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Grapevine yellows: Comparison of different procedures for DNA extraction and amplification with PCR for routine diagnosis of phytoplasmas in grapevine

E. Boudon-Padieu[1)], A. Béjat[1)], D. Clair[1)], J. Larrue [1)], M. Borgo[2)], L. Bertotto[2)] and E. Angelini[1) 2)]

[1)] Epidémiologie des phytoplasmes, UMR 1088 BBCE-IPM, INRA, Dijon, France
[2)] Istituto Sperimentale per la Viticoltura, Conegliano Veneto, Italia

Summary

In order to devise a procedure to be used as reference for detection of grapevine phytoplasmas and monitoring of Flavescence dorée, 12 combinations comprising three methods of plant DNA extraction and 4 procedures for amplification in polymerase chain reaction of phytoplasma DNA were examined in parallel using the same plant tissues infected with phytoplasmas. In a first series tissues of periwinkles (*Catharanthus roseus*) infected with phytoplasma isolates of the Elm yellows group (16SrV) and maintained in the greenhouse, were used. In a second series tissues of grapevines (*Vitis vinifera*) naturally infected with Flavescence dorée or Palatinate grapevine yellows phytoplasma were used. The DNA preparations obtained with each of the three extraction procedures were used undiluted or serially diluted, as target DNA in the 4 nested-polymerase chain reactions. The results showed differences in the efficiency among different methods of extraction as well as in the sensitivity among the DNA amplification procedures, which improved when DNA extracted from field grapevines was diluted. After additional comparative validation on numerous field-collected samples of GY-affected grapevines, the quickest extraction procedure was selected for use in routine diagnosis, with nested-PCR amplification either of ribosomal DNA or of the FD9 DNA fragment specific for Flavescence dorée and other 16SrV group phytoplasmas.

K e y   w o r d s : phytoplasma, grapevine yellows, Flavescence dorée, Elm yellows, 16SrV, diagnosis, DNA extraction, nested-PCR, ribosomal DNA, non-ribosomal DNA, *Vitis vinifera*, *Catharanthus roseus*.

Introduction

Grapevine yellows (GY) are associated with several different phytoplasmas worldwide but cannot be identified on the basis of symptoms. Phytoplasmas are obligate parasitic phloem-restricted bacteria, which are transmitted by insect vectors and propagated by vegetative multiplication of plant material. Flavescence dorée (FD) is the more dangerous GY and FD phytoplasma is a quarantine organism in the European Community (EC directive Nr 77/1993 modified 92/103), because it is transmitted by a vine-feeding leafhopper vector, *Scaphoideus titanus* Ball (Schvester *et al*. 1963) spread out in many parts of the Western Mediterranean area (Boudon-Padieu 2002). FD phytoplasma belongs to the Elm yellows (EY) group (Daire *et al.* 1992, 1993, 1997 a; Seddas *et al.* 1996) or 16SrV group, according to the classification of Seemüller *et al*. (1998) and Lee *et al*. (1998), respectively. Bois noir (BN) or Vergilbungskrankheit (VK), associated with phytoplasmas of the stolbur (STOL) or 16SrXII group, is the second economically important GY in Europe. The respective occurrence of FD and BN/VK are overlapping in southern France, northern Italy and northern Spain. In addition, BN also occurs in FD-free regions where the FD leafhopper vector is nevertheless present (Baggiolini *et al.* 1968; Batlle *et al.* 2000; Seljak 2002). Moreover, other different phytoplasmas have also been associated with GY in Europe (Daire *et al.* 1993; Bertaccini *et al*. 1995; Maixner *et al*. 1995 b; Marcone *et al*. 1996; Davis *et al*. 1997; Skoric *et al*. 1998) and the USA, Australia and Israel (Daire *et al*. 1993; Prince *et al*. 1993; Padovan *et al*. 1995; Davis *et al*. 1997; Tanne and Orenstein 1997; Gibb *et al*. 1999; Constable *et al*. 2002). International exchange of plant material increases the risk of long distance spreading of phytoplasma and of introduction of new vector insects.

Monitoring, control and sanitation measures of GY diseases require sensitive and reliable diagnosis (Maixner *et al.* 1997). Amplification with polymerase chain reaction (PCR), followed by restriction fragments length polymorphism (RFLP) analyses of phytoplasma DNA has been developed in the past decade and is the method of choice for phytoplasma detection and characterization. Numerous PCR–based detection procedures of phytoplasma DNA have been developed, using various primers. Primers are universal or group-specific; they amplify with PCR a ribosomal phytoplasma DNA fragment (Sears *et al*. 1989; Deng and Hiruki 1991; Ahrens and Seemüller 1992; Lee *et al*. 1993; Schneider *et al*. 1993; Gibb *et al*. 1995; Maixner *et al*. 1995 a; Padovan *et al*. 1995). Alternatively, a few primer pairs have been designed to amplify with PCR group-specific fragments of non-ribosomal DNA of phytoplasma (Daire *et al.* 1992, 1997 b; Padovan *et al.* 1996).

However, a prerequisite of PCR amplification is the preparation of good quality DNA with a consistent yield from phytoplasma-infected hosts. The titre and distribution of phytoplasma in different parts of the plant depend on variety, age of infection and date of sampling (Berges *et al.* 2000). In addition, the concentration of phytoplasma can be very low in woody plants such as fruit trees or grapevine (Kartte and Seemüller 1991; Daire 1994). Moreover, electron microscopic studies have shown that phenolic compounds accumulate and cell walls collapse in phloem sieve

Correspondence to: Dr. E. Boudon-Padieu, Biologie et Écologie des Phytoplasmes, UMR 1088 Plante Microbe Environnement, INRA-Université de Bourgogne, Domaine d'Epoisses, BP 86510, F-21065 Dijon Cedex, France. Fax: +33-3-8069-3261. E-mail: boudon@epoisses.inra.fr

tubes containing high numbers of phytoplasma cells (Meignoz *et al.* 1992; Credi 1994). Extraction methods must separate the latter from tannins and cell debris to give access to specific targets of diagnosis, such as phytoplasma DNA (Daire 1994) or outer membrane proteins in ELISA tests (Caudwell and Kuszala 1992). Different methods for the extraction of phytoplasma DNA have been described (Kirkpatrick *et al.* 1987; Ahrens and Seemüller 1992; Prince *et al*. 1993; Daire *et al.* 1992, 1994, 1997 b; Bertaccini *et al.* 1995; Barba *et al*. 1998; Angelini *et al*. 2001; Palmano, 2001). They essentially followed one of the following strategies: (1) an enrichment procedure was used to prepare a phytoplasma-rich pellet subsequently submitted to DNA extraction (Kirkpatrick *et al.* 1987; Ahrens and Seemüller 1992; Daire *et al.* 1992, 1994; Prince *et al.* 1993; Bertaccini *et al.* 1995; Barba *et al*. 1998); (2) a second group of extraction of total DNA was used after grinding tissues in the presence of a strong detergent (Daire *et al.* 1997 b; Angelini *et al.* 2001).

The present work attempts to compare several procedures described in literature and currently used in different laboratories, for extraction of phytoplasma DNA from naturally infected grapevines and for its further amplification with PCR. These procedures were used in all combinations for two limited series of plants in order to discriminate the method of choice for routine use in surveys and control of GY, especially of FD. Criteria of rapidity of treatment and sample capacity of the procedures were also taken into account. Selected methods were then used after further improvements for numerous field-collected samples of GY-affected grapevines.

Material and Methods

Plant and phytoplasma source: A first series of tissue samples was taken on periwinkle (*Catharanthus roseus* L.) or broadbean (*Vicia faba* L.) infected with phytoplasmas and maintained in the greenhouse. Six phytoplasma strains out of the 16SrV group (EY group) were used: EY1, American elm yellows (isolated by W.A. Sinclair, New York); ULW, European elm yellows (isolated by G. Morvan, France); FD70 and FD92, French strains of FD (isolated by A. Caudwell and J. Larrue, France); HD1, hemp dogbane yellows (isolated by H.M. Griffiths, New York); ALY, Italian alder yellows (isolated by C. Marcone, Italy). In addition, AAY phytoplasma, American aster yellows (Florida), belonging to the group 16SrI-B was used for comparisons. FD70 was maintained on broadbean and all other strains were maintained on periwinkle. Healthy control was from tissues of periwinkle seedlings grown in the greenhouse.

A second series of tissue samples was taken from naturally GY-affected grapevines (*Vitis vinifera* L.). Four grapevines of cv. Merlot collected in south-western France (Lot) and 1 grapevine of cv. Cabernet Sauvignon collected in southern France (Hérault) were affected by FD. In addition, a greenhouse-maintained cutting of cv. Scheurebe was naturally affected by Palatinate grapevine yellows (PGY) (kindly supplied by M. Maixner, Bernkastel-Kues, Germany). PGY phytoplasmas belong to the 16SrV group (Maixner *et al.* 1995 b; Angelini *et al.* 2001, 2003), but they are transmitted to grapevine by the alder leafhopper *Oncopsis alni* Schrank (Maixner and Reinert 1999; Maixner *et al.* 2000). All diseased grapevines showed typical symptoms at the moment of sampling. Healthy cuttings of cv. Chardonnay (kindly supplied by V. Tassart, ENTAV, Le Grau du Roi, France) were used as negative control. AAY-infected periwinkle was also taken as positive external control.

In further assays, numerous grapevine samples were collected in GY-affected vineyards in different regions of Italy (Tab. 6).

Groups of 10-20 leaves were taken on both periwinkle and grapevine. The main veins of each leaf batch were carefully separated with a sterile scalpel blade on a disinfected glass plate. They were then cut into 2-3 mm pieces and dispatched in 1 g aliquots that were stored at -80 °C until processing for DNA extraction.

DNA extraction: Three different methods were used for extraction of DNA. The 8 samples in each of the two series of plants described above (7 periwinkles and 1 broadbean or 6 grapevines plus AAY-periwinkle and healthy periwinkle controls) were submitted simultaneously to a single extraction procedure.

Method A was derived from Daire *et al.* (1997 b) with modifications described by Angelini *et al.* (2001): 1 g of tissue was homogenized at room temperature in disposable plastic sachets with a ball-bearing device (Tecam for Bioreba AG, Basel, Switzerland) in 7 ml of CTAB buffer (3 % CTAB, 1 M Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl) with the extemporaneous addition of 0.2 % 2-mercaptoethanol; 1 ml was transferred to an Eppendorf tube and incubated in a water bath at 65 °C for 20 min. After extraction with 1 ml of chloroform, nucleic acids were precipitated from the aqueous phase with an equal volume of isopropanol, collected by centrifugation, washed with 70 % ethanol, dried, dissolved in 150 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and stored at -20 °C until use.

Method B was a modified procedure of Prince *et al.* (1993). It included a first step of phytoplasma enrichment and several stages of purification to obtain high quality DNA; 1 g of tissue was triturated with liquid nitrogen, then with phosphate grinding buffer (PGB) (100 mM K_2HPO_4, 30 mM KH_2PO_4, 10 % sucrose, 0.15 % bovine albumin fraction V, 2 % polyvinylpyrrolidone-10 (PVP-10), 25 mM ascorbic acid, pH 7.6) in a cold mortar maintained on ice. After centrifugation for 20 min at 20,000 *g*, the resulting pellet was resuspended in 1 ml of extraction buffer (100 mM Tris-HCl, NaCl 250 mM, EDTA 100 mM), with the addition of 0.5 % proteinase K and 1 % Sarkosyl. The suspension was incubated at 55 °C for 1-2 h and centrifuged for 10 min at 7,500 *g*. The pellet was dissolved in 0.6 volume of isopropanol, left at -20 °C for 30 min and then centrifuged for 15 min at 7,500 *g*. A new series of purification was performed with TE buffer (750 µl), with the addition of 0.5 % proteinase K and 0.5 % SDS and incubation at 37 °C for 1 h. Incubation lasted 10 min at 65 °C after addition of 0.7 M NaCl (final concentration) and of 100 µl of 10 % CTAB buffer containing 0.7 M NaCl. Extraction was then carried out with three successive wash-

ings with 1 ml of chloroform: isoamyl alcohol:phenol (24:1:25), 1 ml of chloroform:isoamyl alcohol (24:1) and 0.6 ml of isopropanol, respectively. After precipitation overnight at 4 °C, the pellet was washed with cold 70 % ethanol. The final DNA pellet was dried, resuspended in 150 µl of TE buffer and stored at -20 °C until use.

Method C described by Barba *et al.* (1998) is a modification of the method described by Ahrens and Seemüller (1992). This method is a compromise of methods A and B. It includes an enrichment step of phytoplasma but does not use multiple purification steps of DNA; 1 g of tissue was incubated at 4 °C for 15 min in 2 ml of PGB and then homogenized in a cold mortar using a pestle and sterile quartz sand. After centrifugation at 2,500 *g* for 5 min, the pellet was resuspended with 0.5 ml of 2 % CTAB buffer (2 % CTAB, 100 mM Tris, 400 mM NaCl, 20 mM EDTA, pH 8) and was further incubated in a water bath at 60 °C for 1 h. Extraction was carried out with three successive washings with 0.5 ml of chloro-form:isoamyl alcohol (24:1), 1 ml of cold isopropanol and 1 ml of 70 % ethanol, respectively. The pellet was suspended in 400 µl of TE buffer to which 900 µl of 95 % ethanol and 40 µl of 3 M sodium acetate were added. After a final washing with 70 % ethanol, the DNA pellet was dried, resuspended in 150 µl of TE buffer and stored at -20 °C until use.

The total DNA content of final pellets in each extraction procedure for each plant sample was measured using a TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) after dilution in TEN (10 mM Tris, 1 mM EDTA, 100 mM NaCl) containing 10 % (w/v) bisbenzimide.

Primers and PCR conditions: DNA amplification was performed in 20 µl total reaction volume in a Biometra T3 thermocycler. DNA (undiluted or diluted in a 10-fold series up to 1:1,000,000), obtained for each plant sample with each of the 3 extraction methods, was assayed with PCR amplification according to 4 different nested procedures.

Only DNA from AAY-infected periwinkle, used as positive control in the periwinkle series and in the grapevine series, was used undiluted throughout the experiments. The reaction mixture contained as template 1 µl of DNA (undiluted or diluted as specified above) or of the diluted (1:1000) first amplification product, primers as specified below and PCR reagents as described by Schaff *et al*. (1992), Daire *et al.* (1997 a) and Angelini *et al.* (2001).

Three ribosomal DNA fragments were obtained with nested-PCR amplification using universal primers. The first set of PCR primers was P1 (Deng and Hiruki 1991) and P7 (Smart *et al.* 1996). P1-P7 amplimers were then used as target DNA in nested-PCR amplification with the universal primer pair for phytoplasmas U5/U3 (Lorenz *et al.* 1995) (procedure 1) or with 16r758f/M23Sr (Gibb *et al.* 1995; Padovan *et al.* 1995) (procedure 2). The length of the U5-U3 fragment is about 860 bp and that of the 16r758f-M23Sr fragment is about 1,050 bp.

In procedure 3, P1-P7 amplimers were also used as target DNA in a nested amplification assay using the 16SrV group-specific ribosomal primer pair R16(V)F1/R1 (Lee *et al.* 1994). The resulting product R16(V)F1- R16(V)R1 is about 1,100 bp in length.

Procedure 4 was the nested-PCR amplification of the FD9 non-ribosomal DNA fragment specific of 16SrV-group phytoplasmas. Amplimers obtained with primers FD9f2/r were used as target DNA in the nested amplification with primers FD9f3/r2 (Angelini *et al.* 2001). The length of the fragment FD9f3-FD9r2 is about 1,160 bp.

All the 24 DNA samples obtained from one series of plants (8 plants x 3 extraction procedures) were amplified simultaneously in the same thermocycler with one of the PCR protocols described in Tab. 1. Final products (5 µl) were analyzed by 1% agarose electrophoresis, stained with ethidium bromide (EtBr) and visualized under a UV transilluminator.

Validation under routine conditions: A modification of PCR procedure 4 for the amplification of the FD9 fragment was added in further comparisons. In the latter procedure (procedure 4 b), annealing was allowed for 40 s instead of 30 s and elongation was allowed for 90 s instead of 75 s, in both the first and the nested amplification runs. This procedure was applied in comparison with the 4 amplification procedures described previously, on DNA samples obtained with extraction method A with tissues taken from 3 symptomatic grapevines collected in Italy.

In another group of assays, DNA extraction methods A and B and PCR procedures 3 and 4 b were combined on vein tissues taken from 36 grapevines (5 non-symptomatic and 31 symptomatic) collected in different provinces of Italy. Seven of the 31 symptomatic grapevines had been shown

Table 1

PCR conditions and different primer pairs used in the study

Primer pair	Predenaturation	Denaturation	Annealing	Elongation	Final elongation
P1/P7	92 °C 120 s	92 °C 45 sec	57 °C 45 s	72 °C 105 s	72 °C 5 min
U5/U3	”	92 °C 30 sec	57 °C 30 s	72 °C 50 s	”
16r758f/M23Sr	”	92 °C 60 sec	50 °C 120 s	72 °C 180 s	”
R16(V)F1/R1	”	92 °C 60 sec	50 °C 120 s	72 °C 180 s	”
FD9f2/r	”	92 °C 30 sec	46 °C 30 s	72 °C 75 s	”
FD9f3/r2	”	92 °C 30 sec	47 °C 30 s	72 °C 75 s	”

previously to be infected with a stolbur phytoplasma (BN disease). DNA extraction method C was also performed on vein tissues of 20 grapevines (2 non-symptomatic and 18 symptomatic) out of the group of 36 grapevines collected from the field.

Results

As a rule, in all PCR assays, amplimers had the expected size and no amplification was obtained from any healthy control. A typical electrophoresis pattern is shown in the Figure.

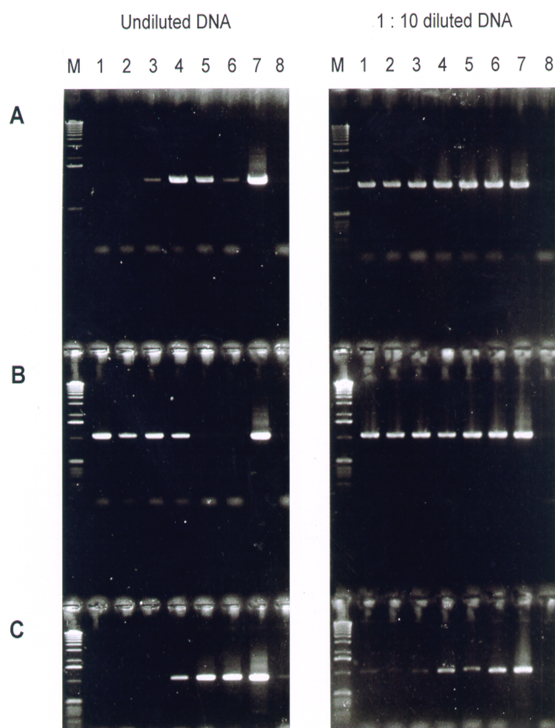

Figure: Agarose electrophoresis patterns of amplimers obtained in nested-PCR procedure 2 (P1/P7 followed by 16Sr758f/M23S primer pairs) with DNA extracted from grapevines and periwinkle control with the three extraction methods A, B and C. Left, undiluted DNA. Right, DNA diluted 1:10. M, 1 kb ladder. Lane 1-5, FD-infected grapevines: 1-4, cv. Merlot; 5, cv. Cabernet Sauvignon. Lane 6, PGY-infected cutting of cv. Scheurebe. Lane 7, undiluted DNA from AAY-infected periwinkle as positive control. Lane 8, healthy cutting of grapevine cv. Chardonnay.

Table 2

Average concentration (ng/ml) and standard deviation of DNA samples obtained from periwinkle and grapevine with the DNA extraction methods A, B and C

Host plant	DNA extraction method		
	A	B	C
Grapevine	123.5±32.5	31.8±34.6	3.7±3.8
Periwinkle	292.4±71.9	107.4±28.3	14.1±8.3
Ratio	42.2 %	29.6 %	26.2 %

D N A y i e l d : Tab. 2 shows the average concentration of DNA samples obtained with each extraction procedure from periwinkle and grapevine. A much higher concentration was obtained with method A, compared with methods B and C which excluded most of nucleus and chloroplast DNA. However, in the three occurrences, the ratio of yield of grapevine DNA to periwinkle DNA was in the same range, with a higher DNA concentration obtained from periwinkle.

C o m p a r i s o n o f t i m e a n d c o s t o f e x t r a c t i o n m e t h o d s : Method A lasted 2 h and allowed to process 80 plant samples in 2 d. Only disposable plastic sachets and a limited series of Eppendorf tubes were used. Method B lasted 36 h and permitted to process 12 plant samples in 2 d. Method C lasted 8 h and permitted to process 24 plant samples in 2 d. The latter two methods required mortar and pestle, several containers and tubes and numerous reagents.

D i l u t i o n t r i a l o f D N A f r o m e x p e r i m e n t a l h o s t p l a n t s : Tab. 3 shows the sensitivity and rate of detection of phytoplasma in periwinkle. All combinations of the three DNA extraction methods A, B and C and of the 4 PCR procedures showed positive results up to dilution 1:1,000 of all DNA samples. When ribosomal primers were used, detection was achieved up to dilution 1:10,000 for all DNA samples and up to dilution 1:100,000 for all DNA samples obtained with extraction methods A and B. With the higher dilution of 1:1,000,000 of DNA samples, the rate of detection with the nested-PCR assay using 16r758f/M23Sr primers (procedure 2) was higher than with the other ribosomal primers and combination with DNA extraction method A was the most efficient.

D i l u t i o n t r i a l o f D N A f r o m g r a p e v i n e s : Tab. 4 shows the sensitivity and rate of detection of phytoplasma in grapevine. Extraction methods A and B always yielded better results than method C, regardless of the PCR procedure used in combination. By contrast to experimental host plants (Tab. 3), a better rate of detection was obtained in all cases when DNA samples were diluted 1:10 prior to amplification (Tab. 4, Figure). When ribosomal primers were used, detection was achieved from dilution 1:10 up to dilution 1:1,000 with all DNA samples obtained with extraction method A and altogether the best rate of detection was achieved with 16r758f/M23S primers (Figure). Non-ribosomal primers were less sensitive with high dilutions of DNA. However, the rate of detection was 6/6 with dilutions 1:10 and 1:100 of DNA obtained with extraction method A.

I m p r o v e m e n t o f a m p l i f i c a t i o n o f t h e F D 9 r e g i o n o f 1 6 S r V - g r o u p p h y t o p l a s m a s : Tab. 5 shows comparison of the sensitivity and rate of detection obtained with the 4 PCR procedures formerly devised and with procedure 4 b in addition, on DNA samples obtained with extraction method A on three grapevine samples collected in a FD-affected vineyard in Treviso (Italy). The rate and sensitivity of detection were similar with procedure 4 b to those achieved with the three procedures using ribosomal primers.

V a l i d a t i o n o n f i e l d s a m p l e s : Tab. 6 shows a comparison of results obtained with the 6 combinations of DNA extraction methods A, B or C and of PCR pro-

Table 3

Number of positive responses obtained among 6 phytoplasma-infected experimental host plants (5 periwinkles[a] and 1 broadbean) with undiluted or diluted DNA preparations, according to the DNA extraction method (A, B or C) and the DNA amplification procedure. Healthy controls showed no amplification product

Primer pair	P1/P7 - U5f/U3r (procedure 1)			P1/P7-16r758f/M23Sr (procedure 2)			P1/P7 - R16(V)F1/R1 (procedure 3)			FD9f2/r - FD9f3/r2 (procedure 4)		
DNA dilution	DNA extraction method											
	A	B	C	A	B	C	A	B	C	A	B	C
1:1 to 1:1,000	6	6	6	6	6	6	6	6	6	6	6	6
1:10,000	6	6	6	6	6	6	6	6	6	5	2	1
1:100,000	6	5	4	6	6	5	6	6	2	0	0	0
1:1,000,000	1	0	1	5	1	1	2	0	0	NT	NT	NT

[a] Positive control DNA from AAY infected periwinkle was used undiluted through all experiments and is not taken into account in the table. All other phytoplasma belonged to group 16SrV and could be detected with all PCR procedures.
NT: not tested.

Table 4

Number of positive responses obtained among 6 FD- or PGY-diseased grapevines[a] with undiluted or diluted DNA preparations, according to the DNA extraction method (A, B or C) and the DNA amplification procedure. Healthy controls showed no amplification product

Primer pair	P1/P7 - U5f/U3r (procedure 1)			P1/P7-16r758f/M23Sr (procedure 2)			P1/P7 - R16(V)F1/R1 (procedure 3)			FD9f2/r - FD9f3/r2 (procedure 4)		
DNA dilution	DNA extraction method											
	A	B	C	A	B	C	A	B	C	A	B	C
1:1	3	4	2	5	4	3	3	4	3	2	4	3
1:10	6	6	3	6	6	6	6	6	6	6	6	2
1:100	6	4	1	6	6	3	6	4	1	6	4	2
1:1,000	6	4	1	6	6	1	6	6	1	3	2	0
1:10,000	4	2	2	6	2	2	4	2	1	NT	NT	NT
1:100,000	2	0	0	2	1	0	3	0	0	NT	NT	NT

[a] Positive signals obtained in all gels with control undiluted DNA from AAY periwinkle are not taken into account in the table.
NT: not tested.

Table 5

Number of positive responses obtained among 3 FD-diseased grapevines from the field with undiluted or diluted DNA preparations obtained with DNA extraction method A and 5 DNA amplification procedures. Healthy controls showed no amplification product

	Nested-PCR amplification procedure				
DNA dilution	P1/P7 - U5/U3 (procedure 1)	P1/P7 - 16r758f/M23Sr (procedure 2)	P1/P7 - R16(V)F1/R1 (procedure 3)	FD9f2/r - FD9f3/r2 (procedure 4)	FD9f2/r - FD9f3/r2 (procedure 4 b)[a]
1:1	3	3	3	3	3
1:10	3	3	3	3	3
1: 100	3	3	3	3	3
1:1,000	3	3	3	0	3
1:10,000	3	3	3	0	3
1:100,000	0	0	0	0	1
1:1,000,000	0	0	0	0	0

[a] In procedure 4 b, annealing and elongation periods were allowed for a longer time as compared to conditions in procedure 4.

Table 6

Comparison of detection of 16SrV-group phytoplasma in GY-infected grapevines from various regions in Italy, using DNA extraction methods A, B and C and nested-PCR procedures 3 and 4 b

Cultivar	Geographic location	Symptom observation	P1/P7 - 16S(V)F1/R1 (Procedure 3)			FD9f2/r - FD9f3/r2 (Procedure 4 b)		
			DNA extraction method (A, B or C)					
			A	B	C	A	B	C
Bianchetta	Belluno - Veneto	A[b]	-	-	-	-	-	-
Pinot nero	Treviso - Veneto	A	-	-	nt	-	-	nt
Chardonnay	Treviso - Veneto	A	-	-	-	-	-	-
Barbera	Alessandria-Piemonte	A	-	-	nt	-	-	nt
Barbera	Asti - Piemonte	A	-	-	nt	-	-	nt
Chardonnay[a]	Aosta - Val D'Aosta	S	-	-	nt	-	-	nt
Chardonnay[a]	Aosta - Val D'Aosta	S	-	-	nt	-	-	nt
Vien de nus[a]	Aosta - Val D'Aosta	S	-	-	nt	-	-	nt
Chardonnay[a]	Belluno - Veneto	S	-	-	nt	-	-	nt
Chardonnay[a]	Treviso - Veneto	S	-	-	nt	-	-	nt
Chardonnay[a]	Treviso - Veneto	VS	-	-	nt	-	-	nt
Chardonnay[a]	Treviso - Veneto	VS	-	-	nt	-	-	nt
Auxerrois	Treviso – Veneto	VS	+	+	+	+	+	+
Cabernet Sauvignon	Treviso – Veneto	VS	+	+	+	+	+	+
Cabernet Sauvignon	Treviso – Veneto	S	+	+	+	+	+	+
Chardonnay	Treviso – Veneto	S	+	+	+	+	+	-
Chardonnay	Treviso – Veneto	VS	+	+	+	+	+	+
Chardonnay	Treviso – Veneto	VS	+	+	+	+	+	+
Chardonnay	Treviso – Veneto	S	+	+	+	+	+	+
Chardonnay	Treviso – Veneto	VS	+	+	+	+	+	+
Gamay teinturier	Treviso – Veneto	VS	+	+	+	+	+	+
Guarnaccia	Treviso – Veneto	VS	-	+	+	-	+	+
Manzoni bianco	Treviso – Veneto	VS	+	-	+	+	+	+
Malvasia	Treviso – Veneto	VS	+	+	+	+	+	+
Nerello	Treviso – Veneto	S	+	+	+	+	+	+
Prosecco	Treviso – Veneto	VS	+	+	+	+	+	+
Prosecco	Treviso – Veneto	S	+	+	+	+	+	+
Prosecco	Treviso – Veneto	S	+	-	+	+	+	+
Prosecco	Treviso – Veneto	VS	+	-	+	+	-	+
Prosecco	Treviso – Veneto	VS	+	+	+	+	+	+
Cabernet Sauvignon	Treviso - Veneto	VS	+	+	nt	+	+	nt
Perera	Treviso - Veneto	VS	+	+	nt	+	+	nt
Prosecco	Treviso - Veneto	VS	+	+	nt	+	+	nt
Prosecco	Treviso - Veneto	S	+	+	nt	+	+	nt
Prosecco	Treviso - Veneto	S	+	+	nt	+	+	nt
Trebbiano	Treviso - Veneto	S	+	+	nt	+	+	nt
Positive for 16SrV-group phytoplasma / FD-infected (tested)			23/24	21/24	18/18	23/24	23/24	17/18

[a] grapevine that tested positive for a stolbur phytoplasma in a separate experiment (data not shown).
[b] A= asymptomatic, S= symptomatic, VS= very symptomatic. nt: not tested.

cedures 3 and 4 b, specific for 16SrV-group phytoplasmas, on grapevines collected in different regions of Italy. According to the results described above, DNA was diluted 1:10 for use in the first amplification run and resulting amplimers were diluted 1:1,000 for use in the nested-amplification run. No signal was obtained from 5 non-symptomatic grapevines and from 7 symptomatic grapevines that had been tested positive for a stolbur phytoplasma in a sepa-

rate assay (data not shown). Out of 24 FD-infected grapevines processed with methods A and B, 23 and 21, respectively, scored positive with DNA extraction methods A or B and ribosomal primers (PCR procedure 3), and 23 and 23 scored positive, respectively, with non-ribosomal primers (PCR procedure 4 b). Out of 18 FD-infected grapevines processed with method C, 18 scored positive with procedure 3 and 17 were tested positive with procedure 4 b.

Discussion

The present work is an exhaustive comparison between combinations of methods for extraction of DNA and procedures for DNA amplification with PCR, applied to detect phytoplasmas in experimental host plants and naturally infected grapevines. The aim was to devise an efficient, sensitive and easy diagnosis method for GY that could be used worldwide as reference in research and extension services for the identification and monitoring of phytoplasmas and especially of Flavescence dorée.

The results confirmed that periwinkle contained a much higher titre of phytoplasma than grapevine and that detection of phytoplasma is more difficult in grapevine than in periwinkle. With periwinkle, DNA extraction method A, which did not use a pre-enrichment step of phytoplasma, was shown to be as efficient as methods B and C, which used one or two cycles of DNA purification after the preparation of a phytoplasma-rich fraction of cell organelles. Palmano (2001) used competitive PCR to compare phytoplasma DNA yield of the same three DNA extraction methods on periwinkles infected with Apple proliferation (AP) and Clover phyllody (CP) phytoplasmas and showed minor variation between methods. Our results on periwinkle are roughly in agreement with the conclusion of the author, who also stated that PCR detection procedures are more critical than extraction methods, as far as specificity is concerned. On the opposite, with grapevine, methods A and B were more efficient in our experiments than method C but all three methods yielded DNA preparations that contained inhibitors of the PCR reaction. This was shown by the better rate of detection with any PCR procedure used, when target DNA was diluted 1:10 than when used undiluted. The latter result is of great relevance, since method A could be selected in the end. In fact, method A is quicker and less expensive than the two other methods and hence it is better adapted to routine diagnosis, without a compromise to less efficiency. Pasquini *et al*. (2001) came to a similar conclusion in a ring test conducted by Italian laboratories using a few Italian grapevine DNA samples extracted also with the same three methods. Their slightly different results on the respective sensitivity of the three methods could be due to the low number of grapevines tested. An apparent discrepancy concerned the use of extraction method C on grapevine samples. As a matter of fact, the rate of detection with PCR procedure 3 on DNA extracts diluted 1:10 was as high in the dilution assay as in the validation trial on random samples from the field. When non-ribosomal primers were used with PCR procedure 4 b, the rate of detection in the validation assay with samples from the field submitted to DNA extraction method C was similar to that obtained with methods A and B, in contrast with results obtained in the dilution trial of grapevine DNA when the original PCR procedure 4 was used.

The comparison between primer pairs combined in nested-PCR assays showed that the highest sensitivity and efficiency of detection, whether on highly diluted periwinkle DNA, or on grapevine DNA, was obtained with primer combinations of procedure 2, using P1/P7 followed by 16Sr758f/M23Sr primer pairs. As a consequence, procedure 2 should be used, or alternatively procedure 1 (P1/P7 followed by U5/U3 primer pairs), if a reliable wide range diagnosis of any GY-associated phytoplasma is required.

Amplification of the non-ribosomal fragment FD9 with FD9f2/r followed by FD9f3/r2 primer pairs (procedure 4) was less efficient and less sensitive than amplification of any of the three ribosomal fragments, especially with grapevine DNA. Nevertheless, the original procedure 4 permitted the detection of phytoplasma in 6 out of 6 diseased grapevines in the first series when target DNA was diluted 1:10 before use. In addition, sensitivity and efficiency were improved in the modified procedure 4 b with longer annealing and elongation periods. Moreover, the latter procedure was as efficient as procedure 3 (P1/P7 followed by R16(V)F1/R1 primer pairs) for detection in grapevine samples from the field. PCR procedures 3 and 4 b allowed the specific detection of group 16SrV phytoplasmas. Hence, when FD is specifically monitored, the non-ribosomal primers for FD9 region might be used with great confidence instead of ribosomal primers.

This latter conclusion is particularly important both for epidemiological studies and for sanitation surveys. Actually, other studies have demonstrated the high potential and relevance of FD9 DNA region of 16SrV-group phytoplasmas for analysis of diversity within this group and especially in molecular epidemiology studies of FD *sensu stricto* and FD-related phytoplasmas (Daire *et al*. 1997 b; Angelini *et al*. 2001, 2003; Martini *et al*. 2002). In addition, a recent work has used the FD9 fragment to devise a nested-multiplex PCR procedure for the simultaneous monitoring of FD- and BN-(stolbur) related phytoplasmas (Clair *et al*. 2003).

As a whole, the present work is an important contribution to the harmonization of efficient diagnosis of grapevine phytoplasmas worldwide and to the development of methods that could be used in certification scheme of vine planting material.

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References

Ahrens, U.; Seemüller, E.; 1992: Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology **82**, 828-832.

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

Angelini, E.; Negrisolo, E.; Clair, D.; Borgo, M.; Boudon-Padieu, E.; 2003: Phylogenetic relationships among Flavescence dorée isolates and related phytoplasmas determined by Heteroduplex Mobility Assay and sequences of ribosomal and non-ribosomal DNA. Plant Pathology **52** (in press).

Baggiolini, M.; Canevascini, V.; Caccia, R.; Tencalla, Y.; Sobrio, G.; 1968: Présence dans le vignoble du Tessin d'une cicadelle néarctique nouvelle pour la Suisse, *Scaphoideus littoralis* Ball. (Homoptera: Jassidae), vecteur possible de la Flavescence dorée. Mitt. Schweiz. Entomol. Ges. **60**, 270-275.

Barba, M.; Boccardo, G.; Carraro, L.; Del Serrone, P.; Ermacora, P.; Firrao, G.; Giunchedi, L.; Loi, N.; Malfitano, M.; Marcone C.; Marzachi, C.; Musetti, R.; Osler, R.; Palmano, S.; Poggi Pollini, C.; Ragozzino, A.; 1998: Confronto di differenti tecniche di diagnosi applicate al rilevamento di fitoplasmi in pomacea. Notiziario sulla Protezione delle Piante **9**, 263-278.

Batlle, A.; Martínez, M. A.; Laviña, A.; 2000: Occurrence, distribution and epidemiology of Grapevine yellows in Spain. Eur. J. Plant Pathol. **106**, 811-816.

Berges, R.; Rott, M.; Seemüller, E.; 2000: Range of phytoplasma concentration in various plant hosts as determined by competitive polymerase chain reaction. Phytopathology **90**, 1145-1152.

Bertaccini, A.; Vibio, M.; Stefani, E.; 1995: Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). Phytopathol. mediterr. **34**, 137-141.

Boudon-Padieu, E.; 2002: Flavescence dorée of the grapevine: Knowledge and new developments in epidemiology, etiology and diagnosis. ATTI Giornate Fitopatologiche 2002, 15-34.

Caudwell, A.; Kuszala, C.; 1992: Mise au point d'un test ELISA sur les tissus de vignes atteintes de Flavescence dorée. Res. Microbiol. **143**, 791-806.

Clair, D.; Larrue, J.; Aubert, G.; Gillet, J.; Cloquemin, G.; Boudon-Padieu, E.; 2003: 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. Vitis **42**, 151-157.

Constable, F. E.; Whiting, J. R.; Gibb, K. S.; Symons, R. H; 2002: A new grapevine yellows phytoplasma from the Buckland Valley of Victoria, Australia. Vitis **41**, 147-153.

Credi, R.; 1994: Occurrence of anomalous Mycoplasma-like organisms in grapevine yellows-diseased phloem. J. Phytopathol. **142**, 310-316.

Daire, X. ; 1994: Détection et différentiation de mycoplasma-like organism (MLO) associés aux maladies de la vigne de type jaunisse. Thèse Doctorat Univ. Bourgogne.

Daire, X.; Boudon-Padieu, E.; Bervillé, A.; Schneider, B.; Caudwell, A.; 1992: Cloned DNA probes for detection of grapevine Flavescence dorée mycoplasma-like organism (MLO). Ann. Appl. Biol. **121**, 95-103.

Daire, X.; Clair, D.; Larrue, J.; Boudon-Padieu, E.; 1997 a: Survey for grapevine yellows in diverse European countries and Israel. Vitis **36**, 53-54.

Daire, X.; Clair, D.; Larrue, J.; Boudon-Padieu, E.; Alma, A.; Arzone, A.; Carraro, L.; Osler, R.; Refatti, E.; Granata, G.; Credi, R.; Tanne, E.; Pearson, R.; Caudwell, A.; 1993: Occurrence of diverse MLOs in tissues of grapevine affected by grapevine yellows in different countries. Vitis **32**, 247-248.

Daire, X.; Clair, D.; Reinert, W.; Boudon-Padieu, E.; 1997 b: Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. Eur. J. Plant Pathol. **103**, 507-514.

Davis, R. E.; Dally, E. L.; Tanne, E.; Rumbos, I. C.; 1997: Phytoplasmas associated with grapevine yellows in Israel and Greece belong to the stolbur phytoplasma subgroup, 16S rXII-A. J. Plant Pathol. **79**, 181-187.

Deng, S.; Hiruki, C.; 1991: Amplification of 16S rRNA genes from culturable and non-culturable Mollicutes. J. Microbiol. Methods **14**, 53-61.

Gibb, K. S.; Constable, F. E.; Moran, J. R.; Padovan, A. C.; 1999: Phytoplasmas in Australian grapevines - detection, differentiation and associated diseases. Vitis **38**, 107-114.

Gibb, K.; Padovan, A.C.; Mogen, B.D.; 1995: Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plants species growing in northern Australia. Phytopathology **85**, 169-174.

Kartte, S.; Seemüller, E.; 1991: Susceptibility of grafts of *Malus* taxa and hybrids to apple proliferation disease. J. Phytopathol. **131**, 137-148.

Kirckpatrick B. C.; Stenger, D. C.; Morris, T. J.; Purcell, A. H.; 1987: Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science **238**, 197-200.

Lee, I. M.; Gundersen, D. E.; Hammond, R. W.; Davis, R. E.; 1994: Use of mycoplasmalike organisms (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology **84**, 559-566.

Lee, I. M.; Gundersen-Rindal, D. E.; Davis, R. E.; Bartoszyk, I. M.; 1998: Revised classification scheme of Phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. **48**, 1153-1169.

Lee, I. M.; Hammond, R. W.; Davis, R. E.; Gundersen-Rindal, D. E.; 1993: Universal amplification and analysis of pathogen 16S rDNA for classification and identification of Mycoplasmalike organisms. Phytopathology **83**, 834-842.

Lorenz K. H., Schneider, B.; Ahrens, A.; Seemüller, E.; 1995: Detection of apple proliferation and pear decline mycoplasma-like organisms by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology **85**, 771-776.

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

Maixner, M.; Daire, X.; Boudon-Padieu, E.; Laviña, A.; Battle, A.; Reinert, W.; 1997: Phytoplasmas. In: Les colloques. INRA Editions, N° 86. Sanitary Selection of the Grapevine. Protocols for Detection of Viruses and Virus-like Diseases, 183-195.

Maixner, M.; Reinert, W.; 1999: *Oncopsis alni* (Schrank) (Auchenorrhyncha: Cicadellidae) as a vector of the alder yellows phytoplasma of *Alnus glutinosa* (L.) Gaertn. Eur. J. Plant Pathol. **105**, 87-94.

Maixner, M.; Reinert, W.; Darimont, H.; 2000: Transmission of grapevine yellows by *Oncopsis alni* (Schrank) (Auchenorrhyncha: Macropsinae). Vitis **39**, 83-84.

Maixner, M.; Rüdel, M.; Daire, X.; Boudon-Padieu, E.; 1995 b: Diversity of grapevine yellows in Germany. Vitis **34**, 235-236.

Marcone, C.; Ragozzino, A.; Credi, R.; Seemüller, E.; 1996: Detection and characterization of phytoplasmas infecting grapevine in southern Italy and their genetic relatedness to other grapevine yellows phytoplasmas. Phytopathol. mediterr. **35**, 207-213.

Martini, M.; Botti, S.; Marcone, C.; Marzachì, C.; Casati, P.; Bianco, P. A.; Benedetti, R.; Bertaccini, A.; 2002: Genetic variability among Flavescence dorée phytoplasmas from different origins in Italy and France. Mol. Cell. Probes **16**, 197-203.

Meignoz, R.; Boudon-Padieu, E.; Larrue, J.; Caudwell, A.; 1992: Flavescence dorée de la vigne. Présence de MLO et effets cytopathogènes associés, dans le liber de la vigne. J. Phytopathol. **134**, 1-9.

Padovan, A. C.; Gibb, K. S.; Bertaccini, A.; Vibio, M.; Bonfiglioli, R. E.; Magarey, P. A.; Sears, B. B.; 1995: Molecular detection of the Australian grapevine yellows phytoplasmas and comparison with grapevine yellows phytoplasmas from Italy. Aust. J. Grape Wine Res. **1**, 25-31.

Padovan, A.; Gibb, K.; Daire, X.; Boudon-Padieu, E.; 1996: A comparison of the phytoplasma associated with Australian grapevine yellows to other phytoplasmas in grapevine. Vitis **35**, 189-194.

Palmano, S.; 2001: A comparison of different phytoplasma DNA extraction methods using competitive PCR. Phytopathol. mediterr. **40**, 99-107.

Pasquini, G.; Angelini, E.; Benedetti, R.; Bertaccini, A.; Bertotto, L.; Bianco, P. A.; Faggioli, F; Martini, M.; Marzachi, C.; Barba, M.; 2001: Armonizzazione della diagnosi della Flavescenza dorato della vite (FD): Risultati di una prova comparativa, 921-940. Atti Progetto POM A32 In: Norme fitosanitarie e commercializzazione delle produzioni vivaistiche, Locorotondo (BA) 4-7 dicembre 2001.

Prince, J. P.; Davis, R. E.; Wolf, T. K.; Lee, I. M.; Mogen, B. D.; Dally, E. L.; Bertaccini, A.; Credi, R.; Barba, M.; 1993: Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-

disease, and elm yellows MLOs. Phytopathology **83**, 1130-1137.

SCHAFF, D. A.; LEE, I. M.; DAVIS, R. E.; 1992: Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. Biochem. Biophys. Res. Comm. **186**, 1503-1509.

SCHNEIDER, B. ; AHRENS, U. ; KIRKPATRICK, B. C. ; SEEMÜLLER, E.; 1993: Classification of plant pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. J. Gen. Microbiol. **139**, 519-527.

SCHVESTER, D.; CARLE, P.; MOUTOUS, G.; 1963: Transmission de la flavescence dorée de la vigne par *Scaphoideus littoralis* Ball. Ann. Epiphyt. **14**, 175-198.

SEARS, B.; LIM, P. O.; HOLLAND, N.; KIRKPATRICK, B. C.; KLOMPARENS, K. L.; 1989: Isolation and characterization of DNA from a mycoplasma-like organism. Mol. Plant Microbe Interact. **2**, 175-180.

SEDDAS, A.; MEIGNOZ, R.; DAIRE, X.; BOUDON-PADIEU, E.; 1996: Generation and characterization of monoclonal antibodies to Flavescence dorée phytoplasma: Serological relationships and differences in electroblot immunoassay profiles of Flavescence dorée and Elm yellows phytoplasmas. Eur. J. Plant Pathol. **102**, 757-764.

SEEMÜLLER, E.; MARCONE, C.; LAUER, U.; RAGOZZINO, A.; GÖSCHL, M.; 1998: Current status of molecular classification of the phytoplasmas. J. Plant Pathol. **80**, 3-26.

SELJAK, G.; 2002: Non-European Auchenorryncha (Hemiptera) and their geographic distribution in Slovenia. Acta Entomol. Slovenica **10**, 97-101.

SKORIC, D.; SARIC, A.; VIBIO, M.; MURARI, E.; KRAJACIC, M.; BERTACCINI, A.; 1998: Molecular identification and seasonal monitoring of phytoplasmas infecting Croatian grapevines. Vitis **37**, 171-175.

SMART, C. D; SCHNEIDER, B.; BLOMQUIST, C. L.; GUERRA, L. J.; HARRISON, N. A.; AHRENS, U.; LORENZ, K. H.; SEEMÜLLER, E.; KIRCKPATRICK, B. C.; 1996: Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Appl. Environ. Microbiol. **62**, 1988-1993.

TANNE, E.; ORENSTEIN, S.; 1997: Identification and typing of grapevine phytoplasma amplified by graft transmission to periwinkle. Vitis **36**, 35-38.

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