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Chapter 1

GRAPEVINE PATHOGENS SPREADING WITH PROPAGATING PLANT STOCK: DETECTION AND METHODS FOR ELIMINATION

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ABSTRACT

The use of healthy propagating material is a key factor in viticulture. Besides causing symptomatic diseases several grapevine pathogens occur in the host plant as systemic latent (symptomless) infections. This phenomenon frequently causes epidemic disease outbreaks in new plantations leading to significant economic losses and regulatory consequences. Therefore, the use of pathogen-free propagating material is a critical component of integrated strategies to manage plant diseases.

At present, more than 70 virus- and virus-like diseases of grapevines are known. Some of them (e.g., grapevine degeneration, leaf roll) can cause significant economic losses or may even be lethal (e. g., Rugose wood). Eight phytoplasmas belonging to five different groups are known to cause severe diseases with the same or very similar symptoms of grapevine yellows worldwide. *Flavescence doree* induced by '*Candidatus* Phytoplasma vitis' of the 16SrV-C group, and the diseases described under different names but caused by phytoplasmas belonging to the 16SrXII group play very important roles in grapevine production.

Crown gall disease caused by *Agrobacterium vitis* occurs in nearly all grape growing countries of the world, while Pierce's disease (*Xylella fastidosa*) and bacterial necrosis (*Xylophilus ampelinus*) have been described in North and Central America, in the Mediterranean region of Europe and South Africa, respectively. Fungal diseases (e. g., Petri disease, Esca) leading to death of canes and trunk have emerged as important factors in viticulture in recent decades worldwide. Several fungal pathogens were found to cause decline in young vines e. g., *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Cylindrocarpon*

spp., while others can cause different trunk diseases in the field as canker agents (e. g., *Eutypa lata*, *Botryosphaeria* spp.) and decay agents such as *Fomitiporia mediterranea*.

Pathogen-free propagating stock material can be obtained by testing existing plant material to select healthy plants and produced by appropriate curative treatments or propagation methods. For identification of virus free plants, testing on woody plant indicators (grapevines that are especially susceptible to a given virus) by tissue grafting is still a basic and important approach. In parallel, ELISA and reverse transcription PCR that provide more rapid results are also widely used. For diagnosis, detection and identification of phytoplasmas, bacteria and fungi various sophisticated PCR-based protocols are now available (e.g. quantitative real-time PCR, multiplex PCR, nested PCR, etc.).

For the elimination of viruses, grape plants are heat-treated by growing at 38^{o}C or shoot tips are frozen in liquid nitrogen prior to starting *in vitro* cultures from apical meristems. Hot water treatment of dormant woody canes kills phytoplasmas, *X. fastidiosa* and *X. ampelinus* but does not completely eliminate *A. vitis* and fungal pathogens, although it strongly reduces the infection rate. To produce bacterium-free plants *in vitro* shoot tip cultures or shoot tip propagation can be used. The pathogen-free plants obtained by either of the above protocols serve as a basic material to establish stock plantations for large scale production of propagating material in vineyards.

Besides the pathogens described above, there are several pests which directly cause damage, contribute to the spreading of pathogens as vectors or promote their infections through causing wounds. The most common of such pests of grapes are nematodes, mites, phylloxera and insect vectors of viruses. They can be eliminated from dormant canes by hot water treatment.

1. INTRODUCTION

Grapevine (*Vitis vinifera*) cultivation, like the production of other crops, is endangered by several diseases. Although the pesticides in use today provide efficient control for certain diseases (e. g., powdery mildew, downy mildew and grey mold), there are still several pathogens which cannot be controlled by traditional chemical methods. A general characteristic of these pathogens is that all they are systemic in the host plants. They live intercellularly in the root system and in the vascular tissues (phloem or xylem) or even intracellularly in the host cells.

Many pathogens, including viroids, viruses, phytoplasmas, bacteria and fungi, are able to infect grapevines systemically. Their infection frequently is

not promptly followed by symptom development, that is, the pathogen remains latent for variable time intervals due to the low level of initial pathogen concentration in the host plant, to unfavorable environmental conditions or to the defense reactions of the host plant. Thus these visually healthy, symptomless plants may carry dangerous pathogens which are spread by vegetative propagating material. This phenomenon frequently causes epidemic disease outbreaks in new plantations leading to significant economic losses and serious legal consequences. Therefore, the causative agents of several grapevine diseases are considered as quarantine organisms, and testing for them and certification of propagating material as being pathogen-free are strongly regulated in most grape growing countries (Frison & Ikin 1991, OEPP/EPPO 2008, Rowhani *et al*. 2005). Therefore the use of healthy propagating material is also an important basic principle for sustainable viticulture.

Here we give a brief overview of the most important grapevine pathogens which are disseminated in vegetative plant material used for propagation. Methods for their detection and identification as well as protocols to cure these pathogens from systemically infected plants are also described.

2. DISEASES & PATHOGENS SPREADING BY PROPAGATING STOCKS

2.1. Viroids

Diseases of grapevine caused by viroids are not as severe as some of the viral or fungal diseases. Most of the viroids do not cause symptoms in grape. However, they can influence the physiology of infected grape plants.

Symptoms

Grapevine yellow speckling disease symptoms include: small chlorotic specks along main veins and veinlets on exposed mature leaves on main canes or small lateral shoots. The yellowish-green speckling is indicative of severe yellow speckle disease (Taylor & Woodham 1972). Symptom severity depends on the condition of the plant, the variety, the climate, the type of viroids, wheather it is a single or mixed infection. Symptoms generally develop on young grape plants by mid-summer. Indexing can be improved by enhancing symptoms by keeping the plants in a growth chamber (Mink &

Parsons 1975). Grapevine cultivars developing symptoms include Sultanina, Cabernet Sauvignon, Grey Riesling, and Cabernet franc. Only grapevine yellow speckle viroid GYSVd-1 and GYSVd-2 cause typical yellow speckling on grapevine. No macroscopic symptoms on grapevines have been associated with *Australian grapevine viroid* (AGVd), *Hop stunt viroid*-grape (HSVd-g), or *Citrus exocortis viroid*-grape (CEVd-g). In general, grapevine viroids produce very few, if any, disease symptoms in grape. Mixed infections of viroids are commonly present in grapevines without symptom development or significant effect on yield and quality of grape (Tabler & Tsagris 2004).

Grapevine vein-banding disease is an exception. It is induced by a synergistic reaction between GYSVd 1 and 2 and *Grapevine fenleaf virus* (GFLV) (Krake & Woodham 1983, Szychowski *et al*. 1995). Grapevine co-infected with HSVd and GFLV were asymptomatic in grape (Uyemoto *et al*. 2009). Recently, symptoms of vein banding, yellow vein, leaf rolling, yellowing and small leaves were observed during a survey to identify grapevine viroids in Turkey on several grapevine varieties in the East Mediterranean region. Of 184 samples, 62 were found to be infected by one or more viroid. *Citrus exocortis viroid*-grapevine (CEVd-g) alone was found in four samples, while in others were infected with GYSVd-1, GYSVd-2 and *Hop stunt viroid*-grapevine (HSVd-g) as a mixed infection (Gazel & Önelge 2003).

Occurrence and Impact

Viroids in cultivated grapevines are common (Hadidi *et al*. 2003, Tabler & Tsagris 2004). CEVd and HSVd are distributed worldwide and infect a large number of hosts (Singh *et al*. 2003). *Australian grapevine viroid* (AGVd), *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Grapevine yellow speckle viroid* 1 (GYSVd1), and *Grapevine yellow speckle viroid* 2 (GYSVd 2) and GYSVd 3 have been isolated from grapevines (Koltunow & Rezaian 1988).

GFLVd and HSVd are the most widespread. HSVd was the first viroid described in grapevines in Japan (Sano *et al*. 1985, Shikata *et al*. 1984). In Brazil, CEVd and HSVd have been detected in grapevine (Fonseca & Kuhn, 1994) and the genetic variability of isolates from *V. vinifera* 'Cabernet Sauvignon' and *Vitis labrusca* 'Niagara Rosada' grapevine was studied (Eiras *et al*. 2006). CEVd was also isolated from symptomless grapevines in Spain, Australia and California (García-Arenal *et al*. 1987, Rezaian *et al*. 1988, Semancik & Szychowski 1992). CEVd and HSVd were detected in mixed infections from scion and rootstock varieties in Hungary (Farkas *et al*. 1999).

Grapevines are the only natural host of AGVd which have been found only in Australia, Tunisia and China (Elleuch *et al.* 2002, Guo *et al.* 2007, Li *et al.* 2006, Rezaian *et al*. 1992).

The economic impact of viroid diseases of grapevines is uncertain (Wolpert *et al*. 1996) or largely similar to viruses (Randles 2003). Impact can vary depending on the climate, the environmental conditions, the variety and interaction with other pathogens. Cultivated grapevines are symptomless reservoirs of HSVd which can be transmitted to hop crops to cause epidemics (Kawaguchi-Ito *et al*. 2009).

Causal Agent

Viroids are subviral pathogens causing infectious diseases spreading systemically in host plants (Diener 1971, 1999). They have short (246–401 nt), single stranded but highly structured, non-protein-coding, naked, circular RNA genome with characteristic domains (Diener 2003, Ding & Itaya 2007, Ding & Zhong 2009, Flores *et al*. 2004, Tabler & Tsagris 2004, Tsagris *et al*. 2008).

Viroids can be classified into two major families, the *Pospiviroidae* (type species potato spindle tuber viroid RNA (PSTVd) which members are replicate in the nucleus and the *Avsunviroidae* (type species avocado sunblotch viroid (ASBVd) that replicate in the chloroplast. Families are subdivided into several genera. Viroids infecting grapevine are classified into three genera within the *Pospiviroidae* family based on sequence homology of their Conserved Central Regions (CCRs). GYSVd-1, GYSVd-2, and AGVd are classified in the *Apscaviroidae,* HSVd-g in the *Hostuviroid* and CEVd-g in the *Pospiviroid* genus (Flores *et al*. 2005, Tabler & Tsagris 2004). Recently a new member of the *Apscaviroidae* group named GYSVd-3 was reported from China (Jiang *et al*. 2009).

Biology and Epidemiology

There have been many efforts to understand the biology of viroids (Ding 2009, Hadidi *et al*. 2003, Owens & Hammond 2009). Viroids are able of replicating and moving through infected plants (Ding *et al*. 2005, Di Serio & Flores 2008, Flores *et al.* 2004, 2009). The movement of viroids (Takeda & Ding 2009) is related to viroid structure and stability (Zhong *et al*. 2008). A quite narrow region in the sequence is responsible for the pathogenicity, however, the biological significance of this is not resolved. Viroids interact with the cellular machinery and modify the enzymatic activities (Ding 2009). Grapevine infecting viroids are synthesized in the nucleus (Gas *et al.* 2007). Similarly to viruses, viroid infections are associated with the accumulation of

viroid-derived 21–24 nt small RNAs (vd-sRNAs) products of RNA mediated plant defense systems (Brodersen & Voinnet 2006, Carbonell *et al*. 2008). The structured viroid RNA is resistant to RNA-induced gene silencing complex mediated degradation (Gòmez & Pallás 2007, Itaya *et al.* 2007). Viroids may interact with host enzymes involved in the RNA-directed DNA methylation pathway (Navarro *et al*. 2009).

Transmission

Viroids are transmitted mechanically and by grafting. The use of contaminated cutting tools during vine management may contribute to the distribution of viroids in vineyards (Szychowski *et al*. 1988). Sodium hypochlorite and formaldehyde can be used to decontaminate pruning tools (Wutscher & Schull 1975). Systemic transmission upon grafting plays a more significant role in viroid spread in grapevines (Staub *et al*. 1995). Seed and pollen transmission have also been reported. Seed transmission of *Grapevine yellow speckle viroid* 1 and *Hop stunt viroid* was confirmed in 11 seedlings of eight grapevine (*V. vinifera*) varieties (Wan-Chow-Wah & Symons 1999). Seed transmission can have an impact on seedling reservoirs of viroids. Natural populations of wild grapes, such as *Vitis sylvestris*, can be invaded by viroids via seed and pollen transmission.

Disease Management

The widespread occurrence of viroids in plantations and their easy spreading by routine propagation techniques makes control generally unpractical. Plants cannot be cured of infection in a plantation. The real danger of viroids is that they can interact with other grape pathogens. Efforts have been made to save germplasm collections and to eliminate viroids from stocks of propagating materials. Somatic embryogenesis is suitable for eliminating viroids from different grape cultivars over meristem culture and thermotherapy (Gambio *et al.* 2011). During maintenance cutting tools should be disinfested before working with viroid-free stocks.

2.2 Viruses

More than 70 virus- and virus-like diseases of grapevines are known. Some of them are latent with minor importance while others alone or in combination with other viruses cause serious diseases of grapevines. Grapevine viral and virus-like diseases have been reviewed recently from virological viewpoints (Martelli and Boudon-Padieu 2006, Martelli 2009),

according to the type of disease and spreading (Uyemoto *et al.* 2009) or field assessment and diagnostic methods (Gambino *et al.* 2010).

Symptoms

Grape viral diseases are often named according to the typical symptoms appearing on grapevine (Table 1.). The disease symptoms associated by the presence of viruses can affect all parts of the plant (leaf, shoot, root, fruit, woody trunk, Figure 1). Severity of symptoms are different and depends on the rootstock and scion varieties, the plant condition and the environmental factors. The manifestations of symptoms has specific period in case of each viral diseases. Typical symptoms are leaf curling, rolling (leafroll), deformation of leaves and shoots (fanleaf), small-sized leaves, discoloration of veins or shoot, yellowing or redding of leaves (leafroll), speckling, interveinal chlorosis and mottling, light green veins with bright yellow vein banding, feathery veins (e.g. caused by TBRV-Canadian strain), weak shoot growth with short internodes (*Grapevine fanleaf virus*, GFLV), abnormal branching and fascination (fanleaf), delayed bud break [(*Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Peach rosette mosaic virus* (PRMV)], poor fruit set, late ripening (leafroll). Some viruses (like *Grapevine Bulgarian latent virus*, GBLV) cause latent infections in *V. vinifera.* Diseases like fleck and vein necrosis are latent in European varieties, others are symptomless and show typical symptoms (Rugose wood complex) on indicator varieties only (Uyemoto *et al*. 2009).

Figure 1. Virus symptoms on grapevines. Grapevine leafroll associated virus 3 (GLRaV-3) symptoms on *Vitis vinifera* cv. 'Pinot noir' (left). Grapevine virus A (GVA) is the putative agent of Grapevine Kober stem grooving, symptoms are shown on the indicator variety 'Kober 5BB' (right). (photos: J. Lázár).

Occurrence and impact

Viruses playing role in most important viral diseases are widespread all over the world. Fanleaf (GFLV), leafroll (*Grapevine leafroll-associated viruses-1, 2, 3*, GLRaV-1, 2, 3), rupestris stem pitting (G*rapevine rupestris stem pitting-associated virus*, GRSPaV), Kober stem grooving (*Grapevine virus A*, GVA), fleck complex (*Grapevine fleck virus*, GFkV) and vein necrosis (GRSPaV-1) are present in several viticultural areas. Other viruses are currently geographically limited like PRMV to South-Western Michigan (USA) and South-Western Ontario (Canada), GBLV to Bulgaria and *Grapevine (Hungarian) chrome mosaic virus* (GCMV) to central Europe (Martelli 2009). Virus infection generally have negative effect on plants reducing plant vigor, delayed bud burst, altered and erratic shoot growth, malformation on leaves, mottling, coloration, necrosis, reduced berry size and quality, canes do not mature. Some viral diseases (e.g., grapevine degeneration, leafroll) can cause significant economic losses (50-70%) or may even be lethal (e. g., Rugose wood, Uyemoto *et al*. 2009).

Causal agents

Viruses are very small (submicroscopic) infectious particles. The particle generally is composed of a protein coat (called capsid) and a nucleic acid core which carry genetic information and typically specifies two or more

proteins (Hull 2002). About 70 grapevine-infecting viruses are known (Fauquet *et al*. 2005; Martelli 2009, Martelli & Boudon-Padieu 2006). The important viruses and virus diseases of grapevine are listed in Table 1. They are classified by particle morphology, host range and information content of the genome, etc. (Hull 2002).

Biology and epidemiology

The existence and replication of viruses completely depend on metabolically active plant cells. Most viruses are restricted to a particular type of host or hosts. To enter the host cell plant viruses depend on injuries or on transmission *via* invertebrates (insects, nematodes, etc.). The waste majority of plant viruses including grapevine infecting viruses have ssRNA genome and have coat protein. The most important ones belong to the *Nepovirus, Closterovirus, Ampelovirus, Vitivirus* genus, but many other type of viruses have been reported from grapevines (Gambino *et al*. 2009). Grapevine infecting viruses have been studied in details in molecular level (Martelli 2009, Minafra *et al*. 1997, Moskovitz *et al*. 2008). The studied characteristics help to classify them and to understand how they can replicate, cause diseases and spread with different vectors.

Nepoviruses like *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic nepovirus* have an almost spherical capsid. There are currently 9 serologically distinct different viruses associated with leafroll. Eight of them *Grapevine leafroll-associated viruses 1* and *3-9* belong to the *Ampelovirus while* GLRaV-2 to the *Closterovirus* genus. *Closteroviruses* are road shaped viruses with long particles and having the largest genome among (+)-strand RNA plant viruses. They are coding different proteins for replicase, coat protein, cell to cell movement and other functions. *Vitiviruses* (like *Grapevine virus A*, GVA) and *Grapevine virus B* (GVB) have monopartite genome. The RNA encodes 5 ORFs, has 5'cap and the 3' is polyadenilated (Hull 2002). G*rapevine rupestris stem pitting-associated virus* (GRSPaV) is also a positive strand, ssRNA virus and belong to the *Foveavirus* genus (family *Betaflexiviridae*; order *Tymovirales*). GRSPaV represents a group of distinct viruses for which mechanisms for viral replication may be substantially different from other RNA viruses (Meng & Li 2010, Zhang *et al.* 1998).

Transmission

Viruses are transmitted by grafting, plant sap, pollen or vectors. Some viruses can be easily transmitted mechanically to indicator plants (Table 1.), while others, like phloem-limited viruses are transmitted by nematodes or

aphids (Uyemoto et al. 2009). Grafting also contributes to virus spreading since the infected rootstock varieties can be symptomless (Martelli 2009).

The name of *Nepoviruses* is an acronym from: nematode-borne polyhedral viruses. They are transmitted specifically by different nematode species, e. g., GFLV by *Xiphinema index and X. italie*, ArMV *by X. diversicaudatum and Longidorus caespiticola*, RpRSV by *Paralongidorus maximus.* TRSVand ToRSV (causing virus-induced grapevine decline) are transmitted by *X. americanum*, and ToRSV by *X.rivesi, X. americanum sensu lato* and *X. californicum. Nepoviruses* are also sap-transmitted to herbaceous indicator plants and are seed borne in weed hosts. *Peach rosette mosaic virus* (PRMV) is vectored by *X. americanum sensu lato, Longidorus diadecturus* and *L. elongatus.*

Closteroviruses have aphid vectors however GLRaV-2 has no known aphid vector yet. Some other members of *Grapevine leafroll associated viruses* (GLRaVs-1, -3, -5 and -9) belonging to *Ampelovirus* genus are transmitted by mealybug (*Pseudococcidae*), and soft scale insects (*Coccidae*). Viruses associated to Rugose wood complex belong to the *Vitivirus* (GVA, GVB and GVD), and *Foveavirus* genera (GRSPaV). GVA mealybug vectors include *Planococcus citri, Pl. ficus, Pseudococcus longispinus. Ps. affinis, Heliococcus bohemicus.* GVB is transmitted by *Ps. longispinus, Ps. affinis* and *Pl. ficus.* Many viruses have no known vector yet (Martelli 2009).

Disease Management

The infected plants should be removed since virus elimination from plants in an established vineyard is not possible. Before replanting vineyards the soil should be freed from vectors. The use of virus-free propagating material is essential. To get such stock material through somatic embryogenesis proved to be more efficient than *in vivo* or *in vitro* heat therapy followed by apical meristem cultures (Gambino *et al*. 2009, 2011).

Table 1. Grapevine viruses, virus-like diseases, and their indicators*

Disease	Virus	Acr.	*Vitis vinifera* cultivars, other *Vitis* indicators	*Herbaceous* indicators
	1. European nepo viruses			
Infectious degeneration	1.1 grapevine fanleaf nepovirus	GFLV	FS-4, *V. rupestris*, Mission	*Chenopodium quinoa, Gomphrena globosa*
Yellow mosaic	~ yellow mosaic strain	GFLV-YM	Chardonnay, *V. rupestris*	*Ch. quinoa, Celosia argentea*
Vein banding	~ GFLV + grapevine yellow speckle viroid	GFLV-VB	Chardonnay	*Ch. quinoa, Ch. amaranticolor*
	1.2 Arabis mosaic virus	AMV	FS-4, Chardonnay	*N. glutinosa, Phaseolus vulgaris*
	1.3 artichoke Italian latent virus	AILV	Cabernet sauvignon, Merlot	*Ph. vulgaris, G. globosa, Cucumis sativus*
	1.4 cherry leafroll virus	CLRV	Pinot noir	*Ch. quinoa, N. clevelandii*
	1.5 grapevine Anatolian ringspot virus	GARSV	Kizlar tahtasi	
	1.6 grapevine Bulgarian latent virus	GBLV	FS-4, Pinot noir, *V. riparia*	*Ch. quinoa, N. megalosiphon, G. globosa*
	1.7 grapevine chrome mosaic virus	GCMV	Pinot noir, Red veltliner	*Ch. quinoa, G. globosa, P. vulgaris*
	1.8 grapevine deformation virus	GDefV	Montepulciano, Chasselas	*Ch. quinoa, Ch.amaranticolor*
	1.9 grapevine Tunisian ringspot virus	GTRSV		
	1.10 raspberry ringspot virus	RpRSV	FS-4,	*Ch. quinoa, N. clevelandii*
	1.11 tomato black ring virus	TBRV	FS-4, Pinot noir	*Ch. quinoa, N. rustica*

Table 1. (Continued).

Disease	Virus	Acr.	*Vitis vinifera* cultivars, other *Vitis* indicators	*Herbaceous* indicators
Joannes-Seyve disease	Joannes-Seyve virus, strain of TBRV		Joannes-Seyve	
	1.12 strawberry latent ringspot virus	SLRSV	FS-4,	*Ch. quinoa, N. rustica, C. sativus*
Grapevine degeneration and decline	2. American nepo viruses			
	2.1 blueberry leaf mottle virus	BLMoV	*V. labrusca*	*Ch. quinoa, N. clevelandii*
	2.2 peach rosette mosaic virus	PRMV	*V. labrusca*	*Ch. quinoa, N. tabacum*
	2.3 tobacco ringspot virus	TRSV	Chardonnay	*Ph. vulgaris, C. sativus*
	2.4 tomato ringspot virus	ToRSV	Carignane, Baco 22A	*Ch. quinoa, Ph. vulgaris, C. sativus*
Grapevine leafroll	3. Grapevine leafroll complex			
	grapevine leafroll associated viruses 1-9	GLRaVs	Pinot noir, other red vines	*Nicotiana sp. (GLRaV-2)*
Rugose wood complex	4. Rugose wood complex			
Rupestris stem pitting	4.1 grapevine rupestris stem pitting-a virus	GRSPaV	*V. rupestris*	
Kober stem grooving	4.2 grapevine virus A	GVA	Kober 5BB	*N. clevelandii*

Disease	Virus	Acr.	*Vitis vinifera* cultivars, other *Vitis* indicators	*Herbaceous* indicators
Grapevine corky bark	4.3 grapevine virus B	GVB	LN-33	*N. benthamiana*
	4.4 grapevine virus C	GVC		
	4.5 grapevine virus D	GVD		
	5. Fleck complex			
Fleck	5.1 grapevine fleck virus	GFkV	*V. rupestris*	
Asteroid mosaic	5.2 grapevine asteroid mosaic-associated virus	GAMaV	*V. rupestris*	
Rupestris vein feathering	5.3 grapevine rupestris vein feathering virus	GRVFV	*V. rupestris*	
	5.4 grapevine redglobe virus	GRGV	*V. rupestris*	
Rupestris necrosis			*V. rupestris*	
	6.Minor virus diseases			
Grapevine yellow mottle	6.1 alfalfa mosaic virus	AMV	Chardonnay, Red Veltliner	*Ch.quinoa, N. megalosiphon, Ph. vulgaris*
Grapevine line pattern	6.2 grapevine line pattern virus	GLPV	Irsai Oliver, Jubileum 75	*C. sativus, N. glutinosa*
Roditis leaf discoloration	6.3 carnation mottle virus + GFLV (mixed)	CarMV	Mission	
Grapevine angular mosaic	6.4 grapevine angular mosaic virus	GAMV	cv. Baresana x Baresana	*G. globosa, Ch. quinoa, Nicotiana spp.*
Yellow line pattern	6.5 raspberry bushy dwarf virus	RBDV	Laski Riesling (~Italian Riesling)	*Ch.quinoa, N. benthamiana*

Table 1. (Continued).

Disease	Virus	Acr.	*Vitis vinifera* cultivars, other *Vitis* indicators	*Herbaceous* indicators
Grapevine berry inner necrosis	6.6 grapevine berry inner necrosis virus	GINV	Takao, Kyoho or Pione	
Grapevine stunt	6.7 anonymous		Campbell Early	
Grapevine Ajinashika disease	6.8 anonymous, and leafroll & fleck		Koshu	
	7. Virus-like diseases			
Grapevine enation disease		GED	LN-33, Kober 5BB	
Grapevine vein mosaic		GVMV	*V. riparia* Gloire	
Grapevine summer mottle		GSM	Cabernet franc, Mission, Sideritis	
Grapevine vein necrosis		GVNV	110R	
	8. Graft incompatibility			
Kober 5BB incompatibility	Grapevine leafroll associated virus-2	GLRaV-2	Cabernet Sauvignon	*Nicotiana sp.*
California's young vine decline	GLRaV-2 + Grapevine virus B		Cabernet Sauvignon, LN-33	*Nicotiana sp.*
Redglobe disease	Grapevine rootstock stem lesion-associated virus	GRSLaV	Redglobe	

Disease	Virus	Acr.	*Vitis vinifera* cultivars, other *Vitis* indicators	*Herbaceous* indicators
Young vine decline in NZ, CL, AU	other molecular variants of GLRaV-2		Cabernet Sauvignon	*Nicotiana sp.*
Australian Shiraz disease	GVA has been detected in affected vines		Shiraz, Merlot	
Syrah decline	RSP strains were associated with SD		Pinot noir, Syrah	
	9. Viroids			
Yellow speckle	9.1 grapevine yellow speckle viroids 1-2	GYSVd-1,2	LN-33, Mission	
	9.2 citrus exocortis viroid	CEVd-g		
	9.3 hop stunt viroid	HSVd-g		
	9.4 Australian grapevine viroid	AGVd	certain viroid-free grapes	*C.sativus, Lycopersicum esculentum*

*Data from: Bovey 1999, Frison & Ikin 1991, Martelli 1992, Martelli & Boudon-Padieu 2006, Neszmélyi *et al*. 1996., Vanek 1992, Vindimian *et al*. 1988, Walter 1997.

2.3. Phytoplasmas

Phytoplasma diseases occurring in grapevines in different continents are referred to collectively as grapevine yellows (GY), referring to the main characteristic symptom. GY diseases cannot be differentiated by visual observation of symptoms as the same or very similar symptoms are induced by different phytoplasma species in *V. vinifera* varieties.

GY diseases include Flavescence dorée (FD), Palatinate grapevine yellows (PGY) and Bois noir (BN) in certain countries of Europe; North American grapevine yellows (including Virginia grapevine yellows, I and III, New York grapevine yellows and grapevine yellows in Canada); Australian grapevine yellows (Australia and New Zealand and Buckland Valley grapevine yellows in Australia; grapevine diseases in South Africa and Chile).

Main Symptoms

Characteristic GY symptoms occur on shoots, flowers, fruit clusters and on the canes of the grapevine. The symptoms frequently develop only on one shoot. In the Northern hemisphere, the first symptoms appear in mid-summer (June-July). The infected young shoots have a fir-like appearance due to their zig-zag growth and shortened internodes, and their leaves are pale yellow and rolled slightly downwards (Figure 2a). The leaf symptoms develop during vegetative growth. The leaves become crispy and brittle; the triangle-shaped rolling becomes increasingly evident (Figure 2b). Discoloration of the leaf blade changes (Figure 2f) reddish to purple (on the red varieties), or to chlorotic and yellow (on the white varieties) (Figure 2c), and necrosis may also occur. The discoloration can be sectorial or it invades the entire leaf blade including the veins (Figure 2e). Due to uneven lignification, the diseased shoots have a weeping appearance (Figure 2d). The rubbery canes become frost susceptible and die during cold winter. Infected flowers wither, may die and fall down. The infected bunches wither, may die or the berries shrivel later in the season (Figure 2g). The taste of these berries becomes sour and/or bitter. The quantity and quality of the crop on the diseased vines are significantly reduced. In case of severe infection the plants die.

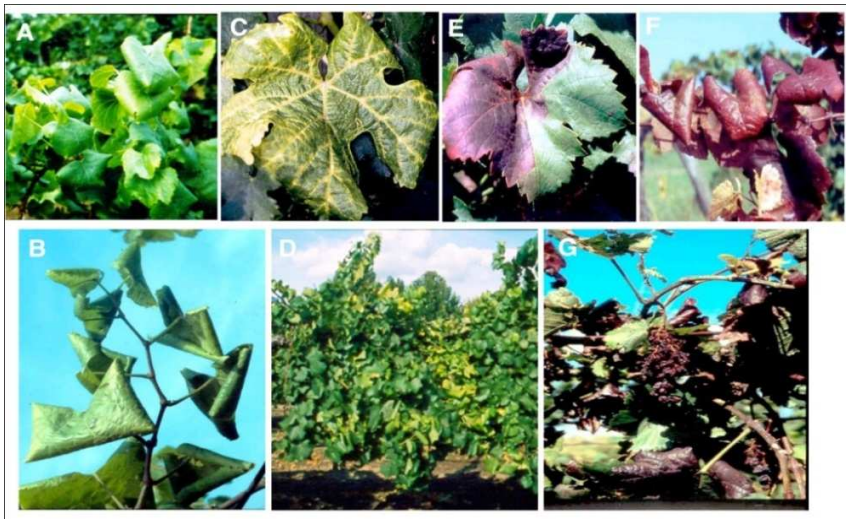

Figure 2. Characteristic symptoms of grapevine yellows on *Vitis vinifera* varieties. Pale, slightly downward rolling of leaves on young shoots with shorthened internodes (a); triangle-shaped rolling of the brittle, crispy leaves in late stages of vegetative growth (b); Discoloration of leaves on white and red varieties (c, e & f); Weeping shoots due to uneven lignification (d); Infected bunches with withered, shriveled berries that die late in the season (g) (photos: M. Kölber).

Causal Agents

Phytoplasmas are phloem-colonizing, non-culturable, gram-positive bacteria lacking cell wall. They belong to the class Mollicutes. They are obligate pathogens of plants and insects. Phytoplasmas have smaller cell (<0.8 µm) and genome (0.5-1.3 Mbp) sizes than culturable bacteria. The biology of phytoplasmas and the changes they cause in host plant gene expression has recently been reviewed (Hogenhout *et al.* 2008, Hren *et al*. 2009). At first these pathogens were thought to be viruses and they were named after the disease symptom they caused. From 1967 to 1993 they were called Mycoplasma-like organisms. In 1993, the International Committee of Systematic Bacteriology introduced the term 'phytoplasma' for these organisms.

Phytoplasmas are classified into groups and subgroups based on their genetic relatedness. Phylogenetic analyses, based on various conserved genes, symptomatology, host range and serology have demonstrated that the phytoplasmas form a homogeneous phylogenetic clade. This clade was

subdivided into 20 distinct phylogenetic groups and subgroups by their 16S rDNA sequence data (Seemüller *et al.* 1998). According to this classification, the causal agents of GY diseases identified so far belong to five groups in the phytoplasma clade. Flavescence dorée (FD) and Palatine Grapevine yellows (PGY) phytoplasmas belong to Elm yellows (16SrV) group. Stolbur phytoplasmas causing GY diseases and described under various names in different countries such as Bois noir (BN), Vergilbungskrankheit (VK) and Schwarzholzkrankheit or Legno nero and the Australian grapevine yellows phytoplasma ('*Candidatus.* Phytoplasma australiense'), belong to the stolbur (16SrXII) group. In Australia GY can be caused also by '*Ca.* Phytoplasma australiense' of the peanut witches' broom (16SrII) group. GY diseases in North America and Israel are induced by phytoplasmas of the X disease (16SrIII) group. Phytoplasmas associated with endemic GY diseases in certain countries of Europe, as well as in the US belong to the aster yellows (16SrI) group *(*Martelli & Boudon-Padieu 2006).

The Phytoplasma/Spiroplasma Working Team-Phytoplasma of the International Research Programme for Cooperative Mycoplasmology proposed to establishment of a new genus-level provisional taxon called '*Candidatus (Ca.)* Phytoplasma' to accommodate plant-pathogenic, non-helical *Mollicutes* (IRPCM 2004). According to the guidelines determined by the Working Team, a novel '*Ca.* Phytoplasma' species description should refer to single, unique 16S rDNA gene sequences of greater than 1200 bp long and sharing less than 97.5% sequence similarity to that of any previously described '*Ca.* Phytoplasma' species unless the phytoplasma under consideration clearly represents an ecologically separate population. Zhao et al. (2010) divided the phytoplasma clade into three subclades on the basis of their 16S rDNA sequence similarities. Causal agents of the GY diseases belong to Subclade I ('*Ca.* Phytoplasma solani', '*Ca.* P. australiense', '*Ca.* P. americanum', '*Ca.* P. Buckland valley grapevine yellows phytoplasma, '*Ca* P. asteris') and to Subclade II ('*Ca.* P. vitis', '*Ca.* P. pruni', '*Ca.* P. aurantifolia' and '*Ca.* P. australasiae').

Transmission

Phytoplasmas inducing GY diseases are spread from plant to plant by hemipteran insects, certain planthopper or leafhopper species that feed in the phloem of the grapevine vascular tissue. Phytoplasmas multiply in the body of the insect, move into the salivary gland and then are injected with the salavia into phloem of the plant when the insect vector feeds. Phytoplasmas spread by vectors for short distances within the vineyard or nearby vicinity. Infected

propagating material is responsible for long distance dissemination of GY phytoplasmas.

Flavescence dorée disease

Flavescence dorée (FD) was the first grapevine yellows disease described as a viral disease in France (Caudwell 1957).

Causal agent

Flavescence dorée phytoplasma belongs to the 16SrV phylogenetic group. Grapevine FD isolates are included into the 16SrV-C and 16SrV-D subgroups. According to the recent classification, the species name is '*Ca.* Phytoplasma vitis'.

Occurrence and Impact

FD is a regulated pest in the European Union (Council Directive 2000/29), in the NAPPO countries (NAPPO 2009), as well as in South Africa and New Zealand. FD is not known to occur in North America. The FD-D isolate is present in Southern and Central France, Northern and Central Italy, North-Eastern part of Spain, Northern Portugal, and in an area of Southern Switzerland. FD-C isolates were identified in France, Italy, Serbia and Slovenia. The disease is highly epidemic causing important crop losses.

All of the *V. vinifera* varieties grown in France, Italy and Spain are susceptible to FD but they show various levels of sensitivity. Highly susceptible varieties (e.g. Nieluccio and Garganega) do not recover after infection. Sensitive varieties (such as Alicante Bouschet, Grenache, Cabernet Sauvignon, Sauvignon Blanc and Chardonnay) may recover when they are protected from new infections. Merlot seems more tolerant, although severely affected plants can be also found. Symptoms are rare on plants on Syrah plants. Infected *Vitis riparia* rootstocks can be found but they rarely develop symptoms (Martelli & Boudon-Padieu 2006).

Main Symptoms

As described previously in subheading grapevine yellows, Angelini *et al*. (2006) observed that the first symptomatic grapevines that appeared early during vegetative growth were FD-infected. Symptoms become more pronounced by the autumn. Leaves remain longer on the affected than on unaffected plants. Generally the symptoms develop throughout the whole plant. North American rootstocks are generally symptomless carriers of the GY-associate phytoplasmas, so they are important reservoirs. In case of certain

rootstock varieties GY causes rolling of leaves and lack of lignification. On rooted cuttings necrosis of the terminal bud may occur.

Transmission

In nature, the FD phytoplasma is transmitted from grapevine to grapevine only by the leafhopper *Scaphoideus titanus* Ball (Homoptera, Cicadellidae), in a persistent manner. This insect was introduced from North America into Europe via infested propagating material in the early 1900s and became established in several countries. This monophagous species feeds only on grapevine. It has one generation per year and overwinters as eggs. The females oviposit under the bark of 2-year-old or older shoots. All larval stages and the imago feed on grapevine and are capable to transmit FD.

Control

Only pathogen-free propagating material can be used for planting (see below). Budwood can be taken only from healthy mother plants standing in vineyards of regions free from FD disease. Control measures against FD are compulsory in France and Italy. An indirect way to manage FD is application of insecticides to control the insect vector. Three treatments with chemical insecticides are efficient in preventing the development of FD epidemics. Rouging of infected vines is important to avoid or reduce epidemics. This is compulsory in France.

Bois noir disease

Bois noir (BN) disease was first described in France (Caudwell 1961) under different names as 'Vergilbunsgskrankheit' (VK) or 'Schwarzholzkrankheit' in Germany and 'Legno nero' in Italy. In the 1990s it was determined that the causal agents of these diseases were closely related and the same vector, *Hyalesthes obsoletus* was identified in different countries. BN and FD are caused by different phytoplasmas (Martelli & Boudon-Padieu 2006).

Causal Agent

The cause of BN is attributed to '*Ca.* Phytoplasma solani'-related strains. These phytoplasma strains belong to the stolbur phytoplasmas (16SrXII-A ribosomal subgroup). Based on *tuf*-gene sequences different stolbur strains were differentiated. Three of them are associated with BN in grapevine,

although they have distinct specificities for different weed host species. *tuf-a* types are specifically associated with stinging nettle (*Urtica dioica)* and *tuf*-b types are specific to field bindweed (*Convolvulus arvensis)*. *tuf*-b types have been frequently found in several countries in Eastern and Southern regions of Europe. *tuf-c* types have been identified in hedge bindweed (*Calystegia sepium)* only in a limited area of Germany so far. The fourth strain was identified in the planthopper *Reptalus panzeri*. According to the combination of *tuf*- and *Stol4*-typing, stolbur isolates from grapevine were classified into VK1, VK2 and VK 3 groups. Currently, ribosomal protein genes, *secY* and *vmp1* gene are also widely used for further characterization of stolbur isolates (Maixner 2011).

Occurrence and Impact

BN is endemic and widely distributed in the Mediterranean region and in countries of Western, Central and Eastern Europe as well as in Lebanon and Israel. Recently, it has been reported in Iran and China but it is not known to occur in North America, Australia, New Zealand or South Africa. In some years, continuous increased incidence of BN has occurred. In Europe severe economic loss occurred by reducing the quality and quantity of yield and vitality of grapevine plants. The majority of the *V. vinifera* varieties are susceptible to stolbur, and Chardonnay seems the most sensitive. Infection of rootstock varieties has not been reported so far but it cannot be excluded. The causal agent of BN spreads slower than FD-associated phytoplasmas, however, its importance is increasing. BN is more widespread and more difficult to control due to the feeding habit of its polyphagous insect vector. Beside grapevine, stolbur phytoplasma infects crops of the *Solanaceae* family (tomato, potato, pepper, eggplant, tobacco) as well as maize and lavender, causing economic damage on them. A wide range of weed species are also hosts of the stolbur phytoplasma.

Main Symptoms

BN symptoms occur on all parts of the grapevine but their severity may vary deepnding upon cultivars. All of the typical symptoms of grapevine yellows can be observed on BN diseased plants. The symptoms remain restricted only to one part of the infected plant. Symptom remission and recovery of the BN-infected vines are common.

Transmission

The known vector of tomato stolbur, *H. obsoletus* the polyphagous Cixiid planthopper is the principal vector also of the BN-associated phytoplasma. It is very abundant in Germany and its role as the vector of the BN-associated phytoplasma in France, Italy, Israel, Switzerland, Serbia and Spain has been also verified. The main perennial hosts of *H. obsoletus*, field bindweed and stinging nettle are natural reservoirs of the stolbur-associaated phytoplasma in several countries. Eggs are laid on the ground at the base of the stem and the first larval instars move to the roots to feed. *H. obsoletus* overwinters as juvenile larvae on roots and stays there until its last developmental stage. The frequency of infective larvae feeding on the roots of diseased weeds increases from the third to the last larval instar. They acquire the stolbur phytoplasma from the roots of the weeds and the adults can transmit it to grapevines during probing as they do not continue to feed on *Vitis*. Grapevine is only an occasional feeding host for the adults. Grape to grape transmission of stolbur phytoplasma has not been observed, so *Vitis* is a dead-end host for the stolbur phytoplasma. Stolbur-associated phytoplasmas are associated with either nettle (*tuf-a* type) or bindweed (*tuf-b* type). In addition, the *H. obsoletus* populations are specifically associated with one of these hosts (Maixner 2010).

H. obsoletus is present in diseased vineyards in several countries although at very low population levels. So, existence of other alternative vectors cannot be excluded. Members of Cixiid or Cicadellid species collected in diseased vineyards were found positive for the stolbur-associated phytoplasma, but only *Reptalus quinquecostatus* and *Euscelis lineolatus* were able to transmit it from artificial feeding medium (Landi *et al.* 2009, Pinzauti *et al.* 2008). Their eventual vectoring of the stolbur-associated phytoplasma to grapevine has to be studied. *Macrosteles quadripunctulatus* and *Anaceratagallia ribauti* were able to transmit the stolbur-associated phytoplasma to herbaceous plants (Battle *et al.* 2008, Riedle-Bauer & Sára 2009). The eventual ability of these species to transmit the stolbur phytoplasma to grapevine remains to be determined.

Stolbur phytoplasma can be transmitted also by grafting so long-distance dissemination of the phytoplasma-associated BN occurs by means of infected propagating material. At present insect vectors with the ability of acquiring Stolbur from grapevine are still not known. So, based on our current knowledge of BN epidemiology, *Vitis* may be a dead-end host. Therefore, BN-affected grapevine propagating material is not considered as an important infection source.

Control

Use of pathogen-free propagating material is important (see below) since vitality and yield of the BN-affected plants as well as the quality of the berries are significantly reduced. Cuttings can be collected only from healthy mother plants standing in vineyards free of BN disease. Insecticide treatments to control *H. obsoletus* alone are not efficient due to the biology and feeding behavior of the insect. Mechanical and chemical weed control may reduce the vector populations.

North American Grapevine Yellows diseases

In North America four GY (NAGY) diseases have been described, three in the US and one in Canada. *Virginia grapevine yellows I (VGYI)* is associated with '*Ca.* Phytoplasma asteris'-related strains belonging to the aster yellows (16SrI-A) phytoplasma subgroup. *Virgina grapevine yellows* III (VGYIII) is associated with '*Ca.* P. pruni'-related strains belonging to the peach X disease (16SrIII-B) phytoplasma. *NewYork grapevine yellows* is associated with a phytoplasma thought to be serologically related to FD-associated phytoplasmas, but this finding has not been confirmed.

In Canada a GY disease is associated with a phytoplasma, '*Ca.* P. asteris'-related strain belonging to the tomato big bud (16SrI-B) phytoplasma subgroup (http://plantpathology.ba.ars.usda.gov/phytoplasma.html). In the 1990s, a severe outbreak of GYs was observed in New York and in Virginia States. The infected plants died in 1-3 years after the appearance of the first symptoms. Recent studies on NAGY in Virginia are focusing on epidemiological questions. The leafhopper species *Agallia constricta* was found as the most frequent vector of NAGY (Beanland *et al*. 2006, Wolf 2000).

2.4. Bacteria

Grapevines are affected by three bacterial diseases worldwide. These are bacterial blight, crown gall and Pierce's disease.

Bacterial Blight

This disease called also bacterial necrosis is an economically serious and destructive vascular disease. Bacterial blight has been known in Europe for about 130 years. Incidence and severity of the disease vary from year to year.

Bacterial blight has also been described as “mal nero della vite” (Italy), “gelivure”, “gomnose bacillaire”,“la maladie d’Oleron” and “nécrose bactérienne de la vigne” (France), “necrosis bacteriana” (Spain), “ulamsickte” (South Africa) or “tsilik marasi” (Greece) and other synonymous names in the different countries (Grall & Manceau 2003) where the disease occurs.

Symptoms

While symptoms associated with bacterial blight are characteristic of the disease, they are not bacterial blight-specific, and may be similar to those of other diseases and disorders of grapevines (e.g., fungal diseases) that commonly occur in vineyards. The symptoms can be observed mainly on young shoots and leaves. Symptoms occur on all aerial parts of infected plants in the field from early summer to late spring.

Leaves may be infected via the petiole and the pathogen migrates into the veins resulting in death of whole leaves (Figure 3a). Necrotic areas surrounded by a halo develop on leaves infected through the petiole. Alternatively, leaves are infected via the stomata resulting in development of reddish-brown, angular lesions. The lesions may become chlorotic and surrounded by a discolored halo. The central dried part of the spots may drop out resulting in a ‘shot hole’ symptom. Infection of leaves through the hydathodes results in reddish-brown discoloration of the tips of leaves. When humidity is high, light yellow bacterial ooze from angular, reddish-brown lesions on infected leaves or at the tips of infected leaves.

Infection usually occurs on the lower two to three nodes of young shoots and spreads upwards. Initially, linear reddish-brown streaks appear, extending from the base to the shoot tip. As the tissue below the discolored areas disintegrate and due to hyperplasia of the cambial tissues sometimes extending into the pith, cankers develop (Figure 3b). Infection spreads along the branches and is manifested as a brown discoloration. Infected shoots that do not die are shorter than uninfected shoots, giving the vine an overall stunted appearance. Also, affected grapevines may be less erect than healthy vines. Lower nodes of young shoots develop pale yellowish-green areas. Shoots wilt, become dry and eventually die. Browning of internal tissues of stems is visible in cross-sections. Buds on infected shoots either fail to sprout or are stunted in the spring. Adventitious buds on infected shoots die. Immature flowers turn black and die. Symptoms, including cankers, similar to those on infected shoots, may occur on primary and secondary flower and fruit stalks. On the stem, longitudinal necrosis can be observed following infection. Cross stem sections also show necrotic areas. Infected grapevines die usually within one-

two years following infection. Roots can also be infected, especially when infected scion material is used for grafting, resulting in retarded shoot growth.

Figure 3. Bacterial blight disease caused by *Xylophilus ampelinus* on leaves (a) and on the stem (b). (photos were kindly provided by M. M. López, IVIA, Valencia, Spain).

Occurrence and Impact

Bacterial blight occurs in the Mediterranean region (France, Greece (including Crete), Italy (including Sardinia and Sicily), Spain, Moldova, Portugal, the Canary Islands, South Africa and Slovenia (Dreo *et al.* 2005, EPPO/CABI 1997a, 1997b, Grasso *et al*. 1979, López *et al*. 1980, OEEP/EPPO 2009a, Panagopoulos 1969, Serfontein *et al.* 1997) and its spread to other geographical regions can be expected in the future. The disease may also occur in Argentina, Austria, Bulgaria, Switzerland and Tunisia. In Asia, bacterial blight of grapevine was reported to occur in Turkey; however, the disease has been eradicated from that country (EPPO/CABI 1997a, OEEP/EPPO 2009a). The causal pathogen, *X. ampelinus* is an internationally regulated quarantine pest and implementation of phytosanitary measures affects the international movement of grapevine materials. Bacterial blight is a chronic, systemic disease that results in reduced productivity and shortened life of diseased grapevines. Long distance dissemination of *X. ampelinus* can occur via infected stock, propagating material, and grafting and pruning.

Planting infected but asymptomatic stock can lead to establishment of the disease.

Causal Agent

Bacterial blight is caused by *Xylophilus ampelinus* (Willems *et al*. 1987) formerly called *Xanthomonas ampelina* (Panagopoulos 1969). The bacterium causes disease only in grapevines, and *V. vinifera* is the only known host of *X. ampelinus* where it is localized in the xylem vessels (Grall & Manceau 2003). *X. ampelinus* belongs to the family *Comamonadaceae* in the Class *Betaproteobacteria*. *X. ampelinus* cells are Gram-negative, strictly aerobic, non-spore forming, straight to slightly curved rod (0.4-0.8 x 0.6-3.3 µm) and motile by a single polar flagellum. The bacterium can be cultured on common laboratory media (e. g. Difco Nutrient Agar), although its growth is very slow that makes its isolation quite difficult due to the presence of faster growing symbiotic bacteria and fungi occurring in grapevine tissues. Formation of yellow colonies (about 1mm in diameter) at 25 ^{o}C usually takes 7-8 days (Dreo *et al*. 2005, Serfonstein *et al*. 1997). Filamentous cells have been observed in older cultures. The bacterium metabolizes sugars oxidatively and produces a yellow, insoluble pigment when grown on some artificial nutrient media. *X. ampelinus* is differentiated from *Xanthomonas* species by the absence of xanthomonadins, very slow growth, a maximum growth temperature of 30^{o}C, presence of urease, utilization of *meso*-tartrate and no production of acid from glucose or sucrose. *X. ampelinus* is catalase and urease positive, utilizes citrate, fumarate, malate and tartrate, growth was also observed on arabinose, glucose, galactose, glutamic acid and on a few other carbon sources (OEPP/EPPO 2009a, Willems & Gillis 2006). *X. ampelinus* forms a relatively homogenous genomic group. However, there may be some genomic diversity within the species resulting in geographic- or cultivar-specific populations (Manceau *et al*. 2000). Pathogenic or other ecological variants of *X. ampelinus* have not been conclusively identified.

Biology and Epidemiology

The biology of *X. ampelinus* is not completely understood. Bacteria enter a plant through natural openings (e.g., stomata, hydathodes) and wounds, e. g. during the pruning and grafting work. The bacterium inhabits, multiplies and survives in the xylem vessels of infected plants where *X. ampelinus* cells aggregate in biofilms, and can be detected in expressed xylem fluid (Grall & Manceau 2003). The bacteria are not uniformly distributed within infected plants. Epiphytic survival and multiplication of *X. ampelinus* under natural

conditions have not been conclusively established. However, epiphytic multiplication of *X. ampelinus* has been suggested as symptoms developed when *X. ampelinus*-containing suspensions were sprayed on leaves, but no symptoms were observed when leaves were infiltrated with *X. ampelinus* under high humidity and environmental conditions were conducive to symptom development (Grall & Manceau 2003). Nevertheless, the importance of an epiphytic phase of *X. ampelinus* in the epidemiology of bacterial blight is not fully understood. *X. ampelinus* can survive in infected plants for several years without inducing symptoms. This may be the result of latency and probably depends upon many factors, including environmental conditions. Environmental conditions conducive to symptom development are warm (around 24^{o}C) temperatures and high relative humidity. Infection is facilitated by naturally-occurring and mechanical (e.g., pruning) wounds. Differential susceptibility-resistance of commercially-grown *V. vinifera* cultivars to bacterial blight has been reported, however, this is poorly understood. The genetic and physiological basis of virulence is yet not known.

Transmission

X. ampelinus is spread primarily via epiphytically-colonized or infected grapevine planting materials. *X. ampelinus* is also spread by grafting from infected sources and pruning via contaminated pruning tools. Bleeding xylem fluid from diseased plants is an epidemiologically important source of *X. ampelinus* (Grall *et al*. 2005). Spread of epiphytic *X. ampelinus* or bacteria in ooze from infected tissues is facilitated by wind, rain and overhead sprinkler irrigation. No insect vector has been reported to be significant factor affecting spread of *X. ampelinus* or bacterial blight epidemiology.

Disease Management

Management of bacterial blight is based on rapid, reliable detection of *X. ampelinus*, use of pathogen-free propagative material and planting stock, hot water treatment of propagating material, pruning and destruction of infected canes or branches, application of copper-containing sprays after pruning until about half expansion of new leaves, avoidance of overhead irrigation, sanitation (e.g., disinfestation of tools and equipment), and regulatory phytosanitary and quarantine measures to reduce the risk of long-distance dissemination of the pathogen.

Pierce's Disease

Symptoms

Climatic conditions affect the timing and severity of Pierce's disease symptoms, but generally not the type of symptoms. Pierce's disease symptoms begin to appear in mid- to late summer initially as yellowing of the leaf margin that progresses inward. The yellowing may progress in concentric zones. Eventually the tissue at the leaf margins and tips of large veins turn brown and rapidly become dry giving affected leaves a scorched or scalded appearance. Scorching is characterized by rapid drying as the leaf that develops progressively inward from the margin with the leaf blade becoming brown and dry. The leaf tissue at the edges of necrotic areas may be yellow or reddish-purple tissue depending upon the variety of grapevine (Figure 4). Severely affected leaves eventually drop, leaving the petiole still attached ('matchstick' symptom) to the shoot or cane. Late in the season, 'green islands' of immature tissue surrounded by brown mature bark may appear on canes. Depending upon when the disease develops during the season, fruit growth may be reduced and fruit may eventually wither. Bud break on severely affected vines in the spring is delayed, and shoot growth is slow and stunted with small leaves. Young leaves may show interveinal chlorosis or mottling, and may also be deformed. Leaf scorching and leaf drop increase during the growing season, and severely affected shoots may die back from the tips. Roots of affected grapevines may also die back.

Figure 4. Pierce's disease symptoms on grapevine leaf. Note the yellow border between the necrotic and green tissues. Photo was kindly provided by Dr. Jianchi Chen (USDA-ARS, Parlier, CA, USA).

Occurrence and Impact

The geographical distribution of Pierce's disease is limited by the presence of suitable insect vectors of the pathogen. Except for a few isolated

reports of the disease in France (Boubals, 1989) and in former Yugoslavia (Kosovo) (Berisha *et al*. 1998), Pierce's disease occurs in warm subtropical and tropical areas of North America (United States), Central America (Mexico) and South America (Brazil).

X. fastidiosa infects many plant species and causes serious economic losses in numerous agriculturally important crops; however, it may be considered a weak or opportunistic pathogen (Schaad *et al*. 2004a). Pierce's disease can severely limit commercial production of table, wine and raisin grapes. However, there is considerable variability in cultivar susceptibility. Among wine grape cultivars, 'Barbera', 'Chardonnay', 'Mission', Fiesta, and 'Pinot Noir' are susceptible to highly susceptible, while Thompson Seedless, 'Cabernet Sauvignon' 'Gray Riesling', 'Merlot', 'Napa Gamay', 'Petit Syrah' and 'Sauvignon blanc' are less sensitive. 'Riesling' and 'Zinfandel' are moderately tolerant to tolerant. Many rootstocks are resistant to Pierce's disease, but the rootstock does not confer resistance to susceptible *V. vinifera* varieties grafted on it. However, field observations suggest that the incidence and severity of Pierce's disease may be reduced in varieties grown on some rootstocks.

Causal Agent

X. fastidiosa is a xylem-limited, Gram-negative, strictly aerobic, nutritionally fastidious bacterium in the family *Xanthomonadaceae* in the Class *Gammaproteobacteria*. *X. fastidiosa* cells are thin, straight rods (0.2-0.4 x0.9-4.0 μm). Genetic and pathogenic variants cause diseases in a wide range of horticultural and ornamental crops, and landscape plants. There are distinct differences in the host ranges of *X. fastidiosa* strains (Hopkins & Purcell 2002). Based on comparative phenotypic, serological, structural protein and genetic analyses of several strains from different hosts, distinct genotypes of *X. fastidiosa* were classified into three subspecies (Schaad *et al*. 2004a; Schaad *et al*. 2004b), namely *X. fastidiosa* subspecies *fastidiosa*, *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *pauca*. *X. fastidiosa* subsp. *fastidiosa* strains cause diseases in grapevines (Pierce's disease) as well as in alfalfa, maple and almonds. Separate genetic groups that include strains that cause oleander leaf scorch disease (Scally *et al.* 2005) and that are associated with leaf scorch symptoms in *Chitalpa tashkentensis* in the Southwestern United States (Randall *et al*. 2009) have also been described. However, the sub-specific names suggested or proposed for these strains were not validly published and, therefore do not currently have standing in the international nomenclature of prokaryotes.

The complete genomes of *X. fastidiosa* subsp. *fastidiosa* (strains Temecula-1, M23), *X. fastidiosa* subsp. *multiplex* (strain M12) and *X. fastidiosa* subsp. *pauca* (strain 9a5c) have been sequenced (Chen *et al*. 2010; Simpson *et al*. 2000; Van Sluys *et al*. 2003). The genomes of two *X. fastidiosa* strains isolated from oleander and almond trees have been partially sequenced and annotated (Bhattacharyya *et al*. 2002). Genomic differences among *X. fastidiosa* strains are largely associated with prophage-related chromosomal rearrangements, insertions and deletions that, at least partially, account for the presence of strain-specific genes in different strains (Van Sluys *et al*. 2003). *X. fastidiosa* genomes also include genomic islands that are known to represent adaptive traits; however, the adaptive functions in *X. fastidiosa* are unknown (Van Sluys *et al*. 2003). Pierce's disease diagnosis based on monitoring *X. fastidiosa*-induced transcripts (plant biomarker genes), as well as *X. fastidiosa* subsp. *fastidiosa* rRNA, was recently described (Choi *et al*. 2010). The system was sensitive enough to detect both host gene transcripts and *X. fastidiosa* subsp. *fastidiosa* rRNA in infected, but asymptomatic grapevines. The host biomarker genes were not induced by water deficit.

Biology and Epidemiology

The geographic distribution of Pierce's disease suggests that winter temperature is a significant factor limiting occurrence of the disease. In areas where the pathogen and insect vectors are endemic, indigenous species of *Vitis* are generally resistant or tolerant to *X. fastidiosa* subsp. *fastidiosa* infection. Some *V. vinifera* cultivars may survive for up to five years depending upon the age of the vine when infected, the variety and environment or other local conditions. However, young vines are particularly susceptible to infection and disease development, and may die within two years. Some vines may recover from Pierce's disease during the first winter following infection depending upon when they were infected. However, it is likely Pierce's disease persists as a chronic disease in asymptomatic grapevines that become infected with *X. fastidiosa* subsp. *fastidiosa* in the spring. Alternate host plants and its insect vectors are reservoirs of *X. fastidiosa* subsp. *fastidiosa*.

The glassy-winged sharpshooter has a very wide host range. The principal breeding habitat for the blue-green sharpshooter is riparian vegetation. In areas where aggressive insect vectors (e.g., glassy-winged sharpshooter; GWSS) do not occur, Pierce's disease occurrence is highest in vines nearest overwintering habitats of *X. fastidiosa* subsp. *fastidiosa* insect vectors. However, vine-to-vine spread of the pathogen can occur in the presence of aggressive insect vectors,

such as the GWSS, that move faster and flies greater distances than other sharpshooter species.

Spatial patterns of Pierce's disease occurrence are usually short or shallow diffuse gradients or no significant gradients with distance from alternate hosts in the surrounding environment, suggesting vine-to-vine spread by the GWSS in southern California (Hopkins & Purcell, 2002). Thus, even in areas were the GWSS does not occur, vines that become infected via inoculative overwintered adult insect vectors that enter vineyards in the spring become chronically infected. Late season and dormant period infections resulting from inoculative GWSS feeding throughout the vines can survive the winter and lead to chronic infections. Such chronic infections can persist from year to year (Hopkins & Purcell, 2002).

Transmission

X. fastidiosa subsp. f*astidiosa* is transmitted by several species of xylem-feeding sharpshooters (subfamily Cicadellinae in the leafhopper family Cicadellidae) and spittlebug (family Cercopidae). Insect vectors do not require a measurable period of time between pathogen acquisition and transmission to efficiently transmit *X. fastidiosa* subsp. *fastidiosa* from grape to grape. *X. fastidiosa* subsp. *fastidiosa* is transovarially transmitted in the insect vectors. Once inoculative, insects continue to transmit the bacterium until they molt (Hopkins & Purcell 2002). After molting, insect vectors must re-acquire the bacterium to transmit it. The biology of *X. fastidiosa*-plant-insect vector interaction has recently been reviewed by Chatterejee *et al*. (2008).

Disease Management

Pierce's disease management is based on insecticide treatments to control insect vectors in vineyards and insect vector habitats adjacent to vineyards. In areas in which Pierce's disease is endemic, varieties that are less susceptible to, or more tolerant of, the disease may be effective. During the dormant season, vines that are severely affected or that have had Pierce's disease for more than one year that are likely to be chronically infected should be removed. Diseased canes can also be pruned out during dormant periods. Cutting back diseased vines to just above the graft union to allow vigorous regrowth the following year may help prolong the life of vines for a short period of time. Phytosanitary and quarantine measures to regulate the movement of infected propagative and infected, as well insect vector-infested, plant material can reduce the risk of long distance dissemination of *X. fastidiosa* subsp. *fastidiosa*. Certain native wild American *Vitis* species carry

genes encoding resistance to Pierce'disease. Such sources are utilized for breeding to combine disease resistance with high fruit quality of European grapevines (Krivanek *et al*. 2006).

Crown Gall

Symptoms

Most pathogenic bacteria and fungi causes decay or necrosis of green or woody parts of grapevines leading to the death of the host plant. However, during the development of crown gall disease plant cells start an undifferenciated growth resulting in tumor formation (Figure 5). The primary symptoms of crown gall (called also black knot) of grapevines are galls (tumors) formed at wounds the base of the crown near the soil line and up the trunk of affected grapevines. Galls develop on all wooden portions and roots of the vine where injuries occur. Interestingly, the natural occurrence of crown gall on young green grapevine shoots has not been reported yet. Symptoms become apparent in early summer, and are initially creamy white or light green, but become brown to black in autumn. Red grapevine varieties produce anthocianins in the tumors. Galls are initially soft masses of disorganized tissue, they become dry and woody-like as they age. Bark cracking and peeling may be associated with gall development. Extensive gall development can cause girdling leading to death of crown-affected vines.

Figure 5. Different forms of crown gall disease symptoms. *Agrobacterium vitis* induce local and solitary tumors (a); extensive tumors covering large surfaces (b); and local cauliflower head-like galls on the woody parts of grapevines (c) (Photos by L. Mugnai (a) and E. Szegedi (b & c).

Occurrence and Impact

Crown gall disease occurs in all grape-growing regions around the world, and is economically significant particularly in areas where cold winter temperatures cause severe wounds that are essential for *A. vitis* infection (Burr *et al*. 1998). Most commercially-grown *V. vinifera* cultivars are highly susceptible to freeze injury and crown gall disease development. Reduced growth, and vine vigor of severely affected grapevines result in reduced productivity and cropping potential of vineyard. Young, crown gall-affected vines can die. Dead vines need to be removed and replaced. Both nurseries and plantations are seriously affected by this disease.

Causal Agent

Crown gall disease on grapevine is caused by *Agrobacterium vitis* (synonym: *Rhizobium vitis*) formerly called *Agrobacterium tumefaciens* biotype 3 (Young *et al*. 2005, Young 2008). While *A. tumefaciens* occurs on a wide range of host plants and in soil, *A. vitis* has been isolated only from grapevines or from grapevine rhizospeheres. *A. vitis* belongs to the *Rhizobiaceae* family in the Class *Alphaproteobacteria*. *A. vitis* cells are Gram-negative, strictly aerobic, non-spore producing rods (0.6-1.0 µm x 1.5-3.0 µm), and are motile by one to six peritrichous flagella. *A. vitis* is generally distinguishable from other *Agrobacterium* species by lack of 3-ketolactose production, no acid-clearing on PDA plus $CaCO_3$ and lack of motility at pH 7.0. Additionally, *A. vitis* is pectolytic at pH 4.5, utilizes sodium L-tartrate and grows at 37^{o}C (Moore *et al.* 2001, Young *et al*. 2005). Occasionally *A. tumefaciens* biovar 1 and 2 strains may also be associated with grape crown gall (Palacio-Bielsa *et al*. 2009b, Szegedi *et al*. 2005)

Pathogenic agrobacteria, among others, harbour a large plasmid called the tumor inducing (Ti) plasmid which carries the genes essential for virulence (Sciaky *et al.* 1978). Besides Ti plasmids, *A. vitis* cells contain an additional large plasmid encoding tartrate utilization (Szegedi *et al*. 1992). Since grapevines contain tartrate the ability of *A. vitis* to utilize this compound as carbon and energy source contributes to its association with grapevines (Kado 1998, Salomone *et al*. 1998). Recently, the genomes of *A. tumefaciens*, *A. radiobacter* and *A. vitis* type strains have been completely sequenced. They contain one or two chromosome(s), one of which has chromosomal, while the other (in *A. tumefaciens* and *A. vitis*) has plasmid (*repABC*) replication origins (Slater *et al*. 2009).

Biology and Epidemiology

Due to its biotechnological impact, crown gall tumorigenesis is one of the most extensively studied plant-microbe interaction. The process starts with induction of the virulence genes located on Ti plasmids by plant metabolites released in wounds, i. e. phenolic compounds and sugars. This leads to the synthesis of virulence proteins and a single-stranded DNA, called T-strand which is transferred to the plant cells. The transfer of T-DNA from the procaryotic bacterial cell to the eucaryotic plant cell is mediated by virulence proteins. A set of proteins (VirB, VirD4) form a very sophisticated type IV transport system in the bacterial membrane through which the T-strand and other Vir proteins are transported as a protein-nucleic acid complex into the plant cell. VirD2 and VirE2 proteins direct the transport of T-strand into the plant nucleus where it becomes stably integrated into the host chromosome facilitated by the VirE3, VirF and several host proteins (e. g. BTI1, VIP1, certain histones, etc.). The biology of *Agrobacterium* and the molecular basis of crown gall tumorigenesis have recently been reviewed in detail in several excellent papers (Citovsky *et al*. 2007, Gelvin 2009, 2010, Matthysse 2006, McCullen & Binns 2006, Tzfira & Citovsky 2008).

The genetic transformation of plant cells by *Agrobacterium* results in two basic physiological changes. First, T-DNA directs the synthesis of plant hormones. The elevated hormone level induces unorganized cell division resulting in tumorous growth (formation of crown gall disease symptoms). T-DNA genes directly contributing to crown gall formation are called oncogenes (Britton *et al*. 2008). Second, the tumorous tissues produce specific amino acid derivatives, called opines. The synthesis of opines is also determined by the T-DNA, and the type of opines induced by the given *Agrobacterium* form a basis for the classification of Ti plasmids. Ti plasmids of *A. vitis* isolates can be classified into three opine groups, namely octopine/cucumopine, nopaline and vitopine (Paulus *et al*. 1989, Szegedi *et al*. 1988). In nature, octopine, nopaline and vitopine isolates/tumors occur approximately at 60, 30 and 10 % frequencies, respectively (Burr *et al.* 1998, Ridé *et al*. 2000, Szegedi 2003). These amino acid derivatives serve as selective nutrient source for the inducing bacterium, and they may also induce conjugal transfer of the Ti plasmids to avirulent *Agrobacterium* cells. Thus the production of opines in plant tumors are beneficial for the propagation and maintenance of the inducing bacterium in nature (Dessaux *et al.* 1992, 1998).

Although agrobacteria are known as soil bacteria, until now *A. vitis* has not been isolated from soil except from the grapevine rhizosphere. There is only one reported exception, but such positive results are also derived from

vineyard soil samples (Lim *et al*. 2009). These observations further confirm the strong association of this bacterium with its host plant. The systemic nature of *A. vitis* infection in grapevine was first shown by J. Lechoczky (Lehoczky 1968, 1971) in Hungary and later confirmed by several independent laboratories (Burr & Katz 1984, Cubero *et al*. 2006, Süle 1986, Tarbah & Goodman 1987, Thies *et al*. 1991).

The presence of the bacterium has been shown not only in the woody aerial parts and bleeding sap of the infected plants, but also in the root system (Lehoczky 1978, Szegedi & Dula, 2006) where it can survive for years in the soil even after removing the plantation (Burr *et al*. 1995). Thus, latent, systemic infection of the propagating material is considered to be the main factor in the spreading of *A. vitis* in nature. Tumorigenic *A. vitis* is introduced into new vineyard soils by planting infected nursery stock. *A. vitis*-free grapevines can become infected when planted in soil with debris remaining after the removal of infected vines. Infected grapevines may remain symptomless until injured. Wounds that are sites for *A. vitis* infection can be caused by freezing, pruning, grafting, vine training and other mechanical devices and practices used to cultivate and maintain vineyards.

Transmission

A. vitis is disseminated via apparently healthy, but infected or infested, propagative materials and planting stock, as well as in soil containing infested grapevine debris. Secondary spread of *A. vitis* in vineyards may occur through pruning and cultivation Irrigation can facilitate dissemination of *A. vitis* in vineyards. Intermediate vectors (e. g. insects) contributing to the spreading of *A. vitis* from infected plants to healthy ones have not been described. However, some observations show that nematodes may promote infections of grapevine plants through the roots system (Süle *et al*. 1995). Thus, they can be considered to be potential *Agrobacterium* vectors in the soil, although it has not been proven that nematodes can transmit agrobacteria from one plant to another.

Disease Management

Management of crown gall is based on the use of *A. vitis*-free planting stock (see below). Additionally, a critical crown gall disease management strategy is to avoid planting new vineyards and mother blocks at sites that were previously planted with grapevines, are prone to frost, and have wet, heavy soils. Treatment of planting sites to control soilborne plant parasitic nematodes can help minimize root wounds. Wounding of trunks and canes

mechanically and via freeze injury, should be avoided as far as possible. Other cultural practices include establishing multiple trunks to allow removal of galled trunks and train suckers as new trunks from non-galled trunks, and removal of infected vines that grow poorly or produce light crops. Biological contol methods have also been developed to manage crown gall disease (Kawaguchi *et al.* 2007, Toklikishvili *et al.* 2010, Zäuner *et al*. 2006) although the effectiveness of their practical application has not been documented yet. The use of resistant varieties obtained by traditional or molecular breeding may provide us further alternatives in the control of *Agrobacterium* on grapevines (Krastanova *et al*. 2010, Süle *et al*. 1994, Szegedi *et al*. 1984).

Rare Bacterial Diseases

Besides the above mentioned bacterial diseases which occur in several countries and/or cause serious economic losses from year to year occasional occurrence of some other bacterial diseases of grapevines have also been published. In the late 1970s leaf necrosis observed on Cabernet Sauvignon grapes was associated with the presence of *Xanthomonas* sp. in diseased plants. The wet season most probably contributed to symptom development, since epidemic outbreaks were not observed in dry years (Burr & Hurwitz 1980). More recently decaying inflorescences with necrotic leaves and shoots observed on *V. vinifera* grapes caused significant economic losses in Australia. The causative agent was identified as *Pseudomonas syringae* pv. *syringae* by physiological characteristics and molecular markers. Like *Xanthomonas* sp. infection was associated with high environmental humidity (Whitelaw-Weckert *et al.* 2011). Wood decay caused also by *P. syringae* was observed in Sardinia (Italy) affecting mainly young plantations (Cugusi *et al.* 1986). Due to the localised and sporadic occurrence of these diseases their impact on grapevine growth and yield and production of grapevine propagating material is not well documented.

2.5. Fungi

During the past 20 years the decline of young grapevine plants increase a dramatically worldwide both in nurseries and new plantations. Fungal pathogens causing the vascular disease within the esca complex and those causing black foot have been primarily responsible for this decline.

Esca Complex

Within the esca complex the tracheomycotic fungi *Phaeomoniella chlamydospora (Pch*) and *Phaeoacremonium aleophilum (Pal*) are responsible for the "dark wood streaking" of self-rooted canes and graftings, often followed by a decline known as Petri disease (Mugnai *et al*. 1999, Surico *et al*. 2008). At the nursery stage there are no visible external symptoms and only changes in the internal woody tissue are indicative of infection which can be observed in cross and longitudinal sections of the rootstock and graft junction. In longitudinal sections the dark-brown streak symptoms develop in the scion and in the rootstock, from the base upwards or from the graft junction downwards, while in the cross sections small black spots are often arranged in a ring around the pith. A gum-like, dark exudate may be secreted from infected, sectioned, vessels. Dissemination of these pathogens is primarily via contaminated planting stocks (Acheck *et al*., 1998, Aroca *et al*. 2010, Graniti *et al*. 2000, Morton 1995, Mugnai *et al*. 1999, Surico *et al*. 2008, Waite & Morton 2007, Whiteman *et al*. 2007).

Petri disease occurs in young, 1-8 years old, grapevines (Ferreira *et al*. 1999, Morton 1995, Mugnai *et al*. 1999, Pascoe & Cottral 2000, Scheck *et al*. 1998). Only non-specific symptoms of decline are reported among which the most characteristic one is the stunted shoot growth with mildly chlorotic leaves. The pith can become dark and isolated black spots or groups of spots forming a dark ring around the pith are formed in the wood (Figure 6a). Secretion of the black, gummy fluid from the black spots, as in "dark wood streaking" can also be observed. The diseased plants show poor growth with a significant death rate. Therefore replacing dead grapevines increases costs of grape-growers. Later, reduction in yield and trunk death leads to increasing economic losses.

Another syndrome linked to infections by *P. chlamydospora* and often *P. aleophilum*, was described as a vascular disease called „young esca" (Mugnai *et al*. 1998, Surico *et al*. 2008), or, more recently, as grapevine leaf striped disease (Surico 2009). Small chlorotic spots develop between the veins which later become necrotic. Later they extend and turn into the so-called „tiger-stripe" symptoms consisting of bands of chlorotic and/or deep red tissue surrounding necrotic interveinal stripes (Figure 6b and c). The so called „tiger-like" striped leaves symptom can appear, with rapidly increasing incidence, all along with vineyard life, even in 2-3 year old vines, leading to lower fruit quality and yields and to the death of the vines. The necrotic foliar symptoms are thought to be caused by fungal phytotoxins (Abou Mansour *et al.* 2004, Evidente *et al*. 2000, Sparapano *et al*. 2000). Symptoms in the wooden tissue

are the same as in the case of Petri disease. However, the streaking due to the vascular fungi colonization necrotic lesions extends, causing the development of central or sectorial brown-red or light brown necrosis usually linked to colonization of pruning wounds (Figure 6).

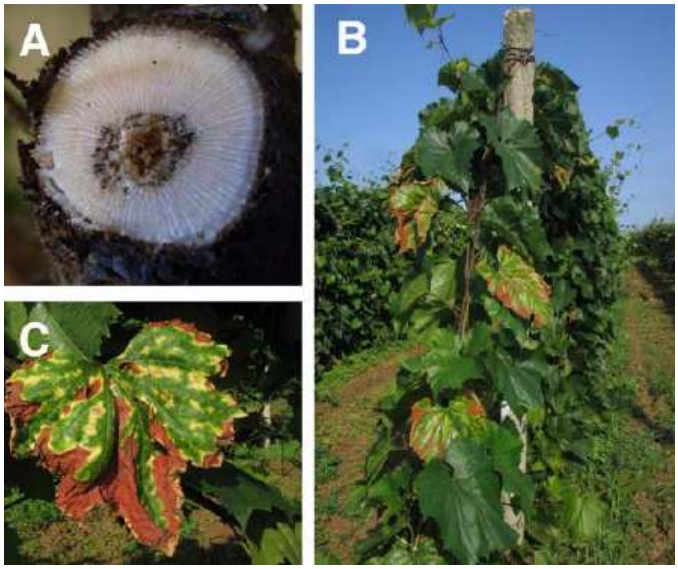

Figure. 6. Woody symptoms (a); infected vineyard (b); and foliar symptoms (c) of 'young esca' caused by *Phaeomoniella chlamydospora (Pch*) on 4 year old *Vitis vinifera* cv. Furmint plants (photos by L. Mugnai).

Within the esca complex another fungal pathogen should be mentioned, even if not transmitted via propagation material: *Fomitiporia mediterranea (Fmed)*, a basidiomycetous fungus causes wood white rot of grapevine, as well as, of many other host plants. In Europe, it is the most frequently isolated fungus causing wood decay, while many other species are found in other parts of affected grapevines (Fischer 2006, White *et al*. 2010). Leaves of the infected plants do not show foliar symptoms, while the infected wooden tissue shows a whitish-yellow rotting and becomes spongy bordered by a dark line (Larignon & Dubos 1997).

As traditionally „esca" is a term linked to the white decay disease, the presence of both the vascular disease causing the striped leaf symptom and the white rot, or decay, on the same vine is referred to esca proper. This syndrome usually appears more and more often in ageing vineyards. In this case both the vascular disease agent(s) and the decay fungus are present in diseased plants.

It is important to note, that the pathogens transmitted and spread via propagation material within the esca complex are the vascular disease agents only, while the occurrence of the decay agent is related to field infections of wounds by aerial spores.

Vines infected by the vascular fungi, *Pch* and *Pal* are frequently colonized also by other fungal pathogens, possibly contributing to trunk decline, as *Botryosphaeria* spp., *Diatrypaceae* species (as *Eutypa lata*), *Phomopsis viticola.* In particular, *Botryosphaeria* spp. have recently been found to infect nursery material (Spagnolo *et al*. 2011). *Pch* has only been isolated from grapevines, with the exception of a recent isolation from a weed in the vineyard (Gramaje *et al*. 2011), and it occurs, on this host all over the world. *Pal* occurs less frequently and infects other woody plants as well (Mostert *et al*. 2006). Currently 34 species of *Phaeoacremonium* have been isolated from different host plants, 25 of which, including *Pal*, have been isolated from symptomatic or asymptomatic grapevine wood.

Still, *Pal* remains the most frequent wood-decay fungus on diseased vines. The teleomorph of *Pch* has not been described yet, while that of *Pal* (*Togninia minima*) was found in several locations (Eskalen *et al*. 2005, Mostert *et al*. 2003, Pascoe *et al*. 2004). The spores of both fungi occur on the surface of rootstocks and scion canes. The spores invade the xylem through wounds at the basal part of stem, at the disbudding sites or at the graft union. In the nursery, infection may occur at any stages of production of propagating material including the hydration and grafting (Aroca *et al*. 2010, Edwards *et al*. 2004a, Fourie & Halleen 2002), via wounds in the rooting area, at the disbudding sites or at the graft union.

Pch can be detected in grapevine soil as well as in infected tissue residues, but the isolation of viable colonies in naturally infested soil was never proved. Therefore, infection of the healthy planting material from infested soil can be hypothesised but it does not appear to be a serious risk (Fourie & Halleen 2004, Rooney *et al*. 2001, Tello & Gonzalez 2010, Whiteman *et al*. 2002). *Pch* and *Pal* can remain latent for several years, and the physiological factors causing symptoms to appear are not fully understood (Di Marco & Osti 2007, Edwards *et al*. 2001, 2004a, Zanzotto *et al*. 2001). Further details on the pathogens, symptom development and disease management have recently been reviewed by Surico *et al*. (2008).

Black Foot Disease

Cylindrocarpon (teleomorph *Neonectria)* species have been reported as causal agents of black foot disease on grapevine (Alaniz et al. 2007, Grasso 1984, Halleen *et al*. 2006, Maluta & Larignon, 1991, Rego *et al*. 1998, 2001, 2006, Scheck et al. 1998). Recent research showed that the causal agents are actually a complex of species belonging both to *Cylindrocarpon* (*Cyl. liriodendri,* as the main species causing black foot on grapevine, *Cyl. destructans*, *Cyl. macrodidynum*) and to *Campylocarpon* (*Campyl. fasciculare* and *Campyl. pseudofasciculare* (Alaniz *et al.* 2007, Halleen *et al*. 2006b).

Black foot agents are soilborne pathogens and they occur in all important viticultural regions of the world. They are frequently isolated from rooted graftings (cuttings) in nurseries (Fourie & Halleen 2001, Rego *et al.* 2000) where they can cause severe losses during rooting, due to root infections by micro-, and macroconidia, chlamydospores or mycelial fragments (Halleen *et al.* 2003, Probst *et al.* 2009) present in the nursery soil. Asymptomatic infected vines usually show decline symptoms once planted in a new vineyard.

The most characteristic symptoms are dark brown to black necrosis at the root crown and sunken necrosis on the roots, causing a decline of the root system. A dark streaking can appear in the woody tissue starting from the basal part of the stem. Diseased plants show slow growth with short internodes and small leaves which become chlorotic or necrotic in the interveinal area. Infected plants die within a short time, and they cannot be immediately replaced as the pathogens are all soilborne resulting in significant losses for the growers. The widely used glyphosate herbicide increases the harmful effect of the black foot agents on moist soils (Whitelaw-Weckert 2010).

3. DETECTION AND IDENTIFICATION OF PATHOGENS

3.1. General Introduction to the Diagnostic Methods

Pathogen-free stock material can be obtained by testing existing plant material to select healthy plants. In this way, visually healthy plants are chosen followed by testing with appropriate protocols. Since certain pathogens (e. g., viruses, phytoplasmas and obligate parasite fungi) cannot be cultured *in vitro*, their detection is carried out directly from the plant material. Bacteria and saprophytic fungi can be enriched or even isolated in pure culture under laboratory conditions for further identification. Although this step increases the time required for pathogen identification, working with pure cultures

makes the pathogen diagnosis more reliable since it eliminates several contaminating materials that may interfere with the identification protocol.

For the detection of the non-culturable grapevine viruses indexing is the most widely used method. This is accomplished by grafting pieces/parts of the plants being tested onto indicator grape varieties which show characteristic symptoms following infection. This can be carried out by transfer of a small piece of wood or dormant chip-bud to the indicator grape (Rowhani *et al*. 2005), by green-grafting (Pathirana & McKenzie 2005a) or micrografting (Pathirana & McKenzie 2005b). Although the novel ELISA- and PCR-based protocols (see below) are more rapid and convenient to use than the laborious and time consuming indexing, this method is still required for certification of virus- and virus-like pathogen-free grapevines (OEPP/EPPO 2008) because it is more reliable for mitigating the phytosanitary risks of quarantine viruses. Besides indexing, immuno-based methods have rapidly become very popular due to their sensitivity and ability for large scale application. ELISA detects pathogen proteins (as antigens) by antibodies produced in animals (e. g., rabbit or goat). The pathogen-specific antibody is conjugated to an enzyme that catalyses a color reaction thereby increasing the sensitivity of the reaction by several orders of magnitude. ELISA kits are now avaiable for most important grapevine viruses (Rowhani *et al*. 2005).

Since 1990s the polymerase chain reaction (PCR) has become the most widely used tool in plant pathology for pathogen detection and identification (Louws *et al*. 1999). This method amplifies specific DNA sequences, if present, by DNA polymerase several million times. The size of amplified fragment is determined by sequence-specific primers that exactly match the start- and endpoints of the target DNA. Thus, new DNA strands are synthesized in both directions. RNAs (RNA viruses) are amplified following reverse transcription ("conversion") into DNA. One cycle of synthesis involves DNA denaturation (92-94 ^{o}C), primer annealing (54-58 ^{o}C) and DNA synthesis (72-75 ^{o}C). The reaction products are usually analysed by visualization in agarose gels and identified according to their sizes. An advantage of PCR over serological methods is that it does not need antigen purification, injecting of animals and serum preparation from animal blood.

In conventional PCR one primer pair directs the synthesis of a well defined single fragment. For nested PCR (that consists of two consecutive reaction steps) and in cooperative PCR more than one pair of primers are designed for the amplification of the specific sequence target. Thus, the reaction becomes more sensitive and specific by eliminating potential false positives. If the detection of more than one pathogen is desired, multiplex PCR

that involves two or more primer pairs designed for detecting different target sequences in a single reaction can be used. In quantitative real-time PCR, the reaction is continuously monitored by the appearance of fluorescent reporter molecules. The level of fluorescence is related to the amount of newly synthetized DNA. Positive reactions can be verified by cloning and sequencing of the amplification products and/or by agarose gel analysis. In recent years, the loop-mediated isothermal amplification (LAMP) has been used increasingly. The reaction is carried out at 65 ^{o}C and DNA amplification is followed directly by a color reaction. Thus, this method can be applied using mobile laboratories in the field since its application requires only a thermal block (Palacio-Bielsa *et al*. 2009a).

The presence or absence of a PCR-amplified target sequences does not always allow the precise identification of a given organism. However, a novel technique called barcoding has been developed that includes sequencing of the amplified fragments. Appropriate genes for barcoding contain short sequences for primer design which border a 600-1500 bp variable region that allows identification of the given organism. Due to the extensive international trade involving plant propagating stock materials, there is an increasing need for rapid and reliable identification of plant pathogens. Accordingly, an EU project has been started to establish and implement a barcoding system for plant pathogens (Bonants *et al*. 2010).

3.2. Virus Identification in Grapevine Stocks

Biological Indexing

The classic method for virus diagnosis, called biological indexing, is based on the specific sensitivity of certain *Vitis* genotypes or herbaceous plants to grapevine viruses. Such plants are called virus indicators. The use of *Vitis* (woody) indicators is still a compulsory step in grapevine certification programs for the identification and detection of certain diseases (OEEP/EPPO 2008). A comprehensive list of virus-, and virus-like diseases as well as their *Vitis* and herbaceous indicators are listed in Table 1. Since isolated virus particles cannot be mechanically transmitted from the tested plants to the indicator, various grafting techniques are applied, e. g., chip grafting, chip-bud grafting, green-grafting or the use or *in vitro* grafting techniques (Pathirana & McKenzie 2005a, 2005b, Rowhani *et al*. 2005, Vindimian *et al*. 1998, Walter *et al*. 1990, 1997). Grafted woody plants are usually transferred to field and

observed for symptom development for 2-3 years, while in the case of green- and *in vitro* grafting symptoms appear within a few months.

Inoculation of herbaceous indicators allows detection of mechanically transmissible viruses, including those of minor or negligible importance (Rowhani *et al*. 2005). The mechanical transmission of viruses to a range of herbaceous indicator plants is used as a preliminary screening of plants designated for indexing on woody indicator plants. This method complements but does not substitute for indexing on *Vitis* indicators. First virus particles can be amplified in *Chenopodium quinoa* indicator plants (bait-plant) and such infected plants that show symptoms are used in a second round to inoculate other indicator species. This two-step method results in a more effective transmission than inoculation made directly from the grape. Herbaceous indicators may develop local or systemic symptoms that appear 10-14 days or a little later after inoculation. A detailed list of grapevine viruses and their *Vitis* and herbaceous indicators are listed in Table 1.

Laboratory Methods

Laboratory methods, such as ELISA and variations of PCR techniques are widely used for detection and identification of viruses in the course of production of virus-free grapevine propagating material.

ELISA, a quick, sensitive and reliable serological method has been routinely applied for large-scale testing in case of several grapevine viruses for about three decades. Commercial kits/sets based on polyclonal antisera and monoclonal antibodies are available for screening of mother plants. Both double antibody sandwich (DAS) and triple antibody sandwich (TAS) ELISA can be used. Cocktails of antibodies successively applied in several combinations to reduce the time and costs of testing. However, the reliability of the ELISA results is influenced by several factors, such as the virus itself, the time and method of sampling, the type of the tissue used for the test, quality of the reagents, etc. (Boscia *et al.* 1997, Gambino *et al*. 2010).

Viruses of infectious degeneration (European and Mediterranean nepoviruses) are reliably detectable by ELISA. The suggested sampling period for detection of GFLV, ArMV, GCMV, ToBRV is when symptoms appear, early summer before flowering (end of May-beginning of June) before the hot summer days. Mature leave blades are used to test for these viruses. Dormant canes are also good sources for virus detection. During hot summer days, the titer of these viruses decreases to below the detection limit. Petioles of the topmost leaves in early summer or phloem tissue from dormant canes are successfully used for grapevine fleck virus testing (Boscia *et al.* 1997).

ToRSV, TRSV, peach rosette mosaic virus (PRMV), causal agents of the degeneration in America, can be detected by ELISA using young leaves collected in the early spring (Rowhani *et a*l. 1992).

For reliable detection of GLRaV viruses, old leaves or dormant canes are generally good sources. The most reliable plant part is the dormant cane but these should not be stored longer than two months as the virus titer significantly decreases during storage resulting in false negatives (Gambino *et al*. 2010). For detection of GLRaV-1 and GLRaV-2 viruses, it is best to use concentrated plant sap extract and biotin-streptavidin amplification. Several commercial ELISA kits are available for GLRaV1 and 2 but they do not detect all strains of these viruses (Bertazzon *et al.* 2002). Detection of GLRaV-3 is the most reliable by ELISA from different plant parts: petioles and midribs of mature leaves or green shoots from June to October. The best source of the virus is the cortical scrapings from mature canes. GVA and GVB are the two viruses of the Rugose wood complex, detectable by ELISA. Dormant canes both of European and American grapevines can be used but the canes should not be stored for more than three months. GVA and GVB kits react also only with a limited number of virus strains (Gambino *et al*. 2010).

During the last decade, different PCR methods were also developed specifically for grapevine viruses where laboratory methods had not existed previously or to increase the sensitivity of detection levels above that of ELISA. Various extraction procedures are used to obtain total RNA of grapevine with or without using commercial extraction kits, e.g. RNeasy (Qiagen) (Dovas & Katis 2003, Osman & Rowhani 2006, Rowhani *et al.* 1995). A rapid CTAB-method was developed by Gambino *et al*. (2008).

Several reverse transcription-PCR protocols were developed for detection of grapevine viruses. References to these are available on the website of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (http://icvg.ch). Polyvalent RT-PCR was applied for the amplification of groups of viruses: for *Vitivirus* (Saldarelli *et al.* 1998), *Nepovirus* (Digiaro *et al.* 2007, Wetzel *et al*. 2002). Dovas and Katis (2003) detected *Vitivirus, Foreavirus* and *Closterovirus* in one reaction tube. Multiplex PCR assays were developed for simultaneous detection of eight or nine viruses (Faggioli & La Starza 2006, Gambino & Gribaudo 2006). Real-Time TaqMan RT-PCR assays provide possibilities to detect viruses/types which have not been detectable previously, such as variants of GLRaV-2 (Beuve *et al*. 2007), GLRaV1-5 and 9 (Osman *et al*. 2007), or the viruses associated with the Rugose wood complex: GRSPaV, GVA, GVB and GVD (Osman & Rowhani, 2008).

Other technology uses DNA microarrays or biochips where solid nonporous surfaces allow the application of 100,000 probes per chip. The short oligonucleotide or the longer cDNA probes are specific to complementary DNA or RNA sequences. Hybridization signals are detected by using labelled complementary sequences. Application of this technology is being extended to the detection of plant pathogens including plant viruses (Boonham *et al*. 2003), and there are a few reports for detection of viruses infecting grapevine (Gambino *et al.* 2010). Due to the multiplex capabilities of the system and the possibility of detecting different pathogens, known and unknown viruses at a time (Osman *et al*. 2008), the power of the microarray technology in the detection and diagnosis of plant pathogens is very high. However the present application is limited due to the high costs associated and the difficulty of adaptation.

High throughput- or deep sequencing technologies allows the analysis of transcriptoms and degradoms. The latter refers to short RNA products of the RNA degrading system of the cells. In purified plant RNAs (total RNAs or mRNAs) RNA products of the pathogens actually infecting the organism are also present. In this way the RNAs of the infectious agents, such as the viral RNAs, can also be detected and identified at the level of sequence specificity. Similarly, degraded short RNAs can be sequenced and data analyzed by computer programs allowing the virtual reconstruction of the possible original RNAs from which the products came. Having viral genome sequence information, specific primers can be synthesised to isolate RNAs of interest. The method is sensitive and is suitable for discovering new pathogens especially new viruses or to distinguish strains and variants in samples. The data produced by deep sequencing require adequate bioinformatic support. The present cost of the technique still limits the number of samples that can be analyzed. Reports have been published for grape virus and viroid detection and identification (Al Rwahnih *et al*. 2009; Navarro *et al*. 2009).

3.3. Detection and Identification of Phytoplasmas

Symptomatology

The same or very similar symptoms are induced by different phytoplasmas on grapevines worldwide. Collectively, these symptoms refer to grapevine yellows (GY) disease. This is a group of serious diseases developing the same typical group of symptoms on leaves, flowers, bunches and canes of *V. vinifera* varieties. However, the diseases and their causal agents cannot be

differentiated based on visual symptoms. According to recent observations in Italy, vines with the earliest appearing symptoms were mostly affected by Flavescence dorée (FD). Later in the season, the incidence of Bois noir (BN) became more frequent (Angelini *et al*. 2006). Visual observation of symptoms is not suitable for the identification of phytoplasma species but the simultaneous presence of the characteristic symptoms on different plant parts plays an important role in the diagnosis of grapevine yellows.

Indexing

Until molecular techniques became available for testing grapevines, phytoplasma detection was difficult. Graft transmission of the varieties onto sensitive indicators (e.g., Baco 22A) was applied. Due to the uneven distribution of the phytoplasmas in the plant, this method is not suitable for reliable detection and cannot be used for specific identification. Therefore, this method is no longer recommended for phytoplasma detection in grapevines (OEPP/EPPO 2007).

Molecular Methods

Grapevine phytoplasmas can be reliably detected from mid-summer through fall. Midveins of leaves, petioles of symptomatic plant parts, as well as phloem scrapings of canes collected in the autumn can be successfully used. Phytoplasmas can be identified also in individual insect vectors or in batches of 5-15 insects (Maixner *et al.* 1997). Diagnosis and characterization of grapevine phytoplasmas is based on DNA amplification by the polymerase chain reaction (PCR), followed by restriction fragments length polymorphism (RFLP) analysis in order to assign them to a '*Candidatus* Phytoplasma' species or to a 16S rDNA group. Several variations of nested-PCR and real-time PCR methods using different universal and group-specific primers have been developed. A number of primers designed on the polymorphic sites of the ribosomal genes have been described for the specific amplification of DNA sequences from FD-, and BN-associated phytoplasma species (Angelini 2010, Galetto & Marczachi 2010). Multiplex real-time PCR assays are successfully used for simultaneous routine detection of FD and BN phytoplasmas (Clair *et al.* 2003, Filippin *et al.* 2006, Pelletier *et al.* 2009, Terlizzi *et al*. 2009).

For the reliable detection of phytoplasmas associated with the GY disease, visual observation of the symptoms in the vineyards combined with use of molecular methods is recommended. For routine screening of grapevines, fast and sensitive real-time PCR is recommended. For further differentiation of phytoplasma strains, nested-PCR followed by RFLP analysis is required

(Angelini 2010). Loop-mediated isothermal amplification has also been developed for phytoplasmas (Tomlinson *et al*. 2010). Application of this protocol for grapevine pathogens would allow the identification of infected plants in the field.

3.4. Detection of Bacterial Infections

Xylophilus ampelinus

Presumptive diagnosis of bacterial blight of grapevines can be made based on symptomatology; however, this should be confirmed by isolation of the pathogen in early spring or late fall (Schaad *et al.* 2001). Isolation of the bacterium may be very difficult because of its slow growth *in vitro*, as fast growing saprophytes occuring in grapevines rapidly overgrow *X. ampelinus* colonies. Visible, pale yellow colonies appear usually after 6-10 days incubation at 24 ^{o}C depending on culture conditions. Colonies can be identified by its cultural and physiological characteristics *in vitro* and pathogenicity tests (OEPP/EPPO 2009a, Willems & Gillis 2006). However, several serological (e.g., immunofluorescence, enzyme-linked immunosorbent assay; ELISA) and molecular assays (conventional, nested and real-time PCR) are also avaliable for rapid and reliable detection and identification of *X. ampelinus* (Hren *et al*. 2010, OEPP/EPPO 2009a, Palacio-Bielsa *et al*. 2009a).

X. fastidiosa

Pierce's disease symptoms resemble drought and other abiotic stresses, as well as some other grapevine diseases. Pierce's disease diagnostic method based on detection and identification of *X. fastidiosa* subsp. *fastidiosa* include isolation of the pathogen on semi-diagnostic media, *in vitro* cultural, physiological and biochemical characteristics; immunoassays, e.g., enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), dot immunobinding assay (DIBA), and various polymerase chain reaction (PCR)-based protocols (Hren *et al*. 2010, Palacio-Bielsa *et al*. 2009a, Schaad *et al*. 2001). Pierce's disease diagnosis based on monitoring *X. fastidiosa* subsp. *fastidiosa*-induced transcripts (plant biomarker genes), as well as *X. fastidiosa* subsp. *fastidiosa* rRNA, was recently described (Choi *et al*. 2010). The system was sensitive enough to detect both host gene transcripts and *X. fastidiosa* ssp. *fastidiosa* rRNA in infected, but not in asymptomatic grapevines. The host biomarker genes were not induced by water deficit stress (Choi *et al*. 2010). Harper and coworkers used the bacterial 16S rRNA processing protein gene

rimM for primer design to detect *X. fastidiosa*. *rimM* specific primers were species-specific and detected all *X. fastidiosa* strains representing the four main subgroups of the pathogen both in LAMP and in real-time PCR (Harpen *et al*. 2010).

Agrobacterium spp.

Although galls on infected grapevines are generally characteristic of crown gall, these may be confused with normal callus tissue. Crown gall tumors caused by diverse types of agrobacteria can be distiguished from healthy callus tissues formed at wounding sites by the presence or absence of opines. Grapevine crown gall tumors induced by *A. vitis* usually contain octopine, nopaline or vitopine, and *A. tumefaciens*-induced tumors may contain octopine, nopaline or agropine depending on the Ti-plasmid types harboured by the disease causing agrobacteria (Szegedi 2003, Szegedi et al. 2005). Opine assays provide simple, rapid and inexpensive protocols to diagnose *Agrobacterium*-infection in grapevines (Fig. 7.). Detailed descriptions of the methods are described by Dessaux and his coworkers (Dessaux *et al*. 1992). Recently, a crown gall diagnostic method based on analysis of the volatile profile of galled plants by gas chromatography-mass spectrometry (GC-MS) was described (Blasioli et al. 2010). Specifically, the presence of styrene was detected in galled, but not in healthy, grapevines. However, while this approach distinguished *A. vitis*-infected and healthy grapevines, it is not clear if the presence of styrene is specific for *A. vitis*-infected, and is not due to other biotic (or abiotic) stress(es). Also, the sensitivity of this approach for detecting *A. vitis*-infected but symptomless plants is not known.

Since tumorigenic endophytes may occur in asymptomatic infected plants, other diagnostic protocols directly target the detection and identification of the pathogen itself. For isolation of agrobacteria from plants and other environmental samples several selective media have been used. Isolated colonies can be identified by their physiological and biochemical characteristics and by virulence assays (Matthysse 2006, Moore *et al*. 2001, Mougel *et al*. 2001, Young *et al*. 2005). Although these classic phytopathological methods are reliable, they are laborious and time-consuming. Thus, they have recently been almost completely replaced by much more rapid PCR-based pathogen detection and identification methods. Total plant DNA extracts or DNA from the isolated colonies can be used. During the past 20 years several chromosomal and Ti plasmid specific primers have been developed for agrobacteria (reviewed in Otten *et al*. 2008, Palacio-

Bielsa *et al*. 2009a). The sensitivity of PCR-based detection can be further increased by nested PCR tests (Lim *et al*. 2009, Peduto *et al*. 2010).

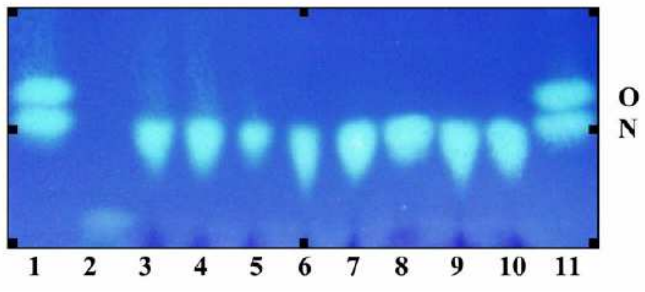

Figure 7. Detection of nopaline in natural grapevine tumors. Lanes 1 and 11: pure octopine (O) and nopaline (N), lane 2: healthy grapevine stem extract, lanes 3-10: independent tumors collected from the same area. The presence of nopaline in tumors distinguishes them from physiological calli and provides clear evidence of their tumorous nature (photo by E. Szegedi).

Although the introduction of molecular assays into plant pathology was a revolutionary break-through, there are still some limiting factors that should be considered during indexing of plant material. The first is caused by the uneven distribution of *Agrobacterium* cells in grapevine plants. Immunofluorescent analysis of canes showed the concentration of bacteria at the nodes, while internodal parts were bacteria-free or contained low cell numbers of the pathogen (Stefani & Bazzi 1989). In one-year old rooted graftings 90% of agrobacteria were found in the roots and 10% in the rootstock, but systemic infection was not detectable in the scion (Szegedi & Dula 2006). The other difficulty comes from the genetic diversity of the pathogen. Grapevines can be infected by various *A. vitis* and *A. tumefaciens* strains (Momol *et al.* 1998, Palacio-Bielsa *et al*. 2009b). Therefore, designing a universal primer pair that can detect all these various agrobacteria in a single conventional PCR reaction is quite difficult. This limitation can be overcome by the combining various primers that direct the amplification of various sequences specific for different groups of agrobacteria. This improved method, called multiplex PCR, allows the rapid detection of diverse types of agrobacteria in a single step (Bini *et al*. 2008, Kawaguchi *et al.* 2005, Kumagai & Fabritius 2008, Pulawska *et al*. 2006). Recently, Lim and coworkers used universal rice primers (URP) to develop SCAR primers to detect *A. vitis*. Although these primers, combined with nested PCR, amplified the appropriate fragment from a wide range of *A.*

vitis strains with high sensitivity, they were not specific for pathogenic strains and did not detect *A. tumefaciens* (Lim *et al*. 2009) that may also occur on grapevines.

3.5. Identification of Fungi Contributing to Esca and Black Foot Disease in Grapevine Wood

In spite of the availability of several modern diagnostic techniques, it still may be difficult to detect and identify pathogenic fungi in the woody parts of grapevines and in the soil. The use of conventional microbiological methods are still essential for isolating fungi *in vitro* for morphological characterization, establishment of type culture collections and for pathogenicity tests. The most commonly used fungal media (e.g., potato-dextrose agar, oat-meal agar, malt extract) are not always appropriate. Thus, several modified media have been introduced to improve the isolation of grapevine fungal pathogens from the microbial communities occurring in plant and soil samples. To suppress the growth of bacteria conventional media are supplemented with antibiotics. Fourie and coworkers (2001) used PDA with chloramphenicol, and Gubler *et al*. (2004) used PDA with tetracycline, while others combined malt extract or PDA with streptomycin-sulfate and ampicillin to facilitate isolation of fungal pathogens (Aroca *et al*. 2010, Romanazzi *et al*. 2009) over bacteria. Tello *et al*. (2009) used two types of semi-selective media, F10S containing PDA, folpet and streptomycin-sulphate, and RB150S based also on PDA supplemented with rose bengal and streptomycin sulphate. These media were appropriate for isolating and identifying *Pch* both from plant tissues and from artificially inoculated soil samples. For *Cylindrocarpon* spp. PDA, „Spezieller Nahrstoffarmer agar" with yeast extract (SNAY) and carnation leaf agar (CLA) was proposed (Rego *et al.* 2001b). Bacterial colonies can also be counter-selected by adjusting the pH of the medium to approx. 5.5 which is optimal for filamentous fungi, while the pH optimum of bacteria is usually 7.0-7.2. After isolation fungal colonies are identified by their morphological characteristics (e. g., fruiting structures, conidia) and molecular markers. The great advantage of isolating colonies on growth media, even if time consuming and often insufficient for a clear identification, is that it detects viable pathogens, while DNA-based methods cannot discriminate between living and dead fungal pathogens.

Among molecular techniques, PCR-based methods are routinely used for the detection and characterization of plant pathogenic fungi. *Pch*, *Pal* and

Fmed were detected by conventional PCR using species-specific or general ITS1/ITS4-specific primers based in genome sequence information available in the GenBank database (Fischer 2002, Overton *et al*. 2004, Pilotti *et al*. 2005, Pollastro *et al*. 2001, Retief *et al*. 2005, Tegli *et al*. 2000, Whiteman *et al.* 2002). Target DNAs are extracted directly from infected woody tissues or from isolated fungal colonies. The molecular techniques are superior to the traditional microbiological methods where endophytic or saprophytic fungi frequently overgrow the pathogen (Retief *et al*. 2005, Romanazzi *et al*. 2009). Martos and coworkers (2011) amplified the rDNA-ITS region by co-operational PCR from several fungi. Of these *P. chlamydospora* was identified by dot blot hybridization using a non-radioactively labelled species-specific probe DNA. *Cylindrocarpon* spp., and also *Pch* and *Phaeoacremonium* spp., were detected from plant and soil samples and from various stages of the grapevine propagation process by nested PCR or by real-time PCR (Aroca *et al*. 2010, Eskalen *et al*. 2001, Nascimento *et al.* 2001b). To isolate *Pch* and *Phaeo* from grapevines, vacuum infiltration of plant tissues was more efficient than placing woody pieces directly on the surface of a culture medium. Using this method rapid processing of high sample number became possible and the infiltration fluid could be used directly for DNA extraction (Aroca *et al.* 2009). For simultaneous identification of *Cylindrocarpon, Eutypa, Botryosphaeria, Phaeomoniella* and *Phaeoacremonium* species causing decline and trunk death from woody tissues, t-RFLP (terminal-Restriction Fragment Length Polymorphism) was also used (Weir & Graham 2009).

Samples were also screened by means of a previously published nested PCR assay specific for *P. chlamydospora*. Based on these assays, it was concluded that: (i) grapevine propagating material play an important role as a source of primary inoculum, not only of *P. chlamydospora*, as previously reported, but also for members of the *Botryosphaeriaceae*, among which *Neofusicoccum parvum*, *Botryosphaeria dothidea* and *Diplodia seriata* are the most common, and (ii) multiple infections by different species belonging to *Botryosphaeriaceae* and/or *P. chlamydospora* occur frequently both in standing vines and propagation material. This last finding supports the hypothesis that at least some of the non-specific symptoms of grapevine decline may be due to the presence of different pathogens within host tissues.

4. ELIMINATION OF PATHOGENS FROM INFECTED PLANTS

4.1. General Description of Curative Methods

Besides selection of healthy stocks from existing plant material by various diagnostic protocols pathogen-free plants can also be produced by curative treatment of plant material or by appropriate propagation methods. To eliminate systemic infections, dormant canes are subjected to hot water treatment (HWT). This is based on the observation that, in a narrow temperature range (usually between 48-52 ^{o}C for grapevines), several (but not all) pathogens and pests are killed when incubated in water for 30-60 minutes while grapevine buds remain viable. Since this method can be applied for large amounts of propagating material, it is now considered to be a general curative treatment for dormant grapevine canes (for details see below).

For growing plant organs (green shoots), "heat therapy" has been used as a classic method for many decades to eliminate systemic virus infection from plants. This process involves growth of target plants in heat chambers at 37-38 ^{o}C for 2-4 months followed by removing the apical meristems for *in vitro* regeneration and propagation. Since this method produces virus-free plants at variable frequencies, individuals should be retested for the absence of viruses (OEPP/EPPO 2008). A novel protocol for virus elimination has been developed from cryopreservation of plant stocks. Pathogen-free plants can be regenerated at very high frequency when dehydrated shoot tips are exposed to ultra-low temperature in liquid nitrogen (-196 ^{o}C) prior to starting *in vitro* cultures. This treatment called "cryotherapy" may yield virus-free plants at frequencies greater than 90 % (Wang & Valkonen 2008, Wang *et al*. 2008).

In conclusion, for the safest and most effective elimination of grapevine pathogens a combination of the above methods including (i) hot water treatment of dormant canes, (ii) heat/cryotherapy of plants with actively growing shoots and (iii) *in vitro* shoot tip/apical meristem cultures is useful. Pathogen-free plants obtained by either of the above described protocols serve as a basic material to establish stock plantations for large scale production of propagating material.

4.2. Production of Virus-Free Plants

For production of virus-free grapevines plants with actively growing shoot are grown at a relatively high temperature (37-38 ^{o}C) for 3-4 months (heat-therapy). Then shoot tips are removed, surface sterilized and apical meristems or shoot tips are prepared to start *in vitro* cultures. Shoots are regenerated on cytokinin (e. g., benzyl-adenine)-containing medium followed by rooting them on hormone-free or auxin (e. g., indole-acetic-acid)-containing medium. Establishment and propagation of grapevine *in vitro* cultures, including their use for obtaining virus-free plants, has been reviewed in detail by Torregrosa *et al*. (2001). This method usually produces virus-free plants at about 20-40 % or higher frequencies and is influenced by several factors. Recently heat-therapy has been adapted also to *in vitro* grapevine plants (Maliogka *et al*. 2009, Panattoni & Triolo 2010). Under hot conditions (e. g., in South Spain) even field samples may be used to start *in vitro* cultures for selecting virus-free plants (Valero *et al*. 2003).

Wang and coworkers (Wang *et al*. 2003) used cryotherapy to eliminate grapevine virus A (GVA) from infected plants. While meristem culture alone produced virus-free plants only at a frequency of 12 %, 96 % of the shoot tips were virus-free following treatment in liquid nitrogen. A further advantage of this method is that laborious and time consuming excision of the apical meristematic tissue (0.5 mm or less) is not necessary, since only virus-free meristematic cells survive the freezing process. An additional advance in the production of healthy grapevine plants was developed in South-Africa. Virus-free plants were rescued through somatic embryogenesis followed by plant regeneration. Regenerated plants were free of symptoms, and their virus-free status was confirmed by ELISA (Torregrosa *et al*. 2001). Today this method is used by several laboratories to eliminate viruses from grapevines with nearly 100% or even to 100% efficiency as shown by ELISA and RT-PCR testing of true-to type regenerated plants (Borroto-Fernandez *et al*. 2009, Gambino *et al*. 2009). Physiological factors that contribute to the elimination of viruses during the induction of somatic embryos have not been determined yet. This may be the result of a hormonal effect during the *in vitro* process, or due to the lack of vascular connection between the embryo and callus tissue (Gambino *et al*. 2009). The lack of embryo-forming capacity of virus infected plant cells may also select the the virus-free cells during the formation of somatic embryos.

Rooted plants can be further propagated *in vitro* as single-node cuttings or acclimatized for greenhouse and field growth. Prior to acclimatization, *in vitro*-grown grapevine plantlets can be further propagated also by rooting

single-node cuttings under inorganic conditions (e. g. in perlite or rockwool moisted with tapwater, pH 6.0). After two-three weeks, they form roots and can be hardened by gradual opening of containers used for culturing. Such plants do not become contaminated due to the inorganic conditions of the *in vitro* culture medium. Using this simple method, 75%-100% of the plants were acclimatized (Zok *et al*. 2007).

4.3. Elimination of Phytoplasmas from Grapevine Stocks

Hot water treatment of dormant canes or rooted plants with dipping in water of 50 ^{0}C for 45 minutes is an efficient method for eliminating both FD (Boudon-Padieu & Grenan 2002, Caudwell *et al.* 1990, 1997, Mannini *et al.* 2009) and stolbur phytoplasmas (Mannini *et al.* 2009) from grapevine propagating material. This treatment also kills the eggs of the vectors (e. g., *S. titanus*, see paragraph 5. in this chapter) that overwinter under the bark. The method has been widely used in France, Italy and Australia for several years. Maintenance of the mother plantations providing the "base" material under insect-proof structures is proposed to avoid phytoplasma infection (Mannini, 2007).

Gribaudo and coworkers (2007) used *in vitro* micropropagation techniques to completely eliminate FD phytoplasmas, while "bois noir'-free lines were selected at a success rate of 40 %. Samples for these studies were collected from the field. Shoot tips started from hot water-treated canes would probably increase the efficiency of *in vitro* techniques. Although cryotherapy has not been tested yet for the production of phytoplasma-free grapevines, this method may be a useful alternative for the existing methods. The causative agent of sweet potato little leaf disease '*Candidatus* Phytoplasma aurantifolia' was eliminated with 100% efficiency using frozen shoot tips for *in vitro* cultures as starting material. Similarly, '*Candidatus* Liberibacter' species associated with citrus huanglongbing (HLB) was eliminated from citrus plants with 98% efficiency by this method (Wang *et al*. 2008).

4.4. Methods for the Elimination of Systemic Bacterial Infections

In contrast to viruses and phytoplasmas, bacteria live intercellularly in grapevines, mostly in the xylem and in the phloem. Thus, their migration in the plant is determined by the differentiation of vascular vessels. The latent

occurrence of the most common bacterial pathogens of grapevine (*X. ampelinus*, *X. fastidiosa* subsp. *fastidiosa* and *A. vitis*) in propagating stocks has been well documented. Thus, freeing plant material of these bacteria is of significant importance to the grape industry.

Thermotherapy of various plant parts has been used for more than a century to kill pests and pathogens to prevent their dissemination by movement of plant material in domestic and international commercial channels. The procedure is based on the observation that, within certain temperature regimes (approx. between 48-52 ^{o}C), dormant vegetative plant parts (e. g., canes) or seeds can survive, while pathogens and pests are killed (Baker 1962, Grondeau & Samson 1994). Hot water treatment has also been adopted for and widely used to eliminate pathogens and pests from dormant grapevine canes (Boudon-Padieu & Grenan 2002). Treatment at 50 ^{o}C for 20 min. completely eliminated the Pierce's-disease causing bacterium from the propagating material of several varieties. The treated plants remained healthy up to two years (Goheen et al. 1973). The same treatment is an appropriate phytosanitary procedure to produce grapevines free of *X. ampelinus*, the causal agent of bacterial necrosis (Psallidas & Argyropoulou 1994). Several experiments have also been carried out to eliminate *A. vitis* from dormant grapevine canes. Although *A. vitis* cells *in vitro* did not survive 30 min in water at 50 ^{o}C under laboratory conditions, they were not completely eliminated from heat-treated canes (Burr *et al*. 1996). The resistance of *A. tumefaciens* that may also occur in grapevine at 50 ^{o}C is an additional limiting factor of the application of hot water treatment to prevent spreading of crown gall disease with propagating material (Burr *et al*. 1989).

A significant improvement was introduced by Burr and coworkers who observed that shoot tips of *V. vinifera* cv. Pinot Chardonnay were completely free of *Agrobacterium* infection even if they were derived from contaminated plants (Burr *et al*. 1988). These results were further confirmed in Germany by Altmayer (1990) and Stellmach (1997). Thies and Graves (1992) started *Vitis rotundifolia* apical meristems from which they regenerated approximately 200 plants. None of these plants were infected. To increase the efficacy this method may be combined with the application of certain antibiotics that do not affect the growth of plant cells but kill agrobacteria (e. g. claforan, carbenicillin, etc.). Such antibiotics are widely used in plant biotechnology during transformation experiments to eliminate *Agrobacterium* cells after co-cultivation. Shoot tip cultures are also an efficient tool for producing plantlets that are free of *X. fastidiosa* subsp. *fastidiosa* (Robacker & Chang 1992). Although these methods (apical meristem culture and shoot tip culture) are

very effective in several laboratories for producing bacteria-free plants, it is still advisable to confirm their pathogen-free status since a few bacterial cells may still be recovered after the *in vitro* procedures (Poppenberger *et al*. 2002). To this end, a three-step screening method is proposed including (i) visual selection, screening (ii) the plant tissue culture media and (iii) pieces of plants for the presence of bacterial colonies (Thomas 2004).

4.5. Curative Protocols for Elimination of Fungal Infections from Grapevine Propagating Material

The primary reason for dramatic increases in early grapevine death caused by systemic fungal infection is a consequence of unproper hygienic conditions during the production of propagating material. Since there are no effective chemical control methods to prevent the spread of these fungi via latent infections, prevention of infection by the use of pathogen-free planting material is a key strategy. An extensive review on all aspects related to spread and control of wood pathogens in the nursery has recently been published (Gramaje & Armengol 2011). Although these fungi are systemic, the surface sterilization of rootstock- and scion canes is still important as spores can contaminate the cane surface. Several attempts have been carried out including physical treatment and the use of biological and chemical agents, to eliminate fungal infections from propagating material. Of these, some satisfactory results were obtained with hot water treatment (HWT), *Trichoderma harzianum* and a few chemical compounds.

HWT has been widely tested to eliminate fungal infection from grapevine propagating material (Crous et al. 2001, Edwards et al. 2004b, Fourie & Halleen 2004, Gramaje et al. 2009a, Retief et al. 2005, Rooney & Gubler 2001, Waite 1998, Whiting et al. 2001) with variable results, often leading to only partial reduction of pathogen viability. It is advisable that such treatment be done before storing the collected dormant canes, and treated material should be cooled gradually in air to prevent heat-stress and bud mortality. There may be differences in heat tolerance among grapevine varieties and other factors related to mother vine and storage conditions that should also be considered. Among *V. vinifera* cultivars, Pinot noir is relatively heat sensitive; Chardonnay, Merlot and Riesling are moderately sensitive, and Cabernet Sauvignon is the most tolerant (Crocker et al. 2002, Waite & May 2005, Waite & Morton 2007, Waite et al. 2001). However, in the effective heat range (50-52 ^{o}C), all varieties survived the HWT (Wample et al. 1991). To provide a

prolonged pathogen-free status of planting material, HWT can be combined with fungicide treatment (e. g., cyproconazole) to prevent rapid re-infection from soil by black foot pathogens and *Pch* (Serra et al. 2009).

Trichoderma species have been widely used as a biocontrol agents against tracheomycotic fungi (Howell 2003). The use of *T. harzianum* and other *Trichoderma* species are highly beneficial for plants since, by colonizing the rhizosphere, these fungi prevent infections through the root system, increase the stress tolerance and resistance of grapevines to pathogens, and promote root growth (Di Marco & Osti 2007, Di Marco et al. 2004, Fourie et al. 2001, Hunt et al. 2001). *Trichoderma* spp. can be effectively applied in several forms, including immersion of propagating material into a suspension of fungal cells during rehydration, soil or root-treatment when a new plant is planted, or irrigation; however the results can be very different or even negative if the application time is not well planned for each single commercial product (Di Marco & Osti 2007).

Various fungicides have also been applied to eliminate fungal infections in grapevine planting material to protect wounds from infections during grafting. Of these, systemic compounds having wide ranges of activity (e. g., benomyl, thiophanate-methyl and thiram) were the most effective against *Pch*, *Pal*, *Cyl* (Alaniz et al. 2011, Fourie & Halleen 2004, 2005). In addition, soaking planting material in didecyldimethylammonium-chloride, captan and carbendazim effectively eliminated these pathogens (Fourie & Halleen 2006, Gramaje et al. 2009b). To increase the effectiveness of the control of fungal infections spread by latently infected grapevine propagating material, an integrated system of physical, chemical and biological methods was proposed (Fourie & Halleen 2004).

Although it has not been documented, application of *in vitro* shoot tip cultures is also a promising and efficient tool to produce grapevines free of latent fungal infections, which are often present in canes from infected mother vines. As such cultures are sterile, it is unlikely that they are latently infected with fungi since the pH (5.8) and the rich organic and inorganic nutrient composition of plant tissue culture media favors the growth of most fungi that may be associated with grapevine. Strains of *Cylindrocarpon* spp., *Diplodia seriata*, *F. mediterranea*, *Neofusicoccum vitifusiforme*, *Phaeoacremonium* spp. and *P. chlamydospora* grew better on Murashige-Skoog (MS) plant tissue culture medium than on potato dextrose agar (PDA) used as conventional fungal medium (Szegedi E., unpublished data). Despite the availability of several possible chemical or biological control methods it is important to pay

particular attention to hygiene during storage, rehydration, grafting and appropriate viticultural practices in the nursery (Gramaje & Armengol 2011).

5. GRAPEVINE PESTS THAT ARE DISSEMINATED BY PROPAGATING MATERIAL

Besides the microbial pathogens described above there are several pests which directly cause significant damage or contribute to the spreading of several pathogens as vectors or promote their infections through causing wounds.

Nematodes live in the soil and invade intercellular spaces or surfaces of the root system (Barker 2003). Some of them (e. g. *Meloidogyne hapla*) cause root-knot, while others (e. g. *Xiphinema americanum sensu lato, X. index, X. californicum, X. diversicaudatum, Longidorus elongatus*) are vectors of certain grapevine viruses such as *Tomato ringspot, Grapevine fanleaf, Arabis mosaic* and *Tomato black ring* (Frison & Ikin 1991, Martelli and Boudon-Padieu 2006, OEPP/EPPO 2009b). Wounds caused by nematodes promote *Agrobacterium* infections of grapevines through the root system (Süle *et al*. 1995).

Another important pest, the grape phylloxera (*Daktulospharia vitifoliae*) also parasitizes the root systems although gall formation can also be observed on leaves. This pest almost completely destroyed the European viticulture during the second half of the 19th century following its importation from North America. Grapevine cultivation was restored by the introduction of phylloxera-resistant American *Vitis* species as rootstocks (Granett *et al*. 2001). Since then, self-rooted grapevines have been replaced by grafted plants worldwide. Self-rooted grapes can be grown only in sandy soils in which this pest cannot exist.

Grapevine mites (e. g. *Colomerus vitis, Calepitrimerus vitis*) colonize the young leaves and their increasing population causes abnormal and delayed leaf and shoot development. Mites usually overwinter in the buds of one year old canes and are also disseminated via propagating stock. The effect of mite damage may be especially harmful in nurseries since shoot development on rootless canes is much slower than in older rooted plants.

Some species of the scale-like pseudococcid mealybugs, coated with a powdery mealy waxy secretion feed on the phloem of the leaves, canes, trunk and also on the bunches of the vine. With their needle-like mouth parts, they

suck out the plant sap and excrete the unused plant materials, the honeydew, onto the leaves, canes and the berries. Beside the unmarketable fruit covered by honeydew and black sooty mold, they indirectly induce severe damage as natural vectors of certain grapevine leafroll-associated viruses e.g. *Heliococcus bohemicus* and *Phaenacoccus aceris* (GLRaV-1); *Planococcus ficus*, *Pseudococcus maritimus* and *Ps. viburni* (GLRaV-3), *Ps. longispinus* (GLRaV-3, 5 and 6). *Planococcus ficus* and *Ps. longispinus* transmit GVA and GVB, the causal agents of the Rugose wood disease (Martelli & Boudon-Padieu 2006).

Due to the waxy cover of the mealybugs and their overwintering under the bark, their chemical control is difficult. However, quite recently promising results have been reported by Pietersen *et al.* (2009) about controlling of the spread of GLRaV-3 in *P. ficus* mealybug-infested vineyards in South Africa. Soft scale insects are also known to transmit grapevine viruses in the nature. *Pulvinaria vitis* and *Neopulvinaria innumerabilis* are vectors of GLRaV-1 and *N. innumerabilis* is also a vector of GVA (Martelli & Boudon-Padieu 2006).

Certain Auchenorrhyncha insects transmit phytoplasmas. For example, *S. titanus* leafhopper (Cicadellidae) is monophagous species that feeds and exists only on grapevine. Besides causing sucking injuries to vines, the major damage is caused by this species as a vector of the Flavescence dorée (FD) quarantine phytoplasma (OEPP/EPPO 2007) threatening the vine industry in Europe and Israel (EPPO/CABI 1997c). *H. obsoletus*, the polyphagous Cixiid planthopper is the vector of the Stolbur phytoplasma to grapevines (Maixner 1994). Further studies are needed to clarify the role of these potential vector species in the epidemiology of Stolbur-phytoplasma (Maixner 2011). New, effective control strategies need to be developed to mitigate the effects of these vectors.

To eliminate the above mentioned pests from propagation material, hot water treatment of planting stocks can be universally applied. Successful applications of this procedure have been described to kill nematodes (Gokte & Marthur 1995), and to eliminate phylloxera (Goussard 1977) and mites (Szendrey *et al.* 1995). During these treatments, dormant grapevine material is immersed in hot water (50-52 ^{o}C) for various intervals (20-60 min). Similarly, the overwintering eggs of the phytoplasma leafhopper vector *S. titanus* (Caudwell *et al.* 1997), as well as mealybugs (*Planococcus ficus*) (Haviland *et al.* 2005), were also killed following such treatment. Hot water treatment is a universal method to free dormant grapevine propagating material of many pests, including pathogen vectors.

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