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DEVELOPMENT OF AN ISOTHERMAL AMPLIFICATION ASSAY FOR IN-FIELD DETECTION OF GRAPEVINE FLAVESCENCE DOREE PHYTOPLASMA

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RÉSUMÉ
La Flavescence dorée est une menace pour la viticulture en Europe et elle est actuellement considérée comme un organisme de quarantaine par la législation européenne. L'inspection visuelle demeure une pratique courante pour la surveillance de cette maladie transmise par le vecteur *Scaphoidus titanus*. Mais cette technique de détection ne permet pas de distinguer la Flavescence dorée d'une autre maladie dangereuse pour la vigne, le Bois noir, qui n'est pas encore inclus dans la liste OEPP des organismes de quarantaine. Seule une analyse ADN permet de détecter la Flavescence dorée d'une manière fiable et sensible, mais cette approche était jusqu'alors réservée aux laboratoires experts. Dans cet article, une nouvelle méthode moléculaire basée sur la technologie d'amplification isothermale de l'ADN est décrite, pour une identification rapide du phytoplasme "Candidatus Phytoplasma vitis" associé à la maladie Flavescence dorée directement sur le terrain.

Mots-clés : Flavescence dorée, phytoplasme, amplification isothermale, bCUBE®, détection précoce.

ABSTRACT
Grapevine flavescence dorée is one of the most important threats in viticulture areas in Europe and it is currently included in the European legislation as a quarantine pest. Visual inspection remains routine practice to determine the presence or absence of this phytoplasma disease transmitted by the vector *Scaphoidus titanus*, but in this way it is not possible to distinguish Flavescence dorée from another dangerous grapevine disease, Bois noir, which is not yet included in the EPPO quarantine pest list. DNA analysis is the only method to detect Flavescence dorée in a reliable and sensitive way but this approach, until now, has been limited to expert laboratories. In this paper, we report a new molecular method, based on isothermal amplification technology, for a rapid and in-field identification of "Candidatus Phytoplasma vitis" associated with Flavescence dorée disease.

Keywords: Flavescence dorée, phytoplasma, isothermal amplification technology, bCUBE®, early detection.

INTRODUCTION

Phytoplasmas are cell wall-less microorganisms which are associated with plant diseases worldwide, and inducing typical symptoms. On the basis of conserved 16S rRNA gene sequence similarity, the currently known phytoplasmas are classified into a number of different 16S ribosomal (16Sr) groups and subgroups (Duduk & Bertaccini, 2011; Disckinson *et al*., 2013). "Candidatus phytoplasma vitis" responsible for Flavescence dorée (FD), which is efficiently transmitted by the sap-sucking insect vector *Scaphoidus titanus* Ball, belongs to the Elm yellows group 16SrV (subgroups -C/D). FD was first described in south-west France in the 1950's by Caudwell (1957) and has since spread into the most important viticulture areas in Europe, including Italy, Spain, Portugal, Switzerland, Austria, Slovenia and Serbia (EPPO, 2014), causing an important economic impact in viticulture. "Candidatus Phytoplasma solani" responsible for Bois noir (BN), another grapevine disease, belongs to the stolbur group 16SrXII-A. The symptoms caused by FD and BN phytoplasma are indistinguishable even though these two diseases are spread by different insect vectors and their epidemiology differs across the most important viticulture areas in Europe. Contrary to BN phytoplasma, the causal agent of FD is classified as a quarantine pest by EPPO (A2 list). Although vineyard monitoring is scheduled yearly by countries where FD occurs, visual inspection remains the routine practice, but this is not sufficient to identify this phytoplasma disease. DNA analysis is the only method to confirm the presence of FD in a reliable and sensitive way.

Many molecular diagnostic methods have been developed for FD phytoplasma detection in the past years (Lee et al., 1994; Daire et al., 1997; Clair et al., 2003; Angelini et al., 2007; Hren et al., 2007; Pelletier et al., 2009). The Euphresco European project 'Grafdepi I' (2010-2014) principally consisted in validating 7 PCR-based methods for FD detection. Singleplex and multiplex Nested End-Point PCR methods as well as Real-Time PCR methods were submitted to an interlaboratory trial consisting of 14 European referent laboratories. The execution of these methods are time-consuming, from 1h40 to 5h00, and it is necessary to work with high quality DNA which implies dedicating a long time to the extraction (from 2h30 to 2 days). Due to these limitations, these methods cannot be used directly in the field by government agencies and technicians in charge of FD monitoring or by the vinegrowers themselves. However, field molecular tools would allow government agencies to make accurate real-time diagnoses.

A new tool for acid nucleic amplification, based on isothermal amplification, has been developed by Notomi *et al*., 2000. The method uses a DNA polymerase which has strand displacement activity and a set of six specifically designed primers that recognized six distinct sequences of the target DNA. Amplification can be performed under isothermal conditions between 60°C and 65°C. The amplification of nucleic acids under these conditions is extremely rapid with an average of amplification time between 8 and 45 minutes.
The aim of the present work was to develop a new isothermal amplification assay for rapid detection of FD and to develop a DNA extraction tool (FAST DNA) that is easy and quick-to-use for in-field FD detection. Grapevine leaf samples collected in different areas of north Italy were used to verify the performance of the new isothermal amplification assay compared to the Qualiplante SAS Triplex Real-Time PCR kit (qPCR.FD.BN-25/100Liq) developed according to the method patented by International Plant Analysis and Diagnostics s.r.l. and which was validated during the 'Grafdepi I' Euphresco European project. The assay can be run on conventional Real-Time PCR machines but it will also possible to run it on a miniaturized thermal cycler (bCUBE®) under development by Hyris Ltd for in-field plant disease detection.

MATERIAL AND METHODS

PLANT MATERIAL

Fourty six grapevine leaf samples, including 9 healthy samples, of cultivars Albarossa, Barbera, Chardonnay, Croatina, Pinot gris and Pinot noir were collected in 2013 and 2014, in July, August and October, in different grapevine areas of Piedmont, Lombardy and Veneto. The leaf veins were excised from symptomatic plants and stored at - 80°C until assayed. In order to verify the presence of FD and/or BN, the collected samples were analyzed using qPCR.FD.BN-kit.

DNA EXTRACTION AND CRUDE SAP PREPARATION

Two different DNA extraction procedures were evaluated. Briefly, 20 leaf samples were extracted using 3% CTAB extraction or FAST DNA extraction method in order to compare the two methods. **3% CTAB extraction method**: each sample was extracted as reported in the French Official method for the detection of FD and BN (MOA06 partie B, version 2a). **FAST DNA extraction method:** approximatively 150 mg of grapevine leaf veins from each sample were inserted in a plastic tube containing an iron bead. The plant material was manually crushed during 1 minute in 1.5 ml of Extraction Buffer (IpadLab Extraction Buffer). Approximately 10 µl of the crude sap were collected with a sterile loop and diluted in 1 ml of Elution Buffer (IpadLab Elution Buffer). Finally, 5 µl of the eluted crude sap were used as a template for the isothermal amplification assay.
The DNA samples obtained using the 3% CTAB extraction method and the crude saps from the FAST DNA method were conserved at -20 °C until assayed.

PRIMER DESIGN

The whole nucleotide sequences of 16SrV operon S10-spc of the strains EY1 (JN851864), PGY-A (JN851865), FD-D (JN851859), FD-C (JN851863) and ALY (JN851860) were used for the design of primers.
Two primer sets were designed using the LAMP Primer Explorer V3 software (http://primerexplorer.jp/elamp3.0.0/index.html); internal primers FIP and BIP were designed manually for both primer sets. The FD1 primer set was designed on the *rps5 gene* while the FD2 primer set was designed on the *rpl14 gene*, as patented by International Plant Analysis and Diagnostics s.r.l. The six primers corresponding to each primer set: F3, B3, F-LOOP, B-LOOP, FIP and BIP were synthesized by MWG-Biotech with HPLC purification. The sequences of each primer of the FD1 primer set are given in Table I (data not shown for FD2 primer set).

Tableau I : Séquences des différentes amorces FD1 dessinées sur le gène *rps5*
Table I: Sequences of the FD1 primer set designed on the *rps5 gene*

Primers	Primers sequence (5'-3')
F3_FD1	GCT TTA GTT GTT GTT GGA GA
B3_FD1	TAT AGG TGT TCT CGA ACC TA
FIP_FD1	GAC AAC TTC GTG AGG AAT AGC AAG TGC TAA AGC TCA AGA A
BIP_FD1	GGT GAG TAT GGT GCT TCT AAG CTG AAA TAT CAC GAA TAC C
F-LOOP_FD1	TAG TTC CTG CAA GCG GAA TT
B-LOOP_FD1	AAG GTA TTA TTG CTG GAG GA

ISOTHERMAL ASSAY

Isothermal reaction: For both primers sets, the isothermal amplification was performed in 8-well strips in a total final volume of 25 µl. The two different extracted materials were used as a template for the assay: 5 µl of DNA template extracted with 3% CTAB method and 5 µl of crude sap diluted to 1:100 in water extracted with the FAST DNA method. The reaction mixture contained the following concentration for each primer: 5 pmol for F3 and B3, 20 pmol for F-LOOP and R-LOOP, 40 pmol for FIP

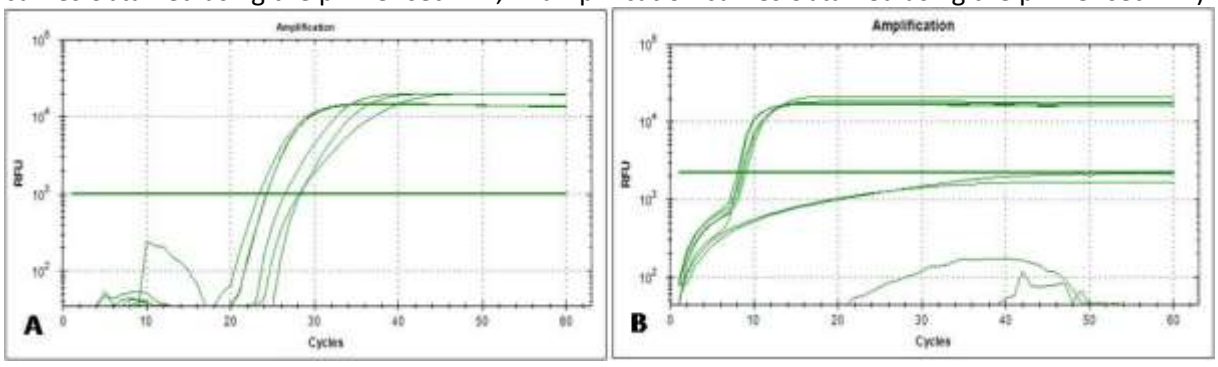
and BIP. Isothermal amplifications were compared using Optigene Genie II Reader and Biorad CFX96 Real-Time PCR thermal cycler.
Real-time PCR assays: 3 µl of DNA extracted using the 3% CTAB method was analyzed using qPCR.FD.BN kit.
Isothermal amplification assay: A gradient isothermal run was performed. Three different temperatures: 60 °C, 62 °C and 65 °C were tested in order to determine the best thermal profile for the amplification reaction. The 2 primer sets (FD1 and FD2) were compared. The 46 samples extracted from grapevine leaves were analysed according to the conditions defined previously: 31 infected with FD, 6 infected with BN and 9 healthy samples. In order to validate the assay, all the collected samples were analyzed, in comparison, using the qPCR.FD.BN-25 kit. Moreover DNA samples of other phytoplasma as *Elm yellows*, *Alder yellows*, *Apple Proliferation*, *Aster yellows, Agrobacterium vitis* and "Candidatus Phytoplasma fraxini" were also tested in order to verify the specificity of the assay.

RESULTS

OPTIMIZATION OF THE ISOTHERMAL ASSAY

Isothermal amplification was performed using DNA samples extracted with 3% CTAB in order to determine the optimal temperature and the best primer set to use for the assays. At 60°C, a decrease assay performance was observed for both primer sets FD1 and FD2. However, no difference in efficiency was observed when the amplification was performed at 62°C and 65°C. We therefore chose to continue assay validation with the 65°C condition and a run time of 30 minutes.
The FD2 primer set was identified as the best compared to the FD1 primer set in terms of Ct values. In more detail, when the assay was performed with the FD2 primer set, only 8 to 9 minutes of reaction were necessary to observe the first amplification signal (Figure 1). On the contrary, use of the FD1 primer set lengthened the amplification time to almost 20 minutes; for this reason, the primer set FD1 was excluded. The melting peak obtained from dissociation step was between 81°C and 81.5°C.

Figure 1 : Courbes d'amplification obtenues par amplification isothermale, Biorad CFX96 (A : courbes d'amplification obtenues avec les amorces FD1 ; B : courbes d'amplification obtenues avec les amorces FD2)
Figure 1: Amplification curves of the isothermal amplification assay, Biorad CFX96 (A: amplification curves obtained using the primer set FD1; B: amplification curves obtained using the primer set FD2)



The Table II compares the differences in results obtained between the FD1 and FD2 primer sets developed in this work and the Real-Time PCR assay (qPCR.FD.BN kit) on the same samples.

Tableau II : Ct obtenues par amplification isothermale (amorces FD1 et FD2) et par Real-Time PCR
Table II: Ct values obtained by using isothermal amplification (primers sets FD1 and FD2) and Real-Time PCR

Infection	Sample	Ct value, temperature condition : 65°C		
		Primer set FD1	Primer set FD2	qPCR.FD.BN kit
FD	0814-004	24.14	8.86	24.31 (FD)
FD	0814-005	24.91	8.25	24.56 (FD)
FD	0814-006	23.97	9.16	25.30 (FD)
FD	0814-007	22.42	8.12	23.24 (FD)
FD	0814-008	22.46	8.58	23.99 (FD)
BN	1012-104	-	-	24.51 (BN)
BN	1012-109	-	-	29.03 (BN)
BN	1012-110	-	-	26.03 (BN)
BN	1012-111	-	-	28.84 (BN)
BN	1012-113	-	-	29.45 (BN)
Healthy	1012-107	-	-	-
Healthy	1012-112	-	-	-

VALIDATION OF THE ISOTHERMAL ASSAY

The results obtained during the validation step underline the capability of the new molecular assay to detect specifically FD in the grapevine samples. All the samples infected by FD showed an amplification signal between 7.86 and 17.19, none amplification signal was observed for the healthy samples and for samples infected by the no target organism, except for the samples infected by other phytoplasma belonging to 16SrV group as *Elm yellows* and *Alder yellows*. In the table III, we report the results (all data not shown).

Tableau III : Résultats issus de la validation de la méthode d'amplification isothermale pour la détection de FD avec les amorces FD2 (+ve: positif; -ve: négatif; N/A : pas d'amplification)

Table III: Results from the validation of the FD2 isothermal molecular amplification assay for the detection of FD (+ve: positive; -ve: negative; N/A: no amplification)

Sample number	Samples	Variety	Infection	16Sr Group	Isothermal amplification assay			qPCR.FD.BN kit	
					Result	Ct	Peak T° (°C)	Result	Ct
1	r0714-001	Pinot gris	FD	16SrV	+ve	8.62	81.5	+ve	27.35
2	r1014-004	Chardonnay	FD	16SrV	+ve	7.86	81.5	+ve	20.83
3	r0714-005	Albarossa	FD	16SrV	+ve	9.26	81.5	+ve	26.09
…	…	…	…	…	…	…	…	…	…
23	0714-065	Barbera	FD	16SrV	+ve	10.86	81.5	+ve	24.53
24	0714-066	Pinot noir	FD	16SrV	+ve	18.56	81.0	+ve	30.54
25	0714-067	Barbera	FD	16SrV	+ve	10.04	81.5	+ve	25.27
26	0714-069	Barbera	FD	16SrV	+ve	9.37	81.5	+ve	25,04
27	0714-073	Barbera	FD	16SrV	+ve	17.19	81.0	+ve	27.91
28	0714-113	Pinot noir	FD	16SrV	+ve	9.26	81.5	+ve	24.50
29	0714-093	Barbera	FD	16SrV	+ve	12.65	81.5	+ve	26.03
30	153/08	-	FD-C	16SrV-C	+ve	12.00	81.5	+ve (PCR)	-
31	154/08	-	FD-C	16SrV-C	+ve	11.50	81.5	+ve (PCR)	-
32	0714-068	Pinot noir	Healthy	-	-ve	N/A	N/A	-ve	N/A
33	0714-070	Pinot noir	Healthy	-	-ve	N/A	N/A	-ve	N/A
34	0714-072	Pinot noir	Healthy	-	-ve	N/A	N/A	-ve	N/A
35	0714-104	Pinot noir	Healthy	-	-ve	N/A	N/A	-ve	N/A
…	…	…	…	…	…	…	…	…	…
41	1012-104	Chardonnay	*Bois noir*	16SrXII	-ve	N/A	N/A	-ve	N/A
42	1012-109	Chardonnay	*Bois noir*	16SrXII	-ve	N/A	N/A	-ve	N/A
43	1012-110	Chardonnay	*Bois noir*	16SrXII	-ve	N/A	N/A	-ve	N/A
44	1012-111	Chardonnay	*Bois noir*	16SrXII	-ve	N/A	N/A	-ve	N/A
45	1012-113	Chardonnay	*Bois noir*	16SrXII	-ve	N/A	N/A	-ve	N/A
46	0714-090	Pinot noir	*Bois noir*	16SrXII	-ve	N/A	N/A	-ve	N/A
	Elm Yellow	-	*Elm Yellows*	16SrV-A	+ve	9.00	81.0	+ve (PCR)	-
	Alder Yellow	-	*Alder Yellows*	16SrV-C	+ve	7.52	81.5	+ve (PCR)	-
	Apple Proliferation	-	*Apple Proliferation*	16SrX-A	-ve	N/A	N/A	-ve (PCR)	-
	Aster Yellow	-	*Aster Yellows*	16SrI-A	-ve	N/A	N/A	-ve (PCR)	-
	A. vitis	-	*A. vitis*	-	-ve	N/A	N/A	-ve (PCR)	-
	Ca. P. fraxini	-	*Ca. P. fraxini*	16SrVII	-ve	N/A	N/A	-ve (PCR)	-

DETECTION OF FLAVESCENCE DORÉE IN THE FIELD

The aim of the present work was to investigate the possibility to perform FD detection directly in field. The limiting step for in-field detection is the preparation of the sample and the DNA extraction. The FAST DNA extraction method permits homogenization and preparation of one sample in only 2 minutes and to obtain a ready-to-use crude sap as template for the amplification step. In comparison, 2 hours were necessary to extract DNA from the samples using the 3% CTAB method.
When FAST DNA extraction was compared to 3% CTAB extraction, no significant differences were observed in terms of Ct values between the two methods. The range of Ct values was between 8.12 and 10.00, as shown in Table IV.

Tableau IV : Comparaison des valeurs de Ct obtenues avec la méthode d'extraction FAST DNA et la méthode d'extraction au CTAB 3%
Table IV: Comparison between Ct values obtained using the FAST DNA extraction method and the 3% CTAB extraction method

Infection	Sample	*Ct values,* temperature condition : 65°C, FD2 primers	
		FAST DNA extraction method	3% CTAB extraction
FD	0814-004	9.15	8.86
FD	0814-005	9.15	8.25
FD	0814-006	9.30	9.16
FD	0814-007	9.00	8.12
FD	0814-008	10.00	8.58
BN	1012-104	-	-
BN	1012-109	-	-
BN	1012-110	-	-
BN	1012-111	-	-
BN	1012-113	-	-
Healthy	1012-107	-	-
Healthy	1012-112	-	-

DISCUSSION

In this work, we have developed a diagnostic assay for FD phytoplasma detection based on a FAST DNA extraction method and isothermal amplification. Two primer sets, designed on the *rps*5 gene (FD1 primer set) and on the *rpl*14 gene (FD2 primer set) were compared using different amplification temperatures. The FD2 primer set gave the best performance at 65°C and permitted to obtain an amplification curve within only 8 minutes. The primer set FD2 showed a high efficiency of reaction and a high specificity for this FD diagnosis. No amplification curve was obtained for the samples infected with Bois noir or for healthy samples. However, *Elm Yellows* and *Alder yellows* phytoplasmas were detected; as for other available methods currently developed for the detection of FD and BN, the isothermal assay was not able to distinguish FD phytoplasma from other phytoplasma of the 16SrV group. Moreover, the results obtained comparing 3% CTAB extraction and FAST DNA extraction did not show any significant difference between the two methods in terms of efficiency of reaction. However, we have taken into account only samples with a high concentration of FD phytoplasma. For this reason, it will be necessary to validate the efficiency of the FAST DNA extraction method on low-infected samples. Following with present work, we are performing a full validation of the isothermal amplification assay at 65°C, with the FD2 primer set, in order to validate this method according to the EPPO standards (PM7/98): analytical sensitivity, diagnostic sensitivity, diagnostic specificity, repeatability, and reproducibility. This second step of validation will permit to finally determine if the FD isothermal assay could be used as a new tool to detect in a sensitive way FD in the field.

CONCLUSION

Up to now the methods used for the identification of phytoplasma associated with Flavescence dorée disease are based on Nested End-Point PCR or Real-Time PCR, which are time consuming and require a high level of specialization of the operator who performs the analysis. Furthermore, the current extraction step and diagnosis assay need to be done in a laboratory and require specific laboratory equipments. The new isothermal assay developed in the present work, in association with the FAST DNA extraction method and the bCUBE® thermal cycler (Hyris Ltd), will allow government agencies and field technicians to monitor themselves, in real time, only the Flavescence dorée phytoplasma directly in the field.

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