Development of a diagnostic protocol for Flavescence dorée phytoplasma (FDp)



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **GWT 1204**

Principal Investigator: **Dr. Fiona Constable**

Research Organisation: **Department of Primary Industries – Victoria.**

Date: **28 February 2013**

CONTENTS		Page
1.	Executive summary	2
2.	Abstract	3
3.	Background	4
4.	Visit centres of expertise for Flavescence dorée phytoplasma (FDp).	7
4.1.	Centro di Ricerca per la Viticoltura (CRA-VIT), Conegliano, Italy 16th-18th September.	7
4.2.	Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) – Patologia vegetale, Alma Mater Studiorum-Università di Bologna, Italy, 19-20th September	8
4.3.	National Institute for Agricultural Research (INRA), Bordeaux, France, during 23-26th September 2012	9
5.	Outcomes	10
6.	References	11
Appendix 1	Presentation given at each of the research facilities	
Appendix 2	Draft Subcommittee on Plant Health Diagnostic Standards (SPHDS) Flavescence dorée phytoplasma detection protocol	

1. Executive summary

Flavescence dorée, which is associated with Flavescence dorée phytoplasma (FDp), is a grapevine yellows (GY) disease that occurs in many countries throughout Europe. This disease is considered one of the most economically important diseases in European viticulture as it is associated with significant loss in production and FDp is a controlled pathogen in many European viticultural regions. FDp does not occur in Australia and this phytoplasma is a high priority pest under the industry biosecurity plan for the Australian viticulture industry.

GY diseases are associated with at least 12 different phytoplasmas worldwide. GY diseases, including Flavescence dorée, can be visually identified on the basis of symptoms but, as all GY diseases display very similar symptoms, these cannot be used to identify specific phytoplasmas that may be present. GY identification of the specific phytoplasma associated with GY disease requires molecular testing using PCR.

The Subcommittee on Plant Health Diagnostic Standards (SPHDS) enhance Australia's biosecurity by facilitating the development of reliable diagnostic protocols for priority plant pests. Protocols are developed against SPHDS *Reference Standard 2 – Development of Diagnostic Protocols – Technical Procedures*, which are consistent with International Plant Protection Convention (IPPC) guidelines for the development of diagnostic protocols.

As a part of this travel grant which was jointly funded by GWRDC and SPHDS, Dr. Fiona Constable visited centres of expertise to gather up-to-date information on Flavescence dorée phytoplasma (FDp) and access to live positive controls. The laboratories visited included the National Institute for Agricultural Research (INRA), Bordeaux, France, Centro di Ricerca per la Viticoltura (CRA-VIT), Conegliano, Italy and Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) – Patologia vegetale, Alma Mater Studiorum-Università di Bologna, Italy. Based on the information that was gathered a draft diagnostic protocol was produced for the detection and identification of FDp according to the SPHDS *Reference Standard 2 – Development of Diagnostic Protocols – Technical Procedures* and has been submitted to SPHDS for endorsement.

2. Abstract

Grapevine yellows (GY) diseases have been reported in most viticultural regions worldwide and these diseases are associated with at least 12 different phytoplasmas species. Flavescence dorée disease, which is associated with Flavescence dorée phytoplasma (FDp), is considered one of the most economically important diseases in European viticulture as it is associated with significant loss in production in many grape varieties. Significant losses due to FD disease are often associated with the incidence of *Scaphoideus titanus*, which lives and feeds solely on grapevine and is the primary vector of FDp.

FDp does not occur in Australia and this phytoplasma is a High Priority Pest under the Industry Biosecurity Plan for the Australian viticulture industry. However, GY diseases do occur in Australia and the symptomatology is identical to that of FD disease. Australian GY diseases are associated with three phytoplasma species that are distinct from one another and FDp, particularly in their biology and epidemiology. Therefore it is critical that an accurate and reliable diagnostic procedure is used for detection and identification of FDp.

A National Diagnostic Protocol for the detection and identification of FDp has been developed for the Subcommittee on Plant Health Diagnostic Standards (SPHDS) using the SPHDS *Reference Standard 2 – Development of Diagnostic Protocols – Technical Procedures*, which is consistent with International Plant Protection Convention (IPPC) guidelines for the development of diagnostic protocols. This protocol incorporates best practice methods that were identified during visits to three laboratories that have expertise in phytoplasma detection. The laboratories that were visited included the National Institute for Agricultural Research (INRA), Bordeaux, France, Centro di Ricerca per la Viticoltura (CRA-VIT), Conegliano, Italy and Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) – Patologia vegetale, Alma Mater Studiorum-Università di Bologna, Italy.

The SPHDS protocol that was developed includes a rapid CTAB based extraction procedure as the primary method for nucleic acid extraction from phytoplasma infected grapevines. The protocol also includes conventional and real-time PCR tests for the generic detection of phytoplasmas and the specific detection of FDp. These methods have been ring-tested to determine their sensitivity in several laboratories in Europe and were found to be reliable for FDp detection. This protocol will enhance Australia's viticultural biosecurity by facilitating the reliable detection of FDp, if an incursion occurs. It will also assist the reliable detection of FDp during post entry quarantine if active testing for this pathogen is required.

3. Background

Grapevine yellows (GY) diseases are found in many countries world-wide and they are associated with more than 12 phytoplasma species (Constable, 2009; Table1). The epidemiology of the GY diseases can vary significantly and this is dependent on the phytoplasma species and their vectors.

The symptomatology of GY diseases is nearly identical regardless of the region in which they are found or the phytoplasma with which it is associated. However the severity of expression of GY diseases is cultivar dependent. For example, Chardonnay and Riesling are more severely affected than many other varieties whilst some phytoplasma infected rootstocks may be symptomless (Boudon-Padieu 1999).

All GY diseases exhibit most of the following symptoms:

- Irregular yellowing in white varieties or reddening in red varieties
- The yellow leaf tissue may become necrotic
- Backward curling of the leaves
- Overlapping of leaves on affected shoots
- Rows of black pustules can develop on the green bark of affected shoots
- Tips of affected shoots may die and shoots may die back
- Affected shoots fail to harden off and remain rubbery
- Flowers on affected shoots may abort
- Berries may shrivel and whole clusters of berries can be subject to early drying

Flavescence dorée (FD) is one of the most economically important GY diseases as it can quickly reach very high incidence in affected regions and is associated with significant loss in production (Caudwell 1964). FD was first described in 1957 from the Gascoyne region of France (Caudwell 1957). The disease is now known to occur in various regions of France, including Corsica, and other European countries including Croatia, Italy, Macedonia, Portugal Spain, Serbia, Slovenia and Switzerland (Boudon-Padieu 2005; Fillipin *et al* 2009; Šeruga Musić *et al* 2011).

FD is associated with the Flavescence dorée phytoplasma (FDp), which is a member of the 16SrV taxonomic group of phytoplasmas. There are three strain clusters of FDp that are differentiated based on sequence analysis of the 16SrRNA, *secY, map* and *uvrB-degV* genes (Arnaud *et al* 2007). These strain clusters include FD1 which has low genetic variability, FD2, which has no genetic variability, and FD-3, which has most genetic variability (Arnaud *et al* 2007). The proposed strain clusters also clarify the naming conventions for the FD strains reported in the literature: FD1 comprises isolate FD70 (FD2000), FD2 comprises isolates FD92 and FD-D and FD-3 comprises isolate FD-C.

Each strain differs in geographic range (Arnaud *et al* 2007). Strain cluster FD-1 occurs in France, with higher incidence in south-western regions, and also occurs in the Piemonte and Lombardia regions of Italy. Strain cluster FD-1 is less frequently detected compared to FD-2. FD-2 occurs in France, Italy and Spain. Strain cluster FD-3 occurs in Italy, particularly in the north-eastern regions, Serbia, Croatia, Macedonia and Slovenia.

Clematis vitalba and *Ailanthus altissima* are alternative hosts for the FD-3 strain cluster (Filippin *et al* 2007; Filippin *et al* 2011). FD-3 infected *C. vitalba* plants have been found in regions where FD phytoplasma has not yet been detected in grapevines. All three FDp strain clusters have also been detected in *Alnus glutinosa* and *A. incana* in Slovenia (Mehle *et al* 2011).

Table 1 Current status of molecular characterization, biology and vectors of phytoplasmas associated with grapevine yellows diseases (after Boudon-Padieu, 2003; 2005; Constable 2009)

Grapevine yellows disease	Phytoplasma name	Ribosomal group (subgroup)	Known insect vector to grapevine	Preferred host plants of vector	Alternative hosts of the phytoplasma	Occurrence
Flavescence dorée	Flavescence dorée (FD; *Candidatus* Phytoplasma *Vitis**)	16SrV (-C, -D) or EY	*Scaphoideus titanus* Ball	*Vitis* sp.	*Clematis alba*	France, Italy, Spain, Serbia, Slovenia, Switzerland
Palatinate grapevine yellows	Palatinate grapevine yellows (PGY)	16SrV or EY	*Oncopsis alni* Schrank	*Alnus glutinosa*	ND	Germany
Bois noir, Legno nero, Vergilbungskrankheit Schwarzholzkrankheit	Stolbur (STOL, *Candidatus* Phytoplasma solani*)	16SrXII-A or stolbur	*Hyalesthes obsoletus* Sign	*Convolvulus arvensis Urtica dioica*, *Ranunculus* sp., *Solanum* sp*, Lavandula* sp.	*C. arvensis U. dioica*, *Ranunculus* sp., *Solanum* sp*, Lavandula* sp.	Europe, Israel, Lebanon, Syria
Australian grapevine yellows	*Candidatus* Phytoplasma australiense	16SrXII-B	ND[†]	ND	*Maireana brevifolia*	Australia
Australian grapevine yellows	Tomato big bud (TBB)	16SrII -D	ND	ND	ND	Australia
Buckland Valley grapevine yellows	Buckland Valley grapevine yellows (BVGY)	16SrI-related or AY-	ND	ND	ND	Australia
Grapevine yellows	Aster yellows	16SrI (-B, -C) or AY	ND	ND	ND	Italy, Chile, Tunisia, Germany
North American grapevine yellows (NAGY)	Virginia grapevine yellows I (NAGY I)	16SrI-A or AY	ND	ND	*Vitis* sp Various herbaceous hosts	Virginia (US)
	Western X Virginia grapevine yellows III (NAGYIII)	16SrIII-I or WX	ND	ND	*Vitis* sp *Prunus* sp	New York (US) Virginia (US)
Grapevine yellows	chayote witches' broom phytoplasmas	16SrIII-J	ND	ND	ND	Chile
Grapevine yellows	*Candidatus* Phytoplasma fraxini	16SrVII	ND	ND	ND	Chile
Grapevine yellows	X-disease	16SrIII	ND	ND	ND	Italy, Israel
Grapevine yellows	Aster Yellows	16SrI	Suspected: *Mgenia fuscovaria*	ND	ND	South Africa
Grapevine yellows	Tentatively related to Clover proliferation phytoplasma	16SrVI	ND	ND	ND	Syria

[†]

All FDp strains are transmitted vegetatively in infected planting material or by insect vectors. They are naturally and persistently transmitted from grapevine to grapevine by the leaf hopper *Scaphoideus titanus*, which is a native species in the USA. *S. titanus* was introduced to Europe in the early-mid 20th century, through infested planting material and has subsequently moved throughout Europe, most likely on infested planting material (Caudwell 1983; Bertin *et al* 2007; Arnaud *et al* 2007). *S. titanus* lives and feeds exclusively on grapevine in Europe, and in the presence of FDp, this can result in the rapid spread of the FD disease leading to epidemics (Schvester *et al* 1969; Boudon-Padieu, 2005; Bressan *et al* 2006). Because of the epidemic nature of the FD disease, FDp is listed as an A2 quarantine pest in the EPPO region.

The plant hopper *Dictyopara europea* was also shown to transmit FDp from clematis to grapevine in glasshouse conditions (Filippin *et al* 2009). However this plant hopper does not occur frequently in vineyards and may not have a significant impact on the epidemiology of the disease (Lessio and Alma 2008). Other potential vectors include the mosaic leafhopper *Orientus ishidae,* in which all three FDp strain clusters have been detected and the leafhopper *Oncopsis alni*, in which FD-1 and FD-3 strain clusters have been detected (Mehle *et al* 2010; Mehle *et al* 2011).The role of *O. ishidae* and *O. alni* in the transmission of FDp has not been determined although both have been shown to be vectors of other phytoplasmas or phytoplasma associated diseases (Rosenberger and Jones 1978; Maixner and Reinert 1999; Maixner *et al* 2000)

FDp can be distributed through planting material. However, not all cuttings from an infected grapevine produce infected plants, which may be due to the uneven distribution of phytoplasmas in the plant (Boudon-Padieu 1999). Dissemination of FDp can occur through infected scion wood and through some rootstocks. The transmission frequency of FDp in rootstock cuttings is between 6-80% (Boudon-Padieu, 1999; Caudwell *et al* 1994). Rootstocks are often symptomless hosts. Infected vines may not show symptoms until the following year after an infection event (Morone *et al* 2007). Movement of both vectors and FDp through planting material into previously unaffected regions represents a risk of the further spread of FD disease.

FDp is a High Priority Pest under the Industry Biosecurity Plan for the Australian Viticulture industry. GY diseases, including FD, can be visually identified on the basis of symptoms, however identification of the specific phytoplasma associated with GY diseases requires molecular testing using PCR. Testing requires generic tests that detect all known phytoplasmas (Table 1) and specific tests that detect particular phytoplasma groups, species or strains. There is currently no Australian diagnostic protocol for FDp that is recognised according to international standards. This travel grant funded Dr. Fiona Constable to visit three centres of expertise to gather information about FDp and develop a national diagnostic protocol for Australia that incorporates internationally recognised diagnostic tools. The development of diagnostic protocols for FDp is consistent with *Sub-program 4 a – Sustainable Production* of the *GWRDC Five Year R&D Plan*.

Objectives of the travel were:

1. Visit centres of expertise and obtain access to live positive controls of Flavescence dorée phytoplasma (FDp). Laboratories visited included the National Institute for Agricultural Research (INRA), Bordeaux, France, Centro di Ricerca per la Viticoltura (CRA-VIT), Conegliano, Italy and Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) – Patologia vegetale, Alma Mater Studiorum-Università di Bologna, Italy.

2. Produce a diagnostic protocol for the detection and identification of Flavescence dorée phytoplasma (FDp) according to the Subcommittee on Plant Health Diagnostic Standards (SPHDS) *Reference Standard 2 – Development of Diagnostic Protocols – Technical Procedures*. These Reference Standards are consistent with International Plant Protection Convention (IPPC) guidelines for the development of diagnostic protocols. Information on SPHDS and the development of internationally recognised protocols can be accessed at http://www.padil.gov.au/Sphds/

3. Give oral presentations at INRA and CRA-VIT about the development of validated diagnostic protocols for exotic pathogens, including phytoplasmas and viruses of grapevines under Australian conditions with an emphasis on SPHDS reference standards.

4. Visit centres of expertise for Flavescence dorée phytoplasma (FDp).

Three research and diagnostic centres with expertise of Flavescence dorée phytoplasma (FDp) were visited during this travel. The purpose of each visit was to discuss the most up to date information about the epidemiology of FD disease and FDp and to identify accurate methods for detection of FDp that can be incorporated into the SPHDS national diagnostic protocol. The draft SPHDS diagnostic protocol is given in appendix 1. The three centres that were visited included:

- Centro di Ricerca per la Viticoltura (CRA-VIT), Conegliano, Italy 16th-18th September
- Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) – Patologia vegetale, Alma Mater Studiorum-Università di Bologna, Italy, 19-20th Spetember
- National Institute for Agricultural Research (INRA), Bordeaux, France, during 23-26th September 2012

A presentation that highlighted the results of previously funded GWRDC projects was given at each of the three research centres and was entitled: An update of phytoplasmas and viruses of grapevines in Australia. The presentation is attached in Appendix 1.

4.1. Centro di Ricerca per la Viticoltura (CRA-VIT), Conegliano, Italy 16th-18th September.

CRA-VIT, Conegliano is located in the Treviso region of Italy. This institute is primarily concerned with viticultural research and one of the activities is pest and disease management, including pathogen detection. Dr. Elisa Angelini has worked with grapevine phytoplasmas and viruses in Italy for more than 12 years and is the regional expert for FDp and FD disease. Discussions were held with Dr. Angelini and Mr. Luigi Miotti as to the best diagnostic tools to use for FDp detection.

- Their approach was to use a generic nested PCR test for all phytoplasmas and sequencing of the resulting PCR product for an initial diagnosis of the phytoplasma that could be infecting a symptomatic grapevine. This is especially important as all grapevine yellows diseases display similar symptoms and therefore cannot be used to differentiate the phytoplasma species that is present.
- A real time PCR using dual labelled (“Taqman”) probe is used for routine screening for FDp as it is specific, quick and less prone to false positive results compared to nested conventional PCR. However this test is slightly less sensitive than nested PCR.
- It was mentioned that while symptomless grapevines may be infected, detection of FDp in these grapevines is difficult, possible due to low titre and location of the phytoplasmas.

During the visit to CRA-VIT some field visits to vineyards were undertaken. The cv. Prosecco is grown extensively in the Treviso region and this cv. is highly susceptible to FDp but is also likely to experience recovery from disease. The definition of recovery is an affected grapevine must have three years without symptoms. The Prosecco vineyard that was visited had previously been severely affected by FD, but was recovering and there was little evidence of typical FD leaf symptoms. This was due to the recovery of affected vines and the mandatory removal of infected grapevines and insecticide application for control for the insect vector, which is done to prevent further spread of disease.

One of the principal problems associated with the incidence of FD diseases in this region is the presence of ‘wild’ rootstocks and vines near vineyards, especially along roadsides, and which are not owned by growers.

- These wild grapevines are a source of infection for vineyards as they host FDp and the vector *Scaphoideus titanus.*

- It was apparent that some of these wild grapevines may be infected due to the presence of grapevine yellows symptoms.

- Although FD disease is subject to official control, the responsibility for these alternative sources of FDp and *S. titanus* was unclear and they were not controlled. Consequently these wild vines present a risk of new and re-infection events to nearby vineyards.

The alternative host of FDp, *Clematis vitalba*, was also observed during the field visit. The significance of *C. vitalba* in the epidemiology of FD disease of grapevines remains unclear.

4.2. Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA) – Patologia vegetale, Alma Mater Studiorum-Università di Bologna, Italy, 19-20th Spetember

Meeting were held with several experts of phytoplasma associated grapevine diseases during the visit to DiSTA, including Professor Assunta Bertaccini, Mr. Rino Credi and Dr. Claudio Ratti.

Research activities associated to phytoplasmas diseases of grapevines at DiSTA include:

- Integrated Management of Phytoplasma Epidemics in different crop systems.

- Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health

- A multi-disciplinary research network focused on innovative tools that enhance the capacity of phytosanitary inspectors to protect European agriculture and forestry sectors from invasive pests

- Specialist support for the conduct of plant health tests and evaluations and epidemiological investigations, on phytosanitary risks related to phytopathogenic organisms.

- Research on phytoplasmas and bacteria of agricultural crops that are significant to quarantine and affect quality

- Specialist support to the Plant Protection Service regional assessments and epidemiological investigations on plant health risks associated with viruses and other quarantine pathogens

During the discussions several important aspects about the epidemiology of FD diseases were highlighted:

- Transmission of FDp in planting material occurs and is an important factor in the distribution of this phytoplasma.

- *S. titanus* is the primary vector of FDp. Control of the vector is important for preventing the dispersal of FDp and FD disease. There is some discussion amongst researchers about the possibility of other vectors.

- Nurseries can play a role in the dissemination of disease and the vector; control strategies must be in place at this level to reduce the risk of their transmission.

- Dissemination of FDp through nursery material is likely to be due to infection events in the nursery rather than transmission in cuttings from diseased grapevines.

- Re-infection events can occur in vineyards and are important in the incidence and continuing presence of disease in vineyards.

- Although recovery of FD affected grapevines occurs, vectors can contribute to re-infection events and recurrence of disease, especially in more varieties that are more susceptible to FDp.

- Recovery from FD disease is cultivar dependent: some varieties are more susceptible than others to FD disease and FDp.

- It is possible but difficult to detect FDp in grapevines recovered from FD disease – this may be associated to location or titre of FDp.

- In vineyards it is important to spray the lower part of the vines as well as the canopy as *S. titanus* may occur on the suckers.

- When testing for FDp, insecticide sprays can interfere with the PCR test. Insecticides may be co-extracted when nucleic acid is isolated from tissue.

- Summer is the best time to test for FDp.

- To reduce costs a quick and easy CTAB based method (Boudon-Padieu *et al* 2003) is used for nucleic acid extraction for phytoplasmas detection. Some researchers think that this method also provides better quality nucleic acid than the commercial DNA extraction kits.

- Prof. Bertaccini recommended the use of the EPPO protocol for FD detection.

- Prof. Bertaccini mentioned the prospect of a new and emerging FDp strain that may be biologically different.

- Dr. Claudio Ratti has developed a quick and easy crude nucleic acid extraction procedure for phytoplasma detection in grapevines. This method allowed them to process large numbers of samples in one day. This method is also used by the local Plant Protection Service for routine phytoplasma detection. The extracts are used in reverse-transcription PCR. This method converts RNA to DNA and then the DNA is amplified. The reasoning behind this method is that a ribosomal RNA gene is used for phytoplasma detection and there should also be a high copy number of the rRNA, and this will increase the sensitivity of the assay. This method is not yet published, but Dr. Ratti hopes to have some ring testing done to confirm the viability of this method. The PCR method developed by Dr. Ratti is also a real-time PCR based method with the advantage of being quicker and having no post PCR processing.

Dr. Constable also visited the local plant protection organisation, who routinely screen for FD and Bois Noir (BNp) phytoplasmas in grapevines, and observed the high throughput extraction protocol for phytoplasma detection developed by Dr. Ratti. We also visited a local experimental vineyard where phytoplasma associated diseases of grapevines could be observed in many different varieties.

4.3. National Institute for Agricultural Research (INRA), Bordeaux, France, during 23-26th September 2012

During the visit to INRA meetings were held with Dr. Xavier Foissac and Dr. Sylvie Malembic-Maher. Dr. Malembic-Maher is involved in a number of research projects specifically about the epidemiology of FD phytoplasma, including a project about the interaction between FDp and *S. titanus* and FDp and grapevines. In particular there are studies to observe levels of resistance in grapevine varieties by measuring FDp titre. Dr. Malembic-Maher has also been instrumental in the development and validation of a multiplex real-time PCR based assay for the simultaneous detection and identification of FDp and BNp in grapevine. Dr. Malembic-Maher provided the current official method of FDp and BNp detection in grapevines for France – this method has been validated by ring-testing in several French laboratories and also by CRA-VIT, Conegliano, Italy.

Dr. Foissac has been involved in a more general project about the sequencing and annotation of full length genomes of many phytoplasma species of different crops, including FDp and BNp. The aim of this research is to identify regions that might be useful for diagnostic development and also to use the information to understand more about the biology of phytoplasmas and their interactions

with plant hosts and insect vectors. Dr. Foissac highlighted the need for multiple genes to taxonomically characterise phytoplasmas. However, at the current time the genes used are not consistent and may differ depending on the taxonomic group.

During the discussions several important aspects about the biology and epidemiology of FD diseases were highlighted:

- Eggs of *S. titanus* can survive hot water treatment, particularly if they are located well beneath the bark. However the phytoplasma is not transovarially transmitted in this vector and the eggs do not represent a risk for transmission of the FDp phytoplasma. *S. titanus* does not occur in Australia and the resistance of eggs to hot water treatment may represent a risk for incursion of this insect into Australia if grapevine material is not adequately treated.

- Low numbers of FDp were observed in tolerant grapevine varieties.

- There were no differences in the titre of the different FDp types in susceptible varieties.

- FDp concentration is greater at 25°C compared to 20°C; however the higher temperature was not ideal for the development of the insect vector and this could be important for the control of FDp spread. At higher temperatures eggs of *S. titanus* take longer to hatch, thereby reducing the numbers of insects emerging and slowing the life cycle.

- For phytoplasma detection a quick and easy CTAB based nucleic acid extraction method (Boudon-Padieu *et al* 2003) was used. For the specific detection of FDp a nested end-point and a real-time PCR method are used (Pelletier *et al* 2009). These methods have been validated during ring testing in several labs.

- Using FDp infectious *S. titanus*, they were able to show that FD symptoms can develop within six weeks of feeding in susceptible varieties. It was also shown that FDp could be detected as early as 5 weeks after feeding and reliably by 10 weeks.

5. Outcomes

- Dr. Constable observed FD disease first hand and, through discussions with European experts, gathered up-to-date information and improved her knowledge of the epidemiology and the best detection methods for FDp.

- The development of a draft diagnostic protocol for detection and identification of FDp that is based on the most current scientific knowledge and written in accordance with SPHDS Reference Standards (Appendix 2). This protocol provides the grape industry with the confidence of having diagnostic procedures for FD disease and FDp that are rapid, accurate and in compliance with international standards.

- Access to FDp DNA was obtained.

- Ties with other international colleagues who are studying FD epidemiology, improving detection and diagnosing FD disease and FDp were established and strengthened.

6. References

Arnaud G, Malembic-Maher S, Salar P, Bonnet P, Maixner M, Marcone C, Boudon-Padieu E and Foissac X. (2007) Multilocus sequence typing confirms the close genetic interrelatedness of three distinct Flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology* 73, 4001-4010

Bertin S, Guglielmino C, Karam N, Gomulski L, Malacrida A and Gasperi G. (2007) Diffusion of the Nearctic leafhopper *Scaphoideus titanus* Ball in Europe: a consequence of human trading activity. *Genetica* 13, 275-285.

Boudon-Padieu E. (1999) Grapevine phytoplasmas. In: First internet conference on phytopathogenic Mollicutes. http://web.uniud.it/phytoplasma//conf.html

Boudon-Padieu E. (2003) The situation of Grapevine yellows and current research directions: distribution, diversity, vectors, diffusion and control. *Extended abstracts 14th Meeting of the ICVG. Locorotondo (BARI), Italy.*

Boudon-Padieu E. (2005) Phytoplasmes de la vigne et vecteurs potentiels/ Grapevine phytoplasmas and potential vectors. *Bulletin O.I.V* 78, 299–320.

Boudon-Padieu E, Béjat A, Clair D, Larrue J, Borgo M, Bertotto L and Angelini E. (2003) Grapevine yellows: Comparison of different procedures for DNA extraction and amplification with PCR for routine diagnosis of phytoplasmas in grapevine. *Vitis* 42(3), 141-149

Bressan A, Larrue J and Boudon-Padieu E. (2006) Patterns of phytoplasma-infected and infective *Scaphoideus titanus* leafhoppers in vineyards with high incidence of Flavescence dorée. *Entomologia Experimentalis et Applicata* 119, 61-69.

Caudwell A. (1957) Deux années d'étude sur la Flavescence dorée, nouvelle maladie grave de la vigne. *Annales d'Amélioration des Plantes* 4, 359–93.

Caudwell A. (1964) Identification d'une nouvelle maladie a virus de la vigne, "la flavescence dorée". Etude des pheno-menes de localisation des symptomes et de retablissement. *Annales des Epiphyties 15 (hors-sér. 1)* p. 193.

Caudwell A. (1983) Origin of yellows induced by mycoplasma-like organisms (MLO) of plants and the example of grapevine yellows. *Agronomie* 3, 103-111.

Caudwell A, Larrue J, Tassart V, Boidron R, Grenan S, Leguay M, and Bernard P. (1994). Caracte`re porteur de la Flavescence dore´e chez les vignes porte-greffes en particulier le 3309 C et le Fercal. *Agronomie* 14, 83–94.

Constable FE. (2009) Phytoplasma epidemiology: grapevines as a model. In: Phytoplasmas: genomes, plant hosts and vectors (Weintraub and Jones, Eds). CABI, Wallingford (GB), 188-212.

Filippin L, Jović J, Forte V, Cvrković T, Toševski I, Borgo M and Angelini E. (2007) Occurrence and diversity of phytoplasmas detected in clematis and their relationships with grapevine “flavescence dorée” phytoplasmas *Bulletin of Insectology* 60, 327-328.

Fillipin L, Jović J, Cvrković T, Forte V, Clair D, Toševski I, Boudon-Padieu E, Borgo M and Angelini E. (2009) Molecular characteristics of phytoplasmas associated with ‘Flavescence dorée’ in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector.- *Plant Pathology*, 58, 826-837.

Fillipin L, De Pra V, Zottini M, Borgo M and Angelini E. (2011) Nucleotide sequencing of *imp* gene in phytoplasmas associated to ‘flavescence dorée’ from *Ailanthus altissima Bulletin of Insectology* 64 (Supplement), S49-S50

Maixner M and Reinert W. (1999) *Oncopsis alni* (Schrank) (Auchenorrhyncha: Cicadellidae) as a vector of the alder yellows phytoplasma of *Alnus glutinosa* (L.) Gaertn. *European Journal of Plant Pathology* 105, 87–94.

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

Mehle N, Seljak G, Rupar M, Ravnikar M and Dermastia M. (2010) The first detection of a phytoplasma from the 16SrV (Elm yellows) group in the mosaic leafhopper Orientus ishidae. *New Disease Reports* 22. [doi:10.5197/j.2044-0588.2010.022.011]

Mehle N, Rupar M, Seljak G, Ravnikar M and Dermastia M. (2011) Molecular diversity of 'flavescence dorée' phytoplasma strains in Slovenia. *Bulletin of Insectology* 64, S29–30

Morone C, Boveri M, Giosue S, Gotta P, Rossi V, Scapin I and Marzachi C. (2007) Epidemiology of flavescence dorée in vineyards in northwestern Italy. *Phytopathology* 97, 1422-1427.

Pelletier C, Salar P, Gillet J, Cloquemin G, Very P, Foissac X and Malembic-Maher S. (2009) Triplex realtime PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. *Vitis* 48 (2), 87-95.

Rosenberger DA and Jones AL. (1978) Leafhopper vectors of the peach X-disease pathogen and its seasonal transmission from chokecherry. *Phytopathology* 68, 782-790.

Schvester D, Carle P and Moutous, G. (1969) Nouvelles données sur la transmission de la flavescence dorée de la vigne par *Scaphoideus littoralis* Ball. *Annales de Zoologie et Ecologie Animale* 1, 445-465.

Šeruga Music M, Škoric D, Haluska I, Krizanac I, Plavec J and Mikec I. (2011) First report of 'flavescence dorée'-related phytoplasma affecting grapevines in Croatia. *Plant Disease*, 95, 353.

Appendix 1: Presentation given at each of the research facilities



An update of phytoplasmas and viruses of grapevines in Australia

Fiona Constable

Victoria
The Place To Be

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research



Overview

1. Who is DPI?
2. Phytoplasmas in Australia
3. Grapevine viruses in Australia



DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

1. Department of Primary Industries-Victoria

Agriculture, Earth Resources, Energy, Fisheries, Forestry

- Farm Services Victoria
- Future Farming Systems Research
- Agriculture and Food Industries Policy
- Office of Science Technology and Commercialisation
- **Biosciences Research**
  - Animal and Plant Biosciences



Plant Microbiology: 14 staff, 4 students

- Plant virology
- Plant bacteriology
- Molecular diagnostics
- Bioinformatics
- Climate change/Modelling
- Plant disease epidemiology
- Plant Biosecurity Services
  - **Largest provider of disease diagnostic services to Australian Temperate Agriculture**
    - Horticulture (Melbourne)
    - Grains (Horsham)

National Leaders in Plant Biosecurity Research for Temperate Agriculture

Australian Biosecurity



Natural protection from exotic threats:

- **Geographically isolated**
- **No shared land borders.**

Active protection:

- **National quarantine system.**

Biosecurity continuum:

Offshore:

- Accredited suppliers
  - Adequate testing

Border:

- Importation
  - Post entry quarantine
  - Illegal importation

Onshore:

- Incursion management
- Interstate biosecurity
- Certification

Requires rapid validated diagnostic tests for the detection of significant pathogens

1. Plant Microbiology R&D Achievements

Set the agenda for Biosecurity Research in Australia

- **National Diagnostic Protocols (E.g. ESFY, PD, Fireblight, PPV, PSTVd etc)**
- **National Diagnostic Capability**

Enhanced molecular diagnostics

- **High throughput diagnostics**
- **Superior primer design for broad-based diagnostics**

Largest provider of Biosecurity Services to temperate agriculture in Australia

- **Horticulture**
  - **Crop Hygiene and Crop Health Services**
- **Grains**
  - **PEQ Pulses**
  - **Diagnostics to support surveillance programs of cereals and pulses in SE Australia**

Accelerated access to new varieties through improved PEQ protocols

- **Pome fruit**
- **Strawberries**
- **Viticulture**
- **Pulses**

2. Phytoplasmas in Australia

- **Six 16Sr groups are certain to occur in Australia**
- **Five additional 16Sr groups may also be present**
- **Three phytoplasmas that may represent new phytoplasma 16Sr groups**

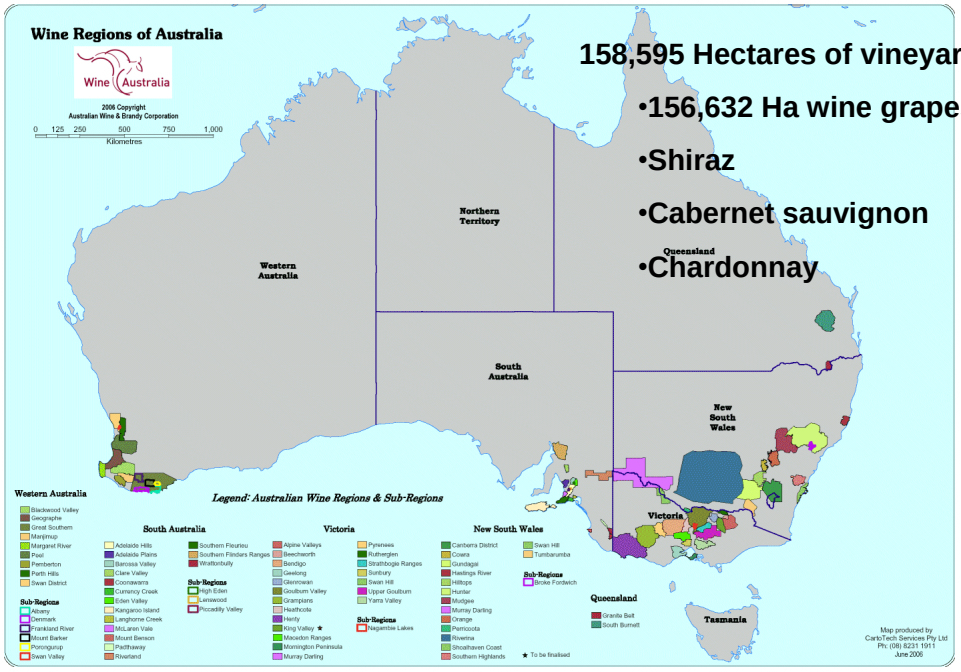
16S rDNA group-subgroup	Phytoplasma/disease
16SrII-A	Sweet potato little leaf-V4
16SrII-D	Tomato big bud • Many strains
16SrXII-B	Australian GY - '*Ca.* Phytoplasma australiense'
16SrXIV	Cynodon white leaf Sorghum grassy shoot phytoplasma variant I
16SrXXIII-A	Buckland Valley GY
16SrXXIV-A	Sorghum bunchy shoot
16SrXXV-A	Weeping tea WB
Phytoplasmas with uncertain taxonomy	
16SrI ?	Sugarcane phytoplasma (AY)
16SrIII ?	Poinsettia branching *
16SrXI?-	Sorghum grassy shoot*
16SrXXIX or IX ?	Vigna Little leaf
16SrXXIX?	Sugar cane phytoplasma
Undetermined	Allocasuarina yellows - '*Ca.* Phytoplasma allocasuarinae'
Undetermined	Stylosanthes little leaf
Undetermined	*Solanum tuberosum* phytoplasma

- **More than 70 plant genera in Australia are affected by 16SrII phytoplasmas – most frequently TBB (16SrII-D) types**
- **14 known plant hosts of *Ca.* Phytoplasma australiense (16SrXII-B) in Australia**
- **Remaining phytoplasmas have only been detected in one or a few hosts**

*Based on ITS sequence only

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

Australian viticulture



158,595 Hectares of vineyards

- 156,632 Ha wine grapes
- Shiraz
- Cabernet sauvignon
- Chardonnay

2. Characterization of phytoplasmas in Australian grapevines

Three phytoplasmas associated with Australian grapevine yellows:







'*Candidatus* Phytoplasma australiense' (16SrXII-B)

- Occurs Australia wide
- 14 known plant host species
- Found in most regions where grapes are grown
  - Less prevalent in cooler climates

Tomato big bud phytoplasma (16SrII-D)

- Occurs Australia-wide
- Less frequently detected in grapevines compared to *Ca.* P. australiense
- Broad host range – more than 70 plant species

Buckland Valley grapevine yellows phytoplasma (16SrXXIII-A)

- Limited distribution – Buckland Valley in Victoria
- No other known alternative hosts

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

2. Transmission and spread of grapevine phytoplasmas in Australia

Candidatus Phytoplasma australiense:

- No known insect vector in Australia
  - Occurs in New Zealand – transmitted by *Oliarus atkinsonii* and *Zeoliarus oppositus*
  - *Ca.* P. australiense has not been detected in grapevines in New Zealand

Tomato Big Bud (TBB) phytoplasma:

- Two known vectors in other crops:
  - Common brown leafhopper *(Orosius orientalis)*
  - Green jassid *(Batrcomorphus angustatus)*
- One suspected vector in other crops:
  - The spotted leafhopper *(Austroagallia torrida)*

Buckland Valley grapevine yellows phytoplasma

- No known vector

Phytoplasmas may also be transmitted through planting material



DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research



Development and validation of diagnostic protocols for the detection of endemic viruses of Australian grapevines

Victoria The Place To Be

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

3. A survey for grapevine viruses in Australia

Tested 218 grapevines for 34 viruses

10 endemic viruses:

Vitivirus

- ***Grapevine virus A*** **(38%)**
- ***Grapevine virus B*** **(1%)**
  - **not associated with corky bark disease**
- **A *Vitivirus* strain most closely related to GVA**

Maculavirus

- ***Grapevine fleck virus*** **(32%)**

Ampelovirus

- **GLRaV- 1 (13%), GLRaV-3 (14%), GLRaV-4 (1.3%), GLRaV-5 (9%) and GLRaV-9 (11%)**
- **Some GLRaV strains most closely related to GLRaV-4,-5 and -9**

Closterovirus

- ***Grapevine leafroll-associated virus 2*** **(6%)**

Foveavirus

- ***Grapevine rupestris stem pitting associated virus*** **(94%)**

Detection is affected by strain variation



24 viruses significant to quarantine:

- **2 GLRaV-6, GLRaV-7, GRGV, 2 tombusviruses, 15 nepoviruses, SLRSV, GVD**
- **None were detected**
- ***Grapevine fanleaf nepovirus*** **- Once occurred in isolated areas of Australia but has since been contained and eradicated**

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

2. Australian grapevine yellows disease

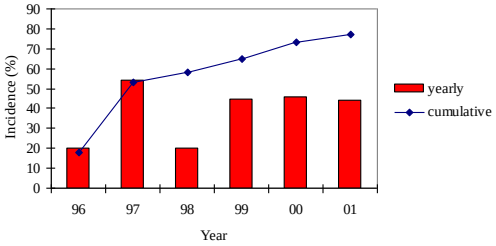
Phytoplasmas in Australian grapevines:

- **Persistant infection**
- **Unevenly distributed**
- **Low concentration**



AGY disease is less prevalent in cooler climates:

- **Occurs more frequently in hot inland areas of Victoria, New South Wales and South Australia**



AGY symptom expression is variable:

- **Within grapevines from year to year**
- **Within and between vineyards**
- **Symptom expression is affected by location of phytoplasmas in the tissue**

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

3. Identify optimal sampling strategies for reliable virus detection by ELISA and RT-PCR



Experimental design:

Replicated trials in two locations:

- Sunraysia (Hot) and Yarra Valley (Cool)
- 25 Shiraz and 25 Chardonnay vines:
  - un-inoculated or graft inoculated with GLRaV-2, GLRaV-3, GVA or GFkV
- Tested monthly by ELISA and RT-PCR

3. Results: Detection of grapevine viruses in Australia by ELISA and RT-PCR in two climates

- **There was no difference in detection between the hot and cool climates**
- **Virus titre can take 2-3 years to build up and allow reliable detection**
- **RT-PCR is generally more sensitive than ELISA:**
  - RT-PCR testing was most reliable using green tissue collected from December until March
    - Inhibitors and extreme weather events may influence virus detection.
- **Symptom expression:**
  - No leafroll symptoms were observed on the Chardonnay vines at both sites
  - Some of the Shiraz vines inoculated with the leafroll viruses have not displayed symptoms at either site
  - No leafroll symptoms were observed in 2009 on Shiraz at either site

3. A Comparison of woody indexing in three climatic conditions



Experimental design:

Replicated trials in three locations:

- Riverland (Hot), Yarra Valley (Cool), Knoxfield (Screenhouse)
- Biological indicators:
  - Cabernet Franc: GLRaV-1, GLRaV-2, GLRaV-3 or GLRaV-9
  - Rupestris St George': GFkV (2 strains) or RSPaV (2 strains)
  - LN33: GVB (2 strains)
  - Kober 5BB: GVA (2 strains)
- Tested by RT-PCR

3. Results: Biological indexing

Virus transmission was not always successful.

- **Affected by success of bud-take or transmission of the virus from the candidate bud to the indicator**
- **GVA was not transmitted**

Biological indexing reliability was affected by different climatic conditions:

- **Symptoms were not always observed in each year at each field trial site even if viruses could be detected by RT-PCR.**
  - **Fleck symptoms observed at the hot climate site but not at the cool climate site**
  - **Leafroll symptoms observed within six months of inoculation at the cool climate site but not at the cool climate site**
  - **In late summer 2009 extreme heat experienced at all sites – no leafroll symptoms observed**
  - **Evene though viruses could be detected, rugose wood symptoms were only observed in a few indicators associated with RSPaV infection**
- **Symptoms were not observed in the screenhouse**
- **Sometimes symptoms were observed but viruses were not detected**

It is recommended that WI be carried out for a minimum of three years in the field.

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

Comparison of diagnostic tests

E.g. Comparison of woody indexing (WI) for grapevines

- Symptoms observed but PCR negative

AND

- Symptomless but PCR positive

→ The diagnostic tests were not 100% efficient in detecting virus

We recommend:

E.g. Comparison of ELISA and PCR for grapevines

- Generally more samples were PCR positive

But

- Occasionally ELISA was positive and PCR was negative

→ • Molecular and biological tests be used in conjunction to verify the presence of a pathogen

→ • Re-testing of grapevines within certification programs

DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

Thankyou for your attention!

Grape and Wine Research and Development Corporation

Department of Agriculture, Forestry and Fisheries – Biosecurity

Department of Primary Industry Victoria

South Australian Research and Development Institute

Australian Vine Improvement Association

Victorian and Murray Valley Vine Improvement Association



DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

Appendix 2: Draft Subcommittee on Plant Health Diagnostic Standards (SPHDS)
Flavescence dorée phytoplasma detection protocol

Diagnostic protocol for Flavescence dorée phytoplasma



Prepared for the Subcommittee on Plant Health Diagnostic Standards (SPHDS)

This version of the National Diagnostic Protocol (NDP) for Flavescence dorée phytoplasma is current as at the date contained in the version control box on the front of this document.
NDPs are updated every 5 years or before this time if required (i.e. when new techniques become available).
The most current version of this document is available from the SPHDS website:
http://www.padil.gov.au/Sphds

Contents:

		Page
1	Introduction	2
2	Hosts	2
2.1	Primary host plants	2
2.2	Alternative host plants	3
3	Taxonomic information	3
4	Detection of Flavescence dorée disease and phytoplasma	3
4.1	The signs or symptoms associated with Flavescence dorée disease and phytoplasma	4
4.2	Recommended Flavescence dorée phytoplasma detection method	12
4.2.1	Quick nucleic acid extraction methods for phytoplasmas in grapevines	12
4.2.2	Conventional PCR	13
4.3	Interpretation of results	17
5	Contact points for further information	17
6	Acknowledgements	18
7	References	18
Appendix 1	Nucleic acid clean-up	22
Appendix 2	Alternative extraction methods	23
A2.1	Phytoplasma enrichment extraction method (Kirkpatrick *et al* 1987 and modified by Ahrens and Seemüller, 1992)	23
A2.2	DNA extraction procedure using the QIAGEN DNeasy® Plant mini kit (Green *et al* 1999)	25
Appendix 3	Alternative PCR detection methods	27

1. Introduction

The Flavescence dorée phytoplasma (FDp) is a member of the 16SrV taxonomic group. Three strain clusters of FDp have been recognised based on sequence analysis of the 16SrRNA, *secY, map* and *uvrB-degV* genes (Arnaud *et al* 2007). These strain clusters include FD1 which comprises isolate FD70 (FD2000) and has low genetic variability, FD2, which comprises isolates FD92 and FD-D and has no genetic variability, and FD-3, which comprises isolate FD-C and has more genetic variability (Arnaud *et al* 2007).

The three FDp strains differ in their geographic range and possible region of origin and perhaps in their means of dispersal (Arnaud *et al* 2007). Strain cluster FD-1 occurs in France, with higher incidence in south-western regions, and in the Piemonte and Lombardia regions of Italy. FD-2 is the most commonly detected strain cluster in France and is also detected in Italy and Spain. Strain cluster FD-3 has been detected in Italy, particularly in the north-eastern regions, Serbia and Slovenia.

Clematis vitalba is an alternative host for FD-3 and infected *C. vitalba* plants were found in regions where FDp infected grapevines have not been observed (Filippin *et al* 2007). Phytoplasmas associated with Palatinate GY (PGYp) in Germany and alder yellows phytoplasma (AldYp) also belong to the 16SrV group and have a high sequence similarity with strains of FDp (Arnaud *et al* 2007). It has been proposed that FDp, PGYp and AldYp have a common origin in Europe.

All FDp strains are transmitted vegetatively in infected grapevine planting material or by insect vectors. They are naturally and persistently transmitted from grapevine to grapevine by *Scaphoideus. titanus*. *S. titanus* was introduced to Europe from the USA in the early-mid 20th century, and has moved throughout Europe, most likely on infested planting material (Caudwell 1983; Bertin *et al* 2007; Arnaud *et al* 2007). *S. titanus* lives and feeds exclusively on grapevine in Europe. The rapid spread and epidemics of FD disease occurs when *S. titanus* occurs in the presence of FDp (Schvester *et al* 1969; Boudon-Padieu 2005; Bressan *et al* 2006). Because of the epidemic nature of the FD disease, FDp is listed as an A2 quarantine pest in the EPPO region.

FD-3 strains have been detected in *Dictyophara europaea* and this leafhopper was shown to transmit FDp from clematis to grapevine in glasshouse conditions (Fillipin *et al* 2007; Filippin *et al* 2009). However this insect does not occur frequently in vineyards and may not have a significant impact on the epidemiology of the disease (Lessio and Alma 2008). All three FDp strain clusters have been detected in the mosaic leafhopper *Orientus ishidae.* FD-1 and FD-3 strain clusters have also been detected *Oncopsis alni* (Mehle *et al* 2010; Mehle *et al* 2011).The role of *O. ishidae* and *O. alni* as vectors is unknown.

2. Hosts

2.1 Primary host plants

The primary host of FDp is *Vitis vinifera*. Whilst all *V. vinifera* cultivars can be infected some differ in their susceptibility/tolerance. Varieties such as Barbera, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Pinot noir, Pinot Gris, Riesling, Sangiovese, Soave and Prosecco are sensitive and Nebbiolo, Merlot, Sauvignon Blanc and Syrah are more resistant (Pavan *et al* 1997; Boudon-Padieu 1999; Belli *et al* 2000, Kuzmanovic *et al* 2003); Vercesi and Scattini 2000; Sancassani and Posenato 1995), *Vitis riparia, V. labrusca* and other rootstocks e.g. 420A (*Vitis* berlandieri x *V. riparia*) and Millardet (*Vitis* interspecific hybrid) may also host this phytoplasma (Maixner and Pearson 1992; Borgo *et al* 2007). Rootstocks may be symptomless hosts and these are considered to play an important role in the dissemination of phytoplasmas into previously unaffected regions (Boudon-Padieu 1999).

2.2 Alternative host plants

Alternative hosts include *Clematis vitalba,* in which only the FD-3 strain cluster has been detected (Angelini *et al* 2001; 2004; Filippin *et al* 2009; Filippin *et al* 2007). All three FDp strain clusters have also been detected in *Alnus glutinosa* and *A. incana* (Cvrković *et al* 2008; Mehle *et al* 2011) and FD-3 has been detected in *Ailanthus altissima* (Fillipin *et al* 2011).

3. Taxonomic information

The taxonomic classification of the phytoplasma associated with Flavescence dorée is:

Bacteria; Firmicutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae; *Candidatus* Phytoplasma; 16SrV (Elm Yellows group); Flavescence dorée phytoplasma.

Three strain clusters of FDp have been recognised based on sequence analysis of the 16SrRNA, *secY, map* and *uvrB-degV* genes (Arnaud *et al* 2007). These strain clusters include FD1 which comprises isolate FD70 (FD2000) and has low genetic variability, FD2, which comprises isolates FD92 and FD-D and has no genetic variability, and FD-3, which comprises isolate FD-C and has more genetic variability (Arnaud *et al* 2007).

Several common names alternative names occur in the literature and include:

Baco 22A disease (English); Flavescencia dorada (Spanish); Flavescenza dorata (Italian); Grapevine golden Flavescence

4. Detection of Flavescence dorée disease and phytoplasma

The presence of symptoms may indicate FD disease, however FD disease symptoms are identical to symptoms of grapevine yellows diseases associated with other phytoplasmas, including those that occur in Australia. Diagnosis of FD disease should be confirmed through PCR detection and sequencing of the 16S rRNA gene of FDp and specific detection and sequencing of other FDp genes including SecY. Most symptoms, particularly if they are observed on their own, may be caused by other biotic and abiotic factors.

Symptomless infections can occur and if this is suspected it is important to thoroughly sample phloem tissue from different shoots of the one plant for phytoplasma detection.

FDp is phloem-limited, however it may infect the phloem tissue of all parts of a grapevine, including roots, trunk, branches and shoots. Phytoplasmas can be unevenly distributed and in uneven titre throughout woody hosts, and symptomatic tissue is optimal for phytoplasma detection (Berges *et al* 2000; Christensen *et al* 2004; Constable *et al* 2003; Necas and Krska 2006).

The location and titre of phytoplasmas in grapevines may be affected by seasonal changes and therefore the timing of sample collection for phytoplasma detection is important (Constable *et al* 2003). In Europe the best time and tissue type for FDp detection is summer and symptomatic tissue is most reliable (Del Serrone and Barba 1996.).

Vascular tissue from symptomatic plant material provides the best opportunity to detect phytoplasmas in grapevines. Leaf petioles, mid veins from symptomatic leaves and bark scrapings from shoots and branches can be used from actively growing plant hosts. If the plant is dormant, buds and bark scrapings from branches, trunk and roots can be used, although these are less reliable for FDp detection. If using bark scrapings from woody material remove the dead outer bark layer, to reveal the green inner vascular tissue.

4.1 The signs or symptoms associated with Flavescence dorée disease and phytoplasma

The following symptoms associated with FDp are often observed on:

FD affected *Vitis vinifera*:

- Irregular yellowing in white varieties or reddening in red varieties (Figures 1-4, 6-9)
- The yellow leaf tissue may become necrotic (Figure 4)
- Backward curling of the leaves (Figures 1-4, 6-9)
- Overlapping of leaves on affected shoots (Figure 6)
- Rows of black pustules can develop on the green bark of affected shoots
- Tips of affected shoots may die and shoots may die back (Figure 3)
- Affected shoots fail to harden off and remain rubbery
- Flowers on affected shoots may abort
- Berries may shrivel and whole clusters of berries can be subject to early drying (Figure 5)

Clematis vitalba:

- yellowing and reddening and rolling of leaves (Figure 10-11)
- may be symptomless

Alder glutinosa (common):

- yellowing, small leaves and reduced foliage
- may be symptomless

Alder incana (grey):

- No symptoms reported

Ailanthus altissima

- Yellowing of the leaves may occur, but no clear association as some plants in may be symptomless

It is important to note that symptoms associated with FD disease and FDp are difficult to distinguish from grapevine yellows diseases associated with other phytoplasmas, including those occurring in Australia. Therefore and accurate diagnosis can only be made via the detection and identification of the specific phytoplasma that is present using molecular methods. *FDp* can be detected using a PCR based on the 16S rRNA gene with universal primers for all known phytoplasmas and identified by RFLP analysis or sequencing (Seemüller *et al* 1998). Specific primers may also be useful in identifying infection by FDp.



Figure 1 Yellowing and leaf curling associated with Flavescence dorée disease in the cv. Prosecco



Figure 2 Yellowing and leaf curling associated with Flavescence dorée disease in the cv. Prosecco



Figure 3 Leaf curling and tip death associated with Flavescence dorée disease in the cv. Prosecco



Figure 4 Irregular yellowing, necrosis and leaf curling associated with Flavescence dorée disease in the cv. Prosecco



Figure 5 Berry shrivel associated with Flavescence dorée disease in the cv. Prosecco



Figure 6 Overlapping leaves with mild yellowing and leaf curling associated with Flavescence dorée disease in an unknown white variety



Figure 7 Irregular reddening and leaf curling associated with Flavescence dorée disease in an unknown red variety



Figure 8 Irregular reddening and severe leaf curling associated with Flavescence dorée disease in an unknown red variety



Figure 9 Flavescence dorée affected wild grape in an unmanaged and overgrown area nearby a vineyard. The circled area shows a portion of the grapevine which is displaying grapevine yellows symptoms, including irregular yellowing and leaf curling.



Figure 10 Leaves of *Clematis vitalba* with yellowing and leaf curling that is associated with FDp infection



Figure 11 Leaves of *Clematis vitalba* with reddening, yellowing and leaf curling that is associated with FDp infection

4.2 Recommended Flavescence dorée phytoplasma detection method

The most reliable method for confirmation of FDp is polymerase chain reaction (PCR), which is used to detect the DNA of the phytoplasma and sequencing of the resulting PCR product. The efficiency of this test is dependent on appropriate sampling of plant tissue and reliable nucleic acid extraction methods.

4.2.1. Quick nucleic acid extraction methods for phytoplasmas in grapevines (Boudon – Padieu *et al* 2003)

Materials and equipment

1. 2 ml centrifuge tubes
2. 20-200 µl and 200-1000 µl pipettes
3. 20-200 µl and 200-1000 µl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Freezer
9. Sterile mortars and pestles or “Homex” grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
10. Scalpel handle
11. Sterile scalpel blades
12. Vortex
13. Water bath or heating block at 65°C
14. Latex or nitrile gloves
15. Buffers:
    - 3% CTAB buffer (Table 2)
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - 70% (v/v) ethanol
    - Sterile distilled water
    - Ice-cold isopropanol

Table 2. 3% cetyltrimethylammonium bromide (CTAB) buffer for DNA purification

Reagent.	**Final concentration**	**Amount needed for 1 L**
CTAB (cetyltrimethylammonium bromide)	3%	30 g
Sodium chloride	1.4 M	56 g
1 M Tris-HCl, pH 8.0 (sterile)	100 mM	100 ml
0.5 M EDTA, pH8.0 (sterile)	20 mM	40 ml
Polyvinylpyrrolidone (PVP-40)	1%	10 g

Method

Perform all operations on ice unless otherwise specified.

1. Grind 1.0 g of plant material in 10 ml of CTAB extraction buffer containing 0.2% β – mercaptoethanol.

2. Transfer 1.5 ml of extract to a 2 ml microfuge tube, close the tube. Centrifuge in a microfuge at room temperature for 10 minutes at 1000 x g.

3. Transfer 1 ml of the clarified extract to a 2 ml microfuge tube, close the tube and incubate at 65°C for 20 min, mixing gently several times.

4. Add an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and mix thoroughly but gently for 5 minutes. Centrifuge in a microfuge at room temperature for 10 minutes at 10000 x g.

5. Transfer the 750 μL of the epiphase into a new 2 ml centrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately. Centrifuge for 15 minutes at 10000 x g. Discard the supernatant and wash the pellet twice with 70% ethanol.

6. Dry the pellet under vacuum or air dry and resuspend in 400 μl of water.

Boudon-Padieu E, Béjat A, Clair D, Larrue J, Borgo M, Bertotto L and Angelini E. (2003) Grapevine yellows: Comparaison of different procedures for DNA extraction and amplification with PCR for routine diagnosis of phytoplasmas in grapevine. *Vitis* 42, 141-149.

4.2.2. Conventional PCR

Laboratory requirements

To reduce the risk of contamination and possible false positive results, particularly when nested PCR is used for phytoplasma detection, it is desirable to set up PCR reactions in a different lab to where nucleic acid extractions have been done. It is also desirable to handle PCR reagent stocks and to set up PCR reactions in a clean room or bio-safety cabinet with dedicated pipettes, PCR tubes and tips that have not been exposed to nucleic acid extracts. Use a separate pipette for the addition of nucleic acids to the PCR reactions. Do not add nucleic acid to reactions in the same clean room or bio-safety cabinet in which PCR stocks are handled.

PCR materials and equipment

1. PCR reagents of choice

2. Primers (Table 3)

3. PCR grade water

4. 0-2 μl, 2-20 μl, 20-200 μl and 200-1000 μl pipettes

5. 0-2 μl, 2-20 μl, 20-200 μl and 200-1000 μl sterile filter pipette tips

6. 1.5 ml centrifuge tubes to store reagents

7. PCR tubes (volume depends on thermocycler)

8. Bench top centrifuge – with adapters for small tubes

9. Freezer

10. Ice machine

11. Latex or nitrile gloves

12. Thermocycler

13. DNA molecular weight marker

Table 3. PCR primers used for phytoplasma detection and internal control primers during conventional PCR

PCR test†	Primer name (direction)	Primer sequence (5´-3´)	Primer annealing temperature	Product size (bp)	Reference
Phytoplasma					
Group specific primers or 16SrV EY (flavescence dorée) single or nested first stage PCR	FD9f (forward)	GAATTAGAACTGTTTGAAGACG	55°C	1,300	Daire *et al* 1997
	FD9r (reverse)	TTTGCTTTCATATCTTGTATCG			
Group specific primers or 16SrV EY (flavescence dorée) nested second stage PCR	FD9f3b (forward)	TAATAAGGTAGTTTTATATGACAAG	56°C	1,150	Claire *et al* 2003
	FD9r2 (reverse)	GACTAGTCCCGCCAAAAG			Angelini *et al* 2001
Universal phytoplasma – single or nested first stage PCR	P1 (forward)	AAGAGTTTGATCCTGGCTCAGGATT	55°C	1,784	Deng and Hiruki (1991)
	P7 (reverse)	CGTCCTTCATCGGCTCTT			Schneider *et al* (1995)
Universal phytoplasma – single PCR or nested second stage PCR	R16F2n (forward)	GAAACGACTGCTAAGACTGG	55°C	1,248	Lee *et al* (1993)
	R16R2 (reverse)	TGACGGGCGGTGTGTACAAACCCCG			
Internal control					
16S bacterial and plant chromosomal	FD2	AGAGTTTGATCATGGCTCAG	55ºC	approx. 1,400-1,500 bp.	Weisberg *et al* (1991)
	RP1	ACGGTTACCTTGTTACGACTT			

Polymerase Chain Reaction

The housekeeping PCR, using the components and concentrations listed in Table 4, is done prior to conducting the phytoplasma PCR, to determine if the nucleic extract is of sufficient quality for phytoplasma detection. The cycling times are listed in Table 6. Electrophoretic analysis of the resulting PCR products is performed as described below. The house keeping PCR is successful if a product of the expected size is observed, indicating the presence of quality DNA in the nucleic acid extract. If no product is observed the nucleic acid extract should be cleaned up or the sample should be re-extracted and a housekeeping PCR conducted on these extracts. If the housekeeping PCR is successful the PCR reactions for phytoplasma detection can be done.

For FDp detection a nested PCR using the specific primers FD9f/FD9r and FD9f3b/FDr2 (Table 3). should be conducted. These primers amplify the SecY gene of FDp and several other phytoplasma species in the 16SrV (Elm yellows) phytoplasma group. Table 4 lists the reagents required and their final concentration for each PCR reaction for FDp detection in single and nested PCR. Table 6 lists the cycling conditions for each PCR. If a positive result is obtained the PCR product should be sequenced to identify the organism that is detected.

To confirm the positive result obtained with the specific primers, or if a negative result is obtained with these primers, a universal phytoplasma nested PCR should be conducted using the primers P1/P7 and r16F2n/r15R2 primers (Table 3). The expected sizes of the PCR products are listed in Table 3. The recommended primers are universal and were developed to amplify all known phytoplasmas. Table 5 lists the reagents required and their final concentration for each PCR reaction for universal phytoplasma detection in single and nested PCR. If a positive result is obtained the PCR product should be sequenced to identify the organism that is detected.

The conventional nested PCR using the specific primers FD9f/FD9r and FD9f3b/FDr2 (Table 3) can be used for detection of FDp in grapevines during surveys if an incursion occurs. During the survey the universal phytoplasma nested PCR may not be required.

A multiplex real-time PCR method is available for simultaneous detection of FDp, Stolbur phytoplasmas and an internal house keeping gene of grapevines in a single sample (Pelletier *et al* 2009; Appendix 2). This method uses dual labelled hydrolysis ("Taqman") probes that are each labelled with a different fluorescent reporter dye for each target. This method is almost as sensitive as nested PCR and could be used for high throughput detection of FDp and Stobur phytoplasmas in grapevines during surveys if an incursion occurs.

Controls for PCR

- Positive control: DNA of known good quality (Housekeeping PCR)
- Positive control: DNA extracted from any FDp infected tissue (phytoplasma PCR)
- No template control: Sterile distilled water

Table 4. Conventional PCR reaction master mix for the specific detection of Flavescence dorée phytoplasma using the primers FD9f/FD9r in the first round and FD9f3b/FD9r2 in the nested PCR.

Reagent	*Volume per reaction*	*Final concentration*
Sterile (RNase, DNase free) water	15.925 µl	
10 X reaction buffer	2.5 µl	1 X
50 mM $MgCl_2$	1.25 µl	2.5 mM
10 mM dNTP mixture	0.625 µl	0.25 mM
10 µM Forward primer†	1.25 µl	0.5 µM
10 µM Reverse primer†	1.25 µl	0.5 µM
5 units/µl Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)	0.2 µl	1 unit
DNA template or control*	2 µl	
Total reaction volume	**25 µl**	

Pipette 23 µl of reaction mix into each tube then add 2µl of DNA template.

† FD9f/FD9r are used in the first round and FD9f3b/FD9r2 are used for nested PCR (Table 3)

*Up to 5µl DNA template may be added, reducing water accordingly, as target DNA may be in low concentration. For the nested PCR only 1µl of the first round PCR product is required, increase the volume of water accordingly.

Non-acetylated molecular biology grade bovine serum albumin (BSA) can be added to the master mix at 0.5mg/ml to reduce the effect of inhibitors on the PCR.

Table 5. Conventional PCR reaction master mix for universal detection of phytoplasmas using the primers P1/P7 in the first round and r16F2n/r16R2 in the nested PCR

Reagent	*Volume per reaction*	*Final concentration*
Sterile (RNase, DNase free) water	17.05 µl	
10 X reaction buffer	2.5 µl	1 X
50 mM $MgCl_2$	0.75 µl	1.5 mM
10 mM dNTP mixture	0.5 µl	0.2 mM
10 µM Forward primer†	1 µl	0.4 µM
10 µM Reverse primer†	1 µl	0.4 µM
5 units/µl Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)	0.2 µl	1 unit
DNA template or control*	2 µl	
Total reaction volume	**25 µl**	

Pipette 23 µl of reaction mix into each tube then add 2µl of DNA template.

† P1/P7 are used in the first round and r16F2n/r16R2 are used for nested PCR (Table 3)

*Up to 5µl DNA template may be added, reducing water accordingly, as target DNA may be in low concentration. For the nested PCR only 1µl of the first round PCR product is required, increase the volume of water accordingly.

Non-acetylated molecular biology grade bovine serum albumin (BSA) can be added to the master mix at 0.5mg/ml to reduce the effect of inhibitors on the PCR.

Table 6. PCR cycling conditions

	Housekeeping primers			Phytoplasma universal and FDp primers		
Step	**Temperature**	**Time**	**No. of cycles**	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	94°C	2 min	1	94°C	2 min	1
Denaturation	94°C	45 s		94°C	1 min	
Annealing	55ºC	45 s	35	55ºC or 56ºC*	1 min	35
Elongation	72°C	1 min 30s		72°C	1 min 30s	
Final elongation	72°C	10 min	1	72°C	10 min	1

*The Primers FD9f3b/FD9r2 used in the nested PCR for specific detection of FDp require a 56°C annealing temperature

Electrophoresis

Separate PCR products (5-10 μl) on a 1% agarose gel containing ethidium bromide or SybR-Safe and visualise using an UV transilluminator (ethidium bromide staining) or blue light box (SybR-Safe staining). Use a DNA molecular weight marker to determine the size of the products. Table 3 lists the expected PCR product size for each primer pair.

4.3 Interpretation of results

Failure of the samples to amplify with the housekeeping primers suggests that the DNA extraction has failed, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The phytoplasma universal and specific PCR tests will only be considered valid if:

(a) the positive control produces the correct size product as indicated in Table 2; and

(b) No bands are produced in the negative control (if used) and the no template control.

Confirmation of the specific phytoplasma species infecting the tree can only be determined through sequence analysis. As sequence similarity of 97.5% or above indicates that the phytoplasma detected is most likely to be a strain of FDp.

5. Contact points for further information

Dr. Fiona Constable
AgriBio
Department of Primary Industries
5 Ring Road
Bundoora
Victoria 3083, AUSTRALIA
Ph +61 3 9032 7000

6. Acknowledgements

This protocol was written and complied by Dr. Fiona Constable, AgriBio, Department of Primary Industries, 5 Ring Road, Bundoora, Victoria 3083, AUSTRALIA, Ph +61 3 9032 7000

Many thanks to the following researchers for providing information and advice for this protocol:

- Dr. Elisa Angelini, Centro di Ricerca per la Viticoltura, Conegliano, Italy.
- Prof. Assunat Bertaccini, Mr. Rino Credi and Dr. Claudio Ratti, Dipartimento di Scienze e Tecnologie Agroambientali – Patologia vegetale Alma Mater Studiorum-Università di Bologna, Italy.
- Dr. Sylivie Malembic-Maher and Dr. Xavier Foissac. National Institute for Agricultural Research, Bordeaux, France.

7. References

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

Angelini E, Squizzato F, Luchetta G and Borgo M. (2004) Detection of a phytoplasma associated with grapevine Flavescence dorée in *Clematis vitalba*. *European Journal of Plant Pathology* 110,193–201.

Angelini E, Filippin L, Michielini C, Bellotto D and Borgo M. (2006) High occurrence of Flavescence dorée phytoplasma early in the season on grapevines infected with grapevine yellows. *Vitis* 45, 3, 151-152.

Arnaud G, Malembic-Maher S, Salar P, Bonnet P, Maixner M, Marcone C, Boudon-Padieu E and Foissac X. (2007) Multilocus sequence typing confirms the close genetic interrelatedness of three distinct Flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology* 73, 4001-4010.

Belli G, Bianco PA, Casati P and Scattini G (2000) Serious and widespread outbreaks of flavescence dorée in vines in Lombardy *Informatore-Agrario*, 56, 56-59.

Bertin S, Guglielmino C, Karam N, Gomulski L, Malacrida A and Gasperi G. (2007) Diffusion of the Nearctic leafhopper *Scaphoideus titanus* Ball in Europe: a consequence of human trading activity. *Genetica* 13, 275-285.

Berges R, Rott M and Seemüller E. (2000). Range of phytoplasma concentration in various hosts as determined by competitive polymerase chain reaction. *Phytopathology* 90, 1145–1152.

Borgo M, Angelini E, Filippin L, Bellotto D, Forte V, Strigher L and Bazzo I. (2007) Critical points in prevention and control strategies against grapevine yellows. XXXth Organisation Internationale de la Vigne et du Vin World Congress (XXXth Congress of Vine and Wine) Budapest, Hungry, 10-16 June 2007.www.OIV2007.hu

Boudon-Padieu E. (1999) Grapevine phytoplasmas. In: First internet conference on phytopathogenic Mollicutes. http://web.uniud.it/phytoplasma//conf.html

Boudon-Padieu E. (2003) The situation of Grapevine yellows and current research directions: distribution, diversity, vectors, diffusion and control. *Extended abstracts 14th Meeting of the ICVG. Locorotondo (BARI), Italy.*

Boudon-Padieu E. (2005) Phytoplasmes de la vigne et vecteurs potentiels/ Grapevine phytoplasmas and potential vectors. *Bulletin O.I.V* 78, 299–320.

Boudon-Padieu E, Béjat A, Clair D, Larrue J, Borgo M, Bertotto L and Angelini E. (2003) Grapevine yellows: Comparaison of different procedures for DNA extraction and amplification with PCR for routine diagnosis of phytoplasmas in grapevine. *Vitis* 42(3), 141-149.

Bressan A, Larrue J and Boudon-Padieu E. (2006) Patterns of phytoplasma-infected and infective *Scaphoideus titanus* leafhoppers in vineyards with high incidence of Flavescence dorée. *Entomologia Experimentalis et Applicata* 119, 61-69.

Caudwell A. (1957) Deux années d'étude sur la Flavescence dorée, nouvelle maladie grave de la vigne. *Annales d'Amélioration des Plantes* 4, 359–93.

Caudwell A. (1964) Identification d'une nouvelle maladie a virus de la vigne, "la flavescence dorée". Etude des pheno-menes de localisation des symptomes et de retablissement. *Annales des Epiphyties 15 (hors-sér. 1)* p. 193.

Caudwell A. (1983) Origin of yellows induced by mycoplasma-like organisms (MLO) of plants and the example of grapevine yellows. *Agronomie* 3, 103-111.

Caudwell A, Larrue J, Valat C and Grenan S. (1990) Les traitements à l'eau chaude des bois de vigne atteints de la flavescence dorée. *Progrès Agricole et viticole*, 107, 281-286.

Caudwell A, Larrue J, Tassart V, Boidron R, Grenan S, Leguay M, and Bernard P. (1994). Caracte`re porteur de la Flavescence dore´e chez les vignes porte-greffes en particulier le 3309 C et le Fercal. *Agronomie* 14, 83–94.

Christensen NM, Nicolaisen M, Hansen M, and Schulz A. (2004) Distribution of phytoplasmas in infected plants as revealed by Real-Time PCR and bioimaging, *Molecular Plant Microbe Interactions* 17, 1175–1184.

Clair D, Larrue J, Aubert G, Gillet J, Cloquemin G and Boudon-Padieu E. (2003) A multiplex nested-PCR assay for sensitive and simultaneous detection and direct identification of phytoplasma in the Elm yellows group and Stolbur group and its use in survey of grapevine yellows in France. *Vitis* 42, 151-157.

Constable FE, Gibb KS and Symons RH. (2003) The seasonal distribution of phytoplasmas in Australian grapevines. *Plant Pathology* 52, 267-276.

Contaldo N, Soufi Z and Bertacccini A. (2011) Preliminary identification of phytoplasmas associated with grapevine yellows in Syria *Bulletin of Insectology* 64 (Suppl.),S217-S218.

Credi R and Callegari D. (1988) Epidemiological profile of grapevine Flavescence dorée in Emilia Romagna: temporal spread, spatial distribution of diseased plants and incidence gradients. *Phytopathologia Mediterranea* 27, 90-98.

Cvrković T, Jović J, Mitrović M, Petrović A, Krnjajić S, Malembic-Maher S and Toševski I. (2008) First report of alder yellows phytoplasma on common alder (*Alnus glutinosa*) in Serbia. *Plant Pathology*, 57, 773. doi: 10.1111/j.1365-3059.2008.01880.x

Daire X, Clair D, Reinert W and Boudon-Padieu E. (1997) Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. *European Journal of Plant Pathology* 103, 507-514.

Del Serrone P and Barba M. (1996) Importance of the vegetative state for phytoplasma detection in yellow-diseased grapevines. *Vitis* 35, 101-102.

Deng SJ and Hiruki C. (1991) Amplification of 16S rRNA genes from culturable and non-culturable Mollicutes. *Journal of Microbiological Methods* 14, 53-61.

Filippin L, Jović J, Forte V, Cvrković T, Toševski I, Borgo M and Angelini E. (2007) Occurrence and diversity of phytoplasmas detected in clematis and their relationships with grapevine “flavescence dorée” phytoplasmas *Bulletin of Insectology* 60, 327-328.

Fillipin L, Jović J, Cvrković T, Forte V, Clair D, Toševski I, Boudon-Padieu E, Borgo M and Angelini E. (2009) Molecular characteristics of phytoplasmas associated with ‘Flavescence dorée’ in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector.- *Plant Pathology*, 58, 826-837.

Fillipin L, De Pra V, Zottini M, Borgo M and Angelini E. (2011) Nucleotide sequencing of *imp* gene in phytoplasmas associated to ‘flavescence dorée’ from *Ailanthus altissima Bulletin of Insectology* 64 (Supplement), S49-S50.

Kuzmanovic S, Starovic M, Tosic M, Stojanovic S and Tomic T. (2003) Phytoplasmas on grapevine in Serbia Abstract 14th ICVG Conference Locorotondo, 12-17 September 2003, pp 93-94.

Lee I-M, Hammond RW, Davis RE and Gundersen DE. (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* 83, 834-842.

Lessio F and Alma A. (2004) Seasonal and daily movement of *Scaphoideus titanus* Ball (Homoptera:Cicadellidae). *Environmental Entomology* 33, 1689-1694.

Maixner M and Pearson RC. (1992) Studies on *Scaphoideus titanus*, a possible vector of grapevine yellows on wild and cultivated grapes in New York. *Proceedings of the 10th Meeting of ICVG, Volos 1990.*

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

Mehle N, Seljak G, Rupar M, Ravnikar M and Dermastia M. (2010) The first detection of a phytoplasma from the 16SrV (Elm yellows) group in the mosaic leafhopper Orientus ishidae. *New Disease Reports* 22. [doi:10.5197/j.2044-0588.2010.022.011]

Mehle N, Rupar M, Seljak G, Ravnikar M and Dermastia M. (2011) Molecular diversity of ‘flavescence dorée’ phytoplasma strains in Slovenia. *Bulletin of Insectology* 64, S29–30.

Morone C, Boveri M, Giosue S, Gotta P, Rossi V, Scapin I and Marzachi C. (2007) Epidemiology of flavescence dorée in vineyards in northwestern Italy. *Phytopathology* 97, 1422-1427.

Necas T and Krska B. (2006) Selection of woody indicators and the optimum plant material and sampling time for phytoplasma ESFY detection. *Acta Horticulturae* 717, 101-105.

Pavan F, Carraro L, Vettorello G, Pavanetto E, Girolami V and Osler R (1997) Flavescence dorée in vineyards of the Trevigiane hills. *Informatore-Agrario*, 53, 73-78.

Sancassani P and Posenato G (1995) Flavescence dorée in Veneto. *Informatore-Agrario.* 51, 109-110.

Pelletier C, Salar P, Gillet J, Cloquemin G, Very P, Foissac X and Malembic-Maher S. (2009) Triplex realtime PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. *Vitis* 48 (2), 87-95.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC. (1995) Phylogenetic classification of plant pathogenic Mycoplasma like organisms or phytoplasmas. In: Razin S and TullyJG (eds). Molecular and Diagnostic Procedures in Mycoplasmology, Vol. 1, pp369-380, Academic press, San Diego.

Schvester D, Carle P and Moutous, G. (1969) Nouvelles données sur la transmission de la flavescence dorée de la vigne par *Scaphoideus littoralis* Ball. *Annales de Zoologie et Ecologie Animale* 1, 445-465.

Seemüller E, Marcone E, Lauer U, Ragozzino A, Göschl M. (1998) Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* 80, 3-26.

Vercesi A and, Scattini G (2000) Spread of flavescence dorée of grapes in the Oltrepo Pavese in 1999 *Vignevini*, 27, 52-55.

Weisberg WG, Barns S, Pelletier DA and Lane DJ. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173, 697-703.

Appendix 1: Nucleic acid clean-up

Materials and equipment

1. 1.5 ml centrifuge tubes
2. 20-200 μl and 200-1000 μl pipettes
3. 20-200 μl and 200-1000 μl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Freezer
9. Vortex
10. Latex or nitrile gloves
11. Reference:
12. Buffers/solutions:
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - Ice-cold isopropanol
    - 70% (v/v) ethanol
    - Sterile distilled water
    - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 or 8.0)

Method

1. Add an additional 100-200 µl of sterile water or TE to the nucleic extract to assist ease of handling.

2. Add an equal volume of chloroform:isoamyl alcohol (24:1) and mix thoroughly by vortexing. Centrifuge in a microfuge at room temperature for 15 minutes at 13000 rpm.

3. Transfer the epiphase into a new 1.5ml microcentrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately by inversion. Centrifuge for 15 minutes at 13000rpm.

4. Discard the supernatant and wash the pellet once with 70% ethanol.

5. Air dry the pellet and resuspend in 20-50 μl of water.

Alternatively the DNA may be purified through a MicroSpin™ S-300 HR column (GE Healthcare Cat. No 27-5130-01) according to the manufacturer's instructions.

Appendix 2: Alternative extraction methods

A2.1 Phytoplasma enrichment extraction method (Kirkpatrick *et al* 1987 and modified by Ahrens and Seemüller, 1992)

Materials and equipment

1. 2 ml centrifuge tubes
2. 20-200 µl and 200-1000 µl pipettes
3. 20-200 µl and 200-1000 µl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Ice
9. Freezer
10. Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
11. Scalpel handle
12. Sterile scalpel blades
13. Vortex
14. Water bath or heating block at 55-65°C
15. Latex or nitrile gloves
16. Buffers:
    - Phytoplasma isolation buffer - The potassium (Table A2.1) and sodium (Table A.2.2) isolation buffers are interchangeable.

      To make the isolation buffer use sterile distilled water or filter sterilise. The phytoplasma isolation buffer can be stored in 50 ml aliquots at -20°C and defrosted for use. Just before use add 0.15% [w/v] bovine serum albumin and 1 mM ascorbic acid.

      Make up 100 mM stocks of ascorbic acid (0.176 g/ml water) and store in 500 µl aliquots at -20°C for up to two weeks. Just before using the grinding buffer, add ascorbic acid at 500 µl/50ml phytoplasma isolation buffer.

      Adjust pH to 7.6 after adding ascorbic acid and BSA.
    - 3% CTAB grinding buffer (Table A2.3)
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - 70% (v/v) ethanol
    - Sterile distilled water
    - Ice-cold isopropanol

Table A2.1 Potassium phosphate phytoplasma isolation buffer

Reagent	Final concentration	Amount needed for 1 L
K_2HPO_4-$3H_2O$	0.1 M	21.7 g
KH_2PO_4	0.03 M	4.1 g
Sucrose	10%	100 g
Polyvinylpyrrolidone (PVP-40)	2%	20 g
EDTA, pH 7.6	10 mM	20 ml of a 0.5 M solution

Table A2.2 Sodium phosphate phytoplasma isolation buffer

Reagent	Final concentration	Amount needed for 1 L
Na_2HPO_4	0.1 M	14.2 g
NaH_2PO_4	0.03 M	3.6 g
Sucrose	10%	100 g
Polyvinylpyrrolidone (PVP-40)	2%	20 g
EDTA, pH 7.6	10 mM	20 ml of a 0.5 M solution

Table A2.3 3% cetyltrimethylammonium bromide (CTAB) buffer for DNA purification

Reagent.	Final concentration	Amount needed for 1 L
CTAB (cetyltrimethylammonium bromide)	3%	30 g
Sodium chloride	1.4 M	56 g
1 M Tris-HCl, pH 8.0 (sterile)	100 mM	100 ml
0.5 M EDTA, pH8.0 (sterile)	20 mM	40 ml
Polyvinylpyrrolidone (PVP-40)	1%	10 g

Method

1. Grind 0.3 g leaf petioles and mid-veins or buds and bark scrapings in 3 ml (1/10; w/v) in ice-cold isolation buffer
2. Transfer 1.5 ml of the ground sample to a cold 2 ml micro centrifuge tube and centrifuge at 4ºC for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 ml micro centrifuge tube and centrifuge at 4ºC for 15 min at 13,000 rpm.
4. Discard the supernatant.
5. Resuspend the pellet in 750 µl hot (55-65°C) CTAB buffer.
6. Incubate at 55-65°C for 30 min with intermittent shaking then cool on ice for 30 seconds.
7. Add 750 µl chloroform:isoamyl alcohol (24:1 v/v), vortex thoroughly and centrifuge at 4°C or at room temperature for 4 min at 13,000 rpm.
8. Carefully remove upper aqueous layer into a new 1.5 ml micro centrifuge tube.
9. Add 1 volume ice-cold isopropanol, vortex thoroughly and incubate on ice for 4 min. Centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm. Discard supernatant.
10. Wash DNA pellet with 500 µl ice-cold 70% (v/v) ethanol, centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm.
11. Dry DNA pellet in a DNA concentrator or air-dry.
12. Resuspend in 20 µl sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.

13. Store DNA at -20ºC for short term storage or -80ºC for long term storage.

Ahrens U and Seemüller E. 1992. Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the16S rRNA gene. *Phytopathology* 82, 828-832

Kirkpatrick BC, Stenger DC, Morris TJ and Purcell AH. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplamsa-like organism. *Science* 238, 197-199

A2.2. DNA extraction procedure using the QIAGEN DNeasy® Plant mini kit (Green *et al* 1999)

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit

2. 1.5 ml centrifuge tubes

3. 20-200 μl and 200-1000 μl pipettes

4. 20-200 μl and 200-1000 μl sterile filter pipette tips

5. Autoclave

6. Balance

7. Bench top centrifuge

8. Distilled water

9. Ice machine

10. Freezer

11. Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)

> If using mortar and pestles, ensure they are thoroughly cleaned prior to use to prevent cross-contamination from previous extractions. To clean thoroughly, soak mortars and pestles in 2% bleach for 1 hour. Rinse with tap water then soak in 0.2 M HCl or 0.4 M NaOH for 1 hour. Rinse thoroughly with distilled water.

12. Scalpel handle

13. Sterile scalpel blades

14. Vortex

15. Water bath or heating block at 55-65°C

16. Latex or nitrile gloves

17. Buffers:

- 3% CTAB grinding buffer (see Table A2.3)
- Absolute ethanol

Method

1. Grind 0.5 g of plant tissue in 5 ml of CTAB extraction buffer (room temperature) containing 0.2% β – mercaptoethanol.
2. Transfer 500 µl of extract to a 1.5 ml microfuge tube and add 4 µl of RNase A (Supplied with the DNeasy kit), cap tube and incubate at 65°C for 25-35 min, mixing gently several times.
3. Add 130 µl of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.
4. Apply lysate onto a Qiashredder column and centrifuge at 20,000 x g (14,000 rpm or maximum speed) for 2 minutes.

5. Transfer 450 μl of flowthrough from QIAshredder™ column to a 1.5 ml centrifuge tube containing 675 μl QIAGEN buffer AP3/E. Mix by pipetting.
6. Transfer 650 µl of extract onto a DNeasy column and spin at 6,000 x g (8000 rpm) for 1 minute
7. Discard flow-through and add the rest of the sample to the column and spin at 10000 rpm for 1 minute
8. Place DNeasy column in a new 2 ml collection tube and add 500 µl of QIAGEN buffer AW (wash buffer) and spin at 10000 rpm for one minute.
9. Discard flowthrough and add another 500 µl of QIAGEN buffer AW and spin at maximum speed for 2 minutes.
10. Discard flowthrough and collection tube. Ensure that the base of the column is dry (blot on tissue if it is not) and place in an appropriately labeled microfuge tube. Add 100 µl of pre-warmed 65°C AE buffer directly to the filter (don't apply down the side of the tube) and spin at 10000 rpm for 1 minute. Discard column and store DNA in Freezer.

The reliability of the PCR test is affected by phytoplasma titre in the plant host (Marzachì *et al* 2004) and low titres can lead to false negative results. If a phytoplasma infection is suspected but phytoplasmas have not been detected using the extraction procedure of Green *et al* (1999) it may be useful to use a phytoplasma enrichment procedure (Appendix 2) to improve detection from symptomless material or from material collected outside the optimum time frame for detection.

Green MJ, Thompson DA and MacKenzie DJ. 1999. Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Disease* 83, 482-485.

Marzachi C, Saracco P and Bosco D. (2004) Multiplication and movement of Chrysanthemum yellows phytoplasma in the host plant *Chrysanthemum carinatum* Journal of Plant Pathology 86, 296-296.

Appendix 3: Alternative PCR detection methods

A multiplex real-time PCR method is available for simultaneous detection of FDp, Bois Noir phytoplasma (BNp) and an internal house keeping gene of grapevines in a single grapevine sample (Pelletier *et al* 2009). This method uses dual labelled hydrolysis ("Taqman") probes that are each labelled with a different fluorescent reporter dye for each target. This method is almost as sensitive as nested PCR and could be used for high throughput detection of FDp and BNp in grapevines if an incursion occurs.

Pelletier C, Salar P, Gillet J, Cloquemin G, Very P, Foissac X and Malembic-Maher S. (2009) Triplex realtime PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. *Vitis* 48, 87-95.

Table A3.1 The primers and probes used for detection of FDp, BNp and the Vitis housekeeping gene by real time PCR

Target	Primer or probe name	Primer or probe sequence	Reference
Phytoplasmas of group 16SrV (flavescence dorée)	mapFD-F	5'-TCA AGG CTT CGG BGG TTA TA-3'	Pelletier *et al* 2009
	mapFD-R :	5'-TTG TTT TAG AAG GTA ATC CGT GAA CTA C-3'	
	Probe: mapFD-FAM	FAM- TTG TAT TTC AGT GAA TGA AG –MGB	
Phytoplasmas of group 16SrXII (Bois Noir)	mapBN-F :	5'-ATT TGA TGA AAC ACG CTG GAT TAA-3'	
	mapBN-R :	5'-TCC CTG GAA CAA TAA AAG TYG CA-3'	
	Probe : mapBN-VIC :	VIC- AAA CCC ACA AAA TGC –MGB1	
Internal housekeeping - *Vitis* genome	*VITIS*-F	5'-AAA TTC AGG GAA ACC CTG GAA-3'	
	VITIS-R	5'-CCC TTG GTT GTT TTC GGA AA-3'	
	Probe: *VITIS*-Cy5	Cy5- CTG AGC CAA ATC C –BHQ	

Controls

Positive control: DNA of known good quality (internal control PCR)
Positive control: DNA extracted from any phytoplasma-infected tissue (phytoplasma PCR)
No template control: Sterile distilled water

Table A3.2 Real-time PCR reaction master mix using the QuantiTect ® Multiplex Kit RT-PCR (Qiagen)for the simultaneous detection of FDp, BNp and the *Vitis* housekeeping gene using dual-labelled hydrolysis probes

Reagent	***Volume per reaction***	***Final concentration***
Sterile (RNase, DNase free) water	3 µl	
2xQT reaction mix	12.5 µl	1 X
10 µM MapFD-F primer	0.5 µl	0.2 µM
10 µM MapFD-R primer	0.5 µl	0.2 µM
10 µM MapFD-FAM probe	0.5 µl	0.2 µM
10 µM MapBN-F primer	0.5 µl	0.2 µM
10 µM MapBN-R primer	0.5 µl	0.2 µM
10 µM MapBN-VIC probe	0.5 µl	0.2 µM
10 µM *VITIS*-F primer	0.5 µl	0.2 µM
10 µM *VITIS*-R primer	0.5 µl	0.2 µM
10 µM *VITIS*-Cy5 probe	0.5 µl	0.2 µM
DNA template or control*	5 µl	
Total reaction volume	**25 µl**	

Table A3.3 PCR cycling conditions fro detection of FDp, BNp and the *Vitis* housekeeping gene by real time PCR

Step	**Temperature**	**Time**	**No. of cycles**
Initial activation and denaturation	95°C	15 min	1
Denaturation	94°C	1 min	45
Annealing, elongation and fluorescence acquisition	59ºC	1 min 30 s	

Interpretation of the results of real-time PCR:

The real-time PCR test is valid if:

- The PCR no template control gives no fluorescence for any of the probes;
- PCR positive controls (FD + BN + Vine) show fluorescence above the threshold line and less than a Ct of 40 (threshold cycle) for each of the probes.

The following decision scheme is recommended to determine when a sample is positive for FDp, BNp or both using the multiplex real-time PCR assay (Pelletier *et al* 2009):

	Ct *Vitis* housekeeping PCR < 25	Ct *Vitis* housekeeping PCR ≥ 25 or not detected
FDp or BNp Ct Values < 40 positive	positive	positive
FD-P or BN-P Ct Value ≥ 40	negative	not interpretable