Vitis **48** (2), 87–95 (2009)

Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control

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Summary

Flavescence dorée (FD) and Bois noir (BN) are the two main yellows of grapevine in Europe and are caused by phytoplasmas of the 16SrV and 16SrXII-A groups respectively. A new triplex real-time PCR assay was developed in order to detect simultaneously the FD and BN phytoplasmas as well as grapevine chloroplastic DNA with TaqMan minor groove binder probes. Each set of designed primers and probes specifically detected the *map* gene of the FD and BN phytoplasmas, respectively and did not detect phytoplasmas from other phylogenetic groups. PCR efficiencies varied from 90 to 110 %. The PCR assay showed good intra-test and inter-test reproducibility. Triplex real-time PCR was compared to the conventional biplex nested-PCR method. The sensitivity of the real-time PCR, tested on several infected periwinkle and grapevine samples, was up to 5 and 100 times higher for the BN-P and the FD-P targets, respectively. Out of 109 grapevine samples analysed 10, which were negative with the nested PCR, turned to be FD-P positive with the real-time PCR. A decision scheme was set up according to the Ct values of the FD-P, BN-P and grapevine targets in order to assess the routine detection results.

K e y w o r d s : Flavescence dorée, Bois noir, TaqMan MGB probe, detection, *Mollicutes.*

Introduction

Grapevine yellows (GY) are disseminated in major vine growing areas of the Mediterranean countries, Eastern Europe, North and South America and Australia (Boudon-Padieu *et al*. 2003). These diseases are caused by phytoplasmas that are non-cultivated plant pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less organisms phylogenetically related to gram-positive bacteria (Weisburg *et al*. 1989).

The most damaging GY in Europe is Flavescence dorée (Caudwell 1957) which is epidemically transmitted by *Scaphoideus titanus* Ball (Schvester *et al*. 1963), a vine feeding leafhopper of American origin (Caudwell 1983). FD phytoplasma (FD-P) is a quarantine organism in the European Community and despite mandatory regulations for control and eradication, FD is still spreading in the contaminated areas of Southern France and Northern Italy. Recently, new outbreaks have been detected in Serbia (Duduk *et al*. 2004), in Northern France (Moyse 2005) and in Switzerland (Schaerer *et al*. 2007). FD-P belongs to the 16SrV taxonomic group, sub-groups C and D (Martini *et al*. 1999, Lee *et al*. 2000). Phytoplasma members of this group share 16S rDNA sequence similarity higher than 97.9 % (Davis and Dally 2001, Lee *et al*. 2004). In Europe, they are mainly "*Candidatus* Phytoplasma ulmi", responsible for yellows of elm species (Lee *et al.* 2004) (16SrV-A), Rubus stunt (RS, 16SrV-E), FD-P (16SrV-C and D), Spartium witches broom (SpaWB, 16SrV-C) and Alder yellows (AldY, 16SrV-C). The latter phytoplasma is transmitted by the leafhopper *Oncopsis alni* which can occasionally inoculate it to grapevine leading to Palatinate Grapevine Yellows (PGY) disease (Maixner and Reinert 1999, Maixner *et al*. 2000). Recent taxonomic and phylogenetic studies based on non ribosomal markers have described three different clusters of FD-P strains (named FD1, FD2 and FD3) presenting differences in genetic variability, distribution and incidence in France and Italy (Arnaud *et al*. 2007). These studies have also demonstrated that PGY and AldY phytoplasma isolates do not constitute a group independent from FD-P isolates; all having a common phylogenetic origin (Angelini *et al*. 2001, Angelini *et al*. 2003, Arnaud *et al.* 2007).

Another important GY in Europe is the Bois noir (BN) disease associated with the endemic Stolbur phytoplasma and mainly transmitted by a polyphagous planthopper, *Hyalesthes obsoletus* (Maixner *et al*. 1995, Sforza *et al*. 1998). Stolbur phytoplasma belongs to the 16SrXII-A group. Members of this sub-group have been mainly reported from Europe and the Mediterranean basin, affecting an important range of crops including tomato, potato, pepper, tobacco, lavender and strawberry. As *H. obsoletus* feeds erratically on grapevine, BN is not epidemic in vineyards but had an increased incidence in the last decade in many countries of the Euro-Mediterranean area. It has recently been detected in grapevines in Canada (Rott *et al*. 2007) and Chile (Fiore *et al.* 2007). The 16SrXII-B sub-group also includes grapevine yellows phytoplasmas described as *Ca* P. australiense and detected in Australia (Padovan *et al*. 1996, Gibb *et al*. 1999, Davis *et al.* 1997). Other grapevine phytoplasmas inducing yellows are classified in other phylogenetic groups (16SrI, II and III) and are

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sporadically detected in vineyards worldwide (Boudon-Padieu 2003, 2005). Due to the differences in aetiology and epidemiology, prevention and control of FD and BN diseases are based on complementary strategies aimed at eliminating infected grapes and control FD-P insect vector. But, as FD and BN induce identical symptoms and are commonly present in the same vineyards, reliable molecular methods to identify and differentiate FD-P and BN-P in plants are required (Clair *et al*. 2003). Sensitive nested-PCR techniques have been developed in order to detect the low concentration of phytoplasmas in the symptomatic vine. Whereas nested-PCR targeting ribosomal DNA of FD-P and BN-P are performed separately and often need post-PCR RFLP analyses to discriminate both phytoplasmas (Maixner *et al.* 1995, Batlle *et al*. 1997, Daire *et al*. 1997, Martini *et al*. 1999, Marzachi *et al*. 2000, 2001), those which target non ribosomal DNA can directly be combined in a multiplex reaction (Daire *et al*. 1997, Clair *et al*. 2003). But nested-PCR analyses are time consuming and are exposed to the risks of contaminations. The real time PCR, with direct and sensitive detection of the amplification in a closed-tube system, has the advantages of being quicker and with a reduced risk of false positive (Heid *et al*. 1996, Walker 2002). Furthermore, TaqMan® techniques provide the possibility of multiplexing by labelling the probes with different compatible fluorescent dyes (Molenkamp *et al*. 2007). Real-time PCR techniques for separate detection of FD-P and BN-P have been developed, but their sensitivity was slightly lower than that of the nested-PCR and they did not include an endogenous control (Bianco *et al*. 2004, Galetto *et al*. 2005). Recently, different TaqMan® assays were developed with a grapevine control which proved to be as sensitive as the nested-PCR (Angelini *et al*. 2007) or more sensitive (Hren *et al*. 2007). But the possibility of multiplexing in a one-step assay the detection of both phytoplasmas has not yet been investigated. Here we present a new sensitive TaqMan method which permits the amplification and the differentiation of three DNA targets in one test: the *map* genes of both FD-P and BN-P related phytoplasmas and a grapevine chloroplastic DNA fragment. The sensitivity and reproducibility of this method is compared with the biplex nested-PCR method established as the official GY diagnosis in France (Clair *et al*. 2003).

Material and Methods

Phytoplasma reference strains: Phytoplasma reference strains used in this work are listed in Tab. 1. Most of them had previously been transmitted to *Catharanthus roseus* 'Cooler' and were maintained in this host by grafting. Some phytoplasma isolates from the16SrXII-A and the16SrV groups were directly collected from their natural host plant: *Vitis vinifera, Ulmus minor, Spartium junceum*. Both FD-P isolates, CAM-05 and PEY-05, were transmitted to broad bean (*Vicia faba* cv. Agua dulce) by infectious *S. titanus* collected in infected vineyards in 2005. CAM-05 and PEY-05 belong to types FD1 and FD2, respectively (Arnaud *et al*. 2007).

Grapevine samples: During 2006 national surveys, grapevine samples were collected in vineyards showing symptoms of grapevine yellows in different regions of France. Leaves with petioles were taken from a maximum of 5 symptomatic vines per affected vineyard and stored at 4 °C (one week maximum) until processing.

DNA extraction: The most symptomatic leaves were selected for each grapevine sample; petioles were detached with a razor blade and pooled. One gram of mixed petioles was used for DNA extraction and the rest was stored at -20 °C for further use. One gram of midribs from periwinkle or broad bean was also used for DNA extraction. Total DNA was extracted using cethyl-trimethyl-ammonium bromide (CTAB) as described in Boudon-Padieu *et al*. 2003. The final total DNA pellet from 1 g of plant material was resuspended in 500 µl of TE buffer (10mM Tris, 1 mM EDTA, pH 7.6). DNA from healthy plants was also collected with each extraction series as negative control. The concentration of DNA in the extracts ranged from 50 $ng \cdot \mu l^{-1}$ to 150 $ng \cdot \mu l^{-1}$.

Oligonucleotides design: Primers used for the simultaneous detection of the FD and BN related phytoplasmas by nested PCR are already described in (Clair *et al.* 2003). Primers and TaqMan® MGB™ probes (Applied Biosystems) used for triplex real-time PCR are detailed in Tab. 2. They were designed using the "Primer Express" software version 5.0 as follows. Primers and probe sequences of the 16SrV group phytoplasmas were determined on the basis of the *map* gene sequence alignment of 19 reference strains comprising HD, EY, AldY, PGY, RS, SpaW, and FD phytoplasmas (Fig. 1). Strains and sequences were as reported in Arnaud *et al*. 2007, accession numbers are AM384884 to AM384902. Amplified fragment was 71 bp long, from position 175 to 245 after the start codon of the *map* gene. Probe was 5' labelled with FAM™ reporter dye. Primers and probe sequences of the 16SrXII-A group phytoplasmas were determined on the basis of the alignment of their *map* gene sequences (Fig. 1). Sequences of the 13 reference strains were obtained in a previous work (Cimerman *et al*. unpubl.); accession numbers are AM990976 to AM990988. The *map* gene sequence of *Ca.* P. australiense (16SrXII-B, AM422018, (Tran-Nguyen *et al.*, 2008)) was also added in the alignment. Amplified fragment was 72 bp long, from position 32 to 103 after the start codon of the *map* gene. Probe was 5' labelled with VIC™ reporter dye. Absence of cross matching between primers and probes sequences of one group and the *map* gene sequences of the other group was verified. Primers and probes sequences for the amplification of an endogenous control (EC) were determined on the sequences alignment of the chloroplast *trn*L-F spacer of different *Vitis* species already described by (Rossetto *et al*. 2001, 2002, Soejima and Wen 2006). Accession numbers are AF300295 and AB235073 to AB235084. Amplified fragment was 73 bp long. Probe was 5' labelled with NED™ reporter dye. All probes were 3' labelled with a non-fluorescent quencher.

PCR assays: Two different PCR methods were tested and compared for FD and BN-related phytoplasma detection: the biplex nested-PCR reaction is described in

Table 1

Specificity of the multiplex real-time PCR assay

Phylogenetic group	Phytoplasma strain / Host plant	Origin	PCR multiplex assay		
			Ct FD[1]	Ct BN[2]	Ct EC[3]
	Healthy/*C. roseus*		-	-	-
	Healthy/*V. faba*		-	-	-
	Healthy/*V. vinifera* cv Pinot noir		-	-	22.4
	Healthy/*V. vinifera* cv Gewurztraminer		-	-	20.7
	Healthy/*V. vinifera* cv Chardonnay		-	-	21.5
	Healthy/*V. vinifera* cv Riesling		-	-	22.4
	Healthy/*V. vinifera* cv Cabernet Franc		-	-	20.9
	Healthy/*V. vinifera* cv Cabernet Sauvignon		-	-	19.6
16SrI	Aster yellow (AYWhitcomb)/*C. roseus*	USA	-	-	-
	Clover phyllody (KVF)/*C. roseus*	France	-	-	-
16SrII	Tomato big bud (TBB)/*C. roseus*	Australia	-	-	-
	Witches' broom disease of lime (WBDL)/*C. roseus*	Oman Sultanate	-	-	-
16SrIII	Peach western X (PeachWX)/*C. roseus*	USA	-	-	-
16SrVI	Brinjal little leaf (BLL)/*C. roseus*	India	-	-	-
16SrVII	Ash yellows (Ash12)/*C. roseus*	USA	-	-	-
16SrX	Apple proliferation (AP-15)/*C. roseus*	Italy	-	-	-
	European stone fruit yellows (ESFY)/*C. roseus*	Italy	-	-	-
	Pear decline (PD)/*C. roseus*	Germany	-	-	-
16SrXII-A	Stolbur (P7)/*C. roseus*	Lebanon	-	27.3	-
	Stolbur (Moliere)/*C. roseus*	France	-	26.9	-
	Stolbur (Charente-1)/*C. roseus*	Charente, France	-	27.3	-
	Stolbur (Charente-2)/*C. roseus*	Charente, France	-	28.7	-
	Stolbur (LG)/*C. roseus*	Lot et Garonne, France	-	27.8	-
	Stolbur (C)/*C. roseus*	France	-	28.7	-
	Stolbur (PO)/*C. roseus*	Pyrenées Orientales, France	-	28.1	-
	Stolbur (Red-Pepper)/*C. roseus*	Serbia	-	28.5	-
	VK (GGY)/*C. roseus*[4]	Pfalz, Germany	-	27.2	34.1
	VK (19-25)/*C. roseus*[4]	Pfalz, Germany	-	26.5	36
	BN (CH1)/*C. roseus*	Italy	-	28.2	-
16SrV	FD (CAM-05) type FD1/*V. faba*	Gironde, France	28.1	-	-
	FD (PEY-05) type FD2/*V. faba*	Gironde, France	29.1	-	-
	FD (VI04-Lig2) type FD3/*V. vinifera*[4]	Veneto, Italy	32	-	19.1
	FD (VI04-C28) type FD3/*V. vinifera*[4]	Veneto, Italy	26.5	-	18.7
	PGY (PGYA)/*C. roseus*[4]	Pfalz, Germany	29.2	-	30.9
	PGY (PGYC)/*C. roseus*[4]	Pfalz, Germany	28.5	-	32.4
	GY (V04-11-1)/*V. vinifera*	Alsace, France	28.6	-	17.3
	AldY (ALY)/*C. roseus*	Basilicata, Italy	29.6	-	-
	RS (RS)/*C. roseus*	Southern Italy	30.3	-	-
	SpaW (SI-04-4-2)/*Spartium junceum*[4]	Basilicata Italy	35.1	-	38.2
	EY (EY1)/*C. roseus*	New York state, USA	-	-	-
	EY (EI04-3-3)/*U. minor*[4]	Campania, Italy	-	-	-

-: undetected; 1: Ct values obtained with primers and probe for the 16SrV group in the multiplex PCR assay; 2: Ct values obtained with primers and probe for the 16SrXII-A sub-group in the multiplex PCR assay; 3: Ct values obtained with primers and probe for the *Vitis* sp. endogenous control in the multiplex PCR assay. 4: samples were kindly provided by M. Maixner, A. Bertaccini, E. Angelini, C. Marcone and G. Cloquemin.

Clair *et al.* (2003) and the TaqMan triplex real-time PCR reaction. The latter was performed in a final volume of 25 µl comprising 12,5 µl of QuantiTect Multiplex PCR buffer (Qiagen), primers and probes (mapFD-F, mapFD-R, mapFD-FAM, mapBN-F, mapBN-R, mapBN-VIC, VITIS-F, VITIS-R, VITIS-NED) at a final concentration of 0.2 µM each and 5 µl of purified DNA. Amplification and detection were done using the ABI PRISM® 7000 (Applied Biosystems) apparatus. The thermal cycle consisted in a pre-step of 15 min at 95 °C for Hot Start Taq DNA polymerase activation, followed by 45 cycles of 60 s denaturation at 94°C and 90 s hybridization and elongation at 59 °C. Each reaction included at least one blank assay without template, two negative controls corresponding to healthy plants and two positive controls corresponding to a FD-P and BN-P positive vine samples. The software sds 1.2.3 (Applied Biosystems) was used for fluorescence acquisition and estimation of threshold cycles (Ct). For this estimation, the baseline was automatically set and the fluorescent threshold was set manually for each individual

Table 2

Primer and probe sequences for simultaneous detection of the FD and BN-related phytoplasmas and of a *Vitis sp.* endogenous control by real-time PCR

Name	Target	Sequence 5' → 3'
mapFD-F	*Map* gene of the 16SrV-C, D and E group phytoplasmas	TCAAGGCTTCGGBGGTTATA
mapFD-R		TTGTTTTAGAAGGTAATCCGTGAACTAC
mapFD-FAM		**FAM**-TTGTATTTCAGTGAATGAAG
mapBN-F	*Map* gene of the16SrXII-A group phytoplasmas	ATTTGATGAAACACGCTGGATTAA
mapBN-R		TCCCTGGAACAATAAAAGTYGCA
mapBN-VIC		**VIC**-AAACCCACAAAATGC
VITIS-F	*Vitis sp.* chloroplast *tRNA*L-F spacer	AAATTCAGGGAAACCCTGGAA
VITIS-R		CCCTTGGTTGTTTTCGGAAA
VITIS-NED		**NED**-CTGAGCCAAATCC

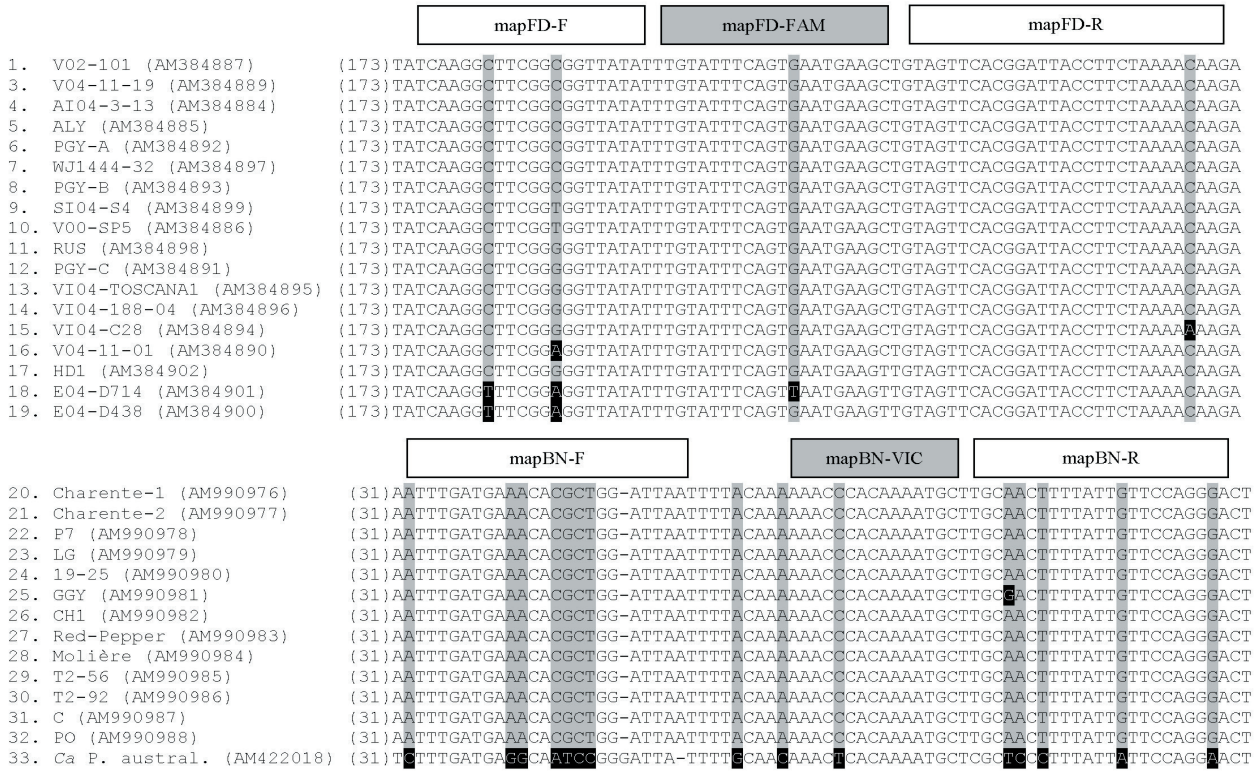


Fig. 1: Alignment of the *map* gene sequences of the phytoplasma reference strains from the 16SrV and 16SrXII groups and positions of the specific sets of primers and probes for the multiplex real-time PCR. Lanes 1 to 19: 16SrV reference strains. Lanes 20 to 33: 16SrXII reference strains. Primers and probes are represented by a white and grey rectangle respectively. Position presenting nucleotide polymorphisms are identified in grey. Mismatches with the sequence of the primers and probes are in black.

target to intersect with the linear part of all amplification curves.

Evaluation of the TaqMan real-time assay and comparison with the nested-PCR: The efficiency and the linear range of each real-time PCR reactions were evaluated by constructing dilution curves of DNA extracts from six different samples: one BN-P and one FD-P infected periwinkle, two FD-P and two BN-P infected grapevines. Five-fold or 10-fold serial dilutions were performed and each dilution was tested in duplicates. The slope (k) of the linear regression line between logarithmic values of the dilution factor (x-axis) and estimated Ct values (y-axis) was used to calculate the amplification efficiency, $E = (10^{-1/k}-1) \times 100$. The squared regression coefficient (R^2) was also calculated. Serial dilutions were also tested in duplicates by conventional nested-PCR targeting BN-P and FD-P DNA (CLAIR *et al.* 2003) in order to compare its sensitivity with that of the TaqMan® assay.

Results

Specificity of the triplex real-time PCR assay: The specificity of the triplex real-time PCR assay was tested on DNA extracts from different healthy and phytoplasma-infected plants (50 $ng \cdot \mu l^{-1}$ total DNA). For the BN-P set of primers and probes, Ct values ranging from 26.9 to 28.7 were measured for all the 16SrX-II-A phytoplasma isolates whereas no significant signal

was observed for the phytoplasmas which did not belong to the 16SrXII-A group and for the healthy plants (Tab. 1). For the FD-P set of primers and probes, Ct values ranging between 26.5 and 35.1 were measured for all 16SrV phytoplasmas tested, except for EY isolates. No amplification curve was observed for healthy plants and for the phytoplasmas which did not belong to the 16SrV group. V04-11-1 and VI04-C28 isolates for which the sequence of the *map* gene presented one mismatch with the sequence of the mapFD-F and mapFD-R primers respectively were also detected. EY isolates which presented two mismatches with the sequence of the mapFD-F primer and one mismatch with the sequence of the probe (E04-714), were not detected. For the EC set of primers and probes, DNA extracts which were obtained from different grapevine cultivars had Ct values ranging from 17.3 to 22.4 (Tab. 1). No EC amplification curve was observed for DNA extracted from broad beans and periwinkles, except for four periwincles which presented an amplification signal with high C_t values (> 30). These DNA extracts might have been slightly contaminated with DNA from grapevine. The infected Spanish broom (*Spartium junceum*) also showed a high Ct of 38 which might be due to slight cross-reactivity of the primers with the plant DNA.

The Fig. 2 shows examples of amplification curves which were obtained by testing field collected grapevine samples with the triplex real-time PCR assay. For healthy grapevines (Fig. 2 a), only the EC amplification curve was observed, the BN-P and FD-P gave no significant signal. For BN-P or FD-P infected grapevines (Fig. 2 b and c), one amplification curve corresponding to the EC and one corresponding to the detection of either the BN-P or the FD-P target were observed. For doubly infected grapevine samples (Fig. 3 d), 3 amplification curves were observed corresponding to the detection of the EC, BN-P and FD-P targets.

Performance characteristics of the Taqman real-time PCR assay in comparison with the nested PCR method: Calibration lines were constructed for each target by analysing tenfold and fivefold serial dilutions of total DNA extracted from 2 infected periwinkles and 4 infected grapevines, respectively. Fig. 3 shows examples of calibration lines obtained for FD-P and BN-P targets in periwinkles (Fig. 3 a and b) and for FD-P, BN-P and EC targets in grapevine (Fig. 3 c and d). Calibration lines had a high $R^2 \geq 0.94$. PCR efficiency values ranged from 94 % to 106.9 % for the EC target, from 97.6 % to 113.5 % for the BN-P target and from 93.7 % to 99.7 % for the FD-P target.

Sensitivities of the real-time PCR assay and the nested PCR (Clair *et al*. 2003) were compared by analysing the same serial dilutions. Fig. 4 shows examples of results obtained for FD-P and BN-P infected grapevines. For the FD-P infected grapevine sample V04-11-17 (Fig. 4 a), amplification curves of the FD-P target were observed up to a dilution factor of 5^7 by real-time PCR whereas FD-P amplicons of 1160 bp were observed up to a dilution factor of 5^4 by nested PCR. No signal was observed for the BN-P tar-

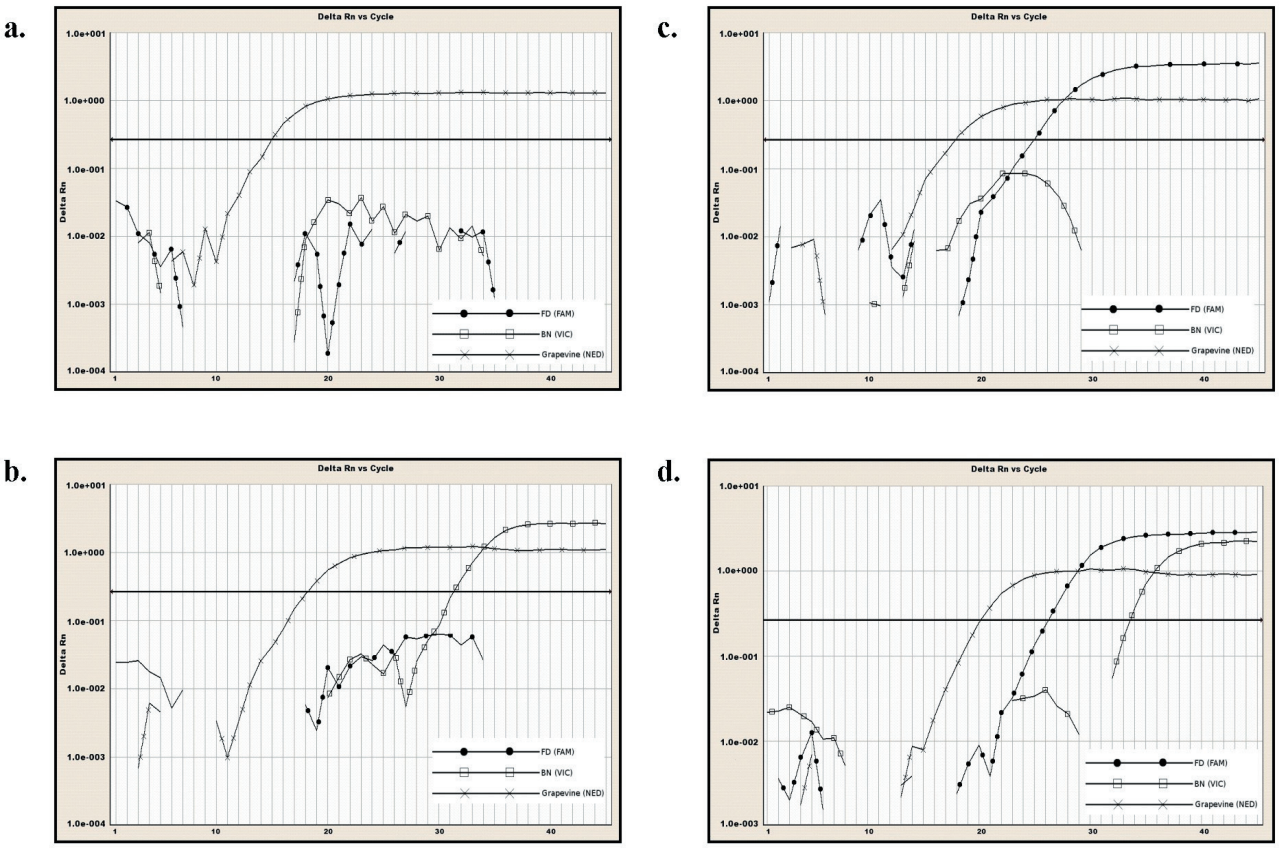


Fig. 2: Examples of amplification curves obtained for field-collected grapevine samples. (**a**) Healthy grapevine; (**b**) BN-P infected; (**c**) FD-P infected; (**d**) FD-P and BN-P doubly infected.

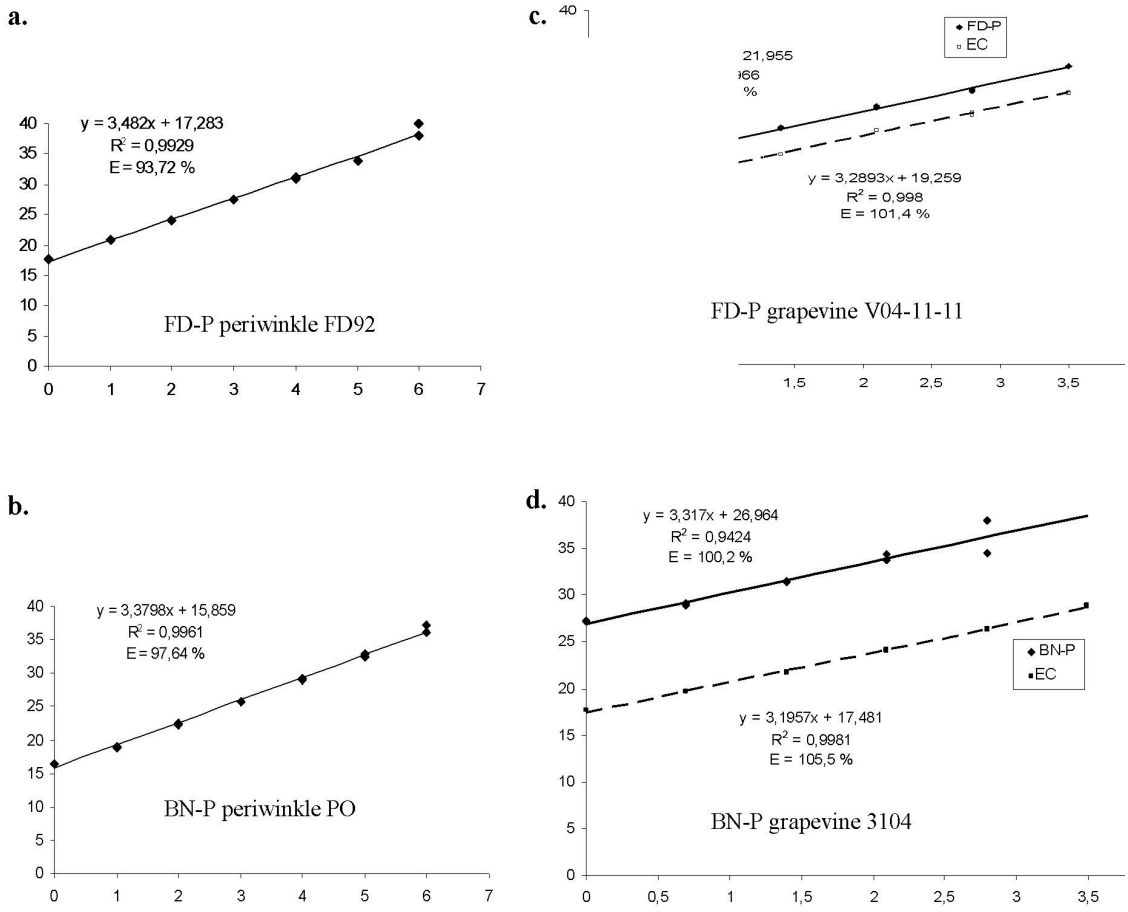
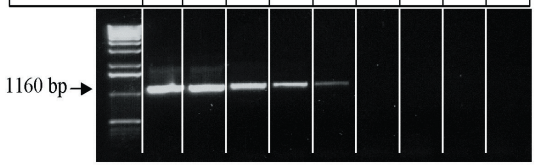


Fig. 3: Calibration lines obtained for FD-P, BN-P and EC targets for FD-P (**a**) and BN-P (**b**) infected periwinkles and for FD-P (**c**) and BN-P (**d**) infected grapevines. y axis: Ct values; x axis: log dilution factor.

a.

Dilution factor	0	5^1	5^2	5^3	5^4	5^5	5^6	5^7	5^8
Mean Ct EC	17.6	20	22.2	24.5	26.7	29.3	31.6	34.1	37.2
Mean Ct BN-P	-	-	-	-	-	-	-	-	-
Mean Ct FD-P	22.7	24.9	27.2	29.5	31.9	34.5	37.8	39.1	-



b.

Dilution factor	0	5^1	5^2	5^3	5^4	5^5
Mean Ct EC	17	19	21.2	23.4	25.7	28.2
Mean Ct BN-P	27.5	29.3	30.8	33.7	35.9	-
Mean Ct FD-P	-	-	-	-	-	-

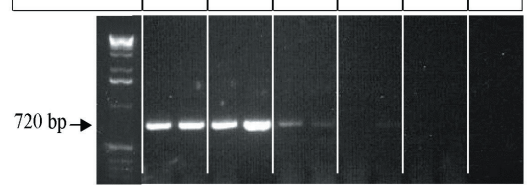


Fig. 4: Comparison of the sensitivity of the triplex real-time PCR and the biplex nested PCR for FD-P V04-11-17 (**a**) and BN-P BN840 (**b**) infected grapevines.

gets by both PCR. For the BN-P infected grapevine sample BN 840, amplification curves of the BN-P target were observed up to a dilution factor of 5^4 by real-time PCR whereas FD-P amplicons of 720 bp were observed until a dilution factor of 5^3 by nested PCR. No signal was observed for the FD-P targets by both PCR. Globally, among the different periwinkle and grapevine samples analysed, the sensitivity of the real-time PCR is up to 100 times higher than the nested PCR for the FD-P target and up to 5 times higher for the BN-P target.

Validation of the multiplex real-time PCR assay for routine GY diagnosis and comparison with the nested PCR method: The intra-test reproducibility of the triplex real-time PCR assay was evaluated on 88 infected grapevine samples which were tested in duplicate. Extracts were analysed either undiluted or diluted 1:10 in order to test different levels of infection. The inter-test reproducibility was evaluated on 11 infected grapevine samples which were tested by two different operators. The Ct deviations values intra-test (between duplicates) and inter-test (between operators) were inferior to 5 %. The Ct values for the FD-P target varied from 23 to 35 with a mean of 28.5 for all the undiluted extracts and from 29.2 to 38.7 with a mean of 31.6 for the diluted extracts. The Ct values for the BN-P target varied from 28.6 to 35.8 with a mean of 32.5 for the undiluted extracts and from 34.5 to 38.1 with a mean of 36.1 for the diluted ones. The Ct values for the

grapevine target were only reported for the undiluted extracts. They varied from 16.1 to 29.9 with a mean of 20.1. On 47 samples tested, 20 had Ct values ≤ 20, 24 ≤ 25 and 3 ≤ 30. The three latter samples showed a signal for the FD-P target with high Ct values between 34 and 38 and no signal for the BN-P target. These high Ct values can be due to the presence of inhibitors or to the partial degradation of DNA.

A decision scheme presented in Tab. 3 was set up for phytoplasma detection in grapevine samples. When Ct values of phytoplasma amplicons were < 40, samples were considered as positive, regardless of the Ct value of the EC amplicon. When FD-P or FD-P Ct values were ≥ 40 or not detectable, samples showing Ct value of the EC amplicon < 25 were considered as negative. Other samples with a Ct of the EC amplicon ≥ 25 were considered as not interpretable as this value may be due to the presence of inhibitors or to the degradation of the DNA. The DNA of such samples should be re-extracted and tested again as undiluted and diluted. The Ct cut-off value of 25, chosen for the EC is 5 units higher than the mean Ct value (20.1). This corresponds to a 30 fold loss in sensitivity. One hundred and nine grapevine field-collected samples were tested with the triplex real-time PCR and with the biplex nested assays by applying the decision scheme. Each test was performed in duplicate. Results are shown in Tab. 4. Negative controls had no amplification signal for the FD-P and BN-P targets. Ninety nine samples gave the same result for both detection tests but ten samples which were FDP-negative (7 BN-P negative and 3 BN-P positive) with the nested PCR gave FD-P positive results with the real-time PCR. The number of BN-P positive samples did not change. Doubly negative were 13.8 % with the nested-PCR and 7.4 % with the real-time PCR. FD-P positive samples were 46.7 % with the nested-PCR and 55.9 % with the real-time PCR. The percentage of BN-P positive samples remained unchanged. None of the samples was “not interpretable”.

Table 3

Decision scheme for FD-P and BN-P detection in grapevine by the triplex real-time PCR assay

	Ct EC < 25	Ct EC ≥ 25 or not detected
FD-P or BN-P Ct Value < 40	positive	positive
FD-P or BN-P Ct Value ≥ 40 or not detected	negative	not interpretable

Table 4

Comparison of phytoplasma detection results between the triplex real-time PCR and the biplex nested PCR assays among 109 grapevine samples

Detection result	Nested PCR	Real-time PCR
FD-P negative / BN-P negative	15	8
FD-P positive / BN-P negative	42	49
FD-P negative / BN-P positive	43	40
FD-P positive / BN-P positive	9	12
	109	109

Discussion

In this study, a new real-time PCR method was developed for the simultaneous detection of FD and BN-related phytoplasmas in grapevine with an endogenous control. The use of the TaqMan technology allowed the multiplexing of three different targets: position 175 to 245 on *map* gene of the 16SrV-C, D and E phytoplasma subgroups, position 32 to 103 of the 16SrXII-A phytoplasma subgroup and the *tRNA*L-F spacer of the grapevine chloroplast. Specificity was promoted by the use of MGB conjugates (Kutyavin *et al*. 2000) and by the choice of a non ribosomal target which presents higher nucleotide variability between phytoplasma phylogenetic groups than does the 16S rDNA used as universal target for phytoplasma detection. It also avoided possible cross-reaction with the plant chloroplastic DNA or with the DNA of saprophytic mollicutes which could be observed when the 16S rDNA was used as a target (Ahrens and Seemüller 1992, Firrao *et al*. 1993). Indeed, no cross-reaction was observed, neither between the three targets nor with phytoplasmas from other phylogenetic groups or with healthy plants.

Variability inside the *map* gene also permitted to discriminate phytoplasmas inside the 16SrV phylogenetic group. Thus, AldY, PGY and SpaW phytoplasmas (16SrV-C and D subgroups) which are phylogenetically very close to FD-P (Angelini *et al*. 2001, 2003, Arnaud *et al*. 2007). were detected, but not EY phytoplasmas which are regarded as different species (Lee *et al*. 2004). It is important to be able to detect phytoplasmas closely related to FD-P in grapevine because it has been shown that AldY phytoplasmas can be transmitted to grapevine (Maixner et al. 2000) and recent genetic studies suggest that alders constitute a possible source of new FD outbreaks (Arnaud *et al*. 2007). Variability of the *map* gene should also permit to discriminate between 16SrXII-A and B subgroups strains. We did not study the detection of Australian grapevine yellows phytoplasmas from the16SrXII-B subgroup by the triplex real-time PCR. But alignment of the sequence of the amplicon with the same region of *Ca.* Phytoplasma australiense *map* gene (Tran-Nguyen *et al*. 2008) shows many mismatches which should prevent the detection of 16SrXII-B phytoplasmas.

Triplex real-time PCR showed a lower detection limit than biplex nested PCR (Clair *et al.* 2003): up to 5 and 100 times lower for the BN-P and the FD-P targets respectively. The increased sensitivity can be partly attributed to the short regions of target DNA that are amplified in TaqMan® MGB probe-based real-time PCR assays: between 10 to 16 times shorter than for the conventional PCR. Therefore, more targets can be amplified in a given time of elongation, and real-time PCR reactions might be less sensitive to DNA degradation or DNA-binding inhibitors present in the samples. This can also partly explain why the increase of sensitivity is higher for FD whose target in conventional PCR is longer than for BN (1160 bp versus 720 bp respectively). It resulted in an improved detection of FD phytoplasma in symptomatic samples coming from the field. On 109 samples tested, 51 where FD positive by conventional PCR while they reached 61 by real-time PCR. Higher sen-

sitivity constitutes a progress for monitoring the disease because plants with very low phytoplasma titre can also constitute a source of inoculum (Boudon-Padieu 2002). But it also implies to take into consideration the risk of generating false positive. Real-time PCR has the advantage over conventional PCR to reduce drastically the risk of contamination by PCR products carry over. But the risk of cross contamination by phytoplasma genomic DNA, especially during the extraction procedure, is not reduced. This risk is not negligible, particularly when only symptomatic samples are tested for routine detection which leads to the manipulation of important amounts of phytoplasma DNA. Special care must be taken at this step.

Acknowledgements

We thank J. Grosman, J. M. Trespaille-Barrau and the Regional Services for Plant protection for the providing of field grapevine samples. We are grateful to J. Berthillier, E. Dégletagne and C. Rigaud from the "Laboratoire Départemental d'Analyses 71" for contributing in the development of the real-time PCR method. We acknowledge F. Ferrer, P. Bonnet and J. L. Danet for maintaining phytoplasma infected plants. We are grateful to B. de Barbeyrac, M. Clerc and S. Raherison from the University of Bordeaux 2 for their help in using the real-time PCR apparatus. We also thank C. Couture for her advises on real-time PCR methods. This research was funded by the "Conseil Général de Saône et Loire" and by the "Institut National de la Recherche Agronomique".

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Received September 3, 2008