Production of healthy grapevine propagating material: pathogens and methods

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Abstract

Grapevine production has been endangered by several diseases against which control methods are not available. Viruses, phytoplasmas, bacteria and fungal infections contributing to esca have significant importance all over the world. These pathogens systemically infect their hosts, thus the use of healthy propagating material is a key factor in the prevention of disease development. Selection of pathogen-free plant material can be achieved by testing for the presence of pathogens. For virus identification a complex protocol including serological and molecular methods followed by testing on indicator plants is used. For the detection of phytoplasmas, bacterial and fungal infections, polymerase chain reaction (PCR) has become the most widely used method. Various treatments and alternative propagation methods can also be used to clean plant material from latent infections. To eliminate viruses, plants are first heat treated followed by *in vitro* multiplication of shoot tips. This protocol is suitable for the efficient elimination of other (phytoplasmas, bacteria and fungi) systemic infections. *Agrobacterium*-free plant material can also be obtained by rooting shoot tips in the greenhouse. Hot water treatment of dormant canes has been successfully used to eliminate phytoplasmas, partially *A. vitis* and several other pathogens and pests.

Introduction

Plant disease control most frequently starts with the use of healthy propagating material which is free not only from disease symptoms but also from latent infections. Like several other crops, grapevine has also a number of pathogens and pests which are transmitted by propagation. Therefore selection or production of pathogen free plant material is a basic step towards efficient disease control. In this review we present a brief summary of the most important grape diseases that spread by propagating material as well as on the methods used for selection of healthy stocks and/or their elimination.

Viruses

The use of virus-free propagating material is an important factor to improve quality and volume of grape production thus methods for detection of grape viruses have been exhaustively studied (Rowhani *et al.*, 2005). In Hungary the detection of virus and virus-like

diseases of grapevine began in 1960's in the Research Institute for Viticulture and Enology by J. Lehoczky and his colleagues. At present, fifteen virus and virus-like diseases of *Vitis vinifera* are known to occur in Hungary (Lázár *et al.*, 2002). Some of them, e. g. Fanleaf and Leafroll cause significant economic loss. Other viruses, e. g. the Rugose wood complex can provoke untimely death of grapevine stocks. A few viruses are latent. Little is known about their effects on grapevine however their occurrence is quite frequent, so they may have high economic importance.

Fanleaf virus and the related strains (Yellow mosaic and Veinbanding) are most widespread and are present in all vine-growing regions of Hungary. Other nepoviruses, e. g. Arabis mosaic, Chrome mosaic, Bulgarian latent, Tomato black ring are less frequent. Symptoms of Enation, Yellow mottle, Line pattern were observed only in one or two cases in the grapevine-growing regions. Rugose wood complex, Leafroll and Vein mosaic are widely distributed in almost all the main grapevine-growing regions. Fleck and Vein necrosis can be often found on the indexed varieties with incidence varying from 50 to 80%.

Regular virological screening of grapevine varieties started in 1972 (Lechoczky *et al.*, 1992). The present system of screening has been established using methods (visual selection, biological indexing, ELISA) with continuous improvement according to recommendations of international organizations (Neszmélyi *et al.*, 1996).

In the first year, symptomless grapevine stocks are selected. Surveys are carried out twice in the vegetation period (at about flowering and in the second half of September). At the time of the first selection, sampling is performed for ELISA. Since 1985 ELISA has been routinely applied for the detection of 7 viruses/strains: GFLV, GFLV-YM, GFLV-VB, ArMV, GCMV, ToBRV and AlMV. Since the spring of 1993 raspberry ringspot and strawberry latent ringspot and since 2004 GLRaV-1, GLRaV-3, GVA and GFkV viruses have also been serologically screened. In November canes of symptomless and ELISA-negative plants are collected for futher investigations.

In the spring of the second year, overwintered canes are checked by woody indexing on 8 indicator species in the field: FS 4, *Vitis rupestris* St. George,*V. vinifera cv.* Pinot noir, *V. vinifera cv.* Chardonnay, *V. berlandieri x V. riparia* Kober 5BB, Couderc x *V.berlandieri* LN 33, *V. riparia* Gloire, *V. rupestris x V. berlandieri* 110 R. In the present system FS 4 and Chardonnay are regularly used but they will be omitted or only occasionally used in the future. Symptoms are recorded in June and September.

In the third and fourth years the nursery is evaluated twice again. At the end grapevine plants giving negative results on all indicators in every case, are considered virus-free. If there are varieties from which it is not possible to select healthy plants, their cuttings are rooted and heat-treated or adapted to *in vitro* culture for the production of virus-free progenies. After heat treatment they are re-tested for the presence viruses.

In autumn of the fourth year the virus-free material is planted out under screenhouse (3-4 plants/variety) and also in a special mother block (nuclear stock) (30 plants/variety) for maintenance and propagation. Plants of the nuclear stock (pre-base) produce propagating material (basic), which will be planted in propagation stocks. The progeny of basic material originating from the propagation stocks is used for nursery propagation. Propagating material derived from mother grapevines established in nurseries is delivered to the growers as certified material.

During these propagation steps, visual observation and random tests by ELISA are done to monitor the virus status of the plants. Propagation is performed under strict official control by the national Plant Protection Service. Trueness to type is also monitored by the inspectors of the National Institute for Agricultural Quality Control.

Mother blocks of virus-free scion varieties have been established on one hectare and those of rootstock varieties on 0,5 ha including the following number of varieties included in

the national list: 73 European scion - and 12 rootstock varieties or variety candidates/clones. Number of other virus-free varieties and clones: 45. Clone propagation and maintenance of the virus-free stocks intended for propagation and nurseries meet the EU standards. In Hungary the existing area is sufficient to produce propagating material for the renewal of all plantations.

Phytoplasmas

Grapevine yellow (GY) diseases caused by phytoplasmas have been known for more than 50 years. Flavescence dorée and other phytoplasmas occur in vineyards of several countries of Europe, North America, Asia Minor and Australia. Phytoplasmas, discovered in 1967, are cell wall-less intracellular bacteria, restricted to phloem sieve tubes and transmitted by insect vectors, in which they multiply. They can spread over long distances by means of infected propagating material. Sanitation of dormant planting material with hot water treatment was first recommended by Caudwell and his coworkers in 1990. Since that time a special instrument has been developed for hot water treatment (at 50^{o}C for 30-45 minutes) of dormant canes and rootstocks and routinely used in France, Australia and some other countries to eliminate phytoplasmas (Boudon-Padieu 2003). For the detection of phytoplasmas only molecular tools are available. Nested PCR followed by restriction fragment length polymorphism (RFLP) provide reliable methods for their identification.

Since 1997 a regular survey started in vineyards to determine the distribution of Grapevine yellows (GY) in Hungary. All types of symptoms, typical for phytoplasma infection, have been observed: leaf yellowing/reddening with or without necrosis of leaf blade, triangle-shaped leaf rolling, shortening of internodes, drooping of shoots, drying of flowers, berries and clusters, as well as decline of grapevine stocks. Molecular analyses resulted in the identification of Bois Noir (BN; 16SrXII-A or stolbur) phytoplasmas in the diseased grapevine plants (Kölber *et al*. 1997). Other phytoplasmas associated with yellows disease such as Aster Yellows (AY), Elm yellows (EY), Clover phyllody (CPh) and European stone fruit yellows (ESFY), as well as mixed infections were recorded in symptomatic grapevine plants in some locations (Kölber *et al*. 2003).

In compliance with the Decree of Ministry of Agriculture and Rural Development (MARD) No. 86/2006 in the frame of the certification programme, mother plants of pre-base and base plantations, as well as the nurseries are visually inspected for the presence of symptoms caused by viruses and phytoplasmas. Symptom-showing mother plants cannot be used for further propagation. Plants suspicious for phytoplasma infection are tested by nested PCR. Phytoplasma-specific universal primers P1/P7 (Deng and Hiruki 1991; Schneider *et al.* 1995) are applied to amplify 16SrDNA, or 16SrDNA plus spacer region followed by R16F2/R16R2 (Lee *et al*. 1995). Then phytoplasma group-specific primers are used: FStol/rStol (Lee *et al*. 1994) and FD9f2/r, FD9f3/2r (Daire *et al*. 1997). The identity of PCR products is confirmed by RFLP analyses by digesting the PCR products with *Tru9*I.

Based on the results of the nine-year survey, it can be concluded that Flavescence dorée phytoplasma, listed by the 2000/29 EC as a harmful organism, has not been detected so far in Hungary, although Stolbur phytoplasma is wide-spread in the vineyards of the country. It was identified, on an average, in 10 % of the tested plants from twenty-one cultivars grown in bearing plantations. The ratio of the infected mother plants is relatively low, due to the strict regulation, i.e. the phytoplasma-diseased plants have to be eliminated from the plantations and nurseries.

Bacterial diseases

Grapevines are affected by bacterial diseases as well that have local or worldwide importance. *Xylella fastidiosa,* the causal agent of Pierce's disease (Almeida and Purcell, 2003; Berisha et al., 1998; Buzkan et al., 2005) is not present, while *Xylophylus ampelinus* responsible for bacterial necrosis (Dreo et al., 2005; Grall and Manceau, 2003) occurs only in a few EPPO countries. In the frame of the certification scheme the mother plants are visually inspected for the eventual occurrence of these two regulated bacteria in Hungary. According to the survey results of the past and resent years none of these bacteria are present in the country. The crown gall bacterium *Agrobacterium vitis* (Burr et al., 1998) is common in nearly all grapevine growing countries. Like viruses and phytoplasmas these bacteria are also systemic in the host plants thus they are transferred by propagating material.

For the detection of *X. ampelinus* Botha *et al*. (2001) developed a nested PCR technique based on the 16S-23S intergenic spacer region. Primers designed specifically amplified the corresponding 277 bp sequence from 38 strains derived from various sources. Serological and molecular methods for the detection of *Xylella fastidiosa* have also been developed for efficient indexing of plant material (Berisha et al., 1998, Buzkan et al., 2005). In order to clean plant material, systemically infected with *X. Fastidiosa,* shoot-tip cultures were established. Grapevine plants regenerated from the 4-5 mm tips did not contain the pathogen anymore (Robacker and Chang, 1992). Hot water treatment of dormant canes proved to be an efficient tool to kill both *X. ampelinus* (Manceau 2006) and *X. fastidiosa* (Goheen *et al*. 1973).

Crown gall caused by *A. vitis* occassionally by *A. tumefaciens* causes serious economic loss in viticulture worldwide (Burr et al. 1998, Szegedi *et al*. 2005). This bacterium, like others, is also systemic in the host plant and may occur in grapes without symptom development. To obtain *Agrobacterium*-free stocks several traditional and molecular diagnostic methods have been tested during the last decades. Among these the PCR-based techniques have become the most popular. Due to the high genetic diversity of *Agrobacterium* spp. single primer pairs usually do not detect all types of agrobacteria occuring on grapevines.

Certain propagation methods are also suitable to eliminate agrobacteria from grapevines. For example, *in vitro* plants obtained from 1-2 cm shoot tips (see also above) proved to be free from *Agrobacterium* (Burr *et al*. 1988). Further studies have shown that not only the tips but the young, green grapevine shoots are still free from systemic *Agrobacterium*. Since single node internodes can bee rooted in aeriated media (Stellmach, 1997; Thomas and Schiefelbein, 2004; Szegedi and Lázár 2005) this "green-multiplication" technique provides us an alternative way for mass production of healthy plants. To obtain *Agrobacterium*-free grapevine propagating material hot water treatment has also been applied (Burr *et al.* 1996). Although this "curative" method has been widely used to eliminate pathogens and pests from dormant canes, it cannot completely kill agrobacteria present in canes.

Fungi

Grapevine plantations are affected by several fungal diseases as well. Although certain diseases, e. g. downy mildew and powdery mildew can be efficiently controlled by a wide range of chemicals, we are still unable to protect grapes from fungi infecting the wood. The latter groups of fungi are responsible for the so called esca disease causing trunk death of old and young, even 1-4 year old plants. Although these diseases have been known for a very long time, the most intensive research on this field started only during the last few decades

(Chiarappa, 2000). In Hungary some *Hyphomycetes* fungi, e. g. *Phaeoacremonium* spp., *Phaeomoniella chlamydospora* (*Cephalosporium*), *Cylindrocarpon* spp., the causal agent of the ”black foot” and wood rotting basidiomycetes fungi *Fomitiporia mediterranea* (=*Phellinus punctatus*) are most frequently found in diseased grapes (Dula, 2003, Rábai 2006). A similar disorder called now Petri-disease which is possible precursor of esca causes also serious decline in younger vines in several countries including Hungary. *Phaeoacremonium* spp., *Phaeomoniella chlamydospora* and *Cylindrocarpon* spp. associated with typical Petri-disease symptoms (Crous *et al.* 1996, Ferreira *et al.* 1994, Morton 1995).

Certain fungal species, e. g. *Botryosphaeria spp.* (Black dead arm disease), *Eutypa spp.* and *Phomopsis spp.* are not closely associated with the esca disease but they may also contribute to the partial or complete early trunk death of grapevines. They are also common in Hungary frequently causing complex infections. Pathogenic fungi causing esca and/or Petri disease, *Botryosphaeria* and *Phomopsis* mainly transmitted by vegetatively propagated plant material (Fourie and Halleen, 2004). Their low growth rate makes their isolation very difficult since fast growing saprophytic fungi overgrow them before the appearance of pathogenic colonies. The complexity of these grapevine diseases is an additional factor limiting their exact diagnosis.

Since the infected propagating material is the most important factor in the spreading of esca and Petri diseases the use of healthy stock material for the establishment of plantations is essential (Fourie and Halleen, 2005), because the diseased trunks cannot be cured. Propagating material should be collected on symptomless healthy plants with disinfected tools and stored under proper conditions to prevent contamination. To eliminate systemic infection hot water treatment is proposed in South-Africa and Australia (Wait and May, 2005). Interestingly, from hot water treated vines viable *P. chlamydospora* could not be isolated although its DNA was still detectable by PCR (Retief *et al.*, 2005). The efficiency of heat treatment was further increased by the application of fungicides or the biocontrol agent *Trichoderma harzianum* (“proactive control”, Fourie and Halleen, 2004).

In compliance with the Decree of Ministry of Agriculture and Rural Development (MARD) No. 86/2006, in the frame of the certification programme, mother plants of pre-base and base plantations, as well as the nurseries are visually inspected for the presence of symptoms. Diseased plants cannot be used for further propagation. Besides the visual selection PCR has also been extensively used for the identification and detection of fungal diseases. For example, *P. chlamydospora* was detected more efficiently from grape wood by PCR than by traditional microbiological protocols (Retief *et al.*, 2005). A database for molecular detection of phytopathogenic fungi is now avaiable (Ghignone and Migheli, 2005).

Other pests

Besides the above mentioned microbial pathogens several pests of grapevine, e. g. phylloxera, nematodes and mites are also transmitted by propagating material. Hot water treatment has been used since 1910 to kill phylloxera present on grape material (Goussard, 1977). Similarly, successful experiments were carried out using this physical method to clean grapes from nematodes (Gokte and Mathur, 1995) as well as to kill overwintering mites in buds (Szendrey et al., 1995) frequently resulting in improved bud-burst and shoot development. Taken together, heat treatment of dormant grapes can be used to eliminate several pathogens and pests including vectors of grapevine viruses and bacteria.

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