EUPHRESCO Final Report

Project Title (Acronym)
Epidemiological studies on reservoir hosts and potential vectors of Grapevine flavescence dorée (FD) and validation of different diagnostic procedures for GFD (GRAFDEPI)

Project Duration:

Start date:	01/02/10
End date:	29/04/14

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2. Executive Summary

Project Summary

Title

Epidemiological studies on reservoir hosts and potential vectors of Grapevine flavescence dorée (FD) and validation of different diagnostic procedures for GFD (GRAFDEPI)

Introduction

Phytoplasmas are cell wall-less microorganisms belonging to the class *Mollicutes*, and are associated with plant diseases worldwide. Typically located in the plant phloem tissue, they are transmitted by sap-sucking insect vectors, and induce typical symptoms (Bertaccini and Duduk, 2009).

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 and Bertaccini, 2011; Dickinson *et al.*, 2013).

Many important food, vegetable and fruit crops can be severely affected by these pathogens with a significant economic impact (Bertaccini and Duduk, 2009).

Flavescence dorée (FD) is one of the greatest threats for grapevine cultivation in Europe and included in European legislation as a quarantine pest (directive 2000/29 EC). It is caused by a phytoplasma belonging to 16SrV group, efficiently transmitted by the insect vector *Scaphoideus titanus* Ball.

More recently some other leafhoppers have been shown to harbour FD phytoplasma: *Dictyophara europaea* (Filippin *et al.*, 2009) and *Orienthus ishidae* (Gaffuri *et al.*, 2011; Mehle *et al.*, 2011). *D. europaea* was also demonstrated to trasmit FD from *Clematis vitalba* to grapevine (Filippin *et al.*, 2009)

Interest has recently been focused on several wild species, found infected by FD, to verify their possible role in FD epidemiology: *Clematis vitalba, Alnus glutinosa* (Malembic-Maher *et al.*, 2009) and *Ailanthus altissima* (Filippin *et al.*, 2010).

Genetic analysis of FD genome with different molecular markers revealed a population variability and the presence of different FD strains in the 16S rDNA, belonging to subgroups 16SrV-C and 16SrV-D (Martini *et al.*,1999; Arnaud *et al.*, 2007).

Main objectives:

- improvement of knowledge on epidemiological cycle of the disease;
- to provide guidelines for the harmonization of FD diagnostic procedures and control strategies within the EC.

Methods

The Project has been organized in three scientific WPs, each focused on different activity, in addition to the WP1, specifically dedicated to the Project management:

WP2 - Epidemiological studies,

The WP2 activity was focused on investigations of disease outbreaks, following specific guidelines, in different viticulture regions to analyse the epidemiology of the disease with respect to alternative host plants, potential vectors and spreading of FD isolates.

WP3 - Validation of diagnostic procedures

An interlaboratory comparison with 14 participant labs was organized to evaluate the

performance criteria of 7 diagnostic methods (including conventional and real time PCRs) for the detection of FD-phytoplasma

WP4 - Design of surveillance systems

The WP4 activity was focused on the release of guidelines for new surveillance schemes for FD control, in view of an harmonization of phytosanitary measures within EC.

Results

WP1:

a) In a broad range of different wild plants tested for the presence of FDp, only *Clematis vitalba, Alnus glutinosa*, and *Ailanthus altissima* resulted to be wild host plants confirming their potential role as reservoir for FDp and as a source of infection for new outbreaks.

b) Among all analyzed insects three insect species were confirmed to harbor FDp: *Scaphoideus titanus, Orientus ishidae,;* three insect species were defined as new potential vectors for FDp: *Phlogotettix cyclops* and *Psylla alni in Austria, Oncopsis alni* for the first time has been demonstrated to harbor FDp strains other than Palatinate grapevine yellows (16SrV-C)

c) A distribution map of FDp strains in grapevines and other hosts have been designed, including isolates with 'mixed profiles' identified in Italy and Austria.

WP2: The ringtest results showed that the real time PCR protocols have performance criteria higher than the conventional PCR protocols. The general view of the results leads to recommend the use of rt PCR methods in phytosanitary laboratories belonging to national and international networks.

WP3: Guidelines for the definition of surveillance schemes for FD have been defined, including:

- Sampling plan (period, number of samples, matrices, etc.)
- Diagnostic protocols
- Monitoring of phytoplasma and vectors distribution
- Novel control strategies

Conclusion

The results obtained within the project GRAFDEPI are very relevant and reliable. The GRAFDEPI Consortium composed by a large number of Contries/Partners allowed to collect data from different geographical areas and phytosanitary experiences, contributing to the improvement of the knowledge of the epidemiology of the disease, to the harmonization of the diagnosis and the control strategies.

3. Report

EUPHRESCO Project:

'Epidemiological studies on reservoir hosts and potential vectors of Grapevine flavescence dorée (FD) and validation of different diagnostic procedures for GFD' (GRAFDEPI)

Topic Coordinator: Sylvia Bluemel (AGES – Austria)

Scientific Coordinator: Graziella Pasquini (CRA-PAV – Italy)

• Foreword

Phytoplasmas are cell wall-less microorganisms belonging to the class *Mollicutes*, and are associated with plant diseases worldwide. Typically located in the plant phloem tissue, they are transmitted by sap-sucking insect vectors, and induce typical symptoms (Bertaccini and Duduk, 2009).

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This disease is included in European legislation as a quarantine pest (directive 2000/29 EC).

More recently some other leafhoppers have been shown to harbour FD phytoplasma: *Dictyophara europaea* (Filippin *et al.*, 2009) and *Orienthus ishidae* (Gaffuri *et al.*, 2011; Mehle *et al.*, 2011). *D. europaea* was also demonstrated to trasmit FD from *Clematis vitalba* to grapevine (Filippin *et al.*, 2009)

Interest has recently been focused on several wild species, found infected by FD, to verify their possible role in FD epidemiology: *Clematis vitalba, Alnus glutinosa* (Malembic-Maher *et al.*, 2009) and *Ailanthus altissima* (Filippin *et al.*, 2011).

Genetic analysis of FD genome with different molecular markers revealed a population variability and the presence of different FD (Martini *et al.*,1999; Arnaud *et al.*, 2007).

The goal of the FD control measures is to prevent introduction and spread of harmful organism. EU had experience of the effects of introduced pests on agriculture, forests or other natural areas. Very often this introduction has occurred

as a consequence of human activities, such as importing or exporting plant products, or other goods. Now, international trade in plants and plant products has become an important part of the economies of individual countries introducing more and more opportunities for the spread of pests.

Thus this important aim of any standard of plant protection and prevention is to promote fair and safe trade between countries ensuring also that it does not put in jeopardy the health and productivity of plants in the importing Countries.

Further aim is to ensure that Countries have not the tools and skills they require to protect themselves from pests that may be inadvertently introduced when people trade in plants and plant products.

• Objectives of GRAFDEPI Project

The **main objectives** of the Project:

- improvement of knowledge on epidemiological cycle of the FD disease;
- to provide guidelines for the harmonization of FD diagnostic procedures and control strategies within the EC.

The objectives have been achieved through a series of activities, covering the following **research topics**:

- studies on transmission mechanisms and dynamics of FD, with particular respect to alternative host plants and potential vectors of FD;
- inter-laboratory trials to compare different diagnostic protocols and calculate their validation parameters.
- definition of new surveillance systems for the control of the disease

- **Methods used and Results obtained**

The Project has been organized in four scientific WPs, each focused on different activity:

WP1 - Project Management and Co-ordination
WP2 - Epidemiological studies
WP3 - Validation of diagnostic procedures
WP4 - Design of surveillance systems

WP1 - Project Management and Co-ordination

Leader: Graziella Pasquini (CRA-PAV, Italy)

WP1 activity have ensured running and accomplishment of the Project activities by:

- Definition of WP leaders
- Coordination of exchanging of information and obtained data among WPs, prevalently based on e-mails
- Management of Consortium with particular regards to an Agreement aimed to regulate the participation of a private Company to the Ringtest (Annex 1).

The Project activities and the partners' interactions have been defined within the Kick-off Meeting, held in Sofia (Bulgaria) on May 7, 2012.
The Project progress has been verified with an intermediate Meeting, held in Lisbon (Portugal) on October 2nd, 2013. During this Meeting the ringtest trials have been also defined.

No major problems were encountered so that all the project objectives were achieved and the deliverables made available according to the contract, except the statistical analysis of ringtest results that will be ready within December 2014.

WP2 - Epidemiological studies

Leader: Helga Reisenzein (AGES – Austria)

Partners involved: 1, 2, 5, 6, 8, 9, 10, 11, 12, 13.

To generate testable hypothesis on transmission mechanisms and dynamics of FD in particular with respect to alternative host plants and potential vectors of FD, case and outbreak studies were performed. Outbreak investigations were performed on the basis of the guidelines from the American Centers for Disease Control and Prevention of human diseases.

In the frame of an environment analysis the presence of wild plants as reservoir for the phytoplasma and potential new vectors were monitored with a defined sampling and monitoring plan in the vineyards and in the surroundings. The presence of FDp had to be verified by lab testing.

From the gathered data new insights were gained with regard to the epidemiology of FD.

A broad range of different wild plants were tested for the presence of FDp, but only *Clematis vitalba, Alnus glutinosa*, and *Ailanthus altissima* were those wild host plants which seem to play a potential role as reservoir for FDp and as a source of infection for new outbreaks. *Clematis vitalba*, a geographically widespread plant and frequently found in the vicinity of the vineyards, was detected in several countries to harbor the phytoplasma. Taking into account that it is also the only known wild plant displaying symptoms, we could now assume that it is the most frequent and important reservoir host plant for FDp.

For defining new potential vectors, insects were caught in vineyards and surroundings in Italy, Spain, Switzerland, Slovenia and Austria.

Among all analyzed insects five different insect species were identified to harbor FDp: *Scaphoideus titanus, Orientus ishidae, Oncopsis alni, Phlogotettix cyclops* and *Psylla alni*.

It is the first finding of FDp in *P. cyclops* and *P. alni*. Psyllids are known to transmit fruit tree phytoplasmas, but it is the first time that FDp could be detected in insects of the genus *Psylla*. It is also the first report of FDp in *P. cyclops*, an invasive species in Europe. It is known that *Oncopsis alni* is the vector for Palatinate grapevine yellows (the causal FDp strain is Palatinate grapevine yellows 16SrV-C), but for the first time it has been demonstrated to harbor also other FDp strains.

Having regard to the taxonomic status of these insect species we are now aware of two new potential vectors (*P. cyclops* and *O. ishidae*) belonging to the same family as *S. titanus* (*Cicadellidae*), the main vector of FD.

In contrast to the alternative host plants we could not build a clear picture of the epidemiological relevance of all these potential vectors.

At present *Dictyophara europaea* is the only known leafhopper beside *S. titanus*, which is able to transmit FDp from *Clematis* plants to grapevines. In this study the epidemiological role of *D. europaea* could not be clarified. In Spain it occurred frequently, but none of the captured individuals were infected. In the eastern countries like Italy, Slovenia and Austria this insect was rarely captured and it was also not infected by FDp.

O. ishidae was frequently found in Italy, Switzerland, Slovenia and Austria. It could be captured on *Salix* species, *Coryllus avellana* and *Alnus glutinosa*, but also in vineyards. In Slovenia and Austria it could be shown that *O. ishidae* frequently harbored the phytoplasma. Nevertheless, it is still not clear if *O. ishidae* has a transmission capability for FDp to grapevine.

Three insect species were defined as new potential vectors for FDp:
- *Phlogotettix cyclops* and *Psylla alni* in Austria
- *Oncopsis alni* in Slovenia.

The high infection rate of *P. cyclops* and *P. alni* indicates a possible role of these insects as phytoplasma vector. *P. cyclops* belongs to the *Deltocephalinae*, which include many phytoplasma vectors, and is closely related to the genus *Scaphoideus*. As polyphagous leafhopper that is known to feed also on grapevine, it might be a potential candidate for the transmission of FDp within the vineyards.

P. alni is a monophagous species on *Alnus glutinosa* and *A. incana*. Therefore it might be together with *O. alni* the missing link for the spread of FDp in alder trees.

To summarize, the monitoring activities revealed that

- FDp is rarely detected in individuals of *S. titanus*, although FDp is present in the vineyards.
- Hence, there is increasing evidence that several other insect vectors than *S. titanus* are also important for the initial outbreak of the disease and in the case of *P. cylops* for the spread within a vineyard.

Drawing on these gathered data a refined hypothesis including the role of wild plants and alternative vectors was elaborated and an improved surveillance system for the control of the disease was derived (WP 4).

Case investigations were done by FDp strain characterization of selected samples. For this purposes grapevines, different wild plants and insects commonly present inside or outside the vineyards were tested with specific diagnostic procedures to verify FDp presence (Annex 2). For the molecular characterization of FDp isolates the 16S gene and *Sec*Y gene were used to define the FDp strains and to get insights into the variability of these genes.

One case investigation from the isle of Ischia (Southern Italy) demonstrated that this isolated disease outbreak was originated by long distance movement of the phytoplasma and the vector.

FDp isolates from Italy and Austria revealed on the SecY gene two different profiles (a mixed profile of the “c” and “d” type). This FD-C type was found in FDp samples from Tuscan and Styria and was previously undescribed. The variability on the SecY can provide information on new emerging FDp strains.

Ultimately, distribution maps of FDp strains in grapevines and other hosts were generated to get an overview on the prevalence and distribution of FDp strains related to the geographic origin and to the host (Fig. 1).

All details on epidemiologica data are reported in the Annex 3.

Data on local and international varieties susceptibility were also collected from involved Partners based on visual inspections and on an evaluation scheme (class 1= no disease, class 2= 1-10%; class 3= 11-25%, class 4 = 26-50%, class 5 = 51-100% of leaf surface area and/or bunches affected per plant).

These data allowed to release a list of varieties susceptibility against FD and BN (Annex 4).

Figure 1 - Map of FDp strains in grapevines

WP3 - Validation of diagnostic procedures

Leader: Marianne Loiseau (ANSES, France)

Partners involved: 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14 15.

The goal of this WP was to obtain diagnostic protocols with validation parameters, according to UNI CEI EN ISO/IEC 17025, for the harmonization of FD detection within the EC.

3.1 *Participants*

Fourteen Partners were involved in the ringtest (Tab. 1). Each of them have chosen the protocols to be tested in their laboratory.

Partner number	Institution	Country
1	CRA-PAV	Italy
2	AGES	Austria
3	CRA-W	Belgium
4	PPRS	Turkey
5	INIAV	Portugal
6	ACW	Switzerland
7	ILVO	Belgium
8	DISTA	Italy
9	DISAA	Italy
11	IPEP	Serbia
12	NIB	Slovenia
13	IRTA	Spain
14	ANSES	France
15	CRA-VIT	Italy

Table 1 – List of Partners involved in the interlaboratory trials

3.2 Samples

An identical series of 24 blind samples target and no target, provided by several partners, has been sent to each lab (Tab. 2).

Among no target samples also grapevine infected by bois noir (BN) have been included. BN is a grapevine disease, symptomatically not distinguishable from FD, induced by a phytoplasma (‘*Candidatus* Phytoplasma solani’), belonging to 16SrXII group.

The tested samples were constituted by extracted DNAs to avoid problems of homogeneity and stability.

Origin	Details	16SrV status	FD status	BN status
JKI Germany	Palatinate grapevine yellows 16SrVC	1	0	0
DipSA USA	Aster yellows 16SrI-B	0	0	0
ANSES France	‘Ca. P. solani’16SrXII	0	0	1

Origin	Details	16SrV status	FD status	BN status
CRA-PAV Italy	CRA-PAV healthy certified material	0	0	0
DipSA USA	*Ca.* P. fraxini 16SrVII	0	0	0
DipSA Italy	FD-C	1	1	0
AGES Austria	FD-C	1	1	0
ANSES France	'Ca. P. solani'16SrXII	0	0	1
INRB Portugal	FD-D	1	1	0
DipSA Italy	16SrV-E	1	0	0
ANSES France	healthy grapevine	0	0	0
NIB Slovenia	FD-D	1	1	0
ANSES France	FD diluted at 1/2 into healthy grapevine	1	1	0
ANSES France	Mixed infection (FD + BN)	1	1	1
ANSES France	FD	1	1	0
ACW Swistzerland	mix of FD infected samples	1	1	0
NIB Slovenia	Healthy grapevine	0	0	0
DipSA Italy	Western X grapevine 16SrIII	0	0	0
DipSA China	16SrV-B	1	0	0
ANSES France	FD+ diluted at 1/5 into healthy grapevine	1	1	1
DipSA Europe	ULW 16SrV-A	1	0	0
ANSES France	mix of healthy grapevine	0	0	0
IPEP Serbia	FD	1	1	0
ANSES France	FD	1	1	0

Table 2 – List of tested samples

3.3 Protocols

Seven molecular protocols were submitted to the interlaboratory trials (Tab. 3):

Method N°	Type of amplification		Primers	Disease detected	Number of partners involved
1	Conventional PCR	Universal direct-PCR + 16SrV specific nested-PCR	- P1 (Deng & Hiruki, 1991) /P7 (Schneider *et al.*, 1995); - R16(V)F1/R1 (Lee *et al.*, 1994)	FD	14
2		Multiplex nested-PCR	- FD9f1/r1 (Daire *et al.*, 1997); - STOL11f2/r1 (Daire *et al.*, 1997); - FD9f3b (Clair *et al.*, 2003)/ FD9r2 (Angelini *et al.*, 2001) - STOL11f3/r2 (Clair *et al.*, 2003)	FD + BN	13
a		Universal direct-PCR + universal nested-PCR + RFLP (*Taq*I)	- P1 (Deng & Hiruki, 1991) /P7 (Schneider *et al.*, 1995); - M1 (Gibb *et al.*, 1995)/B6 (Padovan *et al.*, 1995)	FD-C/ FD-D	6
3	Real time PCR(*)	Simplex	Angelini *et al.*, 2007	FD + BN	7
4			Hren *et al.*, 2007	FD + BN	10
5		Triplex	Pelletier *et al.*, 2009	FD + BN	8
6			Oligonucletides under patent (IPADLAB) Durante *et al.*, 2012	FD + BN	9

(*) The partners involved in the evaluation of the real-time PCR methods were invited to determine the cut-off value with methodology proposed by Mehle *et al.*, 2013 with the same batch of samples, specially received and the same plate plans. The DNA extracts should be amplified in 2 tubes because it became a standard for the molecular biology methods.
All real time PCR protocols included an hendogenous control.

Table 3 – Protocols submitted to the interlaboratory trials

3.4 Analysed validation data

Protocols performance criteria were calculated according with the UNI CEI EN ISO/IEC 17025. The following parameters were calculated:

3.4.1 Analytical specificity

The total number of true positives (TP, a positive result is obtained when a positive result is expected), true negatives (TN, a negative result is obtained when a negative result is expected), false positives (FP, a positive result is obtained when a negative result is expected) and false negatives (FN, a negative result is obtained when a positive result is expected) were determined for each laboratory and each method.

Some indeterminate results (i.e. the operator was unable to determine the status of the sample) were reported by some laboratories. The percentage of those indeterminate results on the total number of results by methods was calculated.

The parameter calculations were performed for each method according the recommendations of EPPO Standard PM7/98.

The **accuracy** is the proportion of accords between the results obtained with a tested method and reference results on identical samples:

$$AC = 100 \times (PA+NA) / (NA+PA+PD+ND);$$

The **diagnostic sensitivity** is the capability of the tested method to detect the contaminated samples (based on the positive samples):

$$SE = 100 \times PA / (ND + PA);$$

The **diagnostic specificity** is capability of the tested method to not detect the non contaminated samples (based on the negative samples):

$$SP = 100 \times NA / (NA+PD)$$

Results

Some results have been removed because laboratories have encountered problems in the implementation of protocols:

- For method 1: the results of partner 4 because the protocol was not respected;
- For method 2: the results of partner 6 because all samples were positive although the test was repeated and the controls were compliant.
- For method a: the results of partner 5 because the RFLP analysis was not possible.
- For methods 5 and 6: the results of partner 7 because there was a problem in the double detection of FAM and VIC and the results of partner 13 because some DNA extracts were diluted before amplification.

The results of analytical specificity were summarized in the table 4.

	Method 1	Method 2	Method a	Method 3	Method 4	Method 5	Method 6
Total of results	**312**	**288**	**120**	**168**	**240**	**144**	**168**
Total TN	98	97	41	39	80	42	55
Total TP	171	144	64	91	143	87	99
Total FN	16	28	8	14	4	2	0
Total FP	13	8	3	20	5	3	0
Indeterminate	4.49%	4.51%	8.33%	6.55%	3.75%	5.56%	4.76%
Accuracy	**90.27%**	**87%**	**90.52%**	**79.27%**	**96.12%**	**96.27%**	**100%**
Diagnostic Sensitivity	**91.44%**	**83.72%**	**88.89%**	**86.67%**	**97.28%**	**97.75%**	**100%**
Diagnostic Specificity	**88.29%**	**92.38%**	**93.18%**	**66.10%**	**94.12%**	**93.33%**	**100%**

Table 4 - Analytical specificity of methods for the detection of 16SrV phytoplasmas group

The best performances were obtained with the three last real-time PCR methods. For those methods, the accuracy was superior to 95%. The best analytical specificity (100%) was obtained with the method 6 (Primers and probes under patent IPADLAB).

The low performances of the method 3 were due, partially, to the positive detections of the two samples “e” (Ca. P. fraxini - 16SrVII) and “r” (Western X grapevine - 16SrIII) by, respectively, four and six laboratories on seven participants to this test.

The analytical specificity of two conventional PCR methods, method 1 and method a, was superior to 90%.

Except for method “a”, none of the methods was able to distinguish the Flavescence dorée phytoplasma of the other phytoplasmas of the 16 SrV group. The results obtained during this collaborative study have confirmed this fact. For those methods results were not synthesized.

However, the results of the method “a” were not consistent because some of laboratories were unable to produce results and it seemed that all laboratories did not performe the assay according to the instructions (for recommended plate plan and/or interpretation of results).

3.4.2 Analytical sensitivity

The analytical sensitivity is the the minimum detectable concentration of the analyte. In the case of non culturable pathogens, as phytoplasmas, it can not be calculated because the initial level of contamination of samples used for this evaluation is not available.

To approximate the analytical sensitivity of methods, three values were provided for each method.

The sensitivity score is an arbitrary score. One point corresponds to a positive result for one repetition of one dilution level of one of the three samples used for this evaluation. Because three samples at five dilution levels in five repetitions in five laboratories were tested for this parameter, the maximum sensitivity score was 375 for a method.

The last dilution level with 100% positive results and the last dilution level with, at least, one positive result were provided for each sample and for each method.

Only five partners were involved in the evaluation of the analytical sensitivity of the methods.

Results

The results of analytical sensitivity of methods 5 and 6 of partner 7 were removed of the final analysis because of a problem in the double detection of FAM and VIC.

The results of analytical sensitivity were summarized in the Tab. 5.

		Method 1	Method 2	Method a	Method 3	Method 4	Method 5	Method 6
Sensitivity score 1 (max score = 375)		277	121	116 (1)	309	325	286(2)	266(2)
Sensitivity score 2 (Sensitive score 1/max score for each method)		0.74 (277/375)	0.32 (121/375)	0.73 (116/150)	0.82 (309/375)	0.87 (325/375)	0.95 (286/300)	0.88 (266/300)
Last level at 100% positive results	**sample A**	less than 1/10	less than 1/10	less than 1/10	less than 1/10	less than 1/10	1/2700	1/300
	sample B	less than 1/10	less than 1/10	less than 1/10	less than 1/10	less than 1/10	1/100	1/10
	sample C	less than 1/10	less than 1/10	less than 1/10	less than 1/10	less than 1/10	1/2700	1/100
Last level with positive results	**sample A**	1/2700	1/900	1/2700	1/2700	1/2700	1/2700	1/2700
	sample B	1/2700	1/2700	1/2700	1/2700	1/2700	1/2700	1/2700
	sample C	1/2700	1/2700	1/2700	1/2700	1/2700	1/2700	1/2700

(1) For this method, only two laboratories gave results and then, the best score should be 150.

(2) For those methods, four laboratories gave interpretable results and then, the best score should be 300.

Table 5 - Analytical sensitivity of methods for the detection of 16SrV phytoplasmas group

3.4.3 Repeatability and reproducibility

The **repeatability** is defined as the percentage chance of finding the same result from two identical samples analyzed in the same laboratory.

To evaluate the repeatability from the results of this study, the probability that two samples gave the same result was calculated for each sample, at each level and each partner in turn, and this probability is then averaged over all laboratories.

The **reproducibility** is defined as the percentage chance of finding the same result for two identical samples analyzed in two different laboratories.

The reproducibility was calculated taking each replicate in turn from each participating laboratory and pairing with the identical results from all laboratories. The reproducibility was the percentage of all pairing giving the same results for all possible pairings of data.

Results

The results of repetability of methods 5 and 6 of partner 7 were removed of the final analysis because of a problem in the double detection of FAM and VIC.

The results of repetability and reproducibility were summarized in the Tab.6.

	Method 1	**Method 2**	**Method a**	**Method 3**	**Method 4**	**Method 5**	**Method 6**
Repeatability	81.65%	92.53%	77.60%	88.05%	91.04%	94.93%	88.27%
Reproducibility	73.80%	60.19%	67.73%	75.59%	84.90%	93.27%	86.73%

Table 6 - Repeatability and reproducibility of methods for the detection of 16SrV phytoplasmas group

The best values of repeatability were obtained with methods 2, 4 and 5 for which the repeatability was superior to 90%.

The best value of reproductibility (93.27%) was obtained with method 5 (Pelletier *et al.*, 2009).

The worst value of reproducibility was obtained with method 2 which was really surprising regarding the good results of repeatability. However, this fact was already reported in the network of French laboratories during the last proficiency test.

3.4.4 Conclusions

The Tab. 7 summarizes the performances for the detection of 16SrV phytoplasmas group of the different methods evaluated during this ring-test.

	Conventional PCR			Real time PCR			
	Method 1	**Method 2**	**Method a**	**Method 3 single**	**Method 4 single**	**Method 5 triplex**	**Method 6 triplex**
Targeted area of the genome	**16SrDNA**	**SecY gene**	**16SrDNA**	**16SrDNA**	**SecY gene**	**map gene**	**gene rpl14**
Nb of laboratories	13	12	5	7	10	6	7
Accuracy	90.27%	87%	90.52%	79.27%	96.12%	96.27%	**100%**
Diagnostic Sensitivity	91.44%	83.72%	88.89%	86.67%	97.28%	97.75%	**100%**
Diagnostic Specificity	88.29%	92.38%	93.18%	66.10%	94.12%	93.33%	**100%**
Repeatability	81.65%	92.53%	77.60%	88.05%	91.04%	**94.93%**	88.27%
Reproducibility	73.80%	60.19%	67.73%	75.59%	84.90%	**93.27%**	86.73%
Possibility to detect 16SrXII phytoplasmas group	no	yes	yes	yes	yes	yes	yes

Table 7 - Performances of methods for the detection of 16SrV phytoplasmas group

All details of ringtest trials are reported in Annex 5.

During GRAFDEPI project, no statistical analysis of the data was possible. Therefore, the results presented in this report should be interpreted with precautions because in the absence of concrete technical errors, some suspected outliers have not been removed of the analysis.

WP4 - Design of surveillance systems

Leader: Piero Attilio Bianco (DISAA, University of Milan, Italy)

The activity of WP4 was mainly based on the results coming from WP2 and WP3 on new scientific knowledge regarding alternative FD control strategies.

Data obtained from WP2 has been considered in order to establish the risk connected with new phytoplasma reservoir plants and possible insect vectors in spreading of the disease.

Activity of WP3 was dedicated to validate diagnostic protocols and to individuate suitable analytic tests to be used in different monitoring situation (commercial orchards, nurseries, mother plant fields, symptomatic and asymptomatic samples).

On the basis of these data, surveillance schemes are here below outlined with the aim to harmonize the containment of disease within the EC.

The design of surveillance schemes aimed to prevent the introduction of alien pathogens and the spreading of native pests is valid also for Flavescence dorée (FD). The disease in EU is so far present in several areas where viticulture is an economically important crop such as France, Italy, Spain, Portugal and most of the Balkan Countries (Tab. 8 and 9).

The prevention of phytoplasma introduction and spread is based on the utilization of healthy plant material and its maintenance, controlling the vector population in vineyard and hampering possible infections from outside, in particular from those uncultivated areas surrounding the vineyard. The presence of *D. europea* in fact should be taken in consideration and carefully monitored while no evidence are so far available for *Orienthus ishidae*, "carrier" of the phytoplasma agent of FD but not demonstrated as its vector.

Concerning the FDp plant sources are confirmed *Clematis vitalba*, *Alnus glutinosa* or *A. incana,* and *Ailanthus altissima.*

Extremely interesting are the results related to the insects, possible vector of FDp in addition to *S. titanus* and *D. europea*. In particular the detection of FDp in *Orientus ishidae* (confirmed), *Oncopsis alni, Phlogotettix cyclops* and *Psylla alni* (new finding of this project) will allow to project suitable experiments in order to evaluate the role of this species in the FD spread.

In addition, despite to the rare finding of *D. europea* its presence should be taken in consideration and carefully monitored.

The monitoring activities for FD surveillance should be distinguished in 2 different plans:

- the regional level (Country, Region, District etc)
- the farm level.

Even if the latter one is extremely important it deserves a specific consideration, with the aim to define fine-tuned and tailored measures.

The aim of this WP is to supply general rules to be used for designing of surveillance systems based on new and latest epidemiological data. Then to project novel strategies for FD containment based on lower impact measures.

4.2 Surveillance scheme

The following aspects have been considered:

- Sampling plan (period, number of samples, matrices, etc.)
- Diagnostic protocols
- Monitoring of phytoplasma and vectors distribution

4.2.1 Sampling plan

The sampling campaign is usually accompanied to the symptom observation in the frame of the in field monitoring activities carried out by the Country and Regional Phytosanitary Services. The surveillance measures should be performed also before the symptom' appearance or case of asymptomatic plants (i.e. rootstocks, tolerant varieties, latent infection etc). It is well known that phytoplasmas have unequal distribution in planta and seasonal variability in phytoplasma concentration. In addition, the tolerance to the phytoplasma presence is probably related to the low titre of FDp in the grapevine plant.

In addition, the late season sampling (after the grape harvest) is to be avoided because the higher Taq polymerase inhibitor content in the leaves. Then, sampling

time to the best period according with the phenological stage The better period is from the veraison to the grape harvest.

Leaves at the lower part of the cane is the better sample to collect. Then, vein leaf separation from the lamina is the preliminar operation to be done in laboratory in order to obtain the phytoplasma enriched tissues such as the leaf phloem.

No reliable results were obtained when phloem from dormant cane was used as matrix.

The number of leaf sample to collect should evaluated on the basis of the number of the grapevine plants, its number per hectare and the presence of possible non-grape hosts (see WP2, *Clematis vitalba*, *Alnus glutinosa* and *Ailanthus altissima*) in the vicinity or surrounding the vineyard.

The Austrian approach here below reported represents an interesting and adaptable tool for the sampling design.

The sampling design and the resulting sample size are defined in order to be appropriate for obtaining accurate, reliable result. For sampling FDp and inspection of nurseries two different strategies are applied:

a) sampling designs for randomly selected samples/nurseries
b) sampling designs for risk based selected samples/nurseries

a) Sampling designs for randomly selected samples/nurseries:

For a minimum sampling scheme for sampling of FDp in a <u>vineyard</u>, suspected to be not infested with FDp
Following parameters have to be defined:

- the number of plants within the vineyard or plot
- the confidence level (95 % or 99%)
- the sensitivity and specifity of the diagnostic method

Result of such calculation: the number of plants which have to be sampled and tested, if a confidence level of 95 or 99% has to be achieved.

For a minimum sampling scheme for inspection of <u>nurseries</u> - randomly selected - following parameters have to be defined:

- the total number of nurseries within the region
- the confidence level (95 % or 99%)
- the sensitivity and specifity of the diagnostic method

Result of such calculation: the number of nurseries which have to be checked, if a confidence level of 95 or 99% has to be achieved.

b) Sampling designs for risk based selected samples/nurseries:

There are 3 different approaches for a risk based sampling design:

1) The allocation approach – which means the distribution of the capacities (sampling and analyzing) proportional to the risk.

2) The stratification approach - the stratification of the sample proportional to the risk enables the calculation of a ratio for the total sample
3) Detection-orientated approach – to discover vineyards or nurseries with the highest risk (find the “black sheep”).

Therefore several risk factors have to be defined:
- Probability of the prevalence of the disease
- Temporal and spatial dynamics of the spread of the disease and its vector
- Potential economic impact

The choice of the approach depends on the topic under discussion and the available resources.

4.2.2 Diagnostic protocols

FDp is so far detected by molecular assays reported in the PM7/79 diagnostic protocols of EPPO (http://archives.eppo.int/EPPOStandards/diagnostics.htm).

The diagnostic protocol PM 7/79 (Grapevine flavescence dorée phytoplasma) published of the EPPO Bulletin, suggests the use of three PCR based assays as:

- Multiplex nested-PCR (for simultaneous detection of flavescence dorée and bois noir)
- Direct generic PCR followed by nested generic PCR followed by RLFP
- Direct generic PCR followed by nested group-specific PCR

The results contained in the WP3 activity report showed the different performances of the so far available protocols for FDp detection and identification. In particular the realtime PCR based procedures were found reliable and suitable for a sensitive and specific detection of the phytoplasmas agents of FD disease: 16SrV-C and 16SrV-D taxonomic subgroups.

4.2.3 Monitoring of phytoplasma/vector distribution:

FDp has been reported in several Countries in Europe (Tab. 8). The role of the propagating material in the FD spread is still under evaluation since the trasmission rate by agamic propagation (cuttings and saplings) is very low. However FDp is a quarantine pathogen and its absence from the new grapevine plantlets is required. For this reason the knowledge of the presence and the distribution of the FDp and its vector, *S. titanus*, is a fundamental information to share among the authorities involved in the grapevine plant movement in Europe and in other Countries.

Here below are summarized the information concerning FDp distribution.

Country	presence of FDp	presence of *S. titanus*
Austria	+	-
Croatia	+	+
France	+	+
Italy	+	+
Portugal	+	+
Romania	+	+
Serbia	+	+
Slovenia	+	+
Spain	+	+
Switzerland	+	+

Tab 8 - Presence and distribution of phytoplasmas agent of FD (Fdp) and its vector *S. titanus*

For the characterization of the FD phytoplamas see Annex 2.

The table here below summarizes the phytoplasma subgroups for FDp and for BNp.

Country	*phytoplasma strains*	*disease*
EU-France	16SrV-C, 16SrV-D, 16SrXII-A	Flavescence doré, Bois noir
EU-Italy*	16SrV-C, 16SrV-D, 16SrXII-A	Flavescence dorée , Bois noir
EU-Spain	16SrV-D, 16SrXII-A	Flavescence dorée , Bois noir
EU-Germany	16SrV-C, 16SrXII-A	Palatinate Yellows, VK
EU-Portugal	16SrXII-A, 16SrV-D	Bois noir, Flavescence dorée
EU-Greece	16SrXII-A	Bois noir
EU-Slovenia	16SrXII-A, 16SrI, 16SrV-C	Bois noir, Yellows , Flavescence dorée
EU-Hungary	16SrXII-A, 16SrX-B	Bois noir, Yellows
EU-Austria	16SrXII-A, 16SrV-C	Bois noir,
Croatia	16SrXII-A, 16SrV-C	Bois noir
Serbia	16SrXII-A, 16SrV-C, 16SrX-B	Bois noir Flavescence dorée, Yellows
Israel	16SrIII, 16SrXII-A	Yellows, Bois noir
USA-Virginia	16SrI-A, 16SrIII-I	Yellows
Chile	16SrI-A, I-B. I-C, 16SrVII-A	Yellows
Australia	16SrXII-B, 16SrII-A	Yellows
South Africa	16SrI-B, 16SrII-B, 16SrXII-A	Yellows, Bois noir
Switzerland	16SrXII-A, 16SrV-D	Bois noir, Flavescence dorée

* Other phytoplasmas were detected in grapevine, often in mixed infection: 16SrI-B, 16SrI-C, 16SrIII, 16SrV-A, 16SrX.

Table 9 - Phytoplasma strains detected in grapevine.

4.3. Novel strategies

Flavescence dorée is a grapevine yellows caused by a quarantine pathogen and its management is regulated by a clear frame of rules mostly included in the Country and Regional Laws. New control strategies are then to be applied taking in account the fulfilment, firstly, of the proper regulation.

Reliable protocols based of therapic treatments (chemicals, microbial and nanopartical etc) against the phytoplasma are not available, so far.

Significant and advanced information is now available for the control of the insect vector, *S. titanus* (Rigamonti *et al.,* 2011) based of a development of "supervised management" of *S. titanus* (Homoptera Cicadellidae) and describes a phenology model designed for improving the understanding of the within-vineyard dynamics and the timing of Insect Growth Regulator (IGR) applications; such model allows to limit the chemical sprays to the treatments only with IGR.

Another interesting approach has been developed in Gironde since 2007. The strategy is based on local organization named GDON (Groupement de Défense contre les Organismes Nuisibles). The GDON are associations financed by winegrowers and covering a small territory with the agreement of plant protection services. Each GDON is in charge of managing FD disease on its own territory. This is a really and interesting pattern of management organization that might be replicated in a suitable scaling-up project.

In fact, since the year 2012, 99% of winegrowers from Gironde are part of a GDON. Each GDON develops in own strategy and particularities.

The data reported by Verpy and colleagues (2013) are based on results obtained by the GDON du Libournais, which is the oldest organization in Gironde (created in 2007). The GDON du Libournais works on a territory covering 16 different towns around the Saint Emilion region, equivalent to a 12,000 acres vineyard and 1,200 different winegrowers. In synthesis, the proposed model for FD control is based on the collaboration between growers and public institutions for the definition and the establishment of a capillary network of vineyards, capable to tailor suitable treatments in relation to the severity of the disease and the entity of the *S.titanus* population. In particular the methodology in based on the localization of the plants infected by FD and BN diseases. The scouting is done by crossing vineyard by walk and by localizing suspect plants with GPS. Samples analyzed in laboratory permitted the mapping and repartition of the disease. Then winegrowers were informed of laboratory results and then suppressed the infected plants.

Interestingly, the different controls done to estimate leafhopper population permitted to count (and localize) the number of winegrowers that do not respect the insecticide spraying program. Depending on year studied, we recorded from 2 % to 5 % controls that clearly indicate failure in insecticide sprayings, which is a low rate compared to other territories without local action supported by GDON.

In addition, the reduction of obligatory insecticide sprayings range from 53 %

up to 82 % compared to sprayings strategy developed in the classical ISA. During the period 2007-2012, this insecticide reduction is equal to 70 000 acres of vineyard untreated.

Concerning the program costs, 18 € is the average amount at acre and it is essentially financed by winegrowers. The final cost depends on the importance of scouting, which is a big expense because it requires human labor.

This action carries of a resounding success with winegrowers because they prefer paying a monitoring than spraying insecticides.

Novel control strategies should include, in addition to a systemic risk analysis, the implementation of early indicators which allow the early detection of the occurrence of *S. titanus* and FD outbreaks so that appropriate measures can be taken as needed (Steffek *et al.*, 2007).

Early indicators can be derived from an intensive monitoring program. This program should encompass beside general monitoring activities in vineyards and nurseries, also a specific larvae monitoring and testing of latent infections in high risk areas. A high number of undetected pockets of latent infested grapevines result in an increasing percentage of the infected vector population and hence a further spread of the disease.

Uncontrolled vine-arbours, vine-hedges or uncultivated vineyards can act as shelter plants for the vector. Several wild plants (e.g. *Clematis vitalba, Alnus glutinosa* and *Ailanthus altissima*) are reservoir host plants for FDp and can be starting points for new outbreaks in vineyards. In the frame of the monitoring program particular focus should be given to shelter plants and reservoir host plants. Regular monitoring and testing of these plants reduce the risk of overlooked pocket of infestations and of unnoticed development of vector populations.

An intensive monitoring program increases the chance of early detection of FD outbreaks and occurrence of *S.titanus.*

Due to the difficult control of natural dissemination of the disease vector, the main management strategy should be preventing the establishment of local population of *S. titanus*. Preventive measurements are larvae monitoring and control, monitoring of vine-arbours and hedges and uprooting of untreated vineyards. A successful vector management should also include regional cooperation with transnational vine growing regions in neighboring countries to prevent the migration of the vector from infested areas and to be informed about the current situation.

The applying of a *scenario* specific pest control option with respect to its efficacy on the spread of the disease and on its cost-effectiveness is another approach for a novel control strategy.

For the development of a scenario specific control option the main factors are the initial disease and pest infestation and the occurrence of vine-arbours and hedges as disease and vector reservoir. In addition, topographic conditions like average acreage of vineyards and the percentage of organic vine growers should be taken into account. The assessment of all these parameters allow to decide in each outbreak-case on the best specific risk reduction option, both with respect to its

efficacy on the spread of GFD and on its cost-effectiveness.

- **Discussion**

The results obtained within the project GRAFDEPI are very relevant and reliable. The EUPHRESCO Project experience has been important and useful. The GRAFDEPI Consortium composed by a large number of Contries/Partners allowed to collect data from different geographical areas and phytosanitary experiences.

Regarding the **epidemiological data** the possibility to share cases and outbreak studies has allowed to define some hypothesis to define the epidemiologic cycle of FD disease.

a) In a broad range of different wild plants tested for the presence of FDp, only *Clematis vitalba, Alnus glutinosa*, and *Ailanthus altissima* resulted to be wild host plants confirming their potential role as reservoir for FDp and as a source of infection for new outbreaks.
b) Among all analyzed insects five different insect species were identified to harbor FDp: *Scaphoideus titanus, Orientus ishidae, Oncopsis alni, Phlogotettix cyclops* and *Psylla alni*.
c) Three insect species were defined as new potential vectors for FDp:
    a. *Phlogotettix cyclops* and *Psylla alni* in Austria
    b. *Oncopsis alni* in Slovenia.
d) A new distribution map of FDp strains in grapevines and other hosts have been designed, including isolates with 'mixed profiles' identified in Italy and Austria.

An important **ringtest** has been planned and performed within the Project with 14 participant labs and 7 diagnostic methods to be tested. The results showed that the most real time PCR protocols tested (Hren *et al.*, 2007; Pelletier *et al.*, 2009 and IPADLAB commercial kit) had a diagnostic sensitivity and a diagnostic specificity higher than 90%, whereas the conventional PCR protocols resulted in less sensitive and/or specific and resulted to be also less reproducible. The general view of the results leads to recommend the use of rt PCR methods in phytosanitary laboratories belonging to national and international networks. Nevertheless, no statistical analysis of the data has not yet been conducted in order to underline outliers and demonstrate statistical performances of each protocol.

On the basis of the data obtained from WP2 and WP3 and from the literature it was possible to indicate **guidelines for the definition of surveillance schemes** for FD, including:

- Sampling plan (period, number of samples, matrices, etc.)
- Diagnostic protocols
- Monitoring of phytoplasma and vectors distribution

- Novel control strategies

The expected benefits and usability of results

GRAFDEPI results could contribute to generate innovative and more sustainable and efficient control of FD, as the project results could have an important exploitation route in the quarantine, prevention and management of FD in the agro-business.

Valuable information will be transferred to NPPOs by each Partner as well as to nursery sector and, at last, to farmers.

Recommendations for future work

It is very important the updating of the results, with particular regards to diagnostic protocols. The set up of new diagnostic strategies is always evolving for new scientific and technical acquisitions. GRAFDEPI ringtest results will be the starting point for a new approved EUPHRESCO Project 'GRAFDEPI2', based on the evaluation of performance criteria of LAMP PCR applied in FD diagnosis.

Dissemination

- GRAFDEPI ringtest result will be presented within 3rd IPWG Meeting that will be held on January 14-17, 2015 in Mauritius.
  The paper 'European interlaboratory comparison of detection methods for "flavescence dorée" phytoplasma: preliminary results' has been presented as a result of 'The EUPHRESCO GRAFDEPI GROUP'.
- Scientific papers have been and will be published by single Partners

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