-Montes and Entre-Douro-e Minho).

Here we reports the results of a the investigation: 54 individual samples of *S. titanus* were collected in vineyard and total nucleic acids were singly extracted from each insect as previously described (2). The PCR and RFLP assays were conducted as elsewhere reported (2, 3, 6) tested by polymerase chain reaction (PCR) assays, using the universal primer pair P1/P7 for the amplification of 16SrDNA gene from all the phytoplasma known. Then nested PCR and subsequent RFLP assays were carried out in order to identify the phytoplasmas detected in the PCR positive samples (5): 9 samples were positive in nested PCR tests. The RFLP assays conducted on the obtained amplicons revealed the presence of 9 samples containing phytoplasmas belonging to the subgroup 16SrV-D and (1, 4, 6). Moreover, in 2002, preliminary assays were conducted on leaf samples collected in selected vineyards: so far, only phytoplasmas belonging to 16SrI-B subgroup were detected in symptomatic grapevine plants.

This is the first report indicating the presence, in Portugal, of phytoplasmas associated with FD and belonging to 16SrV-D. Although no evident spreads of the disease are so far observed in Portugal, nevertheless it is crucial to further investigate the occurrence and distribution in Portugal of such phytoplasma in *S. titanus* and in grapevine, in order to prevent possible outbreaks of FD in Portugal and in neighboring viticultural areas.

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PHYTOPLASMA [STOLBUR-SUBGROUP (BOIS NOIR)] INDUCED CHANGES ON PIGMENTS, RUBPC AND ELECTRON TRANSPORT ACTIVITIES IN FIELD GROWN GRAPEVINE (*VITIS VINIFERA* L. CV. CHARDONNAY)

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In this work we have studied the effect of phytoplasma-induced grapevine yellows on some features of the thylakoids from field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. The level of chlorophyll (Chl), carotenoids (Car), Ribulose 1,5-bisphosphate carboxylase (RuBPC) and soluble proteins were markedly decreased in phytoplasma infected leaves. In isolated thylakoids, phytoplasma caused marked inhibition of whole chain and photosystem (PS) II activity. The artificial exogenous electron donors, DPC and NH2OH significantly restored the PSII activity in both mild and severely infected leaves. The same results were obtained when Fv/Fm was evaluated by Chl fluorescence measurements. The marked loss of PSII activity in infected leaves was evidently due to the loss of 33, 28-25, 23, 17 and 10 kDa polypeptides.

Grapevine yellows (GY) diseases are a group of disorders of *Vitis vinifera* L. with similar symptoms. GY are characterised by yellowing, downward curling of leaves on stunted shoots. By employing the Polymerase Chain Reaction (PCR) or DNA hybridisation techniques GY were associated with phytoplasmas belonging to different groups (3), mainly on the basis of 16S rDNA polymorphisms. Phytoplasmas of the stolbur-subgroup and the elm-yellows-group (Flavescence doree-FD) are widespread in Europe and of great economic importance. Two hopper species, the leafhopper Scaphoideus titanus (vector of FD) and the planthopper Hyalesthes obsoletus (vector of BN and VK) have been identified as vectors of grapevine yellows. The aim of this work is to investigate the effects of phytoplasma infection on the pigments, soluble proteins, RuBPC and electron transport activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay).

The grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves used in this study were taken from naturally phytoplasma (grapevine yellows) infected field grown plants located in Istituto Agrario di San Michele all' Adige vineyards, San Michele all' Adige, Italy. Amounts of Chl, Car and total soluble proteins were determined spectrophotometrically by the methods of Lichtenthaler (5) and Bradford (2). The crude leaf extract isolated and assay of RuBPC activity was measured by Bowes and Ogren (1). All measurements of Chl fluorescence were performed, on detached leaves, with portable PAM-2000 fluorometer (Walz, Effeltrich, Germany). Thylakoid membranes were isolated and reactions of photosynthetic electron transport mediated by whole chain, PSII and PSI were measured as described by Nedunchezhian *et al*. (6). Thylakoid membranes and crude leaf extractes were separated using the polyacrylamide gel system of Laemmli (4).

The contents of total Chl and Car were markedly decreased in phytoplasma infected leaves (Table 1). This is due to phytoplasma probably enhanced the chlorophyllase activity in the leaves. We found an increases of Car/Chl ratio and decrease of Chl *a/b* ratio noticed in phytoplasma infected leaves (Table 1). The content of soluble proteins and RuBPC were reduced markedly in severly infected leaves (Table 1). Such reduction was mainly due to inhibition of protein synthesis and induced by phytoplasma. This is also supported by SDS-PAGE analysis of crude leaf extracts of RuBPC proteins shown by a marked loss of both LSU (55 kDa) and SSU (15 kDa) polypeptides in phytoplasma infected leaves (Figure 1). The control leaves showed a good PSII activity than the infected leaves when measured as the Fv/Fm ratio. Increase of infection in leaves leads to decrease in Fv/Fm ratio; severely infected leaves showed the lowest Fv/Fm ratio (Table 1). Analysis of various electron transport chain, the PSII and whole chain electron transport markedly inhibited in phytoplasma infected leaves (Table 1). Among the various electron donors DPC and NH2OH were found to be more effective in restoring PSII activity in both mild and severely infected leaves (Table). These results clearly indicate that phytoplasma induced changes on the donor side of PSII. Supporting evidence for the damage to PSII activity was obtained from the thylakoid polypeptide analysis: a comparison of thylakoids of phytoplasma infected leaves with those of the control showed specific loss of 47, 43, 33, 28-25, 23, 17 and 10 kDa polypeptides (Figure 1). From the results we concluded that phytoplasma infection causes non-specific, general stress responses and induced rapid senescence or ageing in grapevine leaves.

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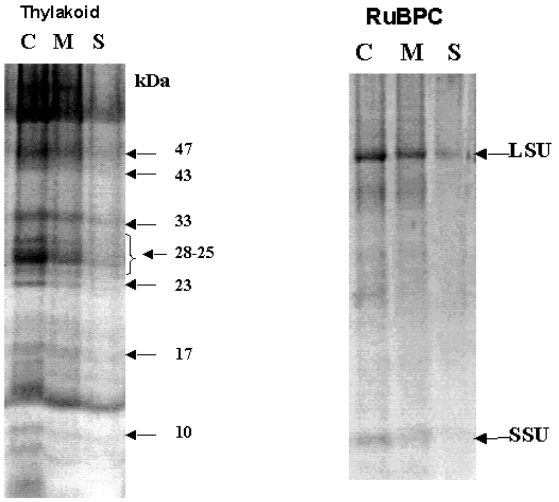
Table 1. Changes in pigments, soluble proteins, RuBPC, Chl fluorescence and electron transport activity in control and different levels of phytoplasma infected leaves. Figures in parantheses are percentage inhibition with reference to respective control leaves. Values are the means ± SE (n=5).

Parameters		Control	Phytoplasma infected	
			mild	severely
Pigments				
Chl a	[mg g^{-1} (f.w)]	2.452±0.10	1.570±0.07 (36)	0.850±0.07 (65)
Chl b	[mg g^{-1} (f.w)]	0.901±0.04	0.710±0.03 (21)	0.424±0.02 (53)
Total Chl	[mg g^{-1} (f.w)]	3.353±0.14	2.280±0.10 (32)	1.274±0.06 (62)
Car	[mg g^{-1} (f.w)]	0.800±0.04	0.625±0.03 (22)	0.384±0.02 (52)
Chl a/b		2.7±0.12	2.2±0.20	2.0±0.22
Car/Chl		0.230±0.010	0.270±0.012	0.300±0.014
Biochemical				
Soluble proteins [mg g^{-1} (f.w)]		44.80±2.0	33.60±1.4 (25)	23.30±1.0(48)
RuBPC [µmol (CO_2) mg^{-1} (prot.) h^{-1}]		55.40±2.5	39.81±1.6 (28)	27.10±1.2 (51)
Chl fluorescence				
Fo		126.0±5.9	123.0± 6.0 (2)	118.0±5.3 (6)
Fv		605.0±28.2	454.0±20.8 (25)	263.0±11.8 (57)
Fv/Fm		0.791±0.033	0.730±0.030 (8)	0.552±0.026 (30)
Electron transport				
Whole chain (H_20 → MV) [(µmol (O_2) $mgChl^{-1}$ h^{-1})]		132.4±6.2	92.6±4.0 (30)	46.3±2.1 (65)
PSI ($DCPIPH_2$ → MV) [(µmol (O_2) $mgChl^{-1}$ h^{-1})]		356.6±16.2	328.0±15.4 (8)	303.1±13.2 (15)
PSII (H_2O → DCBQ) [(µmol (O_2) $mgChl^{-1}$ h^{-1})]		198.0±9.4	190.0±8.5 (6)	180.1±8.0 (9)
PSII (H_2O → SiMo) (µmol (O_2) $mgChl^{-1}$ h^{-1})		110.8±5.1	81.9±3.6 (26)	45.4±2.1 (59)
DCPIP photoreduction				
H2O → DCPIP (µmol (DCPIP red.) $mgChl^{-1}$ h^{-1})		184.5 ± 8.9	140.2 ± 6.9 (24)	71.9 ± 3.4 (61)
DPC → DCPIP (µmol (DCPIP red.) $mgChl^{-1}$ h^{-1})		186.2 ± 9.1	171.3 ± 8.2 (8)	158.2 ± 7.4 (15)
NH2OH → DCPIP (µmol (DCPIP red.) $mgChl^{-1}$ h^{-1})		186.8 ± 8.8	168.1 ± 8.0 (10)	153.1 ± 7.6 (18)
MnCl2 → DCPIP (µmol (DCPIP red.) $mgChl^{-1}$ h^{-1})		184.8 ± 9.0	145.1 ±7.1 (21)	76.0 ± 3.0 (59)

Figure 1



GENOMIC DIVERSITY OF THE FLAVESCENCE DORÉE PHYTOPLASMA

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Phytoplasmas have some of the smallest genomes of any self-replicating organism, ranging from 530 kilobases (kb) to 1350 kb (5). The genome size of four elm yellows (EY) group phytoplasmas, including alder yellows, two isolates of elm yellows from France and the United States and rubus stunt, ranges from 680 kb to750 kb (5). We report the preliminary size estimation of chromosomes and genomic diversity of two strains of the flavescence dorée (FD) phytoplasma, also from the EY group. FD phytoplasma isolates, FD92 and FD2000 were maintained in *Vicia faba* in glasshouse conditions. Grapevines, var. Grenache, infected with FD phytoplasma were collected from the Languedoc region of France. *Vicia faba* and grapevine, in which no phytoplasma were detected by PCR, was used as a control. Phytoplasma chromosomes were prepared as previously described (7) except that 50-100g fresh weight of plant material was ground in 300-600ml of isolation buffer. Plant material included petioles, stems and leaf veins of *Vicia faba* and petioles, leaf veins and phloem scrapings from shoots and cordons from grapevine. To accurately determine the phytoplasma chromosome size, phytoplasma chromosome preparations were incubated with restriction enzymes prior to pulsed field gel electrophoresis (PFGE) to digest the phytoplasma chromosome into smaller fragments. The restriction endonucleases used were *Apa*I, *BssH*II, *Eag*I, *I-Ceu*-I, and *Sal*I (New England Biolabs, USA). PFGE was performed using a CHEF DRIII system (Bio-Rad, USA) at 14°C and 6V/cm. PFGE of undigested chromosome blocks was for 22 hours at a pulse time of 50-90s. PFGE using restriction enzyme digested chromosome blocks was for 20 hours at a pulse time of 2-40s. After electrophoresis the PFG's were stained in 1 µg/ml ethidium bromide solution and viewed with a short wavelength UV transilluminator. The molecular weight of the chromosome DNA was estimated using a lambda DNA concatamer ladder (Lambda ladder PFG marker, New England Biolabs). The DNA was transferred to a Hybond N+ membrane (Amersham Biosciences Europe, France) using Southern blot transfer techniques. The phytoplasma chromosome was detected by hybridization to ^{32}P-dCTP labeled probes of the 16S rDNA PCR product from FD phytoplasma, or *Rsa*I digested FD chromosome that had been isolated after PFGE. The lambda marker ladder was detected using a radioactive labeled probe derived from λ DNA/HindIII markers (Amersham Biosciences Europe). Kodak double emulsion BIOMAX MS film and a BIOMAX MS intensifying screen were used for PFGE autoradiography. Filters were exposed at –80°C for one hour and/or up to seven days

The preliminary chromosome size for both the FD 92 phytoplasma and FD 2000 phytoplasma is approximately 670 kb, smaller than that reported from other EY group phytoplasmas (5). I-Ceu-I did not digest either isolate. *Apa*I, *Eag*I, and *Sal*I produced similar banding patterns in both isolates. *BssH*II produced different banding patterns in each isolate. Digestion of FD 92 with BssHII resulted in two bands of approximately 558kb and 110 kb. Digestion of FD 2000 with *BssH*II resulted in three bands of approximately 540kb, 78 kb and 55kb. Previous research has shown that molecular diversity of isolates of FD phytoplasma exists, based on differences of the 16S rRNA gene sequence and other DNA fragments (1, 3, 4, 6).

Phytoplasma chromosomes can be isolated and detected from grapevine by PFGE (2). However, the FD phytoplasma chromosome was never detected by PFGE from any grapevine sample, positive by PCR for FD phytoplasma, by ethidium bromide staining and UV transillumination or by hybridization with radioactively labeled phytoplasma probes. This indicates that the concentration of this phytoplasma is low in grapevine material.

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DIRECT SENSITIVE DIAGNOSIS OF FLAVESCENCE DORÉE AND BOIS NOIR USING A MULTIPLEX NESTED-PCR ASSAY AND ITS USE IN FIELD SURVEYS

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Flavescence dorée (FD) is an epidemic Grapevine yellows (GY) associated with a phytoplasma in the Elm yellows (EY) group (6) which dramatically affects large winegrowing areas in Southern France and Northern Italy (4, 3) and has been reported in Spain (2). FD is a quarantine organism in the European Community. Its quick progression is due to the presence of its vector *Scaphoideus titanus* Ball, a vine-feeding leafhopper of American origin, which has spread in Europe in a wide area (4). Bois noir (BN) and the similar disease Vergilbungskrankheit (VK) are the second important group of GY in Europe. They are associated with stolbur phytoplasma and transmitted by the planthopper *Hyalesthes obsoletus* Sign., which feeds on grapevine erratically (7, 8). The incidence of BN/VK has also increased in the last decade in several countries of Europe and Asia Minor. Hence, the regions of diffusion of FD and BN are overlapping. Prevention, control and prophylaxis of FD on one hand and of BN/VK on the other hand, rely on very different strategies (5). Because of the similarities of symptoms and possible occurrence of the two diseases in the same area or vineyard, straightforward techniques for analyses of numerous samples are necessary for the monitoring of these two diseases. Up to now, wide-scale diagnosis relied on parallel analyses of samples with two specific procedures or on PCR-RFLP analyses of conserved ribosomal DNA of phytoplasma.

In a preceding work, primers FD9f/r and STOL11f/r which amplify with polymerase chain reaction (PCR), non ribosomal DNA fragments specific for FD and stolbur phytoplasma, called FD9 and STOL11, respectively, have been developed and used in a multiplex PCR assay (6). However, this procedure demonstrated a lack of sensitivity on field samples (unpublished data). The present work was dedicated to the development of multiplex amplification of fragments FD9 and STOL11 with nested-PCR, for greater sensitivity. In addition to primers FD9f/r and Stol11f2/r1 (6), we used for nested amplification the internal primers FD9f3b/r2, modified from primers FD9f3/r2 (1) and the internal primers STOL11f3/r2 constructed on the STOL11 fragment from the aligned sequence of STOL11 fragment in 4 different isolates of stolbur phytoplasma (data not shown). These primers are described in Figure 1. The size of FD9f3b/r2 product and STOLf3/r2 product is about 1160 bp and 720 bp, respectively. Hence, PCR products could be identified directly with agarose gel electrophoresis.

The two external primer pairs FD9f/r and STOL11f2/r1 were used simultaneously in a first PCR run and the two internal primer pairs FD9f3b/r2 and STOL11f3/r2 were used simultaneously in a nested-PCR run. For the exploration of the best PCR conditions, DNA extracted with the CTAB (cethyl trimethyl ammonium bromide) procedure (1) from leaf veins of 3 FD-infected Baco 22A, 3 BN-infected Chardonnay and from healthy Baco 22A cuttings, was used in parallel assays. All reaction mixtures were the same, except for concentration of primers. Two different conditions (a and b) were used in the first PCR run and three different conditions (i, j and k) were used in the second PCR run (Table 1). Among the six combinations, combination [b, j] was selected because it produced a balanced detection of both phytoplasmas and did not show non specific products with phytoplasma-infected samples or healthy control (results not shown). After numerous verifications of the performance of the procedure with the latter conditions on a variety of samples collected in the field, the concentration of primers FD9 and STOL11 in each step, were further modified to obtain more specificity. In the first PCR run, FD9f/r primers were used each at 0.375 µM and STOL11f2/r1 primers each at 0.0625 µM. In the nested-PCR run, primers FD9f3b/r2 and STOL11f3/r2 were used each at 0.375 µM. The latter conditions were evaluated to simultaneously detect and characterize FD and BN phytoplasmas in field-collected grapevine samples (Figure 2A). They were also used on other woody host plants and insect vectors of phytoplasmas belonging to the EY-group and stolbur group.

The procedure was validated for use in routine assays of numerous grapevine samples from the field in the frame of the 2002 national survey at the Laboratoire National de la Protection des Végétaux (LNPV). Leaves with petioles were taken during Summer 2002 on 5 symptomatic grapevines per affected vineyard in each of 2,525 vineyards from different regions of France. The petiole of one leaf of each of the 5 grapevines from one plot were pooled for DNA extraction. The results showed that FD and BN phytoplasma could be detected simultaneously in a number of pooled samples (Figure 2 B). When tissue from individual leaves composing the latter mixed-infected pooled samples, was tested separately, FD and BN could be separately detected, thus confirming that both diseases may be present in the same vineyard and showing that the technique is fitted to large-scale bivalent diagnosis. Mixed infection in individual grapevines was not found.

This new procedure is a sensitive differential assay for simultaneous detection of FD and BN phytoplasma avoiding RFLP analyses, though these two phytoplasmas belong to different ribosomal groups. We chose two DNA fragments of quite different sizes that might be directly identified with agarose gel electrophoresis. Hence, it was necessary that PCR conditions would not favor the copy of the shorter STOL11 fragment with weak or false negative amplification of FD9 fragment as a consequence. A balanced detection system of both phytoplasmas was obtained by using a lower concentration of STOL11 primers in the first step (Table 1) and raising the annealing temperature. The length of the formerly designed primer FD9f3 (1) was increased to permit amplification of FD9 fragment with a hybridization temperature of 55°C. Mixed infections in pooled samples were more easily detected and identified than with RFLP analyses of ribosomal DNA, probably because PCR

conditions in the multiplex procedure permit amplification of the two DNA fragments with balanced efficiency. The procedure was shown to be sensitive and reliable. It is also quick and cheap since it does not require RFLP analyses.

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		1st PCR run		
			a	b
		FD9f - FD9r STOL11f- STOL11r	0.5 µM 0.05 µM	0.5 µM 0.025µ M
2nd PCR run	i	FD9f3b – FD9r2 STOL11f3 – STOL11r2	0.5 µM 0.5 µM	0.5 µM 0.5 µM
	j	FD9f3b – FD9r2 STOL11f3 – STOL11r2	0.5 µM 0.25 µM	0.5 µM 0.25 µM
	k	FD9f3b – FD9r2 STOL11f3 – STOL11r2	0.5 µM 0.125 µM	0.5 µM 0.125 µM

Table 1. Concentration of primers in the 1rst multiplex PCR run (conditions a and b) and in the multiplex nested-PCR run (conditions i, j, k). The six combinations were compared and combination [b, j] was selected.

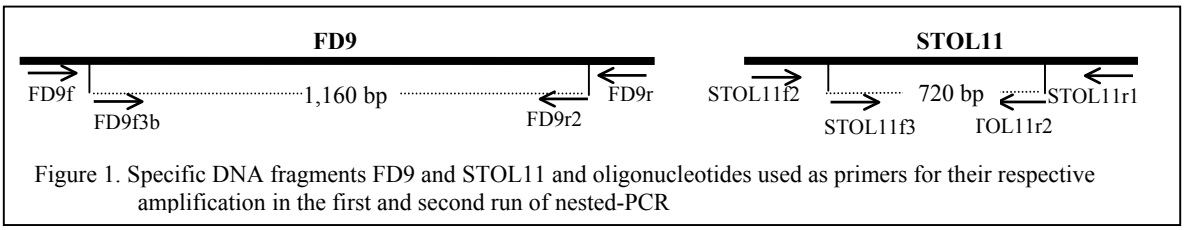

Figure 1. Specific DNA fragments FD9 and STOL11 and oligonucleotides used as primers for their respective amplification in the first and second run of nested-PCR

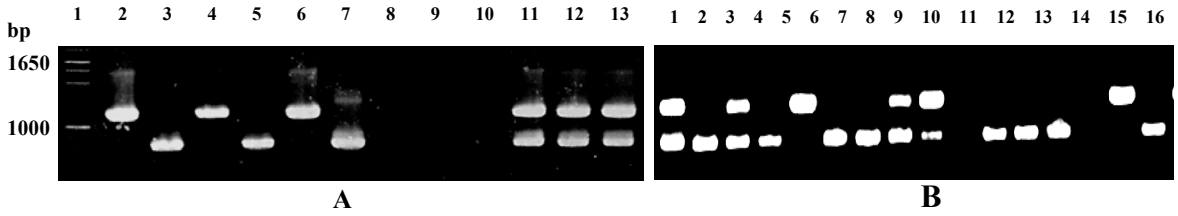

Figure 2 A and B. Agarose gel electrophoresis of amplimers obtained in the multiplex nested PCR with DNA from different plant samples. A, Lane 1, 1kb Ladder (Appligene); 2, FD-infected Chardonnay; 3 and 5, two different BN-infected Chardonnay; 4, FD-infected Pinot noir; 6 and 7, reference phytoplasma strains FD70 and STOL, respectively, maintained in periwinkle; 8 and 9, healthy grapevine seedlings of cultivar Baco 22A; 10, healthy periwinkle seedling.; 11, 12, 13 : double FD+Stolbur graft-infected periwinkle, 3 different extracts. B. Pooled samples (1 petiole from each of 5 grapevines per sample) collected in GY- affected vineyards in 2002 in France. Lane 1, 3, 8 and 9 : vineyard plots showing the presence of both FD and BN; lane 2, 3, 6, 7, 11, 12, 13 and 16 : plots showing BN alone; lane 5 and 15: plots showing FD alone.

GENETIC VARIABILITY AND DISTRIBUTION OF GRAPEVINE PHYTOPLASMAS OF GROUP 16SR-V IN LOMBARDIA (NORTHERN ITALY)

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Flavescence dorèe (FD) is a devastating phytoplasma disease of grapevine that is widespread in several countries such as France, Italy, and Spain. Previous studies indicate that the phytoplasmas associated with this disease belong to elm yellows group (16SrV) and that they are transmitted by *Scaphoideus titanus* (2, 3). Based on the analysis of 16SrRNA gene, FD associated phytoplasmas can be differentiated in two distinct subgroups, 16SrV-C and 16SrV-D (1, 8). Furthermore, the analysis based on a ribosomal protein fragment, containing the 3' end of *rpl22* and the entire rpS3 gene, identified four distinct genotypes among the phytoplasmas associated with FD, that cannot be differentiated based on 16SrRNA gene sequence. The four *rps3* subgroups are consistent with those delineated by precedent analysis of more variable non-ribosomal DNA fragment FD9 (1, 5, 6, 7).

The present work aims to further investigate the possible variability and the role of those phytoplasma strains involved in the spread of FD in Lombardia region. The investigation was carried out in selected vineyards located in three different zones: Oltrepò pavese, Oltrepò mantovano and Valtenesi. Preliminary assays on DNA extracts were conducted on 23 grapevine samples collected in July 2001. PCR and RFLP analyses were carried out on 16SrDNA gene sequence of each amplicon (3, 4). Furthermore, the ribosomal protein gene sequences, coding for the 3' end of a rpl22 and the entire rps3 proteins, were analysed and RFLP tests were done with *Taq*I, *Tsp*509I and *Mse*I enzymes on rpF2(V)/R1 amplicons.

The PCR/RFLP analysis on 16SrRNA gene sequences and the subsequent *rps3* assays conducted on 23 grapevine samples showed the presence of 16SrV-C and 16SrV-D phytoplasma subgroups, and revealed their different geographical distribution. In detail, the subgroup 16SrV-C was found only in Oltrepò pavese whereas the 16SrV-D was found in all the areas examined.

Moreover, the cloning and the sequencing analysis of rpF2(V)/R1 amplicons revealed the presence of two groups of DNA variant sequences. Those related to phytoplasma subgroup 16SrV-C shared a sequence homology of 99% within the same group and a sequence homology of 98% when compared with the sequences of phytoplasma subgroup 16SrV-D.

Also, 16SrV-D sequence group showed a level of homology equivalent to 99% with rps3 gene sequence of the phytoplasma VR2 (AF396951) [subgroup rpE] while 16SrV-C shared a sequence homology of 99% with *rps3* gene sequence of phytoplasmas AL202 and PV259 (AF396945 and AF396942) [subgroup rpG]. Further study will be conducted in order to evaluate the significance on these *rps3* sequence variants and to assess their role in the FD aetiology and epidemiology.

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SURVEY ON PHYTOPLASMAS IDENTIFIED IN CHILEAN GRAPEVINES

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Objectives

To verify association of phytoplasmas with yellows symptoms detected in Chilean grapevines plants in the last two years molecular detection was employed to test samples collected in the grapevine growing region of the state. First years of survey allow to identify 16SrI-C phytoplasmas in some of the symptomatic plants examined (7), during 2003 further monitoring was performed to cover the main grapevine growing areas of Chile.

Material and Methods

Grapevine samples showing typical yellows symptoms were collected starting from March 2002 from the V, VI, VII and Metropolitana Regions (RM) of Chile. The molecular testing was carried out on 14 samples in 2002 and on 41 in 2003. Total nucleic acids were extracted from 1 g of leaf midribs following the protocols described by Prince *et al.* (10) in 2002, and by Zhang *et.al.* (12) in 2003 with further silica particles purification step, diluted to a final concentration of 20 ng/µl in sterile deionized water. One µl of this dilution was used in direct PCR using universal primer pair P1/P7 (6, 11), followed by nested PCR on products diluted 1:30 in sterile distilled water, using primer pair R16F2/R2 (9). Group specific primers R16(I)F1/R1 and R16(V)F1/R1 (9) were finally employed to increase detection sensitivity. Primers M1/M2 (8) were also employed in nested-PCR to cross confirm results. Each 25 µl PCR reaction mix contained 2,5 µl 10X PCR buffer, 0,8 U of *Taq* polymerase (Polymed, Florence, Italy), 0,2 mM dNTPs, 1,5 mM MgCl2 and 0,4 µM each primer. Thirty-five PCR cycles were performed in an automated thermocycler (Biometra, Uno Thermoblock, Gottingen, Germany, EU) under the following conditions: 1 min (2 min for the first cycle) denaturation step at 94oC, 2 min for annealing at 50oC and 3 min (10 min for the last cycle) for primer extension at 72oC. Six µl of PCR products were analyzed in a 1% agarose gel stained with ethidium bromide, and then visualized with an UV transilluminator. To differentiate among phytoplasmas, PCR products were digested with *Tru*I, *Taq*I, *Rsa*I, *Alu*I, *Hha*I, and *Tsp509*I restriction enzymes according with the amplicon studied, and RFLP fragments were separated in a 5% polyacrylamide gel stained with ethidium bromide, and then visualized with an UV transilluminator and profiles were compared with those of described phytoplasma strains.

Results

Molecular analyses demonstrated phytoplasma association with yellows disease in the surveyed vineyards of Chile: phytoplasmas were identified in grapevines collected in the regions studied in both 2002 and 2003. These phytoplasmas were mainly detected with nested PCR using general primers R16F2/R2 and M1/M2 and/or with R16(I)F1/R1 specific primers; identification was obtained by RFLP using the above mentioned enzymes. Phytoplasma identification showed the presence of phytoplasmas belonging to 16SrI-C subgroup (Fig. 1) in 2002 samples, the same subgroup was occasionally detected in grapevine in the past in Italy in both Veneto region (3) (North Italy) and recently in Abruzzo region (5) (Central-South Italy). In 2003 samples, 16SrI-B phytoplasmas were identified together with phytoplasmas belonging to group 16SrVII (Fig. 2) in some cases in mixed infection (data not shown); phytoplasmas of this subgroup were detected in grapevine in both north and south Italy (1, 4). Phytoplasmas of group 16SrVII were never reported before in grapevine; they were however reported to infect ash and lilac in the US and a subgroup 16SrVII-B has been identified in South America (Brazil) in São Paulo state in *Erigeron* and *Catharanthus roseus* plants naturally infected and showing witches' broom symptoms (2). Phytoplasma clearly referable to group 16SrXII-A ("Bois Noir") (3) were also identified together with a previously unreported RFLP profile in sample 28 on amplicons obtained with R16(I)F1/R1 with four restriction enzymes (Fig. 2). This phytoplasma show profiles referable to group 16SrI or 16SrXII-A

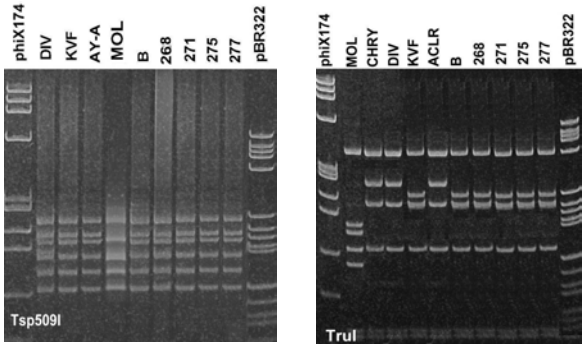

Fig. 1. Polyacrylamide gels (5%) showing the *Tsp509*I and the *Tru*I RFLP patterns of phytoplasma rDNA fragments obtained after nested PCR with primers R16(I)F1/R1 from grapevine samples with yellows symptoms from Chile 2002. Phytoplasma reference strains: MOL, Molière disease (16SrXII-A); CHRY, Chrysanthemum yellows from Germany (16SrI-A); DIV, diplotaxis virescence (16SrI-B); KVF, clover phyllody from France (16SrI-C); AY-A, aster yellows from Spain (16SrI-F). Markers: 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; pBR322 *Msp*I digested; fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34.

with *Hha*I, profiles referable to 16SrXII-A with *Tsp509*I and *Tru*I but it differes from all the employed controls with *Rsa*I. No FD phytoplasmas were identified in the samples analysed. All the samples resulted to be positive were collected from symptomatic grapevine plants located in VI, VII and Metropolitana Regions of Chile. There were not evidence of epidemic spreading of yellows symptoms in the surveyed vineyards.

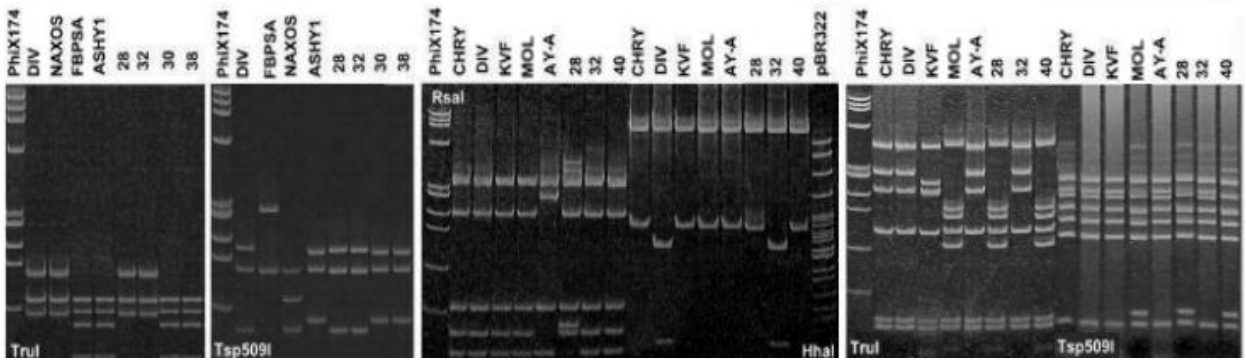

Fig. 2. Polyacrylamide gels (5%) showing the RFLP patterns of phytoplasma rDNA fragments obtained after nested PCR from grapevine samples with yellows symptoms collected in Chile in 2003. RFLP with *Tru*I and *Tsp509*I on the left were obtained on M1/M2 amplicons; profiles with *Rsa*I, *Hha*I, *Tru*I and *Tsp509*I on the right were obtained on R16(I)F1/R1 amplicons. Sample 28 show the undescribed profile referable to groups 16SrI and XII. Phytoplasma reference strains not in fig. 1: NAXOS, periwinkle virescence from Sicily (group 16SrIX-C), FBPSA, faba bean phyllody from Sudan (16SrII-C), ASHY1, ash yellows from New York State, US (16SrVII-A).

Research supported by Fundación para la Innovación Agraria, Gobierno de Chile. Proyecto FIA BIOT-01-A-013.

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MOLECULAR CARACTERISATION AND GEOGRAPHICAL DISTRIBUTION OF FLAVESCENCE DORÉE - PHYTOPLASMAS IN SPAIN

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Objectives

Flavescence dorée (FD) phytoplasma has been detected in Spain since 1996 in the Alt Empordà (NE Catalonia) and *Scaphoideus titanus* Ball. (the FD vector) has been recorded only in the 4 provinces of Catalonia (NE Spain): Barcelona, Girona, Lleida and Tarragona. Since 1996 specific fighting measures are carried out at the Alt Empordà where the first focus was detected; particularly spraying against *S. titanus* was done by helicopter in 1997 over an area of 1900 hectares; since 1999 this area has been increased to 2030 hectares. The present work reports FD disease distribution in Catalonia from 1996 to 2002. Samples of symptomatic grapevine plants from all the vine-growing areas in Catalonia were analysed for phytoplasma presence; once 16SrV phytoplasmas group was detected a finer molecular identification was performed. The phytoplasma strain characterisation at molecular level was also performed on nucleic acid from selected samples FD-positive collected during different years of the epidemic.

Material and Methods

FD presence and plant material. Focuses of FD were located by visual inspection of plots having more than 20% of their grapevine plants with symptoms of yellows. In order to confirm the presence of phytoplasmas, different samples showing symptoms of FD were collected not only in the province of Girona, where the focus of the epidemic was detected, but also in other vine-growing regions (provinces of Barcelona, Lleida and Tarragona)

DNA extraction. Leaf mid-vein tissues from 109 symptomatic grapevine plants obtained mainly between July and September from 1996 to 2002 were examined. Total DNA was isolated from fresh or frozen (-20ºC) tissues using the combined method described by Torres *et al*. (8). DNA from 10 control samples was extracted following the chloroform-phenol procedure described by Prince *et al*. (6) and stored in TE buffer at -20 °C.

Polymerase Chain Reaction. Nested PCR was performed with phytoplasma-universal primer pairs P1 (2) and P7 (7) followed by R16F2/R2 (4) or by $16R_{758f}$/$M23SR_{1804r}$ (=M1/B6) (5). PCR assays were performed using Ready-to-Go® PCR Beads (Amersham-Pharmacia Biotech) as previously described (7). P1/P7 amplicons were used as template in nested-PCR with R16(V)F1/R1 specific primer pairs for group 16SrV and R16(I)F1/R1 specific primer pairs for groups 16SrI and 16SrXII-A (3) performing 35 PCR cycles conducted using the following parameters: 1 min (2 min for the first cycle) at 94ºC, 2 min at 50ºC, and 3 min (10 min in final cycle) at 72ºC. Amplicons for RFLP analyses were obtained in a total volume of 25 µl PCR mixture containing 2.5 µl of 10X PCR buffer, 200 µM of each dNTP, 0.4 µM of primers pair and 0.625 U of Taq polymerase (Polymed, Florence, Italy, EU). The amplification conditions were the same as for amplification with specific primer pairs. Samples, lacking DNA, were run for each experiment as negative controls. After amplification, a 5 µl aliquot from each sample was electrophoresed in a 2% agarose gel and visualized by staining with ethidium bromide and UV illumination.

Restriction Fragment Length Polymorphism analyses. RFLP analyses on 200 ng of DNA from R16(V)F1/R1 and M1/B6 amplicons from positive grapevine samples and the 10 control samples, were performed with *Tru*I, *Taq*I and *Bfa*I. Further RFLP analyses with *Tru509*I were performed on amplicons obtained with primers FD9f3/r2 in nested PCR as described by Angelini *et al.* (1). The restriction patterns were compared after electrophoresis on a 5% polyacrylamide gel followed by ethidium bromide staining, and photographed under UV at 312 nm using a transilluminator.

Sequencing of PCR products and phylogenetic analyses. Amplification products were cleaned using the E.Z.N.A. Clean kit (Omega Biotech) and strands were sequenced using primers P1, P7, R16F2 and/or R16R2 with an ABI Prism 377 genetic Analyzer and the ABI Prism™ BigDye™ terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem). Bioedit™ software was used to identify the consensus sequence from the two strands of each amplification product. To search for the best alignment ClustalW software for multiple sequences was used. Twenty-nine in-group sequences plus one outgroup sequence were aligned at 1261 positions. Pair wise comparisons of phytoplasma 16SrDNA sequences were obtained by optimal global alignment with Bioedit 5.0.6 software. A phylogenetic tree was constructed by the Neighbor-Joining method and distance estimation of van de Peer and de Wachter (9) using the software TREECON 1.3b. A parsimony analysis using the computer program PAUP 4.0b4a (Phylogenetic Program Using Parsimony) was carried out (http://evolution.genetics.washington.edu/phylip/software.html). Branch robustness was estimated by bootstrap analysis of 10000 heuristic replicates using the fast stepwise-addition option.

Results

In the survey carried out from 1996 to 2002 in Catalonia 109 symptomatic samples were analysed (Table 1). Among over the 42 samples positives to phytoplasma presence, 17 gave the PCR products of expected size with specific primers R16(V)F1/R1 for phytoplasmas of Elm Yellows (16SrV) group and were considered FD phytoplasmas since they resulted to be different from Elm yellows phytoplasma after *Bfa*I digestion. A selection of Spanish FD strains was

characterized by RFLP analyses and sequencing of a 16S-23S ribosomal RNA gene fragment, as well as by RFLP analyses on the non-ribosomal DNA fragment FD9f3/r2. The RFLP results indicated that the FD phytoplasmas detected in grapevine samples were molecularly undistinguishable from each others, and were referable to phytoplasmas belonging to the 16SrV group (elm yellows) subgroup D. The phylogenetic distance tree and the parsimony analysis show that Spanish FD strains clustered closely together along with six other established Elm yellows (EY) group member, with a confidence level of 100% (bootstrap) supporting its assignment to the EY (16SrV) group in agreement with RFLP results.

In spite of the fact that viticulture areas in Spain are spread in almost all the country, Flavescence dorée has only reached some vineyards of the Alt Empordà (NE Catalonia). The spatial distribution of affected plots draw a triangle with its base at the Pyrenées which suggest the influence of the Tramuntana wind helping *S. titanus* to reach healthy plots from infected ones. The disease incidence in the affected area (Table 2) has decreased since 1996: in 2002 there was zero plots with more than 20% of symptomatic grapevines and only 23 plots with less than 20% of symptomatic plants.

Table 1. Grapevine samples analysed in this study. Detection of phytoplasmas with R16(V)F1/R1 and R16(I)F1/R1 group specific primer pairs. (*number of positive samples with universal nested-PCR related to total of samples analysed)

	Girona	Tarragona	Lleida	Barcelona
nº positives/nº samples*	23/36	10/32	1/6	8/35
+ R16(V)F1/R1	17	0	0	0
+ R16(I)F1/R1I	6	10	1	8

Table 2. Occurrence of FD, in productive vineyards from Alt Empordà (Girona), from 1996 to 2002.

Year	> 20% symptomatic plants		<20% symptomatic plants	
	Area (Ha.)	Nº plots	Area (Ha.)	Nº plots
1996	23.85	29	77.4	87
1997	15.38	20	135.41	154
1998	0	0	82.81	92
1999	0	0	41.98	45
2000	0	0	31.48	33
2001	0.45	1	28.99	29
2002	0	0	23	23

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IDENTIFICATION OF PHYTOPLASMAS ASSOCIATED WITH GRAPEVINE YELLOWS IN ABRUZZO REGION (ITALY)

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Objectives

Grapevine cultivation in Abruzzo region covers about 38,500 ha, mainly located in the hills near to the seaside of Chieti (more than 75%), Pescara and Teramo provinces. In L'Aquila province grapevine cultivation is less than 4% of the viticultural area of the whole region and is present mainly in Peligna Valley. Wine production is very relevant in the region ranging from 3.5 to 4.0 million of hl per year. In the last decade an improvement of quality was reached with increasing of wine export to Europe, Japan, US, Canada, Australia and Latin America. To prevent the spreading of Flavescence dorée disease, a monitoring of phytoplasma presence by using molecular detection tools was started in the region since 2000. The three Abruzzo provinces having the majority of grapevine cultivation were subjected to surveys to verify yellows symptoms presence and their diffusion. The typical symptomatology was scattered present in these areas, especially in the Southern province of the region.

Material and Methods

Grapevine samples showing typical yellows symptoms were collected starting from August 2000 in the three major provinces growing grapevines in Abruzzo region (Chieti, Teramo, and Pescara). The molecular testing was carried out on 69 samples in 2000, on 50 in 2001 and on 31 in 2002. Total nucleic acids were extracted from 1 g of leaf midribs following the protocol described by Prince *et al*. (7), diluted to a final concentration of 20 ng/µl in sterile deionized water. One µl of this dilution was used in direct PCR with universal primer pair P1/P7 (3, 9), followed by nested PCR on products diluted 1:30 in sterile distilled water, using primer pair R16F2/R2 (6). Phytoplasma group specific primers R16(I)F1/R1 and R16(V)F1/R1 (6) were finally employed to increase sensitivity of Flavescence dorée (ribosomal group 16SrV) and Bois Noir (BN) (ribosomal subgroup 16SrXII-A) detection. In 2002 tests primers M1/M2 (5) were also employed in nested-PCR. Each 25 µl PCR reaction mix contained 2,5 µl 10X PCR buffer, 0,8 U of *Taq* polymerase (Polymed, Florence, Italy), 0,2 mM dNTPs, 1,5 mM $MgCl_2$ and 0,4 µM each primer. Thirty-five PCR cycles were performed in an automated thermocycler under the following conditions: 1 min (2 min for the first cycle) denaturation step at 94oC, 2 min for annealing at 50oC and 3 min (10 min for the last cycle) for primer extension at 72oC. Six µl of PCR products were analyzed in a 1% agarose gel electophoresis, stained with ethidium bromide, and then visualized with an UV transilluminator. To differentiate among phytoplasmas, PCR products were digested with *Tru*I and *Tsp509*I restriction enzymes, RFLP fragments were separated in a 5% polyacrylamide gel stained with ethidium bromide, and then visualized with an UV transilluminator.

Results

Molecular analyses demonstrated phytoplasma association with yellows disease in the majority of surveyed vineyards of Abruzzo region: BN phytoplasmas were identified in grapevines collected in all the three provinces studied, with percentages ranging around 30%, that is similar to the presence reported in other Italian regions (2, 8). No FD phytoplasmas were identified in the 150 samples analysed in the three years survey. It was possible to identify in relevant percentages also the presence of phytoplasmas identified as belonging to subgroup 16SrI-C (clover phyllody and related phytoplasmas), that were mainly present in symptomatic plants. Phytoplasmas belonging to the same subgroup were occasionally detected in the past in Veneto region (1) and recently identified in grapevine with yellows symptoms in Chile (4). These phytoplasmas were mainly detected with nested PCR using R16(I)F1/R1 or M1/M2 primers, identification was obtained by RFLP using the above mentioned enzymes (Fig. 1).

Table 1. Phytoplasmas identified in grapevines in Abruzzo during 200-2002.

Variety	N° of samples per province									Total samples	Phytoplasma detected			% of infected samples
	CHIETI			PESCARA			TERAMO				BN	AY	others	
	2000	2001	2002	2000	2001	2002	2000	2001	2002					
Trebbiano	20	12	3	-	-	-	2	1	1	39	14	6	-	51
Montepulciano	12	13	16	-	-	5	2	7	4	59	9	16	3	51
Chardonnay	10	2	2	4	-	-	2	4	1	25	9	-	-	56
Cabernet	-	-	-	-	-	-	3	3	2	8	1	-	-	13
Sangiovese	-	-	1	-	-	-	6	-	-	7	-	-	-	0
Diverse	2	2	-	-	-	-	3	5	-	12	8	-	-	67
Total	44	29	22	4	0	5	18	20	8	150	41	27	3	47

The percentage of BN identification was always higher than those of other phytoplasmas, in particular 16SrI-C phytoplasmas were detected in about 20% of the examined samples. There were also symptomatic samples that resulted negative and this is possibly due to the very low phytoplasma concentration, as indirectly indicated by the necessity to use specific nested PCR assays for detection. Considering the single provinces, Chieti showed the higher percentages of phytoplasma presence that reached the 83% in 2001. Taking into account that phytoplasma detection rate in the years was very variable, it appears that no epidemic phytoplasma outbreaks are present, probably for the lack of specific vectors and for the low level of biological farms in the viticulture of the area. The molecular monitoring on insects (known and potential vectors) and on grapevine plants started together and did not show the presence of FD or BN vectors in Abruzzo, but a more accurate survey is in program to verify the situation, considering the recent reports of *Scaphoideus titanus* Ball. in the nearby regions such as Basilicata and Umbria (8, 10).

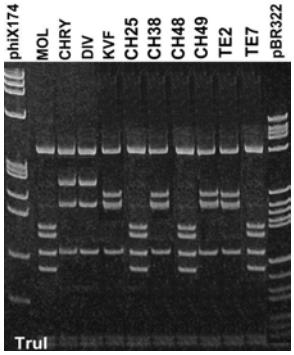

Fig. 1a. Polyacrylamide gel (5%) showing the *Tru*I RFLP patterns of phytoplasma rDNA fragments obtained after nested PCR with primers R16(I)F1/R1 from grapevine samples with yellows symptoms from Abruzzo (CH and TE samples). Phytoplasma reference strains: MOL, Molière disease (16SrXII-A), CHRY, Chrysanthemum yellows from Germany (16SrI-A); DIV, *Diplotaxis* virescence (16SrI-B), KVF, clover phyllody from France (16SrI-C). Markers: 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; pBR322 *Msp*I digested; fragment sizes in base pairs from top to bottom: 622, 527, 404,307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, 9.

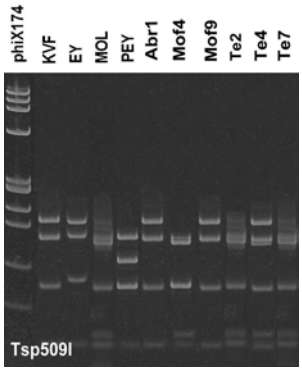

Fig. 1b. Polyacrylamide gel (5%) showing the *Tsp509*I RFLP patterns of phytoplasma rDNA fragments obtained after nested PCR with primers M1/M2 from grapevine samples with yellows symptoms from Abruzzo (Abr, Mof, and Te samples). Phytoplasma reference strains: EY, elm yellows from US (16SrV-A); PEY, *Pichris echioides* yellows (16SrIX-C); KVF, clover phyllody from France (16SrI-C). Markers: 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.

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MOLECULAR CHARACTERIZATION OF A FLAVESCENCE DOREE PHYTOPLASMA INFECTING GRAPEVINE IN SERBIA

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Objectives

Symptoms of grapevine yellows were observed in Serbia since the second part of 1990s, in county of Rasina, south Serbia, but the first published record of phytoplasmalike symptoms on grapevine was in 2000 (4). The observed symptoms appeared on white and red cultivars and included leaf roll, redness and yellowing, vein chlorosis and necrosis, shortness of internodes and lack of lignification. Since then the most severe symptoms appeared on domestic cultivars Plovdina and Smederevka; the infection rate in some cases was so high that some vineyards were uprooted. The disease was registered on young plantations as well as on very old plants; even old vineyards, which were in good condition for more than 30 years, become infected showing typical phytoplasma-like symptoms. The presence, in the symptomatic plants, of phytoplasmas belonging to the Elm Yellows group (16SrV), was confirmed in 2003 (3). Further characterizations of the detected phytoplasmas were obtained by PCR-RFLP analyses of three different DNA fragments: 16S rDNA/spacer region, FD9 fragment and ribosomal protein S3.

Material and Methods

Samples were collected in October 2002 from vineyards in one sub-region of Rasina county, called Zupa Aleksandrovac. Leaf midribs and bark tissues were collected from 10 grapevine plants showing typical phytoplasma symptoms and from 2 asymptomatic plants as negative controls. The grapevine plants belonged to 3 different cultivars called Smederevka, Gamé and Plovdina. Total nucleic acids were extracted from 1 g of leaf midribs and bark tissues from each sample, following the protocol described by Prince *et al* (7), and diluted to a final concentration of 20 ng/µl in sterile deionized water. One µl of this dilution was used in the amplification assays described below. Each 25 µl PCR reaction mix contained 2,5 µl 10X PCR buffer, 0,8 U of *Taq* polymerase (Polymed, Florence, Italy), 0,2 mM dNTPs, 1,5 mM MgCl2 and 0,4 µM each primer. Direct PCR was performed using universal primer pair P1/P7, that allow the amplification of 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA (2, 8). Thirty-five PCR cycles were performed in an automated thermocycler (Biometra, Uno Thermoblock, Gottingen, Germany, EU) under the following conditions: 1 min (2 min for the first cycle) denaturation step at 94oC, 2 min for annealing at 50oC and 3 min (10 min for the last cycle) for primer extension at 72oC. Six µl of PCR products were analyzed in a 1% agarose gel stained with ethidium bromide, and then visualized with an UV transilluminator. To differentiate among phytoplasmas belonging to group 16SrV (elm yellows) subgroups, P1/P7 PCR products were digested with *Taq*I restriction enzyme; the RFLP fragments were separated in a 5% polyacrylamide gel, stained with ethidium bromide, and then visualized with an UV transilluminator. The molecular characterization of the phytoplasmas detected was done also with group 16SrV specific primer pairs: FD9f2/FD9r followed by FD9f3/FD9r2 in nested PCR, which amplify the non-ribosomal FD9 DNA fragment (1), and rpVF1/rpR1 followed in semi-nested PCR by rpVF2/rpR1 for amplification of ribosomal protein fragment (5). RFLP analysis on FD9 fragment was carried out using *Tru*I and on ribosomal protein fragment with *Tru*I and *Tsp509*I.

Results

P1/P7 PCR fragments were obtained from 10 symptomatic samples belonging to all the 3 cultivars tested: 2 samples of cv Smederevka, 4 of cv Gamé, 4 of cv Plovdina; nine symptomatic samples resulted positive when PCR assays were performed on leaf DNA extracts, only one sample of Plovdina resulted positive from bark and not from leaves probably due to the late time of sampling. Both samples from asymptomatic plants were negative (Tab. 1). All the positive grapevine samples tested showed the same restriction profile when subjected to RFLP analysis. Digestion with *Taq*I on the P1/P7 DNA fragments produced identical restriction profiles referable to those of 16SrV-C subgroup (Table 2, Fig. 1A).

Sample			*Bark*	*Leaves*
1	Plovdina	sympt	+	+
2	Plovdina	sympt	+	+
3	Smederevka	asympt	-	-
4	Gamè	sympt	+	+
5	Gamè	sympt	-	+
6	Plovdina	sympt	+	-
7	Smederevka	sympt	+	+
8	Plovdina	asympt	-	-
9	Plovdina	sympt	-	+
10	Smederevka	sympt	+	+
11	Gamè	sympt	-	+
12	Gamè	sympt	+	+

Table 1. Results of PCR FD detection.

Sample	*Ribosomal sugroup*	*P1/P7*	*Rp subgroup*	*rpVF2/Pr2*		*FD9f3/r2*
		*Taq*I		*Tru*I	*Tsp509*I	*Tru*I
PV259	16SrV-C	A	rp-G	A	A	A
TV46	16SrV-C	A	rp-D	B	F	A
FD70	16SrV-C	A	rp-F	A	B	B
SERBIA	16SrV-C	A	rp-D	B	B	A
EY-C	16SrV-A	B	rp-A	C	C	E
ULW	16SrV-A	B	rp-A	C	C	D
JWB	16SrV-B	D	rp-C	D	D	F
ALY	16SrV-C	A	rp-H	E	B	C
RuS	16SrV-E	A	rp-I	F	E	G
FD88	16SrV-D	C	rp-E	G	B	C

Table 2. Molecular characterization of the FD strain from Serbia. Sample acronyms as in Fig. 1

Further RFLP characterization on non-ribosomal FD9 fragment with *Tru*I (Fig. 1B), and on rpVF2/rpR1 amplicons with *Tru*I and *Tsp509*I (Fig. 1C and 1D), allowed to distinguish among phytoplasmas of group 16SrV and, in particular, showed high variability within subgroup 16SrV-C (6). Serbian grapevine samples showed the highest identity, according to the DNA fragments employed, to the grapevine sample from the Italian Treviso province (TV46) from which they differ only for the lack of a low band in RFLP analyses on ribosomal protein gene with *Tsp509*I (Fig. 1D). Since *Scaphoideus titanus* Ball was detected in the area in late Spring 2003 and the phytoplasmas identified belong to subgroup 16SrV-C the Serbian grapevine yellows could be considered a Flavescence dorée strain , closely related to one of those infecting grapevine in Treviso area.

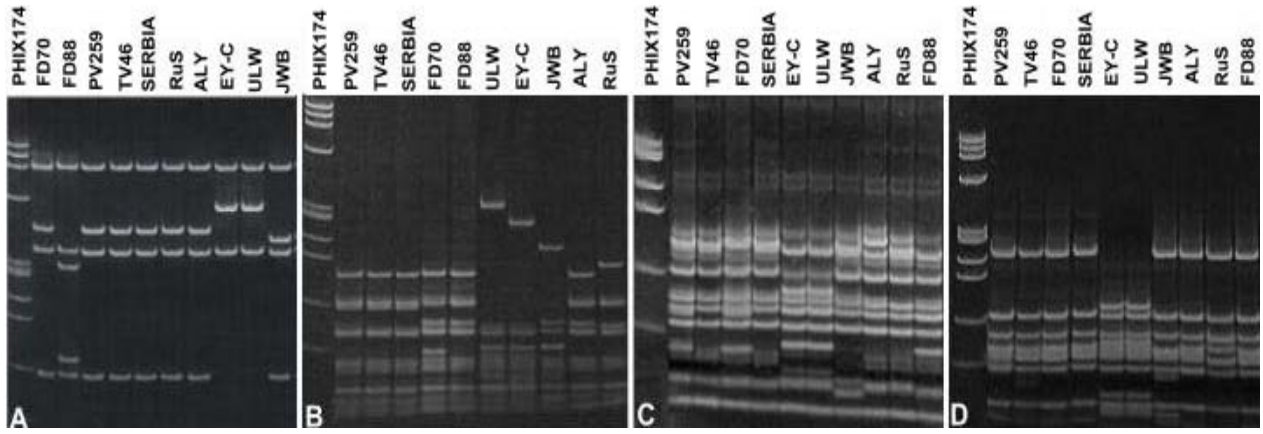

Fig.1. Polyacrylamide gels (5%) showing in A) the *Taq*I RFLP patterns of phytoplasma 16S-23S rDNA fragments obtained with primers P1/P7 from Serbian grapevine samples and from phytoplasma reference strains; in B) phytoplasma FD9f3/r2 amplified DNA fragments digested with *Tru*I; in C) and D) RFLP patterns of phytoplasma ribosomal protein DNA fragments amplified with rp(V)F2/rpR1 and digested respectively with *Tru*I and *Tsp509*I restriction enzymes. PV259, FD from Lombardia, Italy; TV46, FD from Veneto Italy; FD70 and FD88, FD strains from France; ULW, elm yellows from Europe; EYC, elm yellows from USA; JWB, jujube witches' broom from China; ALY, alder yellows from South Italy; RuS, rubus stunt from Italy P, 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.

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PHYTOPLASMAS ON GRAPEVINE IN SERBIA

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In Serbia vine growing and grape production have a long tradition and are of great economic importance. Grapevine covers an area of 90,000 ha (6). Most grapes is processed into wine and a certain quantity is used as raw food. Serbia is also famous after grapevine grafts.

Various pathogens, which are considered to be the causal agents of fungi, bacteria and virus diseases have been determined on grapevine in Serbia. During last years, however, the symptoms, differentiated from aforementioned diseases, have been observed in our vine-growing areas. The dominant symptoms are as follows: colour change and leaf deformation, shoot rubbery and curtailment, their weak woodening and premature vines dying, etc.

The recent research has shown that phytoplasmas (*Phytoplasmas, Mollicutes*) have infected grapevine in some vine-growing areas. Duduk *et al*., (2) proved the occurrence of these diseases applying polymerase chain reaction (PCR) method. Investigating these grapevine diseases, new for us, we applied method of transient electron microscopy, thus confirming that phytoplasmas are the frequent grapevine diseases in Serbia.

The original material for these investigations was collected from an eight year old vineyard in Tules village, Zupa vineyards. Vines with distinctly expressed symptoms are marked, on which the occurrence and development of a disease have been monitored.

During December 2002, from the labeled vines shoots were taken segments which served for hystopathological investigations. The tissue fragments for hystopathological study were cut within the zone of conductive vessels of one-year grapevine shoot. The fragments were further elaborated according to the method of Hopkins et al. (3).

Electron microscope researches of prepared grapevine cross-sections have been conducted in Laboratory of electron microscopy at the Faculty of Medicine, Nis, thanks to Prof. dr Vojin Savic.

The disease symptoms (pathological changes), whose etiology has been investigated in this paper, have been monitored on selected vine-stocks of the following cultivars: Italian Riesling, Pinot Noir, Smederevka, Riesling, Plovdina and Prokupac. At black and red fruited varieties (Pinot Noir, Prokupac and Plovdina) leaf reddness was detected at first. Such symptoms were observed just after flowering and onwards the intensity has increased during vegetation. At white cultivars (Italian Riesling, Smederevka) discoloriation turning into leaf yellows, followed by nerve yellowing afterwards was detected. Apart from colour change, downward rolling af the laminae has been seen, whereas the leaves became britlle. Shoots were undergrown, short- internoded and remained rubbery. These shoots at the base, became bluish, black-dotted. They hardly became wooden and mainly frozen in winter.The diseased vine-stocks sooner or later died. They are usually fruitless or fruits are poor. The berries of such fruits are small, they wilt and dry prematurelly.

At ultrathin cross-sections of conductive vessels at the base and tip of diseased shoots, apperared organisms corresponding to phytoplasmas in shape, size, structure and three-layered membrane.The dimensions of phytoplasmas cells were different, varying from 0.15 x 0.25 μm – 0.6 x 1.0 μm.

The symptoms, detected on phytoplasmas-infected grapevine and described in this paper, corresponded to the changes of this kind of disease, described by other researchers (1, 4, 5, 7, 8). These symptoms, recently found on grapevine in Serbia, are also associated to viral diseases in many cases. Therefore, there is a need for a thorough and detailed investigations of the etiology of all vinegrape symptoms, which imply to either a possible viral or phytoplasmatic nature of these diseases. Duduk et al., (2) have detected Elm Yellows (EY) group – Flavescence doree on grapevine in Serbia.

It is expected that other phytoplasmas, particularly those from stolbur group, will infect grapevine in our country, since this kind of disease has been found on many plants in Serbia for several decades (9).

Further investigations would contribute to both, acquire better knowledge of occurrence and spread of these diseases, and what groups of phytoplasmas are infecting grapevines in our wine-growing areas.

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GRAPEVINE YELLOWS – SPREAD OF THE DISEASE IN CROATIA

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Grapevine yellows diseases (GY) are mainly caused by plant pathogenic prokaryotes – phytoplasmas. Since 1994, when symptoms typical for GY were observed in Zagreb region (4), spreading of the phytoplasmoses was monitored throughout the country (1) Up to date, GY symptoms were observed in all parts of the country except on the islands (Fig. 1). In north-western Croatia the spread of the phytoplasmoses was wide, while in the eastern part it even had an epidemic character, causing significant economic losses. In Ilok and Baranja up to 50% of the vines were infected. The most susceptible cultivar is Chardonnay, followed by Pinot Gris, Pinot Noir, Pinot Blanc, Ugni Blanc and Cabernet Sauvignon. Infection was also confirmed in indigenous cultivars: Debit, Plavina, Maraština and Istarska Malvazija.

The detection of the pathogen in different grapevine samples collected all over the country was based on nested PCR (polymerase chain reaction) that employed phytoplasma generic and/or specific primers amplifying 16S rRNA gene. PCR conditions were as previously described by Šeruga et al. (3) The RFLP (restriction fragment length polymorphism) with a set of restriction enzymes (2) enabled classification of the phytoplasmas from all grapevine samples in the same ribosomal subgroup 16SrXII-A.

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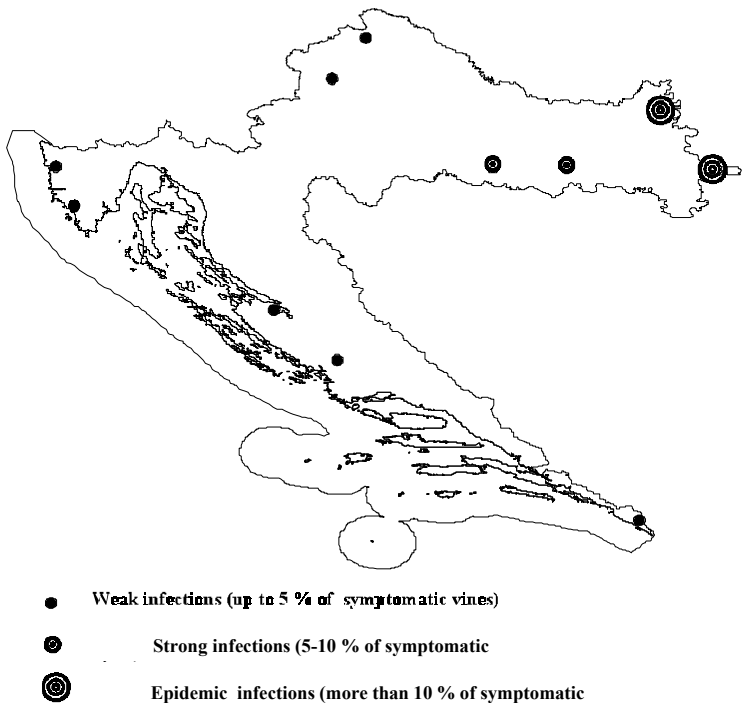
Fig. 1



A COMPARISON OF STOLBUR PHYTOPLASMA ISOLATES FROM CROATIAN GRAPEVINE BY ANALYSES OF RIBOSOMAL AND NON-RIBOSOMAL GENE REGIONS

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So far, the only detected etiological agent of phytoplasmoses in Croatian grapevines is a phytoplasma classified on the basis of the analysis of 16S rRNA gene region as a member of ribosomal subgroup 16SrXII-A (stolbur; bois noir) (7).

Almost fifty stolbur phytoplasma isolates from various cultivars and different geographic regions of the country were used in this study in order to be compared and differentiated. Gene regions other than 16S rDNA were also analyzed: gene for the elongation factor EF-Tu (6) and two stolbur-specific non-ribosomal regions amplified by primer pairs G35pm (2) and STOL11f2/r1 (1).

Nucleic acids used for analyses were extracted by two procedures described in Prince et al. (4) and Daire et al. (1) with some modifications (8). Amplification of phytoplasma 16S rRNA gene was performed in a direct PCR by using R16F1/R0 universal phytoplasma primer pair. The nested PCRs were performed with R16F2/R2, 16R738f/R1232r and R16(I)F1/R1 primers, as already described (7). Major portion of the tuf gene coding for the elongation factor EF-Tu was amplified by employing the fTufu/rTufu primer pair (6). PCR conditions in all experiments were as in Schaff et al (5) with the exception of annealing temperature for the amplification of tuf gene that was 55°C in our experiments. A set of restriction enzymes (Tru9I, TspEI, AluI, KpnI) was used in routine RFLP analyses (7). The problem of finer differentiation of stolbur isolates was approached by using HMA (heteroduplex mobility assay, 9) and SSCP (single-strand conformation polymorphism analysis, 3).

RFLP profiles of 16S rRNA amplicons for all of the isolates tested were the same regardless of the extraction method used. The tuf gene restriction patterns obtained by digestion of PCR product from samples extracted by modified Daire et al. (1) revealed additional bands not resembling any other known phytoplasma profiles. The extra bands were not a result of an unspecific amplification visible in the PCR experiments and could be of non-phytoplasmal origin due to the less selective DNA extraction. Restriction analyses of other amplified regions were not informative enough to make a finer distinction. The results of HMA and SSCP analyses confirmed a very strong relatedness among our stolbur isolates from different grapevine cultivars. It would be interesting to determine their relationship with stolbur isolates from other, especially neighbouring countries.

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THE PRESENCE OF GRAPEVINE YELLOWS AND THEIR POTENTIAL NATURAL VECTORS IN WINE-GROWING REGIONS OF SLOVENIA

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Grapevine yellows (GY) are widespread in all wine growing regions in Slovenia (2, 9, 10). During 2001-02 an epidemic of devastating proportions with 40-80% infected vines, occurred in Drava wine-growing region. In 2002, the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia organized a special survey of GY and laboratory testing for their identification. The main goal was to establish which phytoplasmas are causing GY disease in order to choose proper and efficient measures for their control (5).

The testing in 2002 included only the vines expressing symptoms of GY. Samples were gathered from July 15-30, with the exception of 5, which were gathered in October. Laboratory testing was carried out as PCR analyses of DNA extracts from leaf veins. Universal primers in combination with RFLP, and FD and BN specific primers were used for PCR analysis of all samples (1). Of 79 tested vines 6 were negative, and 73 were positive. In 59 positive vines we could identify Bois noir phytoplasma. Notably, in 14 (out of 79 tested) vines, expressing symptoms of GY, phytoplasma type could not be identified. This could be contributed to poor recovery of DNA, probably due to the improper conservation (4 samples) and too late sampling (5 samples) of plant material. The results showed the widespread presence of the Bois noir phytoplasma in all wine growing regions of Slovenia. A survey confirmed data from previous limited analyses of phytoplasma in GY affected vineyards in Slovenia (4, 8, 9). Heretofore, Flavescence doree phytoplasma was not found to be present in Slovenia. However, it's presence and a possibility for its introduction cannot be ruled out and must be taken in account. Notably, the FD vector, *Scaphoideus titanus* is widespread and abundant in Western Slovenia since mid 1980-ies (6, 7). Therefore, a special survey of GY and their laboratory identification continues in 2003.

In most affected vineyards in Drava region of Northeast Slovenia, investigations on leafhopper and planthopper (Auchenorrhyncha) fauna as potential natural vectors of phytoplasmas, which might be the cause of rapid disease proliferation, were carried out (11). Three vineyards, where the infection rate of BN was very high (40-80 % of the vines), were selected for the further faunistic investigations: Turški vrh v Halozah (UTM - WM83), Strezetina (UTM - WM84, Svetinje – Malek v Slovenskih goricah (UTM – WM94). Two methods were used to find out the presence and frequency of single species:

a) Using yellow sticky traps, which were changed and controlled monthly;

b) Collecting of hoppers with an entomological net in June and at the beginning of September, separately on grapevines and on undergrowth vegetation.

A comparatively large population of planthopper *Hyalestes obsoletus*, which is known as the vector of stolbur phytoplasma (3, 12, 13), was found in the three selected vineyards in the period from the beginning of June till to the end of July. Their main host plants, especially *Convolvulus arvensis* and somewhere *Urtica dioica* too, were also largely present in all three vineyards. A positive correlation was noticed between the capture of *Hyalestes obsoletus* and the abundance of these weeds. Some other leafhoppers mentioned in the literature as possible vectors of the stolbur phytoplasmas on herbaceous plants (e.g. *Aphrodes makarovi*, *Euscelis incisus*, *Anoplotettix fuscovenosus*, *Neoaliturus fenestratus*) were abundantly collected. 43 Auchenorrhyncha species all together were found in the three locations, the majority of them on undergrowth vegetation. The leafhopper *Empoasca vitis* was the only true ampelophagous species, abundantly trapped on yellow sticky plates. The following species were also swept directly from grapevines: *Neoaliturus fenestratus*, *Anoplotettix fuscovenosus*, *Philaenus spumarius* and *Hyalesthes obsoletus*. Only one of them, the *Anoplotettix fuscovenosus* is a true arboricolous species. Another arboricolous species, the *Fieberiella flori*, was only trapped in a very low number on yellow stick plates. The leafhopper *Scaphoideus titanus* the vector of FD (Flavescence dorée phytoplasma) has been found nowhere in this part of Slovenia yet. The present preliminary investigation indicates a possible close relation between *Hyalestes obsoletus* populations and current epidemic occurrence of BN in Drava wine-growing region.

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SIX-YEAR SURVEY OF GRAPEVINE YELLOWS DISTRIBUTION IN HUNGARY

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Objectives

After the first finding of the symptoms suspicious for grapevine yellows (GY) disease in Hungary in the 1970's, GY was found in four counties between 1994 and 1996. Since 1997 a regular survey started to determine the distribution of GY in the country (4). In this paper the results of the survey in the years 1997-2002 are presented.

Materials and Methods

Visual inspection. Grapevine stocks from one hundred to three hundred for each cultivar were surveyed in vineyards of 12 counties, looking for the following types of symptoms typical for phytoplasma infection: leaf yellowing with or without necrosis of leaf blade (reddening in case of red cultivars); triangle-shaped leaf rolling; shortening of internodes; dropping of shoots; drying of flowers, berries and clusters, as well as decline of grapevine stocks. Only the plants showing at least three of the mentioned symptoms were regarded as possibly phytoplasma infected.

Molecular analysis. Participants of the Italian–Hungarian Bilateral Project (I-43/1999) jointly analysed selected samples at the Phytoplasma Laboratory of the Bologna University. Samples from symptomatic plants were tested by nested-PCR and RFLP methods. Nucleic acid extraction was performed by using chloroform-phenol procedure (7). Universal (R16F1/R0) and P1/P7 (6, 3, 8) and group-specific [R16(I)F1/R1, R16(III)F1/R1, R16(V)F1/R1, R16(X)F1/R1] (5, 6), (fStol/rStol) (5) primers were used for amplification of 16S region of ribosomal DNA. Experiments in Bologna were performed using two nested-PCR systems based on 16S-23S ribosomal DNA (9), as well as FD specific primers from non-ribosomal DNA region (FD9f2/r, FD9f3/r2) designed by Daire *et al*. (2) and Angelini *et al*. (1). In order to determine the subgroup affiliation of the detected phytoplasmas, RFLP analyses were applied on PCR products primed by R16F2/R2 using *Mse*I, *Rsa*I, *Ssp*I and *Taq*I enzymes.

Results and Discussion

In the period of 1997-2002 altogether more than 29,800 grapevine plants of 33 cultivars were surveyed in 37 locations of 12 counties. Some 2,354 symptomatic plants of 21 cultivars were observed in 11 counties. Leaf yellows or reddening of leaves, severe triangle-shaped leaf rolling, dropping of shoots due to uneven ripening were the most common symptoms. GY symptoms were recorded on white cultivars Aligote, Chardonnay, Chasselas, Ezerfürtű, Kerner, Muscat lunel, Olaszrizling, Pinot Blanc, Pintes, Rajnai rizling Semillon, Pinot gris and Zöldveltelini. GY symptom-showing red wine cultivars were: Alicante Bouchet, Blauburger, Cabernet Franc, Kékfrankos, Merlot, Pinot Noir, Vranac and Zweigelt (Table 1). No symptoms resembling phytoplasma disease have been found on the following cultivars: Bíborkadarka, Cabernet Sauvignon, Cserszegi fűszeres, Furmint, Hárslevelű, Királyleánya Kövidinka, Medina, Kékoportó, Tramini, Zengő and Zenit. As for the white wine cultivars, Chardonnay proved to be a most susceptible one with the most typical and severe GY symptoms. Among red wine cultivars, Zweigelt, the main one showed the most characteristic and expressed symptoms of phytoplasma infection. Severity of symptoms varied year to year.

Testing ten samples/cultivar with molecular methods, twenty six of one hundred seventy tested samples proved to be positive in nested-PCR using both universal primer pair and Stolbur specific primers. RFLP analysis confirmed that the positive samples were infected with phytoplasmas belonging to Stolbur group (16SrXII-A).

In 1998-1999 phytoplasmas other than Stolbur were also identified when jointly testing eighteen samples from six counties in the Italian laboratory: AY (16SrI-B), ESFY (16SrX-B), EY (16SrV), Stolbur (16SrXII-A) as well as CPh (16SrI-C) + Stolbur (16SrXII-A), AY (16SrI-B) + EY (16SrV), EY (16SrV) + Stolbur (16SrXII-A), AY (16SrI-B) + ESFY (16SrX-B) and AY (16SrI-B) + CPh (16SrI-C) + Stolbur (16SrXII-A). Re-testing of some of the plants from the areas where mixed infection was detected in 2001-2002, only Stolbur phytoplasma could be identified. Phytoplasma controls were positive in all the tests and none of the healthy grapevine samples reacted positively.

Phytoplasma-associated symptoms were observed in eleven of the twelve counties involved in the survey. It can be concluded from symptomatology and molecular studies that grapevine yellows disease is wide-spread on the main cultivars in the Hungarian vineyards. The most frequently identified phytoplasmas belong to the Stolbur group (16SrXII-A). The results of the five-year survey on phytoplasma diseases and monitoring of the Auchenorrhyncha fauna in vineyards confirmed that Flavescence dorée phytoplasmas (16SrV-C and 16SrV-D) and their vector, *Scaphoideus titanus* were not detected in Hungary. Detailed results will be published elsewhere.

Further investigations are in progress in order to

- study the presence and distribution of phytoplasmas other than Stolbur in different grapevine growing counties,
- continue the survey of phytoplasmas in vineyards with cultivars not having been involved or not showing symptoms so far,
- continue the monitoring of Auchenorrhyncha fauna and the molecular testing of specimens collected in GY-diseased vineyards.

Vectorological trials with potential phytoplasma-vector species are planned.

Table 1 Results of the survey of GY disease (1997-2000)

Cultivar	*Number of plants*	*Symptomatic*	*Plants (%)*
Alicante bouchet	241	9	3,7
Aligote	242	51	21
Blauburger	833	14	1,7
Cabernet franc	150	6	4
Chardonnay	9.225	1.371	14,9
Chassleass	850	22	2,6
Ezerfürtű	342	11	3,2
Kékfankos	6.340	452	7,1
Kerner	1.201	24	2,0
Merlot	600	15	1,5
Muscat lunel	322	5	1,5
Olaszrizling	1.150	14	1,2
Pinot blanc	150	1	0,7
Pinot noir	250	3	1,2
Pintes	450	3	0,7
Rajnai rizling	300	20	6,7
Semillon	100	8	8
Szürkebarát	150	12	8
Vranac	100	35	35
Zöldveltelini	1.050	91	8,7
Zweigelt	1.771	187	10,6
Total:	***25.817***	***2.354***	***9,1%***

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Acknowledgements

This collaborative study was the result of a grant made under the Hungarian-Italian Intergovernmental Science & Technology Cooperation Programme in the frame of the bilateral project No. I-43/99.

GRAPEVINE "BOIS NOIR" DISEASE IN LEBANON

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Grapevine (*Vitis vinifera*) which has been cultivated in Lebanon since time immemorial, is one of the most important and widespread fruit crops, covering a surface of about 13.8 10^3 ha with a production of 116 10^3 T, ranking only third to stone fruits and citrus. The great majority of the commercial vineyards (65%) is located in the Bekaa valley. The phytosanitary status of this crop with respect to virus and virus-like diseases is poorly known, the only available information stemming from a preliminary survey conducted 7 years ago (3). However, no research work was carried out to study the presence of phytoplasma diseases on grapevine in Lebanon. Yellows (GY) diseases have been reported in Europe, America, Australia, Israel and New Zealand. In these countries symptoms in *Vitis vinifera* are similar and cause several damages. On the basis of DNA analysis, the phytoplasmas associated with (GY) belong to different groups: Flavescence dorée (FD) phytoplasma is in the elm yellows group (1), "bois noir" and the Vergilbungskrankheit phytoplasmas belong to the stolbur group (2,4), an Australian GY-associated phytoplasma occurs in the Aster yellows group (6), and other grapevine phytoplasmas are in the X-disease group (7).

In view of this, in June 2001, during a survey to evaluate the incidence of phytoplasma diseases in Lebanon, specially after the diffusion of a new phytoplasma associated with an emerging lethal disease of almond trees in the country (10), samples of symptomatic grapevine (i.e. leaf rolling, yellowing or reddening of the leaves depending on the cultivars, and incomplete wood ripening) belonging to the cultivars Chardonnay and Alicante Bouschet were collected in three different vineyards located in the Bekaa Valley in order to characterize the phytoplasmas involved. Samples were representative of the outbreak since the affected area is small. Periwinkle (*Catharantus roseus*) plants with phytoplasma-like symptoms were also collected in various locations of Lebanon.

Nucleic acids were extracted from fresh leaf midveins of diseased and symptomless plants of grapevine and periwinkle, using the CTAB (cethyl-trimethyl-ammonium bromide) extraction protocol described by Maixner *et al*., (5). Phytoplasma-universal primer pair fU5/rU3 (8) was used for the amplification of ribosomal DNA. PCR was performed for 35 cycles using the following conditions: denaturation for 45 s, annealing for 45s at 55ºC and primer extension at 72ºC for 45s. PCR resulted in amplification of an expected 0.8 kbp rDNA fragment from the symptomatic, but not from symptomless samples. The PCR product was sequenced. Digestion with restriction endonuclease *Alu* I according to the manufacturers instructions was also carried out. The restriction products were subsequently separated by 2.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator. The results indicated that the phytoplasmas of all diseased grapevines and 2 periwinkle plants from the Tyr area belonged to the stolbur group (9). The sequences had 98% identity with 2 european stolbur isolates from grapevine and periwinkle (GenBank accession mumbers X76428 and AF24859 respectively).

The fact that periwinkle plants were found to be infected with the stolbur/bois noir phytoplasma suggests that this phytoplasma is transmitted by insect vector especially since *Hyalesthes obsoletus*, the planthopper vector of the stolbur phytoplasma is present in Lebanon.

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OCCURRENCE OF GRAPEVINE YELLOWS AND POTENTIAL VECTORS IN TUNISIA

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In Tunisia Yellows symptoms, characteristic of GY diseases found in other countries, have been detected on imported table grapes varieties. Some samples analysed by molecular techniques have identified presence of phytoplasma. Considering the evolution of symptom diseases, in the first outbreaks foci and aiming to control factors determining spread, an extended survey of potential vectors, in the main grapegrowing regions, has been achieved.

Materials and methods

Biologic material consists on grapevine samples with yellows-like symptoms(1). and leafhoppers captured by D-Vac suction traps, from vines recorded ill and spontaneous plants. Some samples are tested by PCR and RFLP analysis (2)

Indexing by heterografting of symptomatic samples onto periwinkles and inoculation by presumed infectious leafhoppers to healthy Chardonnay and periwinkles, have been achieved to confirm or invalidate presence of GY vectors (3), in Tunisian vineyard .

Results and discussion

- 2,3% showed specific symptoms among 3000 controlled plants, in the first outbreaks foci. Symptoms consist of yellowing along the veins, necroses of main veins and wilting of the bunches. Under climatic conditions, unusually favourable to leafhoppers development, the rate of contamination in this infected field, reached 60%, over the 3rd year. Eradication, trough rooting up, has been undertaken.

- Leafhopper populations surveys carried out more than 40 leafhopper species (4), among which are reported vectors following species:

Fam. Cicadellidae: *Anaceratagallia laevis*, *Cicadulina bipunctella*, *Circulifer haematoceps*, *C. tenellus*, *Euscelis incisus*, *Macrosteles* sp, *Orosius orientalis*, *Psammotettix alienus*, *Thamnotettix* sp. *Neoaliturus* sp., *Balclutha* sp
Fam.Cixiidae: *Hyalesthes obsoletus* , *Pentastiridius* sp, *Philanus spumarius*
Fam. Delphacidae: *Laodelphax striatellus*, *Sogatella vibix*

- Results of transmission by leafhopper populations (captured on symptomatic vines) indicate that 3/5 tested periwinkles showed symptoms consisting on yellowing of leaves, poor coloured flowers and reduced growth. The healthy periwinkles exposed in the infected field show similar symptoms. Inoculated Chardonnay plants by leafhopper populations express no specific symptom of GY, 6 months after inoculation.

- Heterografting of infected vines and micro-injection of infectious plant extract, on periwinkles, showed the same symptoms as induced by leafhoppers transmission on experimented plants. Chardonnay plants express no specific symptoms, 6 months after grafting

- PCR and Nested PCR of the region of consistent ADNr 16S, using primers R16R2/R16F2 and R16(1)R1/R16(1)F1 followed by RFLP analysis permitted to identify phytoplasma 16SrIs of the Aster Yellows group, I-B. Not all affected plants reacted positively to PCR. Similar PCR assays yielded no phytoplasma DNA amplification from inoculative leafhoppers and symptomatic periwinkles

These results, however preliminary, indicate presence of GY symptoms and existence of numerous leafhoppers species, potential agent of dissemination. Comfortable transmission on periwinkles and negative PCR phytoplasma results on leafhoppers, suggest that symptoms were likely to be due to some other transmissible yellows. AY phytoplasma presence could be in mixed infection with other undetermined agents

Acknowledgements

Grateful thanks are expressed to Dr Boudon–Padieu and Mr Larrue (INRA-Dijon), for help

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SURVEY ON BOIS NOIR PHYTOPLASMAS SPREADING IN VINEYARDS OF MODENA PROVINCE (ITALY)

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Objectives

In Modena province (Northern Italy) vineyards, during the last 3 years, together with an epidemic outbreak of Flavescence dorée (FD) disease, a severe spreading of Bois Nois (BN) has been observed. From July to the end of October 2001 grapevine plants with yellows were detected in increasing percentages: in 1.200 farms surveyed there was a 46,8% of disease symptoms increasing. Molecular analyses performed on samples collected in vineyards located across the province showed the presence of 8% of FD positive samples mainly located in the north-west corner of the province, and the presence of 51% of samples positive to BN (3). The aim of the present work was to monitor insects, potential vectors of BN phytoplasmas, and grapevine plants, mainly of Lambrusco, that is the most widespread and important cultivar of this area. It is known that several insects can acquire BN phytoplasmas from diseased plants, but only the Cixiid *Hyalesthes obsoletus* Signoret was demonstrated to transmit BN to grapevine that is a dead end host (1, 6, 11); therefore assessments of this insect populations as well as their contamination by BN phytoplasmas was monitored together with the search for other potential phytoplasma vectors.

Material and Methods

Insects and grapevine samples showing typical yellows symptoms were collected in August 2002 from several farms located in Modena province. Total nucleic acids were extracted from 1 g of leaf midribs following the protocol described by Prince *et al.* (10) and from insect grouped in batches of 1 to 4 according to their sizes following the protocol of Zhang *et al.* (13). Nucleic acids were diluted to a final concentration of 20 ng/µl in sterile deionized water. One µl of this dilution was used in the amplification assays described below. Direct PCR was performed using universal primer pair P1/P7, both for plants and for insects, while nested PCR reactions on direct PCR products diluted 1:30 in sterile distilled water, were performed using primer pairs M1/B6 and M1/M2 (5, 7) on grapevine samples and with primers R16F2/R2 and M1/M2 on insects samples. Each 25 µl PCR reaction mix contained 2,5 µl 10X PCR buffer, 0,8 U of *Taq* polymerase (Polymed, Florence, Italy), 0,2 mM dNTPs, 1,5 mM $MgCl_2$ and 0,4 µM each primer. Thirty-five PCR cycles were performed in an automated thermocycler (Biometra, Uno Thermoblock, Gottingen, Germany, EU) under the following conditions: 1 min (2 min for the first cycle) denaturation step at 94oC, 2 min for annealing at 50oC and 3 min (10 min for the last cycle) for primer extension at 72oC. Six µl of PCR products were analyzed in a 1% agarose gel stained with ethidium bromide, and then visualized with an UV transilluminator. To differentiate among phytoplasmas, PCR products were digested with *Taq*I and *Tru*I restriction enzymes; the RFLP fragments were separated in a 5% polyacrylamide gel stained with ethidium bromide, and then visualized with an UV transilluminator.

Monitoring on insect vectors was carried out in 20 farms (Table 1a and 1b) where grapevine plants were demonstrated to be infected with BN: insect collection was performed with both chromotropic traps and entomological nets with aspirators. Insect collection by using the mowing was also carried out every week between the border grapevine rows and inside the drains nearby to the vineyards, among weed plants belonging to genera *Urtica*, *Convolvolus* and *Medicago*. Insects then were classified and tested by nested-PCR to verify phytoplasma presence.

Results

During 2002 symptomatic grapevines were present starting from the second week of June: more than 100 plant samples were tested and 90% of them was positive to 16SrXII-A (BN) phytoplasmas, while 9% of the sample show the presence of 16SrV-D phytoplasmas (FD). *H. obsoletus* was captured in 17 out of the 20 farms surveyed (Table 1b). The majority of them (655 out of 678) were sample trough mowing on *Convolvolus* and *Urtica* plants located between grapevine rows and at the vineyard borders. Adults were detected starting from the beginning of July till the end of August, with peaks among the second-third decade of July. The more consistent presence of *H. obsoletus* was observed in vineyards having *Urtica* at the borders, confirming the relationship between this vector and BN presence. Comparing the total number of insects collected and the number of treatments performed in the different vineyards it was possible to verify that these treatments did not influence the populations of this vector, confirming data reported in France (12) and in other Italian regions (9).

H. obsoletus is widespread in the province and molecular tests showed that among 59 samples, 6 were positive to BN phytoplasmas corresponding to a percentage of single insect infection of 3.5%. Almost all the monitored vineyards with *H. obsoletus* show the presence of at least one insect sample positive to BN, one of the samples was positive to phytoplasmas of subgroup 16SrX-C (pear decline).

Among the insects reported in the literature as able to acquire BN phytoplasmas *Laodelfax striatellus* Fallén was present in all the vineyards, in some cases also with a high populations; *Metcalfa pruinosa* Say was detected in 15 vineyards, *Neoaliturus fenestratus* Herrich-Schäffer in 8; *Euscelis lineolatus* Brullé and *Macrosteles quadripunctulatus* Kirschbaum was only found in 2 vineyards; no *Pentastiridius beieri* Wagner or *Mocydia crocea* Herrich-Schäffer were captured (Table 1a).

The Auchenorrincha species were tested to verify phytoplasma presence: in 4 out of 28 samples of *L. striatellus* 16SrI phytoplasmas were identified (single insect infection of 4%); some individuals of *M. pruinosa* were positive to 16SrI and/or 16SrXII-A phytoplasmas confirming previous reports in insects collected in fields (4) or in vineyards of other Italian areas (2, 8). Transmission experiments are in progress to verify the ability of these insect species in phytoplasma transmission.

Table 1 a). Number of captured insects potential BN vectors. b). Populations of *H. obosoletus* in the same farm as in a)

Farms	*L. striatellus*	*M. pruinosa*	*N. fenestratus*	*E. lineolatus*	*M. quadripunctulatus*
1	111	1			
2	141		1		
3	351				1
4	73				
5	6	15	18	2	
6	145	5	2		
7	86	1			
8	98				
9	35	19	1	1	
10	123	15	1		
11	100	1			
12	63	5			
13	134				
14	102	3	3		
15	197	1			
16	238	4	1		
17	58	3	1		
18	219	3			
19	267	1			1
20	22	6			

2 July	9-11 July	19 August	22-24 August	29-31 August	5-7 August	13 August	19 August	26-27 August	Total	N° tested
-	-	1	34	11	5	2	1	-	54	3
-	1+*1	-	-	-	-	-	-	-	2	2
-	-	-	-	*1	-	-	-	-	1	2
-	1	-	30	28	7	1	4	-	71	0
-	8	-	1	-	-	-	-	*1	10	2
15	*3	-	1	*5	2	*1	-	-	27	1
-	46+*2	-	16	37+*2	22	6	-	-	131	1
-	-	-	-	-	-	-	-	-	0	2
-	11	-	-	*1	1	-	-	-	13	2
-	5	-	1	-	-	-	-	-	6	2
-	72	42	51	5	14	4	-	-	188	6
-	18	-	1	7	-	-	-	*1	27	2
-	-	7	39	6+*1	-	-	-	-	53	4
-	1	-	4	-	-	*2	-	-	7	Nd
-	4	-	-	-	-	-	-	-	4	Nd
-	20	-	3	-	-	*1	-	-	24	1
-	39	-	7	8	-	-	-	-	54	2
-	-	-	-	-	-	-	-	-	0	2
-	*1	4	-	1	-	-	-	-	6	4
-	-	-	-	-	-	-	-	-	0	1

*Insect detected on chromotropic traps

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BOIS NOIR IN TRENTINO VINEYARDS: TWELVE YEARS VISUAL OBSERVATIONS AND RESEARCH ABOUT ROOTS ANALYSIS

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Eight Chardonnay vineyards (total n. plants 11.049) placed in Valsugana (Trento), bedded from 1989 to 1991, have been observed for twelve years, noting every year the different symptoms of Grapevine Yellows Disease, identified as bois noir (BN). The plants have been divided into five groups:

a) never symptoms = n.9628 plants

b) symptomatic only the last year = n.24 plants

c) showing always or very often the symptoms (from 2 to 8 years)

years	n.plants	2 years	3 years	4 years	5 years	6 years	7 years	8 years
91-'02	11049	82	30	12	8	7	5	1

d) before symptomatic, without symptoms for1-9 years, and symptomatic again

years	n.plants	1 year	2 years	3 years	4 years	5 years	6 years	7 years	8 years	9 years
91-'02	11049	80	47	22	19	10	2	2	1	2

e) in "recovery" since 1-8 years

years	n.plants	1 year	2 years	3 years	4 years	5 years	6 years	7 years	8 years
91-'02	11049	285	139	64	42	27	11	4	1

In fruit trees has been often observed the phenomenon of "recovery" in infected plants after a first shock time (2). "Recovery" is a spontaneous remission of symptoms in plants previously symptomatically affected by a pathogen (3). In recovered apple trees the AP phytoplasma has been found only in the roots (1).

Being the rate of symptomatic plants very low (5) and also that of the vector insect *Hyalesthes obsoletus* (M.Dalrì, unpublished results), as regard the d) group, we presume very unlikely a new infection just in the same plants infected many years before. Considering the groups d) and e), we have supposed a recovery behaviour also in the grapevines.

In autumn 2002, 46 samples of roots samples (about 5 mm diam) were collected from vines belonging to the five groups choosing the DAPI microscopic method (4) to analyse them. As positive control we used a sample surely infected stayed in our "plant lazaret".

Results of DAPI

Group	n.samples	positive	negative	doubt
a)	3	0	2	1
b)	10	7	2	1
c)	8	8	0	0
d)	6	5	0	1
e)	19	13	4	2

Because of abundant rays and starch grains it isn't easy to analyse the grapevine roots.

Positive sample and 33 samples observed early after fixation showed clear phytoplasma presence in sieve tubes. 8 samples were negative and 5 doubtful. Also one of the 3 samples of a) group resulted doubtful, so we repeated some microscopic examinations but the tissues resulted damaged by the long fixation.

Some samples were analysed by RFLP-PCR but till now we haven't had clear results.

These results aren't definite but they suggest the opportunity to deepen this subject.

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Acknowledgements: Luigi Capra for the collection of samples, Elena Rossi and Ivana Battocletti for the samples preparation

SEASONAL PROBABILITY OF FLAVESCENCE DORÉE PHYTOPLASMA TRANSMISSION IN RELATION TO ABUNDANCES OF LEAFHOPPER VECTORS AND SOURCE FOR ACQUISITION.

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Flavescence dorée (FD) is a lethal European grapevine disease associated to phytoplasmas (Flavescence dorée Phytoplasma, FDp) and transmitted by the leafhopper vector *Scaphoideus titanus* Ball.

During 2002 we attempted to identify a pattern of seasonal transmission of FDp, taking into account some important factors influencing the epidemiology of FD. These factors included: 1) transmission efficiency of *S. titanus* after acquisition of FD phytoplasma from field diseased grapevines, 2) pattern of symptom expression of FD in grapevines as factor that affects vector acquisition, 3) pattern of emerging nymphs as an influence on the vector population density in the vineyard, 4) latency and persistency of transmission by the leafhopper vector and 5) abundance of leafhopper vectors and source for acquisition (diseased grapevines).

Material and methods

1) Transmission experiments were planed using eight field grapevines of FD-affected Garganega and White Pinot varieties located in Veneto Region (North-East Italy). Every 20 days starting from May 25th 2002, three groups of about 300 healthy nymphs of *S. titanus* were caged on the canes of infected grapes for an Acquisition Access Period (AAP) of seven days. The leafhoppers were then transferred to healthy grapevines, in cages under glasshouse conditions, to complete the Latent Period (LP). Transmission was done inside the glasshouse using three leafhoppers per grapevine seedling for an Inoculation Access Period (IAP) of seven days. The seedlings were maintained in the glasshouse for symptom expression.

2) The pattern of FD symptom expression was studied in the same vineyards used for vector acquisition. Two blocks were surveyed weekly for FD symptoms starting from May 2002. In the first block 178 grapevines of Garganega variety were surveyed, and in the second block 137 grapes of the White Pinot were surveyed.

3) The pattern of nymph emergence during Spring was evaluated by using published data (1).

4) Latency of FD in the leafhopper *S. titanus* was assumed to have a length period of 30 days, transmission persistency was also assumed occur in the vector.

Results

1) Results of transmission were based on symptom expression of FD on inoculated seedlings and confirmed by using a nested-PCR and RFLP procedure (2). Transmission efficiency increased for the three groups of *S. titanus* as an effect of the increased ability to acquire FDP by older instar nymphs.

Result of transmission were transformed as probability of one *S. titanus* to transmit FDp in one day, using the formula:

$P_{nt}=1-e^{-niEt}$ (3). where P_{nt} was the results of transmission test, n=3 was the number of leafhoppers per seedling during transmission, t=7 was the access time expressed in days for IAP, iE was the probability of FDp transmission by one *S. titanus* per day. The probability of daily transmission of FD over time was calculated from a linear regression.

2) Symptoms of FD were expressed in 54 grapevines of Garganega and White Pinot varieties which showed symptoms starting from the end of May. Data were expressed in a cumulative curve with time.

3) Data of nymphal emergence were expressed in a cumulative curve with time. Time was expressed in days starting from 0 that corresponded to the beginning of egg hatching.

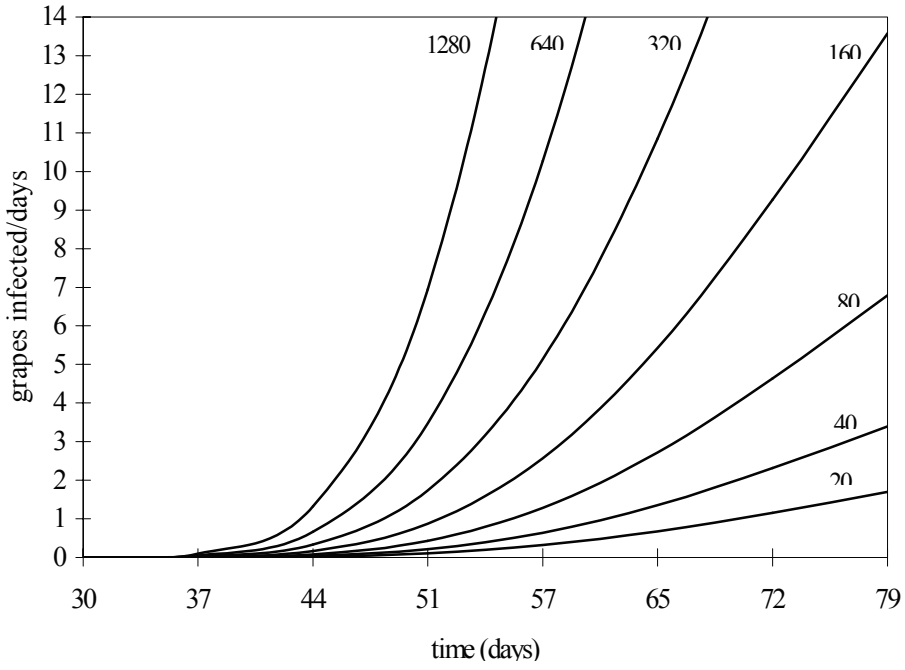
A functional relationship was calculated considering the pattern of three variables: i) daily probability of vector transmission, ii) FD symptom expression and iii) nymphal emergence, which were considered as independent probabilities against time. The calculated curve shows the contingent probability of transmission of FDP by one *S. titanus*.

To estimate the theoretical risk of transmission for more than one leafhopper, the parameter Pressure of infection (Pi) was introduced where: Pi= n p. In this equation n is the average of *S. titanus* per plant and p is the number of symptomatic FD grapevines in the vineyard. Pi represents the average of leafhoppers that fed weekly from diseased grapes and therefore were potentially able to acquire and transmit (at the end of the latent period) FD phytoplasma. To simulate the variation of the probability of FD daily transmission, we applied from the contingent probability of transmission of FDp by one *S. titanus* a range of incremental values of Pi. In figure 1 are reported the results of the application of incremental values of Pi.

On the basis of the above research, the risk of transmission of FDP increases with time due to: a) the incremental ability to acquire FDP by older nymphs, b) availability of sources for acquisition (symptomatic grapevines) and c) nymphal emergence. The probability of transmission is moreover related with the abundance of infected grapevines and density of vector population . It is also possible to estimate a theoretical probability of transmission in a vineyard at any moment of the season if the number of leafhoppers per plant and number of diseased grapes are known.

Other factors may affect the transmission of FD phytoplasma by *S. titanus* in the vineyards such as: vector movement from plant to plant, temperature and inoculation efficiency of different grapevines varieties by the leafhopper vector.

Figure 1. Variation of the probability of daily transmission of FDP per Pi of 20, 40, 80, 160, 320, 640 e 1280. Time is expressed in days, day 0 is the beginning of egg hatch of *S. titanus*.



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COMPARATIVE EXPERIMENTAL TRANSMISSION OF GRAPEVINE YELLOWS PHYTOPLASMAS TO PLANTS AND ARTIFICIAL FEEDING MEDIUM

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Identification of phytoplasma vectors requires the proof of successful transmission of the pathogens since also non-vector species are able to acquire detectable quantities of phytoplasma from infected plants. However, transmission experiments to plants are time consuming and laborious. An alternative technique was developed recently to determine the inoculative ability of potential vectors (7, 8). It is based on the feeding of individual insects on an artificial medium that is afterwards tested for the presence of phytoplasma. Since field transmission of phytoplasmas is influenced to a large extend by the biology of the vectors and their affinity to particular host plants, we intended to compare the inoculative efficiency of vectors of grapevine yellows in artificial and biological transmission assays. The survival of the known vectors and various other potential vector species on the artificial medium was determined.

All experiments were carried out with insects collected from the field. The psyllid *Psylla alni* and three leafhoppers, *Idiocerus stigmaticalis*, *Allygus modestus*, and *Oncopsis alni*, the vector of Grapevine Palatinate yellows (GPY), were collected from black elder, *Alnus glutinosa*, the natural host of GPY. The planthopper *Hyalesthes obsoletus*, the vector of Bois noir (Vergilbungskrankheit, VK), and eight leafhoppers (*Agallia consobrina*, *Agallia ribauti*, *Aphrodes makarovi*, *Euscelis* sp., *Evacanthus acuminatus*, *Issus coleoptratus*, *Macropsis fuscula*, *Neoaliturus fenestratus*) were caught from grapevine or weeds in vineyards. *M. fuscula* transmits the rubus stunt phytoplasma (RS) and occurs occasionally in vineyards where *Rubus* spp. is growing in the vicinity. Feeding of the insects on artificial medium was carried out according to Tanne et al. (7, 8). Insects were kept in the tubes individually until they died. The period of survival of individual insects was recorded and the average survival time for each species was calculated. To avoid bias by damage from sampling only data from insects that survived at least 24 h were used for the calculation. For biological transmission experiments, groups of five or six insects were kept on grapevine and alternative host plants for seven days. DNA was extracted from insects and test plants (4) as well as from the feeding medium (7). PCR tests of insects and test plants were carried out with group specific primers f/rStol (4) to detect VK phytoplasma, fAY/rEY (3) to detect GPY and RS, and with the universal primers fU5/rU3 (3) for unknown phytoplasmas. Feeding medium was tested by a nested PCR procedure using the universal primer pair P1/P7 (1, 5) for the first round and the group specific primers or the universal set fU5/rU3 for the second round. The probability of transmission (pIO) was calculated from the proportion of successful inoculations (R_{IO}) and the number n of insects per test ($p_{IO} = 1-(1-R_{IO})^{1/n}$) (6), and the transmission efficiency was estimated as the ratio of the latter parameter to the proportion of infected vectors (2).

The test insects fed on the medium with different intensity. *O. alni* and *A. consobrina*, for example, sat on the membrane continuously, while *N. fenestratus* and *H. obsoletus* interrupted the feeding frequently and moved around in the tubes. Only *P. alni* did not feed at all and all individuals died within twelve hours. Significant differences between the survival periods were observed between the species. While *Euscelis* sp. and *A. consobrina* lived for 13.8±2.8 days and 12.8±2.7 days respectively, the average lifespan was 9.6±2.4 d for *N. fenestratus*, 6.4±0.9 d for *Macropsis fuscula*, 5.5±0.8 d for *H. obsoletus*, and 3.7±0.5 d for *O. alni*.

Table 1. Results of transmission trials to host plants and artificial feeding medium with insects collected from the field.

Vector	Transmission to…	PCR insects		PCR plant or medium		Transmission efficiency
		pos/tested	%	pos/tested	%	
Hyalesthes obsoletus[1]	Artificial medium	51 / 131	39 %	20 / 91	22 %	56 %
	Vitis vinifera	38 / 100	38 %	4 / 10	40 %	13 %
	Convolvulus arvensis	31 / 63	49 %	9 / 17	53 %	29 %
	Ranunculus bulbosus	41 / 96	43 %	14 / 22	64 %	46 %
Oncopsis alni[2]	Artificial medium	50 / 74	67 %	15 / 48	31 %	47 %
	Vitis vinifera	50 / 526	10 %	4 / 127	3 %	7 %
	Alnus glutinosa	15 / 108	13 %	9 / 64	14 %	20 %
Macropsis fuscula[3]	Artificial medium	15 / 26	58 %	9 / 19	47 %	81 %
Allygus modestus[2]	Artificial medium	14 / 18	78 %	0 / 14	0 %	0 %

[1]Bois noir phytoplasma; [2]Grapevine palatinate yellows phytoplasma; [3]Rubus stunt phytoplasma

Phytoplasmas were only detected in the three known vector species and in *Allygus modestus* (Table 1) which was collected from infected black alder. The ratio of infected insects varied with reference to the source of the field samples. Although 14 of 18 individuals of *A. modestus* carried a phytoplasma of the elm yellows group they were not able to transmit it to the feeding medium. Transmission efficiency of the vectors did not equal 100 % because not all infected individuals passed the phytoplasmas to the medium. The efficiency was 81 % for *M. fuscula*, 56 % for *H. obsoletus*, and only 47 % for *O. alni* (Table 1). Still the feeding medium was infected with a considerably higher efficiency than all host plants. If the principal transmitting capability of potential vectors is checked, it will be an advantage of the artificial test system that feeding preferences of the vectors due to attractant or repellent effects of the host plants do not play a role. Biological tests, on the other hand, are more appropriate as soon as the vectoring efficiency to particular host plants needs to be evaluated. Both vectors of grapevine yellows, for example, transmitted the particular phytoplasma with a substantially lower efficiency to grapevine than to their natural host plants.

O. alni required less time than *H. obsoletus* to transmit detectable amounts of phytoplasma to the artificial medium. Fifty percent of infected *O. alni* that lived less than one day in the test tubes inoculated the medium, but no *H. obsoletus*. The rates were 40 % (62 %) for the hoppers that survived one to three days and 75 % (100 %) for those that lived more than three days. This difference could be due to a stronger secretion of saliva by *O. alni* or to the more continuous feeding of this species compared to *H. obsoletus*. The low inoculative efficiency of H. obsoletus in short time feeding could be one of the reasons that infection pressure to grapevine is low compared to other grapevine yellows in spite of the high levels of infestation of the vector populations in the field.

We found no evidence that other Auchenorrhyncha besides the known vectors transmit grapevine yellows in Germany. Species like *Aphrodes makarovi* or *Neoaliturus fenestratus* are occasionally infected by stolbur phytoplasma, but no positive insects were found during this study. *Allygus modestus* infected by GPY, on the other hand, was not able to transmit the phytoplasma. *Macropsis fuscula* transmitted the rubus stunt phytoplasma to the feeding medium with a high efficiency. Although this species occurs in vineyards we have never detected rubus stunt in grapevine. Biological transmission assays will be carried out to examine whether *M. fuscula* is able to inoculate grapevine, too.

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QUICK AND RELIABLE METHODS TO DETECT FLAVESCENCE DORÉE AND BOIS NOIR PHYTOPLASMAS IN FIELD COLLECTED INSECT VECTORS

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Among grapevine yellows widespread in Italy, Flavescence dorée (FD) is the most threatening, followed by Bois noir (BN). These diseases are associated with phytoplasmas belonging to Elm yellows (16SrV) and Stolbur (16SrXII) groups, transmitted by the leafhoppers *Scaphoideus titanus* Ball and *Hyalesthes obsoletus* Signoret, respectively (1).

Routine analyses to detect these pathogens either in plant tissues or insects still rely on nested PCRs, eventually followed by RFLP analyses. The long time required, the high costs and the risks of contaminations suggest the need of alternative reliable methods.

The aim of this work was to compare the specificity and sensitivity of different quicker methods to detect FD and BN phytoplasmas in field-collected insects.

Total DNAs were extracted as described by Marzachì *et al*. (5) from single adults of both species collected.

Group-specific primer pairs were tested in direct PCR assays: *S. titanus* DNAs were subjected to amplifications driven by the ribosomal primer pairs R16(V)F1/R1 (3) and fAY/rEY (4), whereas the non-ribosomal primer pair M1/P8 (6) was used to detect BN phytoplasmas in *H. obsoletus*. Reactions and cycling conditions were programmed as in the original papers. Alternatively, two molecular probes were used in dot-blot assays for the diagnosis of FD and BN, respectively. Amplicons obtained in direct PCRs with the R16(V)F1/R1 primer pair were hybridised with the oligonucleotide EY-417 (2), designed on elm yellows 16S rDNA sequence. The riboprobe pTS_I224 (6) was used in hybridisation assays on amplicons obtained in direct PCR with M1/P8. Following the good performances achieved in real-time PCR on plant samples (8), preliminary parallel trials were carried out on the insects using fAY/rEY and StolFw1/StolRev1 (6) to detect FD and BN, respectively.

The reliability of the fAY/rEY primer pair in conventional PCR, previously reported for both FD-infected plants and insects (7), was confirmed in the present work. The one-step PCR performed with this primer pair showed a higher efficiency both in comparison with the direct PCR driven by the other group-specific primers, R16(V)F1/R1, and in comparison with the molecular hybridisation of the amplicons with the EY-417 oligoprobe (Table 1).

The diagnostic value of the primer pair M1/P8, already demonstrated on BN-infected plants (2), has been confirmed in this work for the detection of group XII phytoplasmas in the insects. Following the hybridisation assay with the riboprobe pTS_I224, a higher number of samples provided positive signals for the presence of BN phytoplasmas (Table 2).

The direct amplification with fAY/rEY primer pair for the detection of FD phytoplasmas, on one hand, and the molecular hybridisation with pTS_I224, in the case of BN diagnosis, represent two valid tools to overcome the nested PCR in routine analyses, reducing labour and costs as well as further risks of contamination.

Table 1 – Results of PCR and dot-blot detection of FD-phytoplasmas in field collected *Scaphoideus titanus* Ball.

number of FD-positive samples / tested (%)		
F1/R1	**fAY/rEY**	**EY-417**
46/760 (6.10)	82/760 (10.80)	71/760 (9.30)

Table 2 - Results of PCR and dot-blot detection of BN-phytoplasmas in field collected *Hyalesthes obsoletus* Signoret.

number of BN-positive samples /tested (%)	
M1/P8	**pTS_I224**
12/109 (11.01)	37/109 (33.94)

Real-time PCR assays are in progress. The preliminary results obtained on *H. obsoletus* samples showed the sensitivity of this technique: all the infected insects tested gave positive signals, encouraging further trials, also on *S. titanus*.

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POTENTIAL VECTORS OF GRAPEVINE BOIS NOIR PHYTOPLASMA IN SPAIN AND EVALUATION OF THEIR TRANSMISSION CAPACITY

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Bois Noir (BN) has been detected in many countries of Europe and is caused by a phytoplasma belonging to the stolbur group (4), also named 16S rXII-A group. Stolbur phytoplasma appear to be an ubiquitous pathogen that can be hosted by plants of several families. Vegetable crops (Tomato, carrot, pepper, etc), woody plants (fruit trees and grapevine) and wild plants are hosts for Stolbur phytoplasma. Several leafhopper and planthopper species are suspected to be involved in the transmission of stolbur phytoplasma, however, only *Hyalestes obsoletus* has been found to be a transmitter of stolbur in grapevine (2). This vector is a polyphagous species, which overwinters as nymph on the roots of several host plants. However, in the surveys conducted in Spain, this species was only found in Catalonia in plots located in areas where grape production is not commercially important and has not yet been found in the stolbur infected plots sampled in the main viticulture areas, suggesting that the vector in these areas was one of the insects identified as positive to stolbur phytoplasma in previous studies. The aims of this study were to extend the search for vectors and to determine the presence and flight curve of cicadelidae and fulgoridae in a vineyard infected by stolbur phytoplasma where *H. obsoletus* was not present. On the other hand, transmission tests to feeding medium and to grapevine plants have been conducted. The transmission to feeding medium is an alternative method that presents advantages with regard to transmission trials and allows determining the infectivity of insects and its ability to transmit the disease (5).

Insects were captured on sticky yellow traps placed within or near the BN infected vineyards and with a D-vac suction traps. Transmission experiments to feeding medium were carried out with field-collected insects of phytoplasma-carrying species. Insects were maintained in *Avena sativa* for a period of two days and then individually caged on eppendorf tubes with a feeding medium, following the procedure of Tanne *et al*. (5). The PCR technique was used for phytoplasma detection in insects. DNA was extracted by grinding 1-10 insects depending on the species (1).

DNA was extracted from feeding medium adding to 200 µl of the feeding medium, 900 µl of a buffer TNE (SDS 1.1 %, 150mM NaCl, 2mM EDTA, 10 mM Tris-HCl at pH 8, 100 µl of 5M Guanidine Hydrochloride and 100 µg K-Proteinase). The mixture was incubated for 2 h at 58ºC and centrifuged for 10 min at 13000 g. An equal volume of chloroform was added to the supernatant and centrifuged for 10 min at 13000 rpm. The DNA was extracted from the supernatant following the DNA purification kit Wizard Plus (Promega). The DNA was collected with 50 µl of sterile desionized water. Nested PCR was used for a specific detection of the phytoplasma. The universal primers for phytoplasma detection, P1/P7 located at the 16S rDNA and 23S rDNA gene respectively, were used in the first step. The second step was performed with the fstol/r stol specific primers for Stolbur group (3). The negative samples with the specific primers were analyzed in the second step with the universal primer pair fU5/rU3 to determine the presence of phytoplasmas different to Stolbur group. Ten µl of the mixture containing the amplified DNA in the second step were directly digested overnight at 37ºC with 1 unit of enzyme Tru I.

Individuals of cicadelidae, fulgoridae and cercopidae were captured from the beginning of May until October, the maximum population level was obtained in the middle of July, with an average of 60 individuals for yellow trap. The highest number of individuals belonged to the species, *Scaphoideus titanus*, vector of the Flavescence Dorée and *Empoasca vitis*. The species identified as positive for phytoplasmas after nested-PCR were *Agallia laevis*, *Adarrus taurus*, *Cicadula divaricata*, *Hardya tenuis*, *Euscelidius variegatus*, *Macrosteles* sp, *Peragallia sinuata* and *Psammotettix striatus*. Most of the individuals of these species were found infected by stolbur phytoplasma, but some individuals were positive for Aster yellows. Transmission tests to feeding medium confirmed that *P.sinuata*, *P.striatus*, *H.tenuis M.quadriunctulatus* and *E.variegatus* transmitted stolbur to feeding medium. *C.divaricata*, *A.taurus*, *P.sinuata* and *H. tenuis* transmitted Aster yellows also. Both infections were not found together. The presence of the phytoplasma in the feeding medium indicated that phytoplasma was present in the insect in the salivary glands. Preliminary results of transmission tests to *Catharanthus roseus* with *Hardya tenuis* indicating that this species is capable of transmitting the stolbur phytoplama to this plant.

Transmission tests to grapevine plants are currently being carried out to establish the vectorship of these insects.

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A NOVEL APPROACH TO STUDYING THE PHYTOPLASMA GENOME

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Phytoplasms are unculturable, insect-transmissible, plant pathogens belonging to the class Mollicutes. To be transmitted, the phytoplasmas replicate in the insect body and are delivered to the insect's salivary glands, from where they are injected into the recipient plant upon feeding (1, 2). Because phytoplasmas can not be cultured, any attempt to recover phytoplasmal DNA from infected plants or insects has resulted in preparations with a large background of host DNA. Thus, studies of the phytoplasmal genome have been greatly hampered, and aside from the rRNA genes, only a few genes have been hitherto isolated and characterized (3). We developed a unique method to obtain host-free phytoplasmal genomic DNA from the insect vector's saliva and demonstrated the feasibility of the method by isolating and characterizing 78 new phytoplasmal putative open reading frames and their deduced proteins. The technical aspects of the preparation of the genomic DNA libraries and the detailed description of the individual genes are presented as a poster (Construction of Phytoplasmal Genomic Libraries and Characterization of Phytoplasmal Genes).

In order to establish the phytoplasmal specificity of the isolated clones, we have performed a number of Southern, northern and PCR analyses. In all cases the isolated sequences (utilized as probes or source of primers) reacted with plants and insect that had been proven by rRNA assays to carry phytoplasma. None of the phytoplasma-free plants or insects reacted with these clones.

Blast analyses were carried out with each isolated phytoplasma ORF. Most of the highest scored sequences were prokaryotic or mitochondrial (also considered as prokaryotic). Several others were unique sequences without a match at the database and were designated as hypothetical genes. Several ORFs were homologous to eukaryotic genes, albeit, they probably were adapted by the phytoplasma as they resembled phytoplasmal gene by being poor in their G:C content (under 35% in most cases) and most of them were situated on the same physical DNA entity with prokaryotic genes. As expected from such a small genome, the gene density is rather large (83.7%) and many ORFs were superimposing in different (even opposite) frames. Several genes are of special interest. Phytoplasma carries retroelements (probably retrotransposons). A gene which is required for entry and establishment of infection in baculoviruses and entemo-pox viruses (fusilin) was found also in phytoplasma. It is noteworthy that this protein binds to both chitin and cellulose – the outer envelop of insect and plant cells (4).

Phytoplasmas are intra-cellular parasites, devoid of cell-wall and carry a small genome. In these features they resemble mycoplasma and indeed were designated as "mycoplasma-like organisms – MLOs". However, none of the isolated genes was homologous to a mycoplasmal gene, and the gene organization indicated some resemblance to *Bacillus*. The data indicate that phytoplasma probably occupy a unique phylogenetic position, and analysis of a multitude of genes may result in different relationships than the current phylogenetic "trees" based on ribosomal genes.

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SEASONAL FLUCTUATION AND DETECTION OF STOLBUR PHYTOPLASMA IN GRAPEVINE

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Different grapevine yellows phytoplasmas have been detected in vines causing losses in crop and quality. In Spain the stolbur phytoplasma responsible for the Bois Noir disease has been identified in different viticultural areas of Aragon, Catalonia and Navarra. For an accurate detection of the disease it is necessary to have a sensitive detection method. Although both serological and DNA hybridization methods have been used for this purpose in the past, PCR has since proved to be a more sensitive tool for detecting phytoplasmas in their plant and insect hosts.

Due to the low concentration of this phytoplasma in woody plants, nested-PCR is the most suitable technique for its detection. Nested-PCR method sometimes implies DNA contamination, furthermore this procedure is time consuming and expensive for ordinary quality vegetal control and certification analyses. The first objective of this study has been to compare the results obtained with two detection methods, nested PCR and a combined method PCR-dot blot.

On the other hand phytoplasma detection success depends on the season of the year when samples are collected. Several studies in woody plants show that phytoplasma overwinter in the roots of their host, and spread to the aerial parts in the spring. However, some studies done on woody plants revealed that the phytoplasma is also able to survive in aerial parts during winter time (2,4). The other objective of this study was to determine the best period for a reliable detection of stolbur phytoplasma in the aerial parts of the plant. Differences in detection between different types of plant tissue materials (leaf midribs, buds or stems) have also been investigated.

The seasonal variation in the detection of the stolbur phytoplasma was followed in ten plants affected by the disease. Samples were collected monthly from June 2002 to June 2003, except after pruning, from January to March. Stem, buds or leaf samples depending on the season were excised every month from five different parts of the plant and analyzed using the nested-PCR method. PCR-dot blot technique was periodically used in order to compare the sensibility of both methods. The selection of the plants sampled was done taking into account that, all of them were found positive for stolbur in previous studies. Samples from one healthy plant were tested as control.

Plant DNA was extracted from approximately 1.0 g of fresh plant material, using the phytoplasma-enrichment procedure of Ahrens & Seemüller (1992).

Nested PCR was carried out with universal primers for phytoplasma detection, P1/P7, located at the 16S rDNA and 23S rDNA gene respectively in the first step. This procedure amplifies a fragment of about 1800 bp in length. The second step was performed with the fstol/rstol specific primers (3). These primers amplify the 16S rDNA gene from Stolbur group, producing a fragment about 550 bp in length. To verify the results, the nested PCR procedure was performed three times for each sample.

The second method was performed using a single PCR with P1/P7 universal primers. Two µl of the amplification product were spotted onto nylon membrane, fixed at 120 ºC during 30 min and hybridized with a specific stolbur probe.

The probe was obtained from a 550 kb fragment of the stolbur chromosome amplified by PCR with primers fstol/rstol (3) using DNA from a stolbur-infected periwinkle. The probe was digoxigenin labelled using the "PCR dig synthesis kit" (Roche).

Results obtained have shown that occasionally the combined method PCR-dot blot is more sensitive than the nested PCR. This could be due to the fact, that although the PCR is the most sensitive technique, in some instances it has not been able to detect the presence of phytoplasma in diseased plants. Detection failure might be due to low concentrations of the phytoplasma in the plant and also to the presence of PCR inhibitors in the woody plants, that can vary throughout the year.

PCR-dot-blot method may avoid contaminations that often occur when using nested PCR. Furthermore this technique also allows the use of the first product amplified to hybridize with different specific probes, and identify the phytoplasma involved in a short period of time.

The presence of the phytoplasma was detected both in stems, buds and leaf samples. The maximum detection rate of stolbur phytoplasma in grapevine occurred in December, as it has been previously reported in other plants (2). The observations indicate a tendency towards an increase in the number of samples where amplification of phytoplasma DNA was produced since April to December.

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