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A multiplex nested-PCR assay for sensitive and simultaneous detection and direct identification of phytoplasma in the Elm yellows group and Stolbur group and its use in survey of grapevine yellows in France

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Summary

Flavescence dorée and Bois noir (or Vergilbungskrankheit), are two main yellows diseases of grapevines in Europe. The two diseases cannot be distinguished on the basis of symptoms but they are associated with two different phytoplasmas which belong to the Elm yellows (16SrV) group and Stolbur (16SrXII) group, respectively. Their spreading areas are overlapping in France, Italy and Spain but they have different vector insects. Flavescence dorée is an epidemic disease and a quarantine organism. National surveys conducted annually in France require straightforward and sensitive assays to detect phytoplasma that sometimes occur in grapevine with a low titre and to characterize them readily. A bi-specific multiplex nested-PCR procedure was developed, to amplify simultaneously two non-ribosomal DNA fragments, 1150 bp and 720 bp in length, specific for Elm yellows-group and Stolbur-group phytoplasmas, respectively. They were identified using agarose gel electrophoresis of amplification products. The procedure is quick, sensitive and reliable. It was used on 2,525 grapevine samples from the field, in the frame of the French survey in 2002. Mixed samples containing both phytoplasmas displayed a mixed profile in the gel. It was confirmed that the nested-PCR amplimer obtained in the FD9 DNA region with Elm yellows-group phytoplasmas, though shorter than the initial FD9 fragment, nevertheless contained the restriction sites that permit the RFLP identification of geographic phytoplasma isolates already characterized in former studies.

K e y w o r d s : phytoplasma, Flavescence dorée, Elm yellows, 16SrV, Bois noir, Vergilbungskrankheit, Stolbur, 16S rXII, detection, quarantine, non-ribosomal DNA, primers, multiplex PCR.

Introduction

Flavescence dorée (FD) is an epidemic Grapevine yellows (GY) associated with a phytoplasma in the Elm yellows (EY) group (16SrV) (Seemüller *et al.* 1998; Lee *et al.* 1998). It dramatically affects large winegrowing areas in Southern France and Northern Italy (Boudon-Padieu 2002; Borgo and Angelini 2002) and has been reported to occur in Spain (Batlle *et al.* 1997, 2000). FD is a quarantine organism in the countries of the European Community (EC Directive Nr. 77/1993 modified 92/103). Its quick spread-out is due to the presence of its vector *Scaphoideus titanus* Ball. (Schvester *et al.* 1963), an oligophagous vine-feeding leafhopper of American origin, which in Europe found a suitable ecological niche where it can diffuse specifically FD phytoplasmas that are introduced in vineyards (Carraro *et al*. 1994; Mori *et al.* 2002). Such introductions can occur either at long distance with latently infected grapevine planting material (Caudwell *et al.* 1994), or at short distance by migration of infective *S. titanus* leafhoppers. At present *S. titanus* has colonized all vineyards of the Southern half of France (Boudon-Padieu 2000), North of Italy (Alma 2002), some regions of Switzerland (Baggiolini *et al.* 1968; Clerc *et al.* 1997) and of Slovenia (Seljak 2002). It has been reported also in the North of Spain (Batlle *et al*. 2000) and of Portugal (Quartau *et al*. 2001). Hence, a large geographical area is under the threat of FD outbreaks. Reliable and straightforward methods to identify FD in European vineyards and nurseries are necessary to prevent or limit the diffusion of the disease (Boudon-Padieu and Maixner 1998). However, GY have been reported to be associated worldwide with at least 8 different phytoplasmas, nevertheless showing similar symptoms on all varieties (Caudwell *et al*. 1971; Pearson *et al*. 1985; Daire *et al*. 1993, 1997 a; Prince *et al*. 1993; Wolf *et al*. 1994; Bertaccini *et al*. 1995; Bianco *et al*. 1996; Padovan *et al*. 1996; Tanne and Orenstein, 1997; Gibb *et al.* 1999; Maixner *et al*. 2000 b; Constable *et al.* 2002). Bois noir (BN) and a similar disease, Vergilbungskrankheit (VK), are the second important group of GY in Europe. They are associated with a stolbur (16SrXII) phytoplasma and transmitted in Germany, France and Italy by the planthopper *Hyalesthes obsoletus* Sign., which feeds on grapevine erratically (Maixner 1994; Sforza *et al.* 1998; Alma *et al*. 2002). The incidence of BN/VK diseases has also increased in the last decade in several countries of Europe and Asia Minor (Laviña *et al*. 1995; Marcone *et al*. 1996; Davis *et al.* 1997; Skoric *et al*. 1998; Bourquin *et al*. 2000; Maixner *et al*. 2000 a; Varga *et al*. 2000). This could be due to significant changes in climatic and cropping factors that favor pullulation and migration of the planthopper vector *H. obsoletus*, and to the occurrence of weeds that host both, the planthopper and the stolbur phytoplasma (Weber and Maixner 1998). Prevention, control and prophylaxis of FD on the one hand and of BN/VK on the other, rely on very different strategies (Boudon-Padieu and Maixner 1998).

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Hence, monitoring of Grapevine yellows is particularly relevant. It requires straightforward techniques for the analyses of numerous samples, since FD and BN can occur in the same vineyard. Monitoring should also be done on insects and on a diversity of plant tissues. Currently, in France routine surveys of GY rely on FD-specific ELISA (Caudwell and Kuszala 1992; Maixner *et al*. 1997) and on additional examination of ELISA-negative samples with nested-PCR (polymerase chain reaction) of phytoplasma ribosomal DNA, followed by RFLP (restriction fragment length polymorphism) analysis of amplimers in order to detect FD phytoplasma that would occur at a lower titre or alternatively to detect and characterize BN (stolbur phytoplasma) or other possible phytoplasma (Daire *et al.* 1997 b, Maixner *et al.* 1997). These procedures are expensive and time-consuming.

Random-cloned fragments in non-ribosomal DNA of FD and stolbur phytoplasma, called FD9 and STOL11, respectively, have been obtained previously and primers have been constructed for their specific amplification with PCR (Daire *et al.* 1992, 1997 b; Batlle *et al*. 2000). A multiplex PCR assay has been designed to allow the simultaneous amplification of DNA fragments FD9 and STOL11 (Daire *et al.* 1997 b). However this one-step multiplex procedure demonstrated a lack of sensitivity for samples collected in the field (Firrao *et al.* 1999; Boudon-Padieu *et al.* unpubl.). Recently, internal primers on the FD9 fragment have been constructed and PCR conditions for nested amplification of the fragment have been designed, insuring the sensitive detection of EY-group phytoplasmas in grapevine and elms (Clair *et al.* 2000; Angelini *et al.* 2001; Boudon-Padieu *et al*. 2003). In the present work, internal primers were constructed on the STOL11 fragment and a bi-specific multiplex nested-PCR assay was developed. This procedure was evaluated to simultaneously detect and characterize FD and stolbur (BN/VK) phytoplasmas in field-collected grapevine samples. It was also used on other woody host plants of phytoplasmas belonging to the EY- and stolbur groups and on insect vectors. It was further validated for use in routine assays of numerous grapevine samples from the field in the frame of national surveys at the Laboratoire National de la Protection des Végétaux (LNPV).

Material and Methods

Plants and phytoplasma source: In a first set of experiments designed for standardization of the procedure, DNA was prepared from veins and petioles of 3 FD- infected Baco 22A and 3 BN-infected Chardonnay grapevines that had scored positive in former assays. Healthy control were Baco 22A seedlings grown in the greenhouse. DNA was prepared from a periwinkle (positive control) that had been double graft-inoculated with shoots taken from periwinkles infected with FD92 isolate (Daire *et al.* 1997 b) and STOLC (tomato stolbur isolate) phytoplasmas, respectively, while water was used instead of DNA for negative controls.

In assays conducted to validate the final conditions for field surveys, DNA samples were prepared from tissues of GY-affected grapevine cultivars from different sites in France, from cv. Scheurebe affected with Palatinate grapevine yellows (PGY) (kindly obtained from M. Maixner, Bernkastel-Kues, Germany), from elms showing Yellows symptoms (kindly obtained from E. Collin, CEMAGREF, Nogent sur Vergisson, France), from lavender (*Lavandula vera* L.) with decline symptoms in lavender crops in the South-East of France, from experimentally FD-infected *Euscelidius variegatus* Kbm. leafhoppers reared in an insectary (Caudwell *et al.* 1972) and from wild *H. obsoletus* plant-hoppers trapped during former surveys of stolbur phytoplasma in vineyards and sugar beet crops (Sforza *et al.* 1998; Gatineau *et al.* 2001). The phytoplasmas present in all the latter samples had been previously characterized and belonged either to the EY-group (16SrV) or to the Stolbur group (16SrXII). As control, the phytoplasma isolates classified into other groups or subgroups (Seemüller *et al.* 1998; Lee *et al.* 1998), and maintained in periwinkle by serial grafting, were as follows: American Aster yellows (AAY, 16SrI-B), Apple proliferation (AP, 16SrX-A), Beet Leafhopper Virescence Agent (BLTVA, 16SrVI), Plum Leptonecrosis (PLNV12, 16SrX-B), Clover phyllody (CP, 16SrI-C). Healthy controls were DNA prepared from healthy grapevine or periwinkle seedlings and from healthy-reared *E. variegatus* leafhoppers hatched on healthy maize (*Zea maydis* L.) in the insectary.

During the 2002 national survey, grapevine samples were collected from 2,525 vineyards in different regions of France. Leaves with petioles were taken on 5 symptomatic vines per affected vineyard, sent to the Laboratoire National de Protection des Végétaux (LNPV) in Colmar, France, and stored at 4 °C until processing. Leaf petioles were detached with a scalpel and the leaf blades were stored in deep-freezed for control. Petioles of one leaf of each of the 5 grapevines from one plot were pooled in a mixed sample for DNA extraction.

DNA extraction: Total DNA was extracted from vascular tissues of leaf veins or petioles or from individual insects, using cethyl-trimethyl-ammonium bromide (CTAB) as described by Angelini *et al.* (2001) and Gatineau *et al.* (2001). The final total DNA pellet from 1 g of fresh plant tissue was resuspended in 500 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and the final total DNA pellet from individual insect specimen was resuspended in 50-100 µl of the same buffer, depending on the size of the insect.

Primers for PCR amplification of phytoplasma DNA: The two primer pairs FD9f/r (Daire *et al.* 1997 b) and FD9f3/r2 (Angelini *et al.* 2001) have been previously designed on the aligned sequences of FD9 DNA region obtained by Reinert (1999) in 5 EY-group phytoplasmas. The modified FD9f3b primer was constructed by adding 6 nucleotides to the 5' end of FD9f3, using the same sequence data. The sequences of these 5 primers are given in Tab. 1. The STOL11 DNA fragment was amplified with PCR in 4 stolbur phytoplasma isolates using the primer pair STOL11f2/r1 (Daire *et al.* 1997 b; Tab. 1). PCR products were sequenced and accession numbers are given in Tab. 2. Internal primers STOL11f3 and STOL11r2 (Tab. 1) were designed after alignment of the latter sequences. In both occurrences, internal primers were designed as close as possible to each of the primers in the first pair, in order to minimize the size reduction of final products of nested-PCR. The size

Table 1

Sequences of primers constructed on FD9 and STOL11 DNA fragments

Primer name	Sequence	bp	Reference
FD9f	5'-GAA TTA GAA CTG TTT GAA GAC G–3'	22	DAIRE *et al.,* 1997 b
FD9r	5'-TTT GCT TTC ATA TCT TGT ATC G-3'	22	DAIRE *et al.,* 1997 b
FD9f3	5'-GGT AGT TTT ATA TGA CAA G-3'	19	ANGELINI *et al.*, 2001
FD9f3b	5'-TAA TAA GGT AGT TTT ATA TGA CAA G-3'	25	This paper
FD9r2	5'-GAC TAG TCC CGC CAA AAG-3'	18	ANGELINI *et al.,* 2001
STOL11f2	5'-TAT TTT CCT AAA ATT GAT TGG C-3'	22	DAIRE *et al.,* 1997 b
STOL11r1	5'-TGT TTT TGC ACC GTT AAA GC-3'	20	DAIRE *et al.,* 1997 b
STOL11f3	5'-ACG AGT TTT GAT TAT GTT CAC-3'	21	This paper
STOL11r2	5'-GAT GAA TGA TAA CTT CAA CTG-3'	21	This paper

Table 2

GenBank accession numbers of sequence of STOL11 DNA fragment in different stolbur phytoplasma isolates

Stolbur phytoplasma isolates in periwinkle	Origin	Accession number of STOL11 sequence
STOL C	Tomato, France, obtained from M.T. COUSIN	AF447593
P3L	Inoculation with *Hyalesthes obsoletus*[a)] (SFORZA *et al*. 1998)	AF447594
Stol-Bett	Inoculation with *Pentastiridius* sp.[b)] (GATINEAU *et al*. 2001)	AF447595
LAV	Decline of lavender, France, obtained from M. T. COUSIN	AF447596

[a)] Vector of stolbur phytoplasma trapped during monitoring of BN diffusion in a vineyard in the Rhône valley, France.

[b)] Natural vector of stolbur phytoplasma to sugarbeet, trapped in Burgundy, France.

of the FD9f3b-FD9r2 fragment was 1160 bp and the size of the STOL11f3-STOL11r2 fragment was 720 bp.

DNA amplification: DNA amplification was performed in 20 µl total reaction volume in a Biometra T3 thermocycler. The reaction mixture contained as template 1 µl of extracted DNA or of the diluted (1:1000) first amplification product, 150 µM each dNTP, 10 mM Tris-HCl buffer pH 9, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X100, 0.2 mg ml^{-1} BSA and 0.2 U *Taq* polymérase (Q. Biogene); primers as specified below. For exploration of multiplex conditions, assays were conducted in parallel on the same DNA samples. In the first amplification, two parallel assays ("a" and "b") used both FD9f and FD9r primers at a final concentration of 0.5 µM, while STOL11f2 and STOL11r1 primers were each used at final concentrations of (a) 0.05 µM or (b) 0.025 µM, respectively. The conditions for amplification were a predenaturation step at 92 °C for 90 s, then 30 cycles with denaturation at 92 °C for 40 s, hybridization at 55 °C for 40 s and elongation at 72 °C for 70 s. In the nested amplification, products "a" and "b" were used each in three comparative assays ("i", "ii" and "iii") using each FD9f3b and FD9r2 primer at a constant concentration of 0.5 µM, while each STOL11f3 and STOL11r2 primer was added at a final concentration of (i) 0.5 µM, (ii) 0.25 µM or (iii) 0.125 µM, respectively. All other PCR conditions were identical with the first amplification, except that 35 cycles were allowed. The amplimers obtained in these 6 different nested assays were run on 1.2 % agarose gels, stained with ethidium bromide (EtBr) and visualized with UV light.

The procedure, combining conditions ["b", "ii"], was further used on a variety of samples collected in the field. Final conditions for optimum detection were obtained with additional modifications. The final procedure used each of the FD9f/r primers at 0.375 µM and each of the STOL11f2/r1 primers at 0.0625 µM in the first multiplex amplification, then FD9f3b/r2 primers as well as STOL11f3/r2 primers at 0.375 µM each in the nested-multiplex amplification. The latter procedure was used for the national survey at the LNPV, Colmar.

Results

Design of multiplex PCR conditions: Results of the first series of assays

conducted on 3 BN-infected and 3 FD-infected vines are shown in Fig. 1. A balanced detection of the two phytoplasmas with respect to band intensity was obtained with conditions [a, i], [a, ii] and [b, ii]. However, non-specific amplification of one product at the position of the STOL11 fragment in FD-infected samples and healthy control could be observed in lanes run with products [a, i] and another product at the position of the FD9 fragment in BN-infected samples was present in lanes run with products [a, i] and [a, ii] (Fig. 1). Thus, the combination [b, ii] using FD9 and STOL11 first pairs of primers at a 1:20 ratio of their respective concentrations and second pairs of primers at a ratio of their respective concentrations of 1:2, was selected first.

Further assays on a variety of phytoplasma-infected and healthy control samples (not shown) showed that more specificity was obtained when the concentration of FD9 primers was decreased. Fig. 2 shows the amplimers obtained when FD9 primers were used at a concentration of 0.375 µM each in both the first and second amplification and STOL11 primers were used at a concentration of 0.0625 µM (1:6 with respect to FD9 primers) in the first step and 0.375 µM in the second step (1:1 with respect to FD9 primers). Under these conditions, only specific products were amplified in DNA samples from infected grapevines formerly characterized

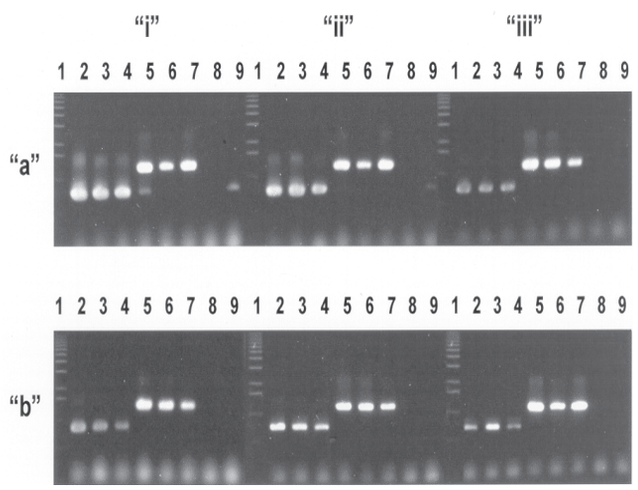

Fig. 1: Amplimers obtained with DNA from 3 Baco 22A and 3 Chardonnay that had been tested positive formerly for FD and BN, respectively, with 6 different combinations of final concentrations of FD9 and Stol11 primers in PCR mixtures of the first ("a" and "b") and nested ("i", "ii", and "iii") amplification runs. Lane 1, 1kb ladder; lane 2-4, 3 different BN-infected Chardonnay; lane 5-7, 3 different FD-infected Baco 22A; lane 8, control using water instead of DNA; lane 9, healthy seedling of Baco 22A. Combination [b, ii] was first selected.

(Fig. 2, lanes 2-4 and 6-8). A positive amplification for an EY-group phytoplasma was also obtained from diseased elm (lane 9) and from cv. Scheurebe infected with PGY (Fig. 2, lane 10). A band corresponding to the size of the STOL11 fragment was obtained from lavender showing decline (Fig. 2, lane 5). No amplification product was obtained with template DNA from periwinkle infected with other, non-related phytoplasmas (Fig. 2, lanes 11-15) nor with DNA from healthy grapevine (Fig. 2, lane 16). Two bands were obtained from the double (FD + STOLC) -infected periwinkle (Fig. 2, lane 17).

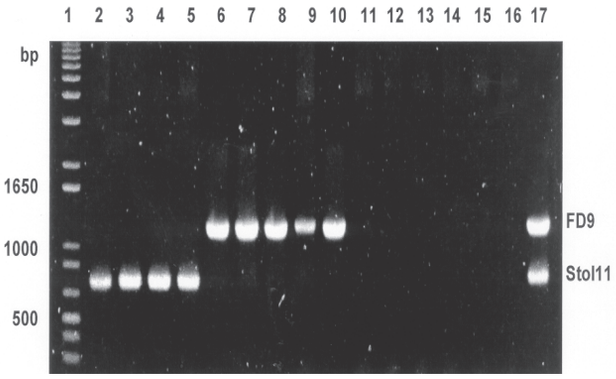

Fig. 2: Amplimers obtained in multiplex nested PCR with DNA from plant samples containing phytoplasma characterized previously and healthy control, with a slight modification of PCR conditions [b, ii]. The concentration ratio between FD9 and STOL primers was brought to 1:6 in the first run and to 1:1 in the second run of PCR. Lane 1, 1 kb ladder. Lane 2-4, BN-infected grapevines: lane 2, Chardonnay from Burgundy; lane 3, Cabernet-Sauvignon from the Loire region; lane 4, Carignan from Languedoc. Lane 5, Stolbur-infected lavender with decline symptoms from South-eastern France. Lane 6-8, FD-infected grapevines: lane 6, Ugni blanc from Armagnac; lane 7, Jacquère from Savoie; lane 8, Alphonse Lavallée from Tarn et Garonne. Lane 9, elm with decline from Loiret. Lane 10, cv. Scheurebe affected with PGY from Palatinate, Germany. Lane 11-15, control phytoplasmas maintained in periwinkle: lane 11, CP; lane 12, AAY; lane 13, AP; lane 14, PLNV12; lane 15, BLTVA. Lane 16, healthy seedling of grapevine cultivar Baco 22A. Lane 17, double-infected (FD+STOL) periwinkle.

Validation on field samples and insects: Fig. 3 shows detection obtained under the latter conditions from a series of grapevine samples originating from various regions of France and with template DNA from insects. Fig. 4 shows detection obtained in LNPV. Each lane contains the amplimers obtained from the mixed sample (1 petiole from each of the 5 grapevines) of a single vineyard. Among the 46 vineyards examined in Fig. 4, 6 vineyards were tested negative for both FD and BN, 8 vineyards were tested positive for FD alone, 25 vineyards were tested positive for BN alone and 7 vineyards were tested positive for both FD and BN. Material (petiole or leaf tissue) from each grapevine in the 7 mixed-infected pooled samples was again separately analyzed after an independent DNA extraction. Results are shown in Tab. 3. The presence of both phytoplasmas in individual vineyards was confirmed, except for one vineyard (A 20) where BN could not be detected. However, no mixed infection was found in individual grapevines.

Molecular epidemiology: It was verified that FD, PGY and EY isolates previously characterized by analyses of the FD9f-FD9r DNA fragment (Daire *et al.,* 1997) and of the shorter FD9f3-FD9r2 fragment (Angelini *et al.,* 2001, 2003) were similarly identified with restriction profiles of the final FD9f3b/r2 PCR product (data not shown).

Discussion

The understanding of presence, incidence and progression of FD and BN/VK diseases of grapevine have greatly

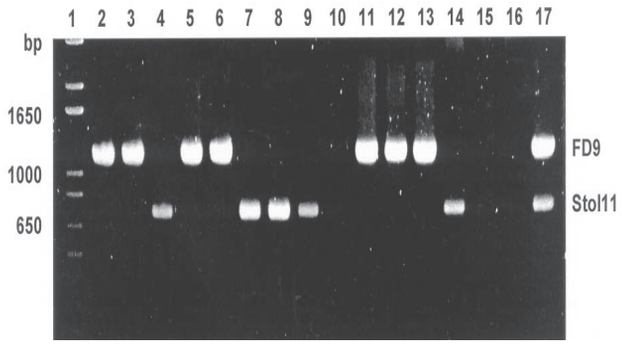

Fig. 3: Validation of multiplex nested-PCR conditions shown in Fig. 2, on DNA from a variety of grapevines showing GY symptoms from different French regions, previously not analyzed, and on insect vector specimen. Lane 1, 1 kb ladder. Grapevine cultivars: lane 2, Ugni blanc; lane 3, Jacquère; lane 4, Chardonnay; lane 5, Alphonse Lavallée; lane 6, Carignan; lane 7, Cabernet-Sauvignon; lane 8, Carignan; lane 9, Gamay; lane 12, Négrette; lane 13, Colombard. Insect vector specimen: lane 10-11, two *E. variegatus* leafhoppers reared in the insectary, lane 10, healthy, lane 11, FD-infected; lane 14-15: two wild *H. obsoletus* planthoppers trapped in lavender fields in South-eastern France. Lane 16, healthy periwinkle. Lane 17, double-infected (FD+STOL) periwinkle.

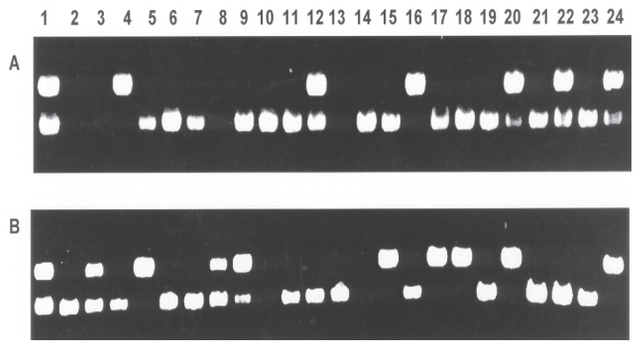

Fig. 4: Amplimers obtained with the same multiplex nested PCR conditions shown in Fig. 2 and 3, from pooled samples of grapevines collected during the 2002 French survey of GY-affected vineyards. Each sample was obtained from tissue taken on 5 symptomatic grapevines in a single vineyard. Lane 1 in **A** and **B**: double-infected (FD+STOL) periwinkle control. Lane 2-24 in **A** and **B**: samples from 46 different vineyards.

progressed during the last decade, due to the development of sensitive characterization methods which showed that the diseases differ both in their associated phytoplasmas and in their vectors. However, during the same period, a dramatic diffusion has been observed for FD in Southern France and Northern Italy. Simultaneously, BN/VK have progressed in the same regions, in non-FD-affected vineyards in regions colonized by *S. titanus* and also in a wider area in Europe and Asia Minor. Thus reliable and quick characterization methods are necessary. Our objective was a sensitive differential assay for FD and BN phytoplasma avoiding RFLP analyses, though these two phytoplasmas belong to different ribosomal groups.

Design of a multiplex procedure requires that the same PCR conditions amplify two different DNA fragments with balanced efficiency. In this particular case, a nested procedure was required for sensitivity. 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 and raising the annealing temperature. The length of the formerly designed primer FD9f3 was increased to obtain conditions permitting amplification of FD9 fragment with a hybridization temperature of 55 °C.

The procedure allowed the specific and sensitive simultaneous detection of the two major grapevine phytoplasmas in Europe and their immediate identification with agarose gel electrophoresis of PCR products. It also proved to be specific on insect vectors. Moreover it was used in a large-scale survey and permitted pooling of samples for rapidity of monitoring, nevertheless allowing the detection of mixed-infected samples. Control on the reliability of results in routine conditions was quite sufficient, taking into account the heterogeneous distribution known for phytoplasma in woody plants.

Mixed infections with two or several phytoplasmas have been reported in different perennial plant species, including grapevine (Lee *et al*. 1994; Bertaccini *et al*. 1995). Such situ-

Table 3

Phytoplasma detection in individual grapevines from vineyards showing the presence of both FD and BN phytoplasmas in Fig. 4

Vineyard (lane Nr in Fig 4)	grapevine 1	grapevine 2	grapevine 3	grapevine 4	grapevine 5
A 12	BN[a)]	BN	FD	FD	Neg
A 20	Neg	Neg	FD	FD	FD
A 22	FD	BN	FD	BN	/ [b)]
A 24	FD	FD	FD	BN	BN
B 3	Neg	BN	Neg	FD	BN
B 8	BN	BN	Neg	BN	FD
B 9	FD	BN	BN	FD	BN

[a)] BN/FD/Neg for detection of either BN, FD or no detection in individual grapevines.
[b)] Only 4 grapevines had been sampled in the corresponding vineyard.

ations could be detected only with two or more independent nested-PCR assays for amplification of ribosomal DNA using different group-specific primers, because of the very low titre of the ”second” phytoplasma. Whatever the pathological significance of the latter phytoplasma, it might nevertheless represent a "silent" reservoir with potential danger. The present study did not allow the detection of mixed infection in single grapevines, though it could be achieved in control double-grafted periwinkle. However, the detection of mixed infections in pooled samples confirmed that such detection is possible with the new nested-multiplex procedure, thanks to the use in the first and second PCR run, of specific primers for each phytoplasma. Its sensitivity will be verified when one of the two phytoplasmas is present at a very low titre compared to the other phytoplasma and the procedure will be used to monitor the frequency and epidemiological significance of multiple infections.

In addition, other work has shown the relevance of FD9 DNA region of 16SrV group phytoplasmas for the study of diversity of strains within this group (Daire *et al*. 1997; Angelini *et al*. 2001, 2003; Martini *et al*. 2002). In the present work, it was also confirmed that the shorter fragment FD9f3b – FD9r2 showed RFLP patterns after digestion with restriction enzymes which were consistent with the previous differentiation of isolates within EY phytoplasma group. Hence, FD-DNA PCR products obtained in the multiplex procedure from samples from diverse regions will be used in the future for molecular epidemiology studies of the diffusion of FD disease.

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