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PATHOGEN TESTING AND CERTIFICATION OF *VITIS* AND *PRUNUS* SPECIES*

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■ **Abstracts** Strategies to screen horticultural crops for graft-transmissible agents, particularly viruses and phytoplamas, have advanced substantially over the past decade. Tests used for *Vitis* and *Prunus* are reviewed in detail, including both biological indexing procedures and laboratory-based assays. Despite advances in laboratory molecular-based detection techniques, a strong case is presented for the continued use of slower biological tests in programs requiring high level of confidence in detection of pathogens that must be excluded from valuable germplasm.

INTRODUCTION

Freedom from viruses and other pathogens in deciduous planting stocks is important because nearly all plants for plantings are produced by vegetative propagation. If present, disease agents will be readily perpetuated, albeit unwittingly, in the progeny. Moreover, once diseased plants are established in commercial orchards or vineyards, they are not amenable to any curative or therapeutic control measures. In most instances, the most effective disease control option is removal of infected plant or plants. Further, several disease agents are spread secondarily by natural vector species, i.e., aphids, mealybugs, mites, leafhoppers, and nematodes, or even, in some cases, by pollen, and infected stocks can serve as sources of inoculum.

First and foremost, the principal method proven most efficient in controlling virus and virus-like diseases in perennial crop plants involves the application of

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pathogen exclusion protocols in advance of wholesale plant propagations. These protocols are often performed in the framework of clean stock/certification programs. This review focuses on assay methods used in sanitation programs to exclude virus and virus-like diseases and to identify healthy source plants of the genera *Vitis* and *Prunus*.

Certification schemes worldwide share a common objective: to identify healthy sources for propagation through the application of time-tested indexing procedures as well as more recently developed molecular assays. Even so, the actual procedures and protocols can vary widely depending on the specific pathogens being targeted, the endemic disease agents in a production region, the availability of techniques and financial resources, and the expectations of industries served. The first step is the establishment of foundation or nuclear source plants: plants, testing free from all known harmful viruses and professionally identified for true-to-type phenotype.

Foundation Plant Services (FPS) is a service department in the College of Agricultural and Environmental Sciences, University of California at Davis. FPS produces, maintains, and certifies nuclear stock materials, which are available to nurseries and growers in California, in the United States, and in foreign countries. In many instances, FPS-certified stocks qualify as primary sources for commercial increase. Most certification programs are monitored by state or federal government agencies in accordance with precise regulations. The California Department of Food and Agriculture (CDFA) administers the statewide California Registration and Certification (R&C) program for *Vitis* and *Prunus*. To maintain planting materials in the program as foundation registered stock or as commercial registered increase block, the program participants, i.e., FPS, nurseries, and licensed propagators, are required to comply with promulgated rules and regulations governing the CDFA R&C programs.

In this review, we discuss the pathogen tests used internally by FPS to screen new accessions of *Vitis* and *Prunus* for the FPS foundation vineyards and orchards. Many of these tests are not currently required to qualify in the CDFA R&C programs but are likely to be included in future revisions of the regulations. This review builds upon previous descriptions (4, 57, 59, 66, 103), with emphasis on the California R&C programs and the role played by FPS.

BIOLOGICAL INDEXING

Two different groups of indicator plants are used in the California R&C program and in other comparable programs implemented elsewhere in the world. Plants in the first group are herbaceous, maintained in the greenhouse, and used in assays for sap-transmitted viruses. These assays may be completed in a matter of weeks. The second group is comprised of woody plants, which require a lengthier incubation period, i.e., up to two to three years (or more when wood or fruit symptoms are involved) to complete. The woody indicators, commonly belonging in the same genera as the accession under test, were selected for their development of

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readily distinguished, diagnostic symptoms. In this section, we review the various indicator-based test methods for detecting native and exotic pathogens in domestic and foreign accessions.

Grapevine

FPS is the source of all grape stock in the CDFA Grapevine R&C program. It also serves as source materials for nursery blocks in other state certification programs. There are no federal certification programs for horticulture crops in the United States, but the tests used to qualify candidate selections for the California R&C program also qualify selections for release from federal quarantine as required by the departmental permit for grape importation issued by the Animal and Plant Health Inspection Service (APHIS) to the director of FPS.

All grape accessions in the FPS foundation vineyards are processed through a complete disease-indexing protocol (described below). When a new grape accession or accessions of foreign imports or domestic sources test positive, disease elimination therapy is performed using heat treatment and tissue culture. The plantlets resulting from therapy are then re-tested and planted in the foundation vineyard as assays warrant. Visual inspections by pathologists and regulatory biologists take place twice a year. Professional identification of cultivars is accomplished through the use of both traditional ampleography (the science of description and identification of *Vitis* species and its cultivated varieties) and molecular DNA fingerprinting techniques. When all disease testing has been completed, the selection can be planted in the foundation and distributed as provisional stock. Stocks become registered when professional identification is complete (Figure 1, see color insert).

HERBACEOUS HOSTS Sap-transmitted viruses of concern belong in the genus *Nepovirus* and, to a lesser extent, to the genera *Vitivirus* and *Closterovirus*. Indicator plants used in the assays are *Chenopodium quinoa*, *C. amaranticolor*, *Nicotiana clevelandii*, and *Cucumis sativus*. Only *C. quinoa* is required for the CDFA R&C program. For the inoculation process, succulent tissues (young leaves and shoot tips) are triturated in 10 mM phosphate buffer pH 7.5 containing 2% nicotine (1:10, wt tissue:ml buffer) and rub-inoculated. Nicotine is used to neutralize the inhibitory effects of polyphenolic compounds and other host cell inhibitors on virus infectivity as well as to facilitate virus transmission (61). When virus transmissions are successful, one or more indicator plants may develop primary symptoms (local lesions, ringspots, etc.) after a few days incubation. Thereafter, systemic symptoms appear (mosaic, vein clearing, leaf deformation, tissue necrosis, etc.) (13, 53).

WOODY INDICATORS The known grapevine viruses in the families *Closteroviridea*, *Flexiviridae*, and *Tymoviridae* (56a), other unidentified graft-transmissible agents (GTAs), and phytoplasmas are phloem-limited. These pathogens are

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refractory to sap transmissions and their detection is accomplished by bud-chip grafts onto a panel of woody indicators that are prescribed in the CDFA Grapevine R&C program but also are used in other sanitary assessment programs worldwide. The indicators used are *Vitis rupestris* St. George, LN33, Kober 5BB, and Cabernet franc. Kober 5BB was added to the panel, in the mid-1990s when it became clear that the disease Kober stem grooving was present in both domestic and foreign grape stocks. This assay, however, is not currently required in the CDFA R&C program or by federal quarantine regulations.

V. rupestris St. George produces diagnostic symptoms in response to infections by *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine asteroid mosaic associated virus* (GAMaV), and *Rupestris stem pitting associated virus* (RSPaV). Symptoms induced by GFLV infections appear as leaf vein clearing, chlorotic ringspots, oak leaf patterns, and/or distortion of leaf blades (12, 13, 46, 53, 77). These symptoms, developing in early spring, are ephemeral in nature and slowly disappear with rise in ambient temperatures. With GFkV, leaf symptoms consist of a "clearing" of third- and fourth-order veinlets and localized translucent spots. In severe cases, leaves may wrinkle, twist, and curl upward. Further, a diffuse mosaic pattern may develop on mature leaves (12, 13, 46, 53, 77). With GAMaV, chlorotic star-shaped spots, which may cluster irregularly, develop on leaves (53, 77). Finally, symptoms ascribed to RSPaV involve stem markings, i.e., as distinct basipetal pitting extending downward from the grafted chip bud. Occasionally, stem pits encircle the woody cylinder (53, 77). RSPaV does not show symptoms on the other grapevine virus indicators, LN33 and 5BB. Currently, a positive test for grapevine rupestris stem pitting disease will not disqualify a selection from advancement to foundation status in the CDFA R&C program.

LN33 is a hybrid cross of Couderc 1613 $\times$ *V. vinifera* cv. Thompson seedless. It is an indicator for corky bark disease, which is associated with *Grapevine virus B* (GVB). Symptoms include grooves and pits on the woody cylinder, trunk bark split, and red leaves due to swelling of canes and proliferation of spongy callus tissues (hence the name corky bark) (53, 77). GVB is symptom-less in Kober 5BB and St. George.

The indicator Kober 5BB, a cross between *V. berlandieri* $\times$ *V. riparia*, is an indicator for Kober stem grooving disease associated with *Grapevine virus A* (GVA). Symptoms include wood necrosis, pits, and grooves; often accompanied by yellowish spots on the leaves (53). St. George and LN33 are latent hosts.

V. vinifera cv. Cabernet franc is diagnostic for leafroll disease. Other leaf roll indicators used elsewhere are Pinot noir, Mission, Cabernet Sauvignon, and Barbera (all *V. vinifera*); choice of indicator depends upon personal preferences and/or climatic conditions under which the indicator is grown (46, 53, 77). On Cabernet franc, symptoms are interveinal reddening of the leaf blade, beginning in early fall and intensifying thereafter, with primary veins prominently green, although these symptoms become less so late in the season. Leaf margins may roll downward. Often internodes are shortened and stunting is apparent. The currently characterized viruses associated with leaf roll are members in the family *Closteroviridae*.

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To date, nine *Grapevine leafroll-associated viruses* (GLRaV) have been reported; two currently lack molecular data for further classification (1, 54, 56).

GRAFT-INDEXING Dormant bud chips removed from canes of accessions are placed onto matching cut areas on stems of self-rooted indicator plants, overlaid with a plastic strip and secured with budding rubber. Two or three bud chips are grafted onto each of three indicator plants. A set of healthy plants and plants grafted with known disease positives (excluding exotics) must be included. Chip-bud grafts are done in late winter or early spring. After a month in the greenhouse, bud chips are evaluated for viability, then exposed to ambient conditions before transplanting in the field. The indicators are visually inspected in spring and fall annually for two to three seasons. A final examination is made for wood markings by lifting plants from the soil and removing bark tissues to expose the stem.

Stone Fruits

Indexing protocols have been developed and applied at both FPS and the National Research Support Program 5 (NRSP5, http://nrsp5.prosser.wsu.edu/) Washington State University Irrigation and Extension Center. *Prunus*, which has been qualified by testing of NRSP5 program, qualifies for inclusion in the FPS foundation. Many imports and new commercial cultivars that enter the FPS program are obtained from NRSP5.

Visual inspections of the foundation orchard by pathologists and regulatory biologists take place twice a year. Professional identification of cultivars is accomplished with the assistance of university and industry experts. When all disease testing has been completed, the selection can be planted in the foundation and distributed as provisional stock. Stock becomes registered when professional identification is completed (See Figure 1).

HERBACEOUS HOSTS Testing *Prunus* species from domestic sources on herbaceous hosts is not required by CDFA R&C program. However, members of genera *Nepovirus*, *Ilarvirus*, and *Trichovirus* infecting *Prunus* species are readily sap-transmissible to the herbaceous indicators *C. quinoa*, *N. occidentalis*, *N. benthamiana*, and *C. sativus*. Candidate agents are recognized by procedures described above for grape by symptoms developing after incubations of a few days to three weeks and subsequent serological and/or molecular tests (13, 29, 73).

WOODY INDICATORS (GREENHOUSE AND FIELD) At NRSP5, assays are performed largely on indicator plants grown in greenhouses. Potted trees are graft-inoculated by chip-budding (see grape indexing) or T-budding, where the bark is cut in a configuration of the letter T and the inoculum chip is inserted in between the bark and woody cylinder. A plastic strip is placed over the grafted bud and tied with budding rubber. Positive disease (exclusive of exotics) controls and nongrafted plants are included.

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After 30 days, the inoculum chips are visually inspected and recorded as alive or dead. At that time and up to 90 days post inoculation, the indicators themselves are inspected for disease symptoms and readings recorded. All indicators are observed for two years, i.e., following a dormancy treatment and further inspections for leaf and wood stem symptoms.

Several *Prunus* species and varieties are used as indicator hosts for the detection of graft-transmitted diseases. The indicators include *P. armenica* cv. Tilton apricot; *P. avium* cvs. Bing, Sam, and Canindex 1 cherries; *P. salicina* hybrid Shiro plum; *P. serrulata* cvs. Kwanzan and Shirofugen flowering cherries; *P. persica* cv. Elberta peach; and *P. tomentosa* Nanking cherry. These are used to index for apricot ring pox (syn. cherry twisted leaf), various nepo- and ilar-viruses, peach mosaic, cherry necrotic rusty mottle, little cherry, peach wart, cherry green ring mottle, apple chlorotic leaf spot, plum pox, and several phytoplasma diseases, such as X-disease, little peach, peach yellow leaf roll, and peach yellows (21, 22, 28, 33, 40, 41, 48, 70, 73, 76; nrsp5.prosser.wsu.edu).

The peach seedling GF305 is a useful universal indicator as it is highly susceptible to infection and reacts differentially to a number of viruses (24). The above indexing procedures are essentially the same as those used in the European Union and in other advanced *Prunus*-growing countries.

At FPS, accessions submitted by in-state breeders and selectors from the University of California, USDA-Agriculture Research Service, and private sector are graft-indexed on indicators. These indicators are maintained in the field for two to three years post inoculation and observed for symptom development. The indicators include Tilton apricot, Bing and Nanking cherries, Shirofugen and Kwanzan flowering cherries, Fay Elberta peach, and Shiro plum. Assays of each accession are complemented by ELISA tests for *Prunus necrotic ringspot* virus (PNRSV) and *Prune dwarf virus* (PDV). If the accessions test positive for one or more pathogens, the materials are sent to NRSP5 for virus elimination. Healthy materials are returned to FPS and planted in the foundation orchards.

Logistically, in the FPS foundation orchards, Shirofugen flowering cherry is planted at the ends of the tree row and used to index for PDV and PNRSV with bud chips collected from in-row trees. Annually, one third of the entire *Prunus* collection is indexed in this fashion and the remainder by serology (see below). The assays are done annually to monitor for possible pollen-borne spread of both viruses. Finally, the orchards are inspected in spring and summer for disease symptoms. Infected trees are removed as they are discovered.

MOLECULAR ASSAYS

Serology

Serology was the first method adopted in the evolution of rapid plant pathogen detection and identification (15, 63, 102). This technique is based on the recognition of antigens with antibodies produced to them. In its initial application by plant

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virologists, serology had been used routinely to identify virus species and strains but was not amenable to high throughput assays. The enzyme-linked immunosorbent assay (ELISA) (16–18, 20, 99) is based on a nearly decade earlier demonstration by Avrameas (3) that glutaraldehyde cross-linked enzyme-antibody conjugates retained both the specificity of the IgG molecule and the catalytic properties of the enzyme. ELISA allows qualitative and quantitative analysis, high throughput, and high sensitivity and was adopted rapidly and widely (17, 19, 63, 86).

Virions of viruses that are sap-transmissible to herbaceous hosts usually can be purified in milligram amounts and high purity for serial injection into rabbits or goats and recovery of polyclonal antibody from serum, or into chickens, for recovery from yolk. Examples are members of the genera *Nepovirus*, *Ilarvirus*, *Trichovirus* and *Vitivirus*. For non-sap-transmitted viruses infecting *Prunus* and *Vitis* species, purified virion antigens have been obtained in microgram quantities and with greater problems of contamination. These preparations have been used for the production of polyclonal antibodies and also to inject into mice to produce monoclonal antibodies (Mabs), the production of which requires special facilities and protocols (11, 31, 32, 36, 96, 98, 107). Mabs often have less avidity than polyclonal antibodies, but the high specificity of Mabs allows strain differentiation and eliminates the problem of cross-reaction with host material (72, 78). If strain specificity is not desired, a broad-spectrum reagent can be produced by combining Mabs generated form several cell lines. Where sequence information is available but purified virions have not been obtained, antibodies with reactivity to the virion may be raised against synthetic peptides (46a, 63a, 80a).

Two classes of ELISA protocols are used for surveillance (20, 44). Direct methods such as double antibody sandwich (DAS)–ELISA involves enzyme attachment to the antibody probe (5, 6, 43, 81, 100). In the indirect method [(DASI)-ELISA], the antibody probe remains unlabeled. Instead, the enzyme is attached to a second antibody or Protein A reactive specifically to the probe antibody (44, 83, 88). DASI-ELISA is favored over DAS-ELISA for its greater sensitivity, broader reactivity and convenience. Only a single enzyme conjugate is needed for assays of different viruses, and usually a suitable conjugate is available commercially.

To date, ELISA has been developed for most of the economically important and widespread viruses characterized in grapevine (10, 19, 47, 68, 83, 90, 96, 99, 106). At FPS, ELISA is routinely performed on grapevine materials entering the program or under therapy. This is in addition to use of biological indicators. The list of viruses tested by ELISA includes GFLV; GFkV; ArMV; *Grapevine leafroll associated virus* (GLRaV) 1, 2, 3, 4, 5, and 6; *Tomato ringspot* virus (ToRSV); and GVA. With the exception of antisera to GLRaV-5 and -6 purchased from commercial vendors, all others were produced in-house. Tests are available for a very similar range of viruses, with few exceptions depending on the local sanitary situation (e.g., ToRSV, which is not known to occur in Europe) for countries in the European Union that have implemented certification schemes, as well as in Canada, Australasia, some Latin American countries, and a few other viticultural countries. ELISA kits are available from commercial sources.

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Similarly, ELISA protocols were developed for viruses in deciduous fruit trees (2, 7, 8, 35, 39, 49, 62, 67, 79, 80, 97). In the NRSP5 program, ELISA is used to detect *Apple mosaic virus* (ApMV), *Cherry leafroll virus* (CLRV), PNRSV, PDV, *Plum pox virus* (PPV), and ToRSV; at FPS, testing is for PNRSV and PDV. The very same viruses are of concern in the European Union (again with the exception of ToRSV) and in other deciduous fruit tree-growing regions worldwide.

At FPS, ELISA is performed annually on the foundation vineyards (on 25% of the collection) and orchards (two thirds of the collection) for pathogens known to spread naturally. In grapevines, the assays include the following: nepoviruses GFLV, ToRSV, and ArMV; the ampeloviruses GLRaVs 1, 3, and 4; the closterovirus GLRaV-2; the vitivirus GVA; and the maculavirus GFkV. Although the biological vectors for GLRaV-2, -4 and GFkV are unknown, screening for these viruses is ongoing to monitor for possible reinfection.

Nucleic Acid-Based Assay

POLYMERASE CHAIN REACTION (PCR) PCR was developed in the mid-1980s (69, 92) and was rapidly adopted to identify pathogens through their DNA genetic materials. PCR assays are extremely sensitive, reliable, fast, and highly versatile. Reverse transcriptase-PCR (RT-PCR) was developed for pathogens with RNA genetic materials (14, 34, 75), i.e., all known *Vitis* and *Prunus* viruses. At FPS, RT-PCR is used routinely in the grape program in testing all post-entry quarantine materials and advanced breeder selections (A. Rowhani, unpublished data). The viruses assayed for are GLRaV-1, 2, 2RG, 3, 4, 5, 6, 7 and 9, GVA, GVB, *Grapevine virus D* (GVD), GFLV, ToRSV, ArMV, GFkV, *Tobacco ringspot virus* (TRSV) and RSPaV.

Preparation of plant extracts is a critical aspect of RT-PCR. Most bark and leaf tissues of woody plant species contain high levels of polysaccharides and phenolic compounds that impede the activities of enzymes used (9, 85). To circumvent the effects of these compounds, different approaches have been reported, including additions of inhibitors and absorbents of contaminants and special resin-packed columns that differentially bind viral RNAs (14, 51, 81a, 84, 85). An alternative approach to virion purification is immuno-capture (IC), referred to as IC-RT-PCR (64, 71, 74, 87, 104). In IC-RT-PCR, virion-specific antibodies are bound in wells of microtiter plates or tubes, and the extract is incubated to allow attachment of virions to the antibodies. Subsequent wash steps remove contaminants derived from the extract before RT-PCR of the well- or tube-bound material. Another simple approach is to dilute the extract to minimize the effects of host inhibitors on RT-PCR (64, 65, 104). Most approaches are labor intensive and limited to processing small sample numbers per day. However, automated RNA extraction instruments and protocols have been developed (103a).

Several variations of RT-PCR has been developed, including nested-, one-step-, multiplex- and real-time-RT-PCR. Nested-PCR is designed for high specificity detection of templates present in very low amounts (25–27). An external primer

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pair is used for an initial amplification. Then, a second primer pair, designed to hybridize within the initial amplified product, is used to prime a second round of PCR to further amplify the targeted segment.

In one-step RT-PCR, all reagents required for reverse transcription and amplification are combined in a single thermocycler tube, and the thermocycler program accomplishes first reverse transcription and then multiple cycles of PCR (60, 84). Sucrose and marker dye may also be included in the reaction mixture so that the contents of the tube may be applied directly to the electrophoresis gel (84). These modifications save time and effort and minimize opportunities for cross-contamination between samples.

Multiplex-PCR has a distinct advantage in that it allows the concurrent identification of viruses in plants with mixed infections, all in a single PCR experiment (26, 82, 91, 93, 105). This technique requires use of multiple pairs of primers, each designed to amplify a different target template. Typically the product of each template is distinguished by its size or fluorescent tag. Multiplex-PCR lowers cost per test through savings of reagents and labor.

In real time-PCR, a fluorescent-labeled oligonucleotide (e.g., TaqMan fluorescent probe) in the reaction mixture and a laser-excited fluorescence detection monitor are utilized to assess the quantity of PCR product at the end of each PCR cycle. The TaqMan probe set consists of a pair of oligonucleotide primers and a TaqMan probe designed to hybridize to a site between the two primer binding sites. The TaqMan primer has a 3′-phosphate to prevent it from priming DNA synthesis, a 5′ terminal "reporter" dye and a 3′ "quencher," located on the 3′ nucleotide residue. During the extension reaction of each PCR cycle, the $5' > 3'$ exonuclease activity of the polymerase cleaves the probe. This cleavage separates the reporter dye from its quencher, resulting in an incremental increase in fluorescence after a cycle has completed. This method eliminates the need for product detection by gel electrophoresis. It is quantitative and highly sensitive (42, 45, 50, 52, 89). Standardization and normalization of background controls are critical in real-time PCR.

NUCLEIC ACID HYBRIDIZATION Specific hybridization between nucleic acids has been used in viroid detection (23, 37, 58, 94) with modifications reported (37, 38, 58, 95). This assay, used extensively in the NRSP5 program to detect *Peach latent mosaic viroid* and *Hop stunt viroid*, employs a complementary RNA (cRNA) as a probe, which forms duplexes of greater stability with the target RNA (a viroid) than DNA probes form with to the same RNA target.

STATUS OF CERTIFICATION PROGRAMS

Certification is a procedure whereby nursery stocks and commercial production undergo controls for securing trueness-to-type and absence from specified pathogens, as directed by official regulations or endorsed by competent governmental agencies

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(57). The practical application of such conceptually simple measures constitutes a most powerful means for the sanitary upgrading of the grapevine and tree fruit industries worldwide. However, there little has been done so far to promote internationally recognized certification schemes that, upon application, would allow free trading of high-quality nursery materials among participating countries. Various political, commercial, and technical impediments stand in the way of international agreement on certification protocols.

The grapevine is the only woody crop that, since 1968, is the object of a compulsory certification by the European Union (EU) (Directive 68/93 EEC). However, this regulation prescribes only the absence of "harmful virus diseases, notably fanleaf and leafroll" from nursery material. It is unlikely that production with such a low sanitary status would be acceptable to any viticultural country aware of, and concerned with, the serious virological problems associated with this crop. Although some EU member states (France, Germany, Italy, Portugal, and Spain) have implemented certification schemes with sanitary requirements that are more restrictive than those of the extant Directive, and use the same virus detection procedures described above, national protocols are still far apart (30, 55). A recently issued Directive (2002/11 EU) is supposed to harmonize the system, but enforcement will depend on the contents of the still unpublished technical annexes.

The EU has taken one initiative (Directive 93/34 EEC) to favor the production and free marketing within its boundaries of nursery stocks with a minimal sanitary status, encompassing the absence, ascertained primarily through visual inspections, of quarantine organisms and other pathogens that could affect their quality. To this end, a new category of materials denoted CAC (Conformatis Agraria Communitatis) has been envisaged for a number of woody crops, comprising all *Prunus* species but not *Vitis* (4). Although compulsory, CAC is not a true certification. Nursery productions are not propagated from registered nuclear stocks under the surveillance of governmental phytosanitary services. Over a decade after its promulgation, the EU is now reevaluating the efficiency of the CAC system that has now been questioned.

Even though no EU certification system for *Prunus* exists, several member states have developed their own schemes. As with the grapevine, all the protocols are not entirely consistent with each other with regard to the kind of infectious agents to be excluded. Nonetheless, national schemes provide the basis for a common system in future, especially if the excellent certification outlines published in 1991–1992 by the intergovernmental agency EPPO (European and Mediterranean Plant Protection Organization) (4) are taken into account.

The North American Plant Protection Organization (NAPPO) is the regional plant protection organization represented by members from the national plant protection organizations of Canada, the United States, and Mexico. Like EPPO, it is one of many regional plant protection organizations whose primary responsibility is to develop regional plant protection standards, which would protect the member states from the entry and establishment of pests, while facilitating trade. In addition, NAPPO participates with other regional plant protection groups within the

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western hemisphere and globally to develop international standards. For *Vitis*, the document "Guidelines for the Importation of Grapevines into a NAPPO Member Country RSPM #15 Part 1: Viruses and Virus-like Pests, Viroids, Phytoplasmas, and Bacteria" was developed by a committee of experts and signed on October 20, 2002. This document is the initial regional guideline for the development of harmonized North American Standards for grapevine nursery stock. For *Prunus* (and *Malus*), the parallel document is "Guidelines for the International Movement of Pome and Stone Fruit Trees into a NAPPO Member Country RSPM #25 Part 1: Viruses and Virus-like Pests, Viroids, Phytoplasmas, and Bacteria." Both are available on the NAPPO website at http://www.nappo.org.

In the area of *Vitis* and *Prunus* certification, Canada has a formal national certification program, which is voluntary. Mexico has no national grape certification program, but the majority of grape nursery stock comes from California and must meet California certification standards to be imported. The United States operates under voluntary state certification programs for both *Vitis* and *Prunus*, which combined with strict quarantine regulations have resulted in high-quality nursery stock with a minimum of regulatory infrastructure. However, as regional organizations like NAPPO and international agencies such as the Food and Agriculture Organization of the United Nations work to harmonize standards for the international movement of plant materials, a more formal, coordinated national and international system may need to be considered to insure that growers and industry are protected from non-quarantined damaging diseases that can be transmitted in planting stocks.

CONCLUSIONS

Molecular assays have proven invaluable in the rapid detection and identification of pathogens. Even so, biological assays must remain as the fundamental assay for the continued success of clean stock/certification programs. This is because uncharacterized GTAs and unknown pathogens abound. Also, development of laboratory assays has been and likely will continue to be in response to biological objectives related to pathogenesis.

An example is the Redglobe virus, described as *Grapevine leafroll associated virus*-2RG (GLRaV-2RG), that was discovered recently by indexing on seldom-used grape rootstocks comprised largely of hybrid with varied speciation. Although serologically related to leafroll type 2, GLRaV-2RG did not incite leafroll disease in the standard indicators, Cabernet franc or Cabernet sauvignon, it did cause an acute reaction on certain grape rootstocks (101). GLRaV-2 induced leafroll symptoms in both indicators, whereas it was latent in grape rootstocks. On the molecular level, GLRaV-2 and –2RG share 74% nucleotide sequence homology (A. Rowhani, unpublished observations). Note that Redglobe is a table grape with foundation status, i.e., it passed the required assays of the CDFA R&C program. Problems arose, however, when Redglobe scion materials were bench-grafted onto

different rootstocks and planted in commercial vineyards. On acutely sensitive rootstocks, all grafted plants died within two growing seasons.

Even though molecular techniques enabled the eventual identification of GLRaV-2RG, its pathology in sensitive rootstocks led us to examine Redglobe plants more closely. In subsequent rootstock trials, we have identified more GTAs, which are also lethal on a different range of rootstocks.

In addition to biological indicators, there is a further need for certification programs to officially recognize and integrate laboratory-based assays (serological and nucleic acid-based methods) into the program. This is because the newer assays can provide more rapid detection and identification of known disease agents; especially of regulated and exotic pathogens in the confines of a laboratory rather than as live specimens in grafted indicator plants held in greenhouses or in open fields.

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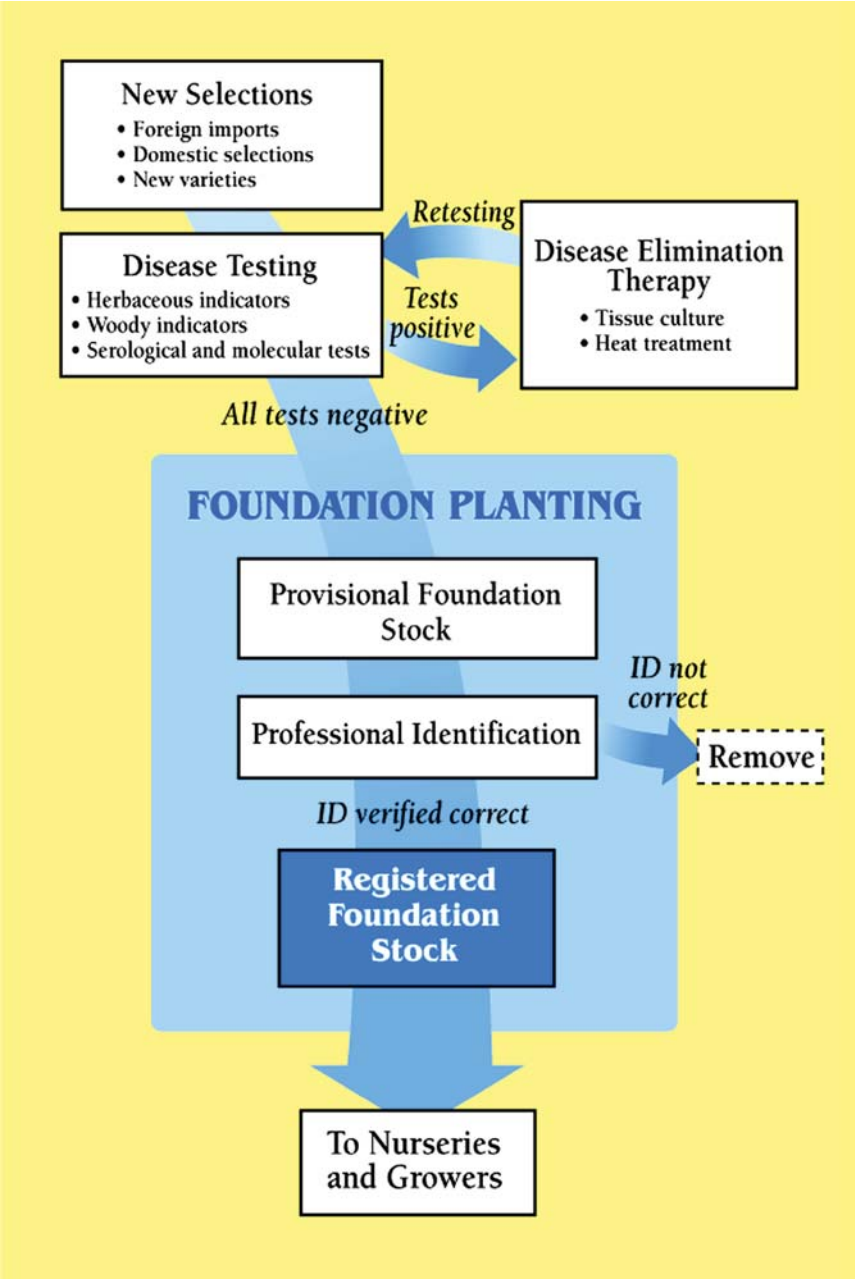

Figure 1 Scheme for testing and establishment of foundation plantings.