

International Journal of Horticultural Science 2011, 17 (3): 45–49.
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

Bacterial diseases of grapevine

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Summary: Grapevines are affected by three major bacterial diseases worldwide, such as bacterial blight (*Xylophilus ampelinus*), Pierce's disease (*Xylella fastidiosa*) and crown gall (*Agrobacterium vitis*). These bacteria grow in the vascular system of their host, thus they invade and colonize the whole plant, independently on symptom development. Latently infected propagating material is a major factor in their spreading. Therefore the use of bacteria-free planting stock has a basic importance in viticulture. Today several innovative diagnostic methods, mostly based on polymerase chain reaction, are available to detect and identify bacterial pathogens of grapevines. For production of bacteria-free plants, the use hot water treatment followed by establishment of *in vitro* shoot tip cultures is proposed.

Keywords: *Agrobacterium vitis*, bacterial blight, crown gall, Pierce's disease, *Vitis vinifera*, *Xylella fastidiosa*, *Xylophilus ampelinus*

Grapevine as a host for bacteria

Bacteria require several environmental factors for their *in planta* growth, including availability of appropriate nutrients, optimal pH, relative humidity and temperature. Grapevine xylem sap contains large amounts of various inorganic and organic compounds including sugars (glucose, fructose) organic acids (tartrate, malate, citrate) and the complete range of amino acids of which glutamine may reach 90% of total amino acid content (Andersen & Brodbreck 1989, Glad et al. 1992, Prima-Putra & Botton 1998, Roubelakis-Angelakis & Kliwer 1979). The pH varied between 5.7 and 6.9 (Glad et al. 1992, Roubelakis-Angelakis & Kliwer 1979) which is appropriate for several microbes. Among the organic acids, tartrate is a characteristic compound of grapevine (Ruffner 1982). These materials serve as potential nutrients for, and support the growth of a wide range of bacteria. In a Canadian survey, the number of endophytic bacterial cells in xylem fluid exceeded 10⁴ cells/ml. Most of them were Gram-negative bacteria in the genera *Pseudomonas* and *Enterobacter* (Bell et al. 1995). The nutrient-rich environment of grapevine also provide favourable conditions for bacterial pathogens, that is they are able to use the nutrient sources provided by this host. For example, the ability of *Agrobacterium vitis* to utilize tartrate contributes to its association with grapevines (Kado 1998, Salomone et al. 1998). Tartrate utilization by *Xylophilus ampelinus in vitro* has also been reported; however, its role in the host specialization of this pathogen has not been documented yet (Dreo et al. 2005, Willems & Gillis 2006). Other xylem compounds, e. g., glucose, citrate and glutamine support the growth of *Xylella fastidiosa* subsp. *fastidiosa in vitro* (Almeida et al. 2004, de Macedo Lemos et al. 2003).

Besides providing nutrients, grapevine sap contains certain, yet undetermined signal compound(s), which induce(s) a differential gene expression in *X. fastidiosa* subsp. *fastidiosa* resulting in biofilm formation (Shi et al. 2010, Zaini et al. 2009).

General features of bacterial diseases

Among grapevine diseases, those caused by various bacterial species are significantly important in most grape-growing countries. They are characterized by relatively slow symptom development compared to foliar and fruit cluster diseases caused by fungi (e. g., downy mildew, powdery mildew, grey mold). Chemical or biological control methods, that could efficiently prevent symptom development are not available for bacterial diseases. Bacterial pathogens associated with grapevine diseases occur in the vascular system and intercellular spaces of their host plant and they can be cultivated on laboratory media. Thus their detection, identification and use for experimental purposes is far easier than those of obligate parasites. All bacteria causing grapevine disease are systemic in the vascular tissues, and this frequently leads to latent infections. *A. vitis* and *X. fastidiosa* subsp. *fastidiosa* produce polygalacturonases, plant cell wall degrading enzymes, that virulence factors facilitate bacterial spread in the xylem (Rodriguez-Palenzuela et al. 1991, Roper et al. 2007). Indeed, Pierce's disease symptom development was delayed in grapevine plants which expressed a polygalacturonase-inhibiting protein gene (Agüero et al. 2005). A consequence of their systemic nature is that they are disseminated by propagating material. Therefore, use of healthy plant stocks for grapevine

propagation is a basic prerequisite for minimizing economic losses caused by these bacterial pathogens.

Three major bacterial diseases are known in several grapevine-growing countries worldwide. These are bacterial blight (*X. ampelinus*), Pierce's disease (*X. fastidiosa* subsp. *fastidiosa*) and crown gall (*A. vitis*). These bacteria live in close association with their grapevine host and (in the case of *X. fastidiosa* subsp. *fastidiosa*) with their insect vector. The sporadic occurrence of *Pseudomonas* sp. (Whitelaw-Weckert et al. 2011) and *Xanthomonas* sp. (Burr & Hurwitz 1980) associated with inflorescence decay and leaf necrosis, respectively, have also been described. However, their economic importance in viticulture has not been conclusively determined, therefore we do not discuss them in detail here.

Bacterial blight

This disease is also known as bacterial necrosis. Bacterial blight is caused by *X. ampelinus*, a Gram negative bacterium that is a member of the Class β -Proteobacteria. The bacterium infects grapevines through wounds or natural openings, such as stomata and hydrathodes. On the stems longitudinal reddish-brown streaks usually appear that develop into cankers. Leaves may be infected via the petioles and veins resulting in death of the whole leaf. When infection occurs through the stomata, *X. ampelinus* cause local necrotic symptoms.

Bacterial blight occurs mainly in the Mediterranean region of Europe (Italy, Greece, Spain), in South Africa and more recently in Slovenia (Dreo et al. 2005, Grall & Manceau 2003, OEPP/EPPO 2009, Serfontein et al. 1997). Isolates derived from different sources show very little physiological and biochemical differences (Dreo et al. 2005, Willems & Gillis 2006). The physiological and molecular bases for virulence of *X. ampelinus* have not been determined.

X. ampelinus is closely associated with grapevine. No other host plant or intermediate vector has been described. Infected propagating material is the primary source of its dissemination. Therefore, diseased plants should be removed from plantations. Resistant *Vitis* species and *V. vinifera* varieties are not known.

Pierce's disease

Pierce's disease is caused by *X. fastidiosa* subsp. *fastidiosa*, a Gram-negative bacterium that belongs to the Class γ -Proteobacteria. The leaves become brown, beginning at the tips and margins, and progress inward. Necrotic lesions usually are surrounded by a yellow border that separates the brown and green leaf tissues. As the disease develops, leaves drop prematurely so that the petioles remain attached to the stem, appearing like „matchsticks”. Hot and arid weather increases symptom development that finally lead to decline.

Pierce's disease of grapevine is common in the southern part of USA, in Central America and in South America

including some islands in the Caribbean region (Aguilar et al. 2008, McGaha et al. 2007, Stover et al. 2008). A few reports describe Pierce's disease in Europe (Berisha et al. 1998, Boubals 1989), although it has, so far, not become epidemic probably due to the lack of appropriate vectors.

During the disease progression, the pathogen invades the grapevine xylem where it attaches to the plant cell wall. The bacterium extensively colonizes these vascular elements forming biofilms in the xylem vessels. This process finally leads to xylem occlusion resulting in blockage of water and nutrient transport (Chatterjee et al. 2008). The pathogen has an extremely wide host range. Besides grapevine, *X. fastidiosa* subsp. *fastidiosa* occurs in several wild plant species that highly promote its survival and spread (Costa et al. 2004, McGaha et al. 2007). *X. fastidiosa* subsp. *fastidiosa* cells are transmitted from infected plants to healthy ones by xylem-feeding insects, e. g., leafhoppers and sharpshooters. To reduce economic losses caused by Pierce's disease, it is necessary to remove diseased grapevines from the plantation and potential host plants (weeds) from areas closely surrounding vineyards, and to apply insecticides to control vectors. European (*V. vinifera*) grapevine cultivars are susceptible to this disease, but a few native American *Vitis* species (e. g., *V. aestivalis*, *V. arizonica*) are resistant (Krivanek et al. 2006).

Crown gall

Crown gall disease is caused by the Gram-negative bacteria *A. vitis*, and occasionally by *Agrobacterium tumefaciens* (Burr et al. 1998, Palacio-Bielsa et al. 2009a, Szegedi et al. 2005). The genus *Agrobacterium* belongs to the Class α -Proteobacteria. Pathogenic agrobacteria contain large plasmids called Ti plasmids (pTis) that carry the genes for their tumorigenicity. pTis of *A. vitis* form three distinct groups on the basis of their opine-inducing ability, as octopine/cucumopine, nopaline and vitopine groups (Burr et al. 1998). Opines are various amino acid derivatives produced exclusively in crown gall tumors. Plant tumors secrete these compounds which are selective carbon and energy sources for the inducing agrobacteria. Besides pTis, *A. vitis* cells contain an additional large plasmid coding for tartrate utilization, that contributes to the adaptation of *A. vitis* to grapevine (see above).

In nature, tumors always appear on the woody parts of the infected grapevines. They may cover the whole surface of trunk, or arise from local wounds exhibiting a cauliflower-like morphology. Natural symptoms on young, green shoots have not been reported. Since they are highly susceptible to infection, the absence of crown galls on young stems may be explained by the bacterium-free status of the green shoots.

This disease causes economic losses not only in the plantations but in nurseries as well. Crown gall is the most widely distributed bacterial disease of grapevine. Its occurrence has been reported in nearly all grape-growing regions of the world, including several European countries.

During crown gall tumorigenesis, a part of the pTi plasmid, called T-DNA, is transferred from the bacterium to the plant cell, and it becomes integrated into the host plant's chromosomal genome. Expression of T-DNA genes in the plant cells results in two basic physiological changes. First, undifferentiated cell division occurs as a result of hormonal overproduction. Second, tumorous cells produce specific amino acid derivatives (opines). The type of opines produced by crown galls is determined by the inducing *Agrobacterium*. Details on the molecular basis of crown gall tumorigenesis have been reviewed (Gelvin 2009, 2010, Tzfira & Citovsky 2008.).

A. vitis, like *X. ampelinus*, is closely associated with grapevines. The occurrence of this bacterium in other plants or in virgin soils has not been reported. Similarly, vectors that disseminate *A. vitis* are not known. Although nematodes promote *Agrobacterium* infection of grapevine (Süle et al. 1995), it is not known if they can mediate the transmission of this bacterium from one plant to another. European grapevines (*V. vinifera*) are susceptible to *Agrobacterium*-infection, but certain rootstock varieties derived from *Vitis riparia* (Gloire de Montpellier, 3309C) show high levels of resistance to tumorigenesis (Süle et al. 1994). Certain *V. amurensis* genotypes were also reported to be resistant, and that resistance was inherited by a simple Mendelian way (Szegedi & Kozma 1984).

Production of bacteria-free grapevine stocks

As noted above, the use of healthy planting material is a key factor for preventing the spread of bacterial pathogens of grapevine. Bacteria-free grapevine stocks can be selected by visual selection of symptom-free true-to-type plants; indexing existing plant material for the presence of pathogens; and laboratory assays. In recent years, polymerase chain reaction (PCR)-based protocols have become increasingly predominant for detection and diagnosis of bacterial plant pathogens. This method amplifies relatively short (usually 200–1000 bp) specific genomic sequences determined by homologous „starter“-sequences, called primers, that direct forward and reverse DNA synthesis. Although the method is highly sensitive and specific, it still has limiting factors, that must be considered for the application of PCR. These include the presence of polymerase-inhibitors in DNA samples, uneven distribution of the pathogen in the host plant, or genetic diversity of the target pathogen. To test plant propagating materials for the presence of bacterial pathogens, total DNA can be isolated (1) directly from the plant or (2) from the isolated (purified) bacterial colonies grown on artificial nutrient media. Although the latter method takes some additional days or even weeks, it is much more reliable than the first method since isolation of bacterial colonies eliminates several polymerase inhibitors (polysaccharides, polyphenols) that occur in extracts of plant tissue samples, as well as results in enrichment of the target pathogen. Due to their very slow

growth, *in vitro* isolation of *X. ampelinus* and *X. fastidiosa* subsp. *fastidiosa* may still be difficult, since endophytic bacteria that grow faster than these pathogens may overgrow them *in vitro* on nutrient agar during isolation. Methods for isolation of these grapevine pathogens have been described, e. g., by Dreo et al. (2005), Serfontein et al. (1997) and Willems & Gillis (2006) for *X. ampelinus*, and by Aguilar et al. (2008), Almeida et al. (2004) and Fry et al. (1990) for *X. fastidiosa* subsp. *fastidiosa*. Some authors prefer gellan gum (GelRite) to agar as gelling agent in the nutrient medium for isolation of *X. fastidiosa* subsp. *fastidiosa* (Almeida et al. 2004).

Several conventional and real-time PCR-based protocols have been described for the detection and identification of these bacterial pathogens (Hren et al. 2010, Palacio-Bielsa et al. 2009b). The application of multiplex PCR allows the identification of genetically diverse types of bacteria in a single reaction (Bini et al. 2008, Kawaguchi et al. 2005, Pulawska et al. 2006), while the use of nested or semi-nested PCR (Peduto et al. 2010, Pulawska & Sobiczewski 2005) increases the specificity and sensitivity of detection. Introduction of simple nucleic acid sampling tools, such as FTA cards (Grund et al. 2010) and loop mediated isothermal amplification, for plant disease diagnosis (Harper et al. 2010) allows simple sample collection and rapid pathogen detection without the need for expensive laboratory equipment and other related costs.

Despite the availability of highly sophisticated molecular diagnostic and detection protocols, these methods alone are not always appropriate for reliable selection of pathogen-free propagating material due to the uneven distribution of bacterial cells in the host plant. Thus, there is a need also for efficient curative methods that are able to eliminate pathogens completely from plant stocks.

Due to the systemic nature of bacterial infection in grapevine propagating material, chemical treatments are insufficient to kill endophytic bacteria in canes. Hot water treatment (HWT; immersion of dormant canes in water at 50 °C for 30 mins) eliminated the Pierce's bacterium, *X. fastidiosa* subsp. *fastidiosa* so that no symptom development was observed for two years following treatment (Goheen et al. 1973). The level of *X. ampelinus* cells was also reduced below the detection limit in grapevine canes using HWT (Psallidas & Argyropoulou 1994). However, these results have not been confirmed. *A. vitis*, surviving in dormant grapevine cutting was only partially eliminated by HWT since treated canes still contained some surviving pathogen cells (Burr et al. 1996). Since HWT kills several other pathogens (e. g., phytoplasmas) and pests (mites, nematodes, etc.), it is advisable to pass propagating material through this treatment.

Grapevine plants which are completely free from *Agrobacterium* spp. and *X. fastidiosa* subsp. *fastidiosa* are achieved by establishing *in vitro* cultures started from apical meristems or shoot tips (Burr et al. 1988, Robacker & Chang 1992, Thies & Graves 1992). In practice, plant material for *in vitro* cultures is started from hot water-treated canes as

described above to ensure, as much as possible, pathogen-, and pest-elimination. Rooted plants can be multiplied *in vitro* and acclimatized for greenhouse and field growth. Details of this process are shown on *Figure 1*. In spite of the fact that plant lines obtained by this way are usually sterile, they should be tested by one or more of the diagnostic methods described above to ensure they are really bacteria-free. Such lines then can be used to establish stock plantations for large scale propagation.

Acknowledgements

E. Szegedi was supported by the HU-SRB 0901/214/123 EU project.

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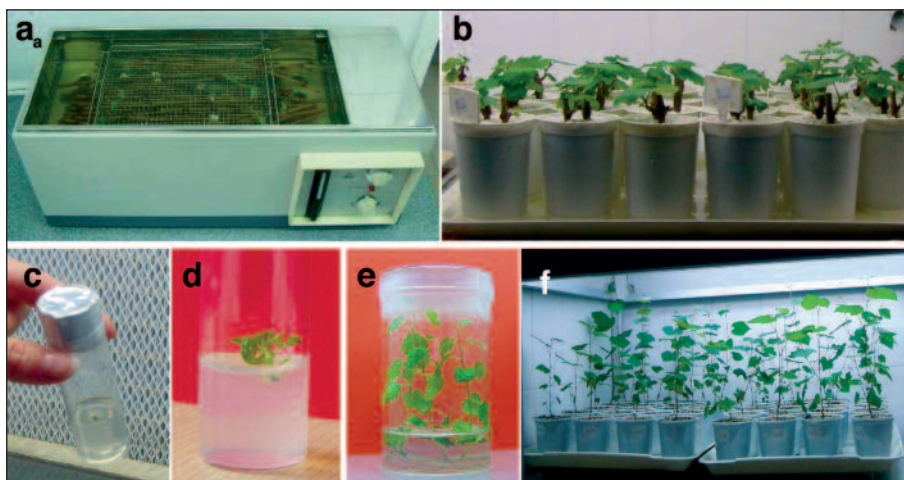


Figure 1. Process of production of bacteria-free plants. (a) Canes are hot water-treated at 52°C for 45 mins, (b) followed by shooting in perlite which is practically a sterile medium. Shoot tips are removed, disinfested and (c) approximately 0.5–1.00 mm long tip pieces are used to start *in vitro* cultures on Murashige-Skoog medium with 0.5 mg/l benzyl-adenine. (d) small shoots are transferred (e) to hormone-free medium to root and (f) finally the plantlets are acclimatized to greenhouse conditions.

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