Chapter 27
Improvement of Grapevine Planting Stock Through Sanitary Selection and Pathogen Elimination

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Abstract Sanitary selection is the most economic, prophylactic strategy to reduce the presence of viruses in propagation material and limit their prevalence in newly established vineyards through the production of clean stocks from which high-quality planting material is derived. The selection of clean stock requires efficient therapy methodologies and rigorous screening of elite accessions of scion and rootstock material for economically important viruses. Several therapeutic methodologies have been developed to sanitize infected accessions, among which microshoot tip culture is one of the most commonly employed for its effectiveness, ease of implementation, and reduced potential to regenerate off-type vines. Efforts at clean plant centers throughout the world to select and produce clean stocks are contributing directly to increasing the quality of the planting material, augmenting the profitability of vineyards, and sustaining the development of the grape and wine industry.

Keywords Clean stock • Disease • Economic value • Grapevine • Microshoot tip culture • Sanitary selection • Therapeutics • Virus

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Introduction

The key to improving the sanitary status of vineyards around the world lies in selecting and supplying the highest possible quality propagating material, which has been rigorously screened for economically important viruses and tested negative. The quality of elite propagating material depends upon the accuracy of diagnostic tools (discussed elsewhere in this volume) and the ability to perform therapy for sanitizing valuable grape selections when they are infected. A multitude of virus elimination techniques have been used experimentally and some are used routinely. Therapeutic methodologies are reviewed in this chapter, and the economic value of clean programs is discussed.

Sanitary Selection

Sanitary selection is an important tool in the field of grapevine improvement. The objective of sanitary selection is to propagate scion clones and rootstock genotypes free of important viruses and to protect them from infection in foundation vineyard blocks which serve as a source for propagation material. In its most primitive form, grape growers have practiced sanitary selection by the simple practice of propagating their highest quality, apparently disease-free vines. In North America, formal recognition of the importance of selecting vines to improve performance began in the mid-twentieth century. While this selection process resulted in increased vigor and production, the clean plant programs were not intended to evaluate vine performance related to wine quality (Boidron 1995). In Europe, it is traditionally preferred to carry out clonal and sanitary selection simultaneously including the evaluation of wine quality potential of individual selections; but elsewhere, grapevine selection basically means virus sanitation: testing selected materials for important viruses and maintaining the selection free of virus through the certification process (Mannini 2000).

In the late nineteenth century, clonal selection began in Germany when Gustav Froelich selected single Silvaner [sic] vines based on visual appearance and performance. Progeny vines were kept separate and evaluated for their performance, which led to high-performing, uniform mother blocks and large-scale virus elimination (Rühl et al. 2004; Schmid et al. 1995). By the 1950s, high-performing clonal propagation material of most traditional cultivars was available, and since the 1970s, only clonal material has been planted (Rühl et al. 2004). German breeders at the Geisenheim Research Center are working to maintain a range of genetic resources to prevent gene erosion by selecting a large number of clones of each cultivar to preserve genetic variability between and within cultivars (Rühl et al. 2004). In addition, researchers are looking to old vineyards, found mainly on the steep slopes in the Mosel region, with non-clonal plantings as a source of new clones (Rühl et al. 2011). Material is identified based on visual appearance and virus testing. If, after

laboratory and field evaluations, the new clone is virus-free, it is registered with the German Federal Variety Office (Rühl et al. 2011).

French methods of clonal selection are based on two factors: sanitary selection and genetic selection. In the 1960s and 1970s, the focus of selection was based on improving yields that had been reduced by *Grapevine fanleaf virus* (GFLV). Then in the 1980s and 1990s, the selection focus was directed toward genetics while still maintaining sanitary selection, as consumer preferences and the wine trade evolution favored qualitative criteria (Grenan et al. 2000). Sanitary selection begins with visually selecting material in the vineyard, which may be based on several years of observation (Boidron 1995). Plant material is then tested for important viruses: the viruses and testing techniques change over time as knowledge and advances in diagnostic tools become available. The genetic selection component of the selection process provides growers with knowledge of the production (yield) and quality performance (sugar content, acidity, aromas, etc.) of the material (Boidron 1995). The French Institute of Vine and Wine (IFV) is responsible for selecting clones, evaluating for viruses, processing material through sanitary selection, and selecting for agronomical characteristics including tasting. After clones have undergone sanitary and genetic evaluation, the selection is registered and certified by the Committee of Selection of Cultivated Plants (CTPS) of the French Ministry of Agriculture. Certified material in France is sold and distributed under the name ENTAV-INRA®, which is managed by the IFV (Audeguin 2016).

Clonal and sanitary selection methods are also well established in Italy. The first reports of grapevine clonal selection from this country date back to the 1960s when the need to improve the quality of the propagation material was recognized. The National Grapevine Certification Scheme began in 1969 and includes registration, preservation, pre-multiplication, and multiplication of certified stocks (Mannini 1995). Selected vines are registered in the National Catalog of Grapevine Varieties after approval by the National Committee for the Evaluation of Grapevine Varieties. Vines are then maintained at a foundation nursery (*Nucleo di premoltiplicazione*), which is the source of materials of the basic category (pre-multiplication). Commercial production in nurseries then starts with the establishment of mother plant vineyards from basic material (Mannini 1995).

The present European national grapevine certification schemes conform to European Union (EU) Directives, although they may differ by country with reference to regulated viruses (Maliogka et al. 2015). The six main influential viticultural countries in the EU are France, Italy, and Spain, which are followed by Germany, Portugal, and Greece. A unique aspect of the EU certification schemes is that they not only ensure clean stock but also trueness to type, for sources with a well-established clonal nature are registered and certified (Martelli 1992; Maliogka et al. 2015). Standards adopted by the European and Mediterranean Plant Protection Organization (EPPO) Certification Scheme are selection of vines for viticultural and enological quality, production of nuclear stock, maintenance of nuclear stock, production of propagation stock, and production of certified plants. This process is carried out by officially registered, specialized nurseries and laboratories (EPPO 2008). Specific requirements and standards of certification nurseries and

micropropagation facilities in Europe are currently directed by the EPPO. The standards address general conditions of the facility and specific measures necessary for growing plants of candidate nuclear stock, nuclear stock, or propagation stock (EPPO 2001). Each participating country has developed its own governing organization to administer and monitor the certification scheme.

The first grape sanitary program in North America was developed at the University of California at Davis, known for its strong programs in both viticulture and plant pathology. During the 1940s, as the state's grape and wine industry had developed and plantings expanded, new knowledge and methods of disease detection gradually made clear to scientists just how widespread virus disease problems were in the state's vineyards. The first published observations documenting virus-induced crop losses involved a table grape, cv. 'Emperor,' which had originally been introduced from Iran. Professor H. Olmo and his colleagues demonstrated that undesirable variation in the color of this grape, from deep to very pale red, was likely caused by a virus because the condition was graft transmissible (Olmo and Rizzi 1943). Olmo partnered with the plant pathologist W. B. Hewitt to raise grower awareness of the need for a sanitary selection scheme for grapevines (Olmo 1951, 1975). This leads to further research which demonstrated that there were widespread virus infections in all of the vineyards of California, regardless of whether the grapes were used for wine, fresh fruit, or raisins (Hewitt 1954). By 1956, the State of California had its first regulations in place, thereby creating the California Grapevine Registration and Certification (CGR&C) Program. Over the years, this program was modeled to create similar programs in Oregon, Washington, New York, and Virginia.

Unlike Europe, the United States has never had a national program for the production of grape clean stock. Certification programs in the United States are managed individually by the states (see 826). However, there is a US Department of Agriculture (USDA) program known as the National Clean Plant Network (NCPN), which provides funding for sanitary selection programs for a variety of specialty crops including grapevines, fruit and nut trees, berries, hops, roses, and sweet potatoes. This funding goes to selected centers which create virus-screened collections. Five grapevine centers are currently funded by NCPN: Foundation Plant Services (FPS), University of California at Davis; Clean Plant Center Northwest, Washington State University, Prosser, Washington; Cornell University, Geneva, New York; Midwest Grape Tissue Culture and Virus-testing Laboratory, Center for Grapevine Biotechnology, Missouri State University; and Center for Viticulture and Small Fruit Research, Florida Agricultural and Mechanical University, Tallahassee, Florida. Both California and Washington States have voluntary "registration and certification" at the state level which govern the production of what is called foundation stock. There are, however, no national regulations in the United States governing the production of grape nursery stock. Plant regulatory aspects of grapevine nursery production are discussed in Chap. 28 of this volume.

Commercial nurseries that produce certified grapevines and participate in the California Grapevine R&C program obtain their clean stock from FPS at the University of California, Davis. UC Davis has a foundation vineyard for major

grape cultivars and clones, as well as rootstocks. Before being planted in the foundation vineyard, all entries are tested across biological indicators, by ELISA and RT-PCR. The foundation vineyard is monitored by visual inspections in spring and fall, and a portion of it is retested by ELISA and RT-PCR on an annual basis to monitor for the possibility of reinfection (Rowhani et al. 2005).

It is clear that using high-performing healthy grapevine propagation material is invaluable for productive vineyards for which a reliable source for clean, pathogen-tested material (rootstock and scion) is essential. While sanitary and clonal selection programs vary by country, the prominent programs described here have evolved to include sanitary selection methods and the establishment of foundation blocks for the production of certified material. These blocks require regular monitoring and testing for vector-transmitted viruses as well as newly discovered viruses of economic importance.

The production of clean cultivars and selections, as well as rootstock genotypes, relies on the implementation of various virus elimination methodologies. Some have been extensively used experimentally; others are used routinely by clean plant centers worldwide.

Heat Treatment

Heat therapy was utilized by Kunkel (1936) for pathogen elimination when infected trees were subjected to dry heat or a hot water treatment for inactivation of peach yellows. In 1950, the first report of virus elimination was by B. Kassanis who used heat therapy to eliminate leafroll virus from potato (Kassanis 1950). Heat therapy to eliminate virus disease in plants was being successfully used during the 1950s on an experimental basis with stone fruits, potatoes, strawberries, and other crops (Nyland and Goheen 1969). W.B. Hewitt and A.C. Goheen, UC Davis plant pathologists, felt that heat therapy would offer promise in the elimination of grapevine virus diseases and, in 1959, began experimenting with this technique using a hot air treatment of plants in a closed growth chamber (Gifford and Hewitt 1961; Goheen et al. 1965). Unfortunately, the only climate control chambers available between 1959 and 1969 had been designed to meet the specific requirements of plants other than grapevines and, although they offered opportunities for experimentation, were not of great use in grapevine disease therapy. Only in 1969, when a chamber with capacity adequate to meet the needs of the grapevines became available, A.C. Goheen began to make headway in pioneering the use of heat therapy to eliminate grapevine virus diseases. In brief, A.C. Goheen experimented with taking cuttings to the very edge of their heat tolerance, exposing the cuttings to high temperatures for extended periods of time to retard or inactivate viruses without also killing the plants. When new, ostensibly clean buds began to appear on the cuttings, they would be removed and utilized to propagate new, clean stock. Thus, A.C. Goheen ultimately settled on an optimum treatment temperature of 38 °C for a period of approximately 60 days (Luhn, personal communication). This heat treatment eliminated 100% of GFLV in

the tested plants (Goheen 1989). Other viruses, however, were more heat stable. Goheen (1989) reported eliminating 42% of corky bark infections, 25% of leafroll infections, and 14% of rupestris stem-pitting infections. This breakthrough in heat therapy set a standard that was widely used. The success of heat therapy depends, in most cases, upon removing a portion of the treated plant post-therapy (Nyland and Goheen 1969). Alternately, Goheen and Luhn (1973) grafted individual buds from candidate vines onto healthy LN-33 (Couderc 1613 x *V. vinifera* cv Thompson Seedless) rooted cuttings followed by the 60-day heat treatment. Virus was eliminated from 77% of the shoots (Goheen and Luhn 1973).

Savino et al. (1985) found that heat therapy reduced the incidence of the following viral diseases: leafroll (from 50% to 34%), vein necrosis (from 71% to 36%), and fleck (from 46% to 12%). While heat therapy is beneficial in reducing incidence of disease, if used alone it may not be sufficient for a successful clean stock program. Combining heat therapy with meristem shoot tip culture has proved to be highly effective, especially for non-heat-labile viruses. A study by Salami et al. (2009) showed that heat treatment for 7 weeks at 40/30 °C (day/night temperatures) eliminated GFLV in 70% (cv. Bidaneh Sefid) and 90% (cv. Shahroodi) of the treated plants. In the same study, meristem tip culture was 80% efficacious in eradicating GFLV. However, when heat therapy was combined with meristem tip culture, 100% of GFLV was eradicated from plants.

Although heat therapy continues to be used for successful elimination of viruses for a number of crops, including hops, stone fruits, potatoes, strawberry, and ornamentals, switching to microshoot tip culture has proven successful for many high-volume clean grapevine stock programs.

Microshoot Tip Culture

Viruses have been eliminated successfully using microshoot tip culture at different institutions worldwide. Between 1993 and 2015, at the Department of Plant, Soil and Food Sciences of the University of Bari (DPSFS-Uniba), more than 1200 selections belonging to 240 cultivars from different Italian regions (Marche, Abruzzo, Apulia, and Calabria) and foreign countries (Portugal, Lebanon, Malta, Albania, Croatia, and Serbia) have undergone successful microshoot tip therapy to eliminate virus infections (G. Bottalico, personal communication). At the Cornell University, microshoot tip culture has been the preferred therapeutic methodology for the elimination of viruses from a dozen accessions since 2005 (Fuchs, unpublished observation).

Based on 25 years' experience in an active grapevine clean plant center, FPS at UC Davis, microshoot tip tissue culture is the method of choice to eliminate virus(es) and other pathogens from grapes (Golino, unpublished observation). At FPS, microshoot tip culture successfully eliminated viruses in 90% of the processed selections (Fig. 27.1). Since 2000, FPS has processed over 1000 grapevine selections through microshoot tip culture. Many of these selections were processed even when the

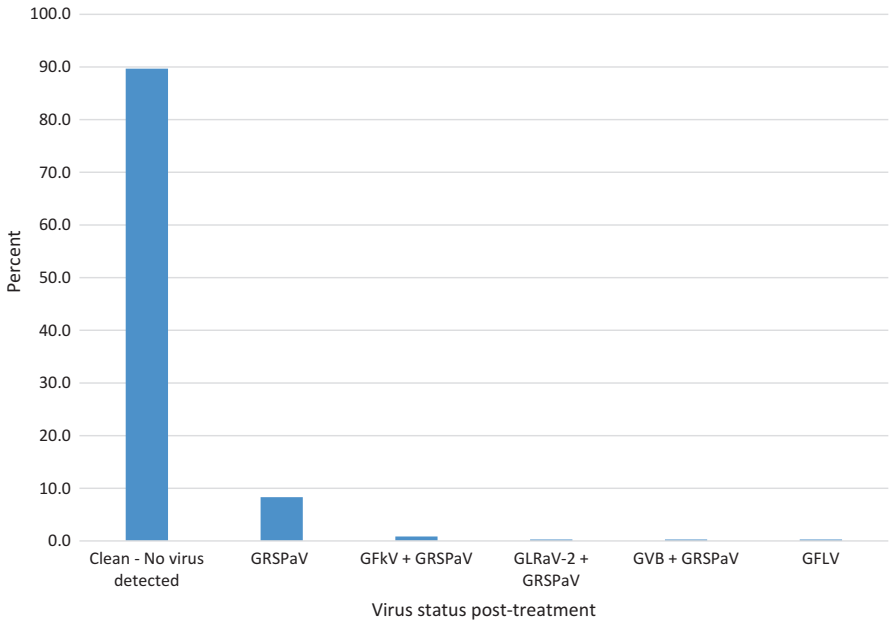

Fig. 27.1 Virus status of 349 selections after processing through microshoot tip tissue culture at FPS. Before treatment selections were often infected with multiple viruses; afterwards, GRSPaV alone was the most common virus detected, indicating the relative difficulty in eliminating this virus. Selections were tested to meet Protocol 2010 standards. Number above *bar* indicates the number of selections in that category. [Note to printer: actual percents are (*left* to *right*) 89.7, 8.3, 0.9, 0.3, 0.3, 0.3%]

original plant material tested negative for viruses. This was done to meet a new rigorous standard that FPS set for grapevine foundation material, referred to as Protocol 2010. Protocol 2010 standards require that vines must be generated using microshoot tip culture and be tested for an extensive list of pathogens. Vines meeting Protocol 2010 standards are planted in a foundation vineyard at Russell Ranch on the UC Davis campus. Since new pathogens may be identified at any time, microshoot tip culture may be considered a preventive measure to limit the presence of viruses and other pathogens in foundation stock. This may have been the case for two recently characterized viruses, *Grapevine red blotch-associated virus* (GRBaV) and *Grapevine Pinot gris virus* (GPGV). Despite not having a test for the viruses at the time of planting in Russell Ranch, high-throughput sequencing has demonstrated that these new viruses have been excluded from the foundation vineyard.

Overall, microshoot tip culture has been a very effective method for eliminating viruses and creating foundation planting material. Microshoot tip tissue culture has the advantage of regenerating a single plant from a single, minuscule (approximately 0.4–0.5 mm in size) explant including the meristem and one to three pairs of leaf primordia (Fig. 27.2). The survival rate of these microshoot tips is higher than that of a meristem as the presence of leaf primordia increases the survival rate.

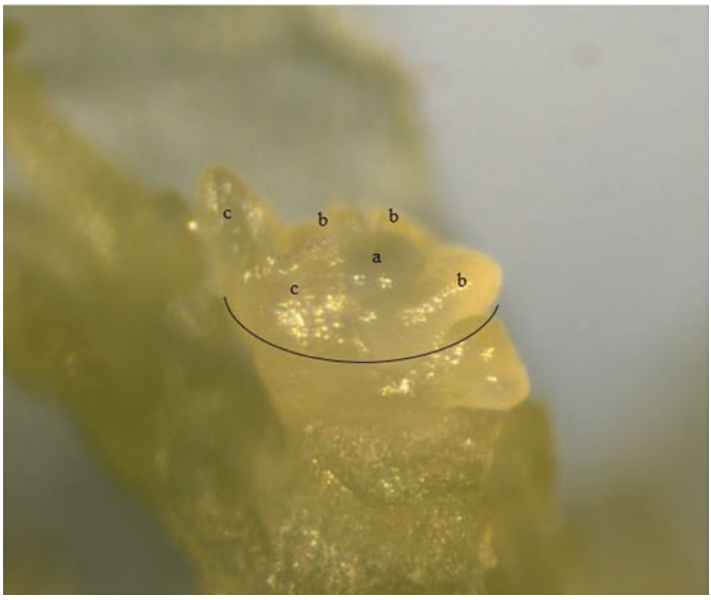

Fig. 27.2 A grape microshoot tip measures less than 0.5 mm and consists of the meristem dome (**a**) and two to three pairs of leaf primordia (**b**). Leaf primordia (**c**) are removed before the final excision cut is made, indicated by the *line*, and the microshoot tip is placed into growth medium

Survival is highly dependent on cultivar; approximately 75% of tips excised survive to form a plant with roots and shoots (Sim et al. 2012). The combination of low hormone levels and a minimum time in culture reduces the chance of mutation and regeneration of an off-type plant. Microshoot tip tissue culture also avoids the production of plants from callus, which can lead to regeneration of an off-type plant, a serious drawback of other types of virus therapy such as fragmented shoot tip and somatic embryogenesis (see below).

One question that is frequently asked about microshoot tip tissue culture is whether the procedure results in off-types or mutations in a cultivar. This is a subject of concern when using tissue culture for mass increase of propagation material, referred to as micropropagation, and other tissue culture techniques but has not proved to be a concern with microshoot tip culture for virus elimination. As mentioned above, microshoot tip culture produces a single plant from a single microshoot tip; micropropagation involves repeated increases to produce thousands of plants in culture for years. It is the mass increase, long periods of time in tissue culture, and specific techniques, such as somatic embryogenesis and fragment shoot tip culture, that may lead to mutations in micropropagation. These types of mutations, known as somaclonal variation, have been studied extensively and are of interest for plant breeding. Cases of detrimental and beneficial mutations in micropropagated plants have been documented in other crops. There are several excellent

review articles on somaclonal variation and factors that affect it (Leva et al. 2012; Rani and Raina 2000; Ruffoni and Savona 2013).

In several older studies described in a review by Monette (1988), micropropagated grapes exhibited juvenile morphology, described as lack of tendrils, leaf shape, and other characteristics similar to a seedling after field establishment. However, it was later concluded that pruning was responsible for artificial maintenance of juvenility (Grenan 1984). In other studies, juvenile characteristics led to a lower yield in micropropagated vines (Martinez and Mantilla 1995; Deloire et al. 1995). Pruning may have been a factor in maintaining juvenility in these cases also. It was reported that all differences progressively disappeared after 7 years (Deloire et al. 1995). Another study observed that vegetative growth differences were not significant after some years and micropropagated plants had higher yield than conventional plants (Gribaudo et al. 2000). Thomas and Prakash (2004) found that vines that were planted in the field after 8 years of micropropagation initially exhibited juvenile characteristics but they disappeared in 6–8 months.

Finally, micropropagation in grapes is studied periodically for commercial increase and breeding purposes. Genetic homogeneity of grapes that were mass propagated by various methods in vitro was assessed using molecular techniques including microsatellite markers, ISSR, and AFLP. In all cases, there was no difference between and among the micropropagated plants and the mother plant. Additionally, there was no difference when plants were grown in a greenhouse or field (Nookaraju and Agrawal 2012; Baránek et al. 2009; Schellenbaum et al. 2008; Gribaudo et al. 2009). In a review article, Bouquet and Torregrosa (Bouquet and Torregrosa 2003) concluded that tissue culture was best used for pathogen elimination and for embryo rescue in seedless cultivars. However, the usefulness of tissue culture for micropropagation was questionable because of the high inputs needed but could be successful if the number of subcultures was limited and phenotype was monitored diligently. Since then, micropropagation has been investigated for many different cultivars especially for use in breeding programs with no apparent problems. It is worth repeating that there have been no reports of somaclonal variation in plants subjected to microshoot tip tissue culture for virus elimination.

For microshoot tip tissue culture, rapidly growing shoots in the spring and early summer provide the best tissue for excision. Microshoot tips are excised aseptically in a laminar flow hood using a 50X stereoscope magnification. The initial and maintenance medium is full-strength Murashige and Skoog (MS) salts (Murashige and Skoog 1962) and vitamins with 1.0 ml/l 6-benzylaminopurine (BA), 3% sucrose, and 6.0 g/l agar adjusted to pH 5.8. Explants are incubated in a growth chamber at 25 °C, 50% relative humidity, and 16-h daylight under cool white fluorescent and incandescent bulbs. Explants are transferred to fresh medium every 2–3 weeks. When the explants develop a shoot, they are transferred to rooting medium [half-strength MS salts and vitamins with 1.0 mg/l indole-3-acetic acid (IAA), 1.5% sucrose, and 6.0 g/l agar adjusted to pH 5.8]. When roots are well developed, plants are transplanted to sterilized potting mix (Golino et al. 2000).

Over the course of 7 months or more, the tips grow into a small plant with shoots and roots (Fig. 27.3). From there, they must produce enough plant material for

Fig. 27.3 Microshoot tip culture of grapevines at stages from 1 day to 6 months old. The microshoot tip on the *left* is less than 0.5 mm; when the explant shoot is approximately 2 cm high, it is transferred from medium with 1 mg/L BA to rooting medium containing 1 mg/L IAA. The rooted 6-month-old explant, far *right*, is ready to transplant to soil in the greenhouse

retesting to see if the targeted virus was successfully eliminated. In most cases, the virus is eliminated, but careful retesting is necessary, and success varies depending on virus and cultivar (see below; Sim et al. 2012). As described elsewhere in this volume, molecular detection techniques for grapevine viruses have improved, making it possible to test young plants regenerated from tissue culture, greatly improving the speed and accuracy of the virus-screening process. However, it is important to test the health status after at least one dormancy as viruses can remain below the detection threshold in young plants.

There has been speculation by many authors on the mechanism of action by which microshoot and/or meristem culture eliminates virus(es) in horticultural crops (Panattoni et al. 2013). Most hypothesize that the lack of vascular tissue in the meristem and immediately adjacent cells of the shoot tip prevents or impedes the movement of virus into those cells. There is also speculation about the presence of possible inhibitors of virus replication in the tissues.

A variation on microshoot tip culture technique is used routinely by IFV in France. Apical tips that are approximately 0.2–0.4 mm in size are excised and grafted onto hypocotyls of grapevine seedlings that were germinated in vitro. Using this method, approximately 90% of the cultivars survive and test negative for viruses (Spilmont et al. 2012; Spilmont 2016, personal communication).

Other Therapy Strategies

Other tissue culture strategies have been used to eliminate viruses from grapevines but are not widely or currently used, due to low survival or virus elimination rates, technical difficulties, or concerns about somatic mutations. These strategies include fragmented tip culture, chemotherapy, somatic embryogenesis, electrotherapy, and cryotherapy or a combination of several of them. As with the microshoot tip and heat treatment techniques described above, survival and virus elimination success depends on the cultivar as well as the virus species. These techniques may be useful in special cases if microshoot tip therapy is not successful.

Fragmented shoot tip culture was developed in 1978 (Barlass et al. 1982) and involves regenerating a plant from shoot tips that are aseptically cut into small pieces of tissue measuring less than 0.5 mm. Virus elimination was highly successful; 100% of plants regenerated tested negative for viruses using detection technology available at the time. Careful observations and electron microscopy revealed that plants grew from adventitious buds formed from leaf primordial fragments (Barlass et al. 1982). Concerns about mutations in the adventitious buds leading to off-types are the reason this technique is not widely used.

Chemotherapy has been investigated with mixed success. In most chemotherapy strategies, 1–2-cm-long green shoot tips are established in vitro in a medium containing antiviral compounds for at least 30 days. Plants are then subcultured to a medium without antiviral chemicals to recover. Ribavirin, oseltamivir, tiazofurin, and mycophenolic acid are commonly used as antiviral chemicals. Survival and virus elimination rate ranges widely depending on which antiviral compound is used and its concentration, cultivar, and virus (Panattoni et al. 2006, 2013; Skiada et al. 2013; Guta et al. 2014). The main obstacle to the regular use of chemotherapy is the high phytotoxicity of the antiviral compounds.

Electrotherapy has been attempted in several studies with limited success. It involves subjecting 1–2 node green cuttings to an electric current for 15–30 min and then establishing them in vitro. The theory is similar to that for heat treatment – the electrical field heats the tissue, inactivates virus particles, and prevents the viral genome from replicating. Survival rate is approximately 60%, and virus elimination rate of cuttings that survive is approximately 40%. Concerns about abnormal morphology developing have been expressed (Guta et al. 2010; Bayati et al. 2011). Electrotherapy is relatively quick and simple and could be a useful technique after more long-term studies are carried out to observe plants for off-types and virus status.

Somatic embryogenesis has also been investigated as a strategy for virus elimination from grapevines with excellent results. In this technique, anthers and/or ovaries are cultured and induced to form callus tissue; calli are then induced to form embryos. Embryos are cultured to regenerate into plants. Virus elimination was 100% in 97 selections in one study, including *Grapevine rupestris stem pitting-associated virus* (GRSPaV), one of the more difficult viruses to eliminate by microshoot tip culture and thermotherapy (Gribaudo et al. 2006). However, somatic

embryogenesis is technically more difficult and time-consuming and has an increased risk of off-types due to somaclonal mutations and variations.

Cryotherapy is the process of freezing shoot tips in liquid nitrogen for a short period of time then thawing them and regenerating a plant. It is the same reasoning as meristem tip culture, except that instead of excising the meristem, freezing temperatures are used to kill cells other than meristematic cells. Due to the fact that meristem cells have very dense cytoplasm with few vacuoles and less water relative to other cells, they are able to survive the freezing, while other cells burst. Tissue requires some type of preconditioning before freezing, either encapsulating in beads and dehydration or culturing medium with increasing sucrose concentration for osmotic protection. The process is reported to yield high survival and a 100% success rate at virus elimination (Wang et al. 2003). Again, cryotherapy is technically difficult and time-consuming.

In summary, plant survival, cultivar genotype, virus species, and technical skills and resources are important considerations for determining which strategy to use to eliminate viruses from grapevines. All strategies rely on regenerating clean plants to create foundation stock plants. From these, clean cuttings are provided to nurseries for establishing increase blocks to harvest cuttings for the production of material for actual planting of productive vineyards. Since there is no cure for infected vineyards, the importance of prevention and the production of clean stock are apparent.

The Place of Grape Clonal Variation in Clean Plant Programs

There are two critical areas that need to be considered in developing a superior grape cultivar collection. The first is disease status. Until a new selection is assayed for viruses and shown to test negative, vine performance is impossible to evaluate because vigor, yield, and fruit quality are all affected by viruses. By using planting material derived from certified, virus-tested stock, grape growers can reduce uncertainty about vine performance. Secondly, as selections of the same cultivar from different sources are compared, subtle performance differences become apparent. These differences are caused by mutations in genes that control characters such as characteristics of the leaf lobes, cluster size and compactness, berry color, disease resistance, and ripening date, among other factors. Over time, mutations accumulate and lead to greater diversity in older cultivars or selections. Selections propagated from single vines that differ in these ways and have been evaluated are known as “clones” of a cultivar. Planting superior clones can improve a cultivar’s production and winemaking characteristics. In the wine grape industry in particular, clonal variability is the subject of numerous studies and discussions.

There have been ongoing debates in the viticultural community about the relative merits of heat treatment versus microshoot tip culture for the elimination of viruses from infected grapevines. A commonly held belief among wine grape growers from California and elsewhere is that heat treatment produces high-yielding clones that

are excessively vigorous for the highest quality winemaking (neither table grape growers nor raisin growers have ever expressed a concern over high yields). Little scientific evidence exists for this theory. The FPS program at UC Davis produced the majority of heat-treated grape selections grown in grape collections around the world. At the time that the "heat treatment clones" were sourced from commercial vineyards by the grape breeder H. Olmo and others for inclusion in the FPS collection, high yield, cluster size, and vigor were important characteristics which were consciously sought (Alley and Golino 2000). Therefore, it is not surprising that many of the early FPS selections were high yielding but that is regardless of whether or not the selection received heat treatment. For example, cv. Zinfandel Selection 1A in the FPS collection never received heat treatment but is reported to be vigorous and productive as is the heat-treated selection Zinfandel Selection 6 (Wolpert 1996).

When A.C. Goheen performed heat treatment, he normally maintained the original preheat treatment selection as well as sequentially numbered selections, which represented varying treatment durations. In the FPS collection, there are multiple selections with varying heat treatment time, which have been produced from the same original source vine. These selections can be expected to be genetically identical in most cases and are not likely to be the source of significant clonal diversity (Christensen et al. 1995). However, because there is some statistical possibility of change and/or different disease profile, individual sources propagated from the same original accession with varying heat treatment history (number of days) are maintained under separate selection numbers at FPS. In more recent years, the same cautious approach has resulted in each tissue culture explant from the microshoot tip therapy program receiving a unique selection number when it is made available to the nurseries.

Variation in Therapy Success Rates Due to Virus Taxa

Successful virus elimination depends on virus taxa. Experience has shown that the leafroll viruses, which are phloem limited, are easier to eliminate than other viruses, perhaps due to the fact that the microshoot tip has no vascular connection to phloem tissue. The following sanitation rates were recorded at the DPSFS-Uniba relative to the analysis of nearly 3000 accessions of different cultivars derived from microshoot tip therapy: *Grapevine leafroll-associated virus 1* (GLRaV-1; 100%), *Grapevine leafroll-associated virus 2* (GLRaV-2; 98%), *Grapevine leafroll-associated virus 3* (GLRaV-3; 98%), *Grapevine virus B* (GVB; 99%), *Grapevine virus A* (GVA; 92%), *Grapevine fleck virus* (GFkV; 94%), and GFLV (84% heat therapy alone; 92% meristem tip culture and heat therapy) (La Notte et al. 2006; Morelli et al. 2015; Bottalico, personal communication). At Cornell, a dozen clean accessions of six cultivars each were obtained after elimination of GLRaV-1, GLRaV-3, and Grapevine red blotch-associated virus (GRBaV) by microshoot tip culture and testing after two dormancy periods. At FPS, 90% of the selections were successfully cleaned through microshoot tip culture. Many of these selections were

infected with more than one virus. After processing, approximately 8% were infected only with GRSPaV. Other viruses that were detected after processing in the remaining 2% of selections were GVB, GFLV, GFkV, and GLRaV-2. This confirms other studies in which GRSPaV was less likely to be eliminated (Maliogka et al. 2009; Gribaudo et al. 2006). GRSPaV is considered a minor virus with no documented economic impact.

Economic Value of Clean Stock

Grapevine viruses and related pathogens have no cure in a vineyard and impose high costs on nurseries and crop producers. Viral diseases are typically disseminated through infected planting stock and plant-propagation material as a consequence of a careless selection of budwood. However, virus dissemination can be minimized if virus-screened stocks are used. Documenting the value of creating virus-screened "clean" planting stock is critical to insure the public sector funding of this expensive and time-consuming work. The development of meaningful data about the economic value of clean plant programs is challenging. The prospect of documenting benefits may seem overwhelming to those knowledgeable about the diversity of the viruses infecting grapevines, the documented variability in the impact of the various virus diseases, demonstrated diversity in the effect of different strains of individual viruses, the frequency of multiple virus infections in field situations, and the role of *Vitis* genotype in the response of the vine to infection.

Nonetheless, several studies in recent time have been able to estimate the economic impact of leafroll viruses. Atallah et al. (2012) found that, using data from a Cabernet franc vineyard in the Finger Lakes region of New York, if no control measures were implemented, the cost of GLRaV-3 ranged from $25,407/ha with 30% yield loss and no quality penalty to $41,000/ha with 50% yield loss and 10% quality penalty. They further found that initially planting GLRaV-3-screened vines rather than unscreened vines was financially rewarding over a 25-year horizon, even under the assumption that GLRaV-3-screened vines cost 25% more than unscreened vines. Among practices they evaluated, the removal of individual infected vines (roguing) was the most efficient and could reduce the losses to between $3000 and $23,000 per hectare if the vineyard contained less than 25% leafroll-infected vines, and replacing with GLRaV-3-free vines would reduce losses further, down to approximately $1800 per hectare (Atallah et al. 2012).

In a related article, Atallah et al. (2014) examined various control strategies using a plant-level spatial dynamic model of the disease. In their simulation analysis, they found that a strategy of roguing and replacing symptomatic vines and testing their four immediate neighbors was economically superior to all other strategies evaluated; compared with a no-control strategy, it yielded benefits over 50 years having a net present value of $59,000 for a 2-ha vineyard. They found that incorporating the less-than-perfect detectability of diseased vines and allowing for a time lag before the vine becomes symptomatic added substantially to the measured disease costs over 25 years, a net present value of $25,000 versus $4000 per hectare.

In California, another economic study estimated the costs and benefits of a virus-screening program for GLRaV-3 in the North Coast region of California (Fuller et al. 2015). Grower costs and benefits from using GLRaV-3-free vines were computed and extrapolated to the North Coast industry as a whole. Economic benefits from the GLRaV-3 testing and cleaning program were found to be in excess of $50 million per year for the region and to substantially outweigh its costs. The results showed potential benefits from removing and replacing diseased vines rather than leaving them in the vineyard where they can be foci for disease spread. In addition, significant costs are associated with disease entering from virus-infected vines in neighboring properties.

A recent study focusing on three major grape-growing counties (Napa, Sonoma, and Northern San Joaquin Valley) in California estimated the economic impact of leafroll on Cabernet Sauvignon (Ricketts et al. 2015). Estimated costs of leafroll with no disease control ranged from $29,902 to $226,405 per ha depending on region, yield reduction, quality reduction, and varying levels of initial infection. The results of the study also found that if disease prevalence is between 5 and 10%, roguing symptomatic vines, replanting with certified vines, and controlling for mealybugs can minimize losses to leafroll. However, if disease incidence is greater than 25%, full vineyard replacement should be considered.

In New Zealand, a 2004 study produced a model that showed the cumulative cost of leafroll virus spread under three different (high, moderate, and low) infection scenarios to be NZ$30,000 per ha by years 12, 15, and 17 (Walker et al. 2004). Furthermore, by year 11, infection might sufficiently justify vineyard replacement when the cost of leafroll infection exceeds the cost of vineyard establishment. In another New Zealand study, the economic impacts of leafroll were investigated by comparing the net present value of healthy and diseased vineyard blocks under three different management strategies: vineyard replacement, roguing and replacing symptomatic vines, and roguing and replacing symptomatic vines along with immediately adjacent vines. Over an 8-year period, roguing and replacing symptomatic vines reduced disease impact by 30% compared to no treatment in both varieties studied (Anonymous 2006). An economic impact model based on a net present value method has been developed and made available to New Zealand winegrowers. The model allows growers to set inputs of some parameters specific to an individual vineyard and provides them with the estimated costs and benefits for various treatment scenarios. For example, forecast revenue lost to removal of infected vines in the year of infection and replacing them the following year is 5% compared to a 28% revenue loss by waiting 6 years to replace all vines after one fallow year (Anonymous 2015).

South Africa is another country where an integrated control strategy of leafroll disease is being successfully implemented. This strategy is essentially based on the use of certified virus-free planting material, systemic insecticide treatments, removal of infected vines by roguing, careful cleaning of agricultural implements, and clothing for reducing passive transport of vectors between vineyards (Pietersen 2006; Pietersen and Walsh 2012).

Conclusive Remark

Sanitary selection of grapevines is a viable and reliable approach to limiting the damage done to vineyards by infection with grapevine viruses. With a combination of robust virus detection technology and the success of microshoot tip culture for virus elimination, programs around the world continue to provide virus-screened grapevine cultivars and clones, as well as rootstock genotypes. This is a tremendous benefit to growers of grapevines, which are used for many purposes, including winemaking, fresh table grapes, raisins, and juice.

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