CHAPTER 6. PATHOGEN DETECTION AND ELIMINATION

SECTION 1. OBJECTIVES

The following objectives are consistent with the mission of NCGR-Corvallis to collect, conserve, characterize and distribute healthy plant materials:

- To obtain accessions from “clean” sources free of viruses and other pathogens when possible.
- To maintain plants in a healthy condition and to prevent the vector transmission of pathogens to plants in the repository base collections.
- To detect infected plants by periodic indexing.
- To keep accurate records on virus indexing results for each accession, and to make this information readily available to the NCGR staff and to germplasm users.
- To eliminate viruses from high priority infected clones using appropriate therapy procedures.
- To verify the identities of plants generated following therapy.
- To inform recipients of the known pathogen status of distributed material.
- To distribute pathogen tested plant material to the extent possible.

SECTION 2. INTRODUCTION

2.1 Why Test for Viruses?

Clonally propagated plant materials may accumulate virus and other "germplasm borne" diseases which can be latent or symptomless. Although free of obvious symptoms, plant vigor, productivity, hardiness, graft compatibility and other horticultural characteristics may be affected. Viruses can be detected by bioassay (inoculating sensitive indicator plants) by seroassay (ELISA) when specific antisera are available), and by laboratory procedures such as PCR. ELISA is the most commonly used seroassay. Some viruses produce distinct symptoms and can be detected by simple observation. Each genus differs in the number and identity of important viruses, in the probability of healthy plants becoming infected, and in the tests used to detect latent infections. Pathogen tested plant germplasm tends to be more productive and consistent in growth characters, and better able to satisfy quarantine requirements of other nations, thus promoting reciprocal international exchange of plant genetic resources.

2.2 Priorities

Plants received as clones should be tested for:

a. Viruses which may have quarantine significance in the US

b. Viruses which may have quarantine significance outside the US

c. Viruses for which reliable tests exist.

d. Viruses common in the plant's place of origin.

2.3 Incoming Plant Materials

- Accessions received from foreign sources will be handled according to all state and federal quarantine regulations.
- Arriving packages, especially those from foreign sources, should be opened in a Bio-safety hood to determine if contents are secure and as expected. All packaging should be autoclaved or incinerated.
- The autoclave should be calibrated annually and tested with bacteria ampoules.
- Detailed records will be kept on quantities, locations, and pertinent dates of quarantine and permitted material, and plants will be clearly labeled to indicate their quarantine or permit status.
- Appropriate APHIS and OR Dept. of Agriculture personnel will be notified when plants are received from a restricted or permitted source, or when material is released from quarantine.
- Contact between permitted plant material and its residue, other plants, or contact surfaces should be minimized to prevent the spread of pests. Permitted material should be handled, processed and transported in a dedicated tray which is disinfected after each use.
- Permitted or quarantine plants will be segregated from other closely related plants, using isolation distances and conditions as specified in the permits.

2.4 Isolate Infected Plants

Known pathogen infected plants will be segregated from healthy or untested plants to the extent possible. An intensive vector control program must be maintained in all greenhouses and screenhouses. Flowers should be removed from *Corylus*, *Rubus*, *Fragaria* and *Vaccinium* accessions to prevent spread of pollen borne viruses as well as contamination of

clones with seedlings. Persons working with protected collections should be certain they are not carrying insects. Persons should not enter screen/greenhouses after visiting field plots. Outer doors should be closed before inner doors are opened. Screen/greenhouses shall be checked periodically to assure they remain insect proof.

Imported accessions will be observed for a period of time in the isolation greenhouse and will not be distributed until they test negative for important virus diseases. Imported accessions which test positive for viruses will undergo therapy, and the infected plants will be destroyed.

SECTION 3. INDEXING RECOMMENDATIONS FOR NCGR GENERA

The tests listed below are recommended to detect important viruses for NCGR genera. Bolded tests are recommended as high priority for the repository. A list of viruses detected by each indicator is presented in the appendix by genus.

3.1 Corylus

ELISA for:

Apple mosaic virus

PCR for:

Hazelnut stunt phytoplasma

Other:

Possible occurrence of prunus ringspot, tulare apple mosaic virus, and other phytoplasma diseases.

3.2 Fragaria

ELISA for:

Arabis mosaic
Fragaria chiloensis latent
Strawberry mild yellow edge
Strawberry necrotic shock (Tobacco streak)
Tomato ringspot

Graft inoculation of:

UC-5 or UC-4 UC-
11 or UC-10
Alpine Seedlings

Sap inoculation of:

Chenopodium quinoa
Cucumber

Viruses Reported to Infect Strawberries (from Martin & Tzanetakis, 2006)

Virus name	Acronym	Transmission	Genus	Laboratory detection
Apple mosaic	ApMV	Pollen, seed	Ilarvirus	ELISA, RT-PCR
Arabis mosaic	ArMV	Nematode, seed	Nepovirus	ELISA, RT-PCR
Beet pseudo-yellows	BPYV	Whitefly	Crinivirus	RT-PCR
Fragaria chiloensis cryptic	FClCV	Unknown	Unknown	RT-PCR
Fragaria chiloensis latent	FClLV	Pollen, seed	Ilarvirus	ELISA, RT-PCR
Raspberry ringspot	RpRSV	Nematode, seed	Nepovirus	ELISA, RT-PCR
Strawberry chlorotic fleck	StCFV	Aphid	Closterovirus	RT-PCR
Strawberry crinkle	SCV	Aphid	Cytorhabdovirus	RT-PCR
Strawberry feather leaf	NA	Unknown	Unknown	NA
Strawberry latent	StLV	Unknown	Cripavirus	RT-PCR
Strawberry latent C	SLCV	Aphid	Nucleorhabdovirus	NA
Strawberry latent ringspot	SLRSV	Nematode, seed	Sadwavirus	ELISA, RT-PCR
Strawberry mild yellow edge	SMYEV	Aphid	Potexvirus	ELISA, RT-PCR
Strawberry mottle	SMoV	Aphid	Sadwavirus	RT-PCR
Strawberry necrotic shock	SNSV	Thrips, pollen, seed	Ilarvirus	ELISA, RT-PCR
Strawberry pallidosis associated	SPaV	Whitefly	Crinivirus	RT-PCR
Strawberry pseudo mild yellow edge	SPMYEV	Aphid	Carlavirus	ELISA
Strawberry vein banding	SVBV	Aphid	Caulimovirus	PCR
Tobacco necrosis	TNV	Oomycete	Necrovirus	ELISA, RT-PCR
Tomato black ring	TBRV	Nematode, seed	Nepovirus	ELISA, RT-PCR
Tomato ringspot	ToRSV	Nematode, seed	Nepovirus	ELISA, RT-PCR

3.3 Humulus

ELISA for:

American hop latent
Apple mosaic
Arabis mosaic
Cucumber mosaic
Hop latent
Hop mosaic
Prunus necrotic ringspot
Tomato ringspot

3.4 Mentha (low priority at NCGR)

Viruses Reported to Infect Mint (from Tzanetakis et al. 2010)

Virus name	Acronym	Genus	Transmission	Detection	Distribution
Alfalfa mosaic	AMV	Alfamovirus	Pollen,aphid	ELISA, RT-PCR	Worldwide
Arabis mosaic	ArMV	Nepovirus	Nematode, seed	ELISA,RT-PCR	Scotland
Cherry raspleaf	CRLV	Cheravirus	Nematode, seed	ELISA, RT-PCR	Worldwide
Cucumber mosaic	CMV	Cucumovirus	Aphid	ELISA, RT-PCR	Europe,China
Impatiens necrotic spot	INSV	Tospovirus	Thrips	ELISA, RT-PCR	USA, Italy
Lychnis ringspot	LRSV-M	Hordeivirus	Seed?	ELISA	Hungary
Mint veinbanding associated	MVBaV	Unassigned	Aphids	RT-PCR	Worldwide
Mint virus-1	MV-1	Closterovirus	Aphid	RT-PCR	USA
Mint virus-2	MV-2	Vitivirus	Aphid	RT-PCR	USA
Mint virus X	MVX	Potexvirus	–	RT-PCR	USA
Peppermint latent	PeLV	Cheravirus	Nematode?	RT-PCR	USA
Peppermint stunt	PmSV	Vitivirus	Unknown	Hybridization	USA
Strawberry latent ringspot	SLRSV		Nematode,seed	ELISA, RT-PCR	Worldwide
Tobacco mosaic	TMV	Tobamovirus	Mechanical	ELISA,RT-PCR	India
Tobacco ringspot	TRSV	Nepovirus	Pollen, nematode	ELISA, RT-PCR	USA
Tomato aspermy	TAV	Cucumovirus	–	–	China
Tomato leafcurl Pakistan	ToLCPKV	Begomovirus	Whitefly	PCR	India
Tomato spotted wilt	TSWV	Tospovirus	Thrips	ELISA, RT-PCR	USA, Italy
Unidentified filiform	–	?	?	?	Bulgaria
Unknown Rhabdovirus	–	Cytorhabdovirus?	?	TEM	Germany

3.5 Pyrus

ELISA for:
- Apple chlorotic leafspot
- Apple stem grooving
- Apple stem pitting

Molecular probes for:
- Apple scar skin viroid
- Pear blister canker viroid

Graft inoculation of:
- *Malus micromalus* (indicator for ASGV)
- ***Pyrus communis* Noveau Poiteau**
- Virginia Crab (replaced by *M. micromalus*)
- ***Pyronia veitchii***
- *Pyrus communis* Bosc (field test for Stony Pit disease)
- *Pyrus communis* Bartlett (field test for bark disorders)

Sap inoculation of:
- *Chenopodium quinoa*

Related genera including *Malus, Cydonia, Mespilus, Crataegus, Sorbus, Amelanchier* may be indexed on the above indicators as well as on Russian apple and Radiant Crab.

3.6 Ribes (low priority, field collection only)

ELISA for:
- Alfalfa mosaic (=Lucerne mosaic)

Arabis mosaic
Cucumber mosaic
Raspberry ringspot (European Nepovirus)
Strawberry latent ringspot (European Nepovirus)
Tomato ringspot

Graft inoculation of:
Laxton No. 1
Baldwin
Amos Black (redundant)

Sap inoculation of:
Chenopodium quinoa
Cucumber

3.7 Rubus

ELISA for:
Apple mosaic (uncommon, self indicating)
Arabis mosaic
Cherry leafroll
Cucumber mosaic (uncommon)
Peach rosette mosaic (uncommon)
Raspberry bushy dwarf
Raspberry ringspot (European Nepovirus)
Strawberry latent ringspot (European Nepovirus)
Tobacco ringspot
Tobacco streak (Strawberry necrotic shock)
Tomato blackring (European Nepovirus)
Tomato ringspot

Graft inoculation of:
***Rubus occidentalis* Munger**
Malling Landmark red raspberry
Norfolk Giant red raspberry
Rubus henryi
Alpine strawberry

Sap inoculation of:
Chenopodium quinoa

3.8 Vaccinium

ELISA for:
Blueberry leaf mottle (uncommon)
Blueberry mosaic
Blueberry scorch carlavirus
Blueberry shock ilarvirus
Blueberry shoestring
Peach rosette mosaic (uncommon)
Red ringspot (when antiserum is available)
Tobacco ringspot
Tomato ringspot

Graft inoculation of:

Cabot Blueberry

Sap inoculation of:

Chenopodium quinoa (Detects many sap transmissible viruses including Blueberry leaf mottle, Peach rosette Mosaic, Tobacco ringspot, Tomato ringspot).
Cucumber (Detects Blueberry leaf mottle, Peach rosette Mosaic, Tobacco ringspot, Tomato ringspot.)

SECTION 4. PATHOGEN TEST PROCEDURES

Each genus at the genebank requires specific indexing procedures. Accessions should be evaluated for visible symptoms. Latent infections can be diagnosed by ELISA or other laboratory methods for appropriate viruses, and by inoculating a range of indicator plants. The base collection (primary location used for distribution), or core collection should receive first priority for indexing. If more than one plant represents an accession, samples from each should be pooled for virus tests. Two replicate wells should be used for ELISAs and two replicate indicator plants should be used for graft inoculations. Three to four replicate indicator plants should be used for sap inoculation assays. Healthy and infected checks must be included in each test.

4.1 Bioassays

4.1.1 Mechanical Transmission

Sap inoculation of herbaceous indicators is a good general screen for viruses with a wide host range, and can detect a large number of known and potentially unknown viruses. Symptoms may be local, occurring in the inoculated leaves within 1-5 days, or they may be systemic when the virus infects the indicator plant and produces symptoms in new growth. Systemic symptoms generally occur 1-3 weeks after inoculation. Many herbaceous species are used. *Chenopodium quinoa* and Cucumber are commonly used herbaceous indicators for the fruit crops grown at NCGR-Corvallis.

Procedure For Sap Inoculation

- Inoculum (leaves, young shoots, buds) is collected from new growth in early spring.
- Tissue is ground in a roller press or mortar and pestle with .05 M phosphate buffer (pH 7.0) + 2% PVP (see Martin & Converse 1982; recipe in appendix).

Tissue:Buffer = approx. 1:5 (W:V)

A small amount of celite is added as an abrasive. Alternatively, celite or carborundum (600 mesh) can be dusted on indicator leaves prior to inoculation. 2% nicotine in water is a good alternate extract buffer.

- Young, vigorously growing indicator plants are inoculated by dipping a finger, pipe cleaner or glass rod into sap preparation, and rubbing each leaf 3 - 4 times.
- Indicator plants are lightly rinsed to remove inoculum and are placed on glasshouse bench to await symptom development.
- Indicator plants may react to virus better if they are kept in the dark for 8-24 hrs. prior to inoculation.
- Local symptoms should develop within 2 - 5 days, systemic symptoms within about 2 weeks. Results are recorded and plants are discarded after 2 - 3 weeks.

4.1.2 Graft Transmission

Graft inoculation to plants of the same or a related genus is used to detect viruses that have a narrow host range and which generally cannot be mechanically-transmitted. Chip budding (double budding) is the inoculation method generally used with tree fruits. Leaflet grafting is the inoculation method generally used for small fruits. A bottle graft is an alternative to the leaflet graft, and may be the procedure of choice for graft inoculation of *Ribes* and *Vaccinium* indicator plants. Symptoms may develop within several weeks for greenhouse inoculations, or may take several years for certain field inoculations which produce only fruit or stem symptoms.

Procedure For Double Budding in Glasshouse

(see: Fridlund 1989; Fridlund 1976; ISHS 1985)

- Budding should begin in January or February when indicator wood has received adequate chilling.
- Seedling rootstocks (3/16 to 1/4 inch caliper) are planted in plastic Dee-pots.
- One indicator bud is chip budded, or scion grafted onto each rootstock. Two inoculum bark chips are budded immediately below the indicator graft. For budded indicators, rootstocks are cut back above indicator bud about 1 week after budding.
- Survival has been much better, particularly with small diameter scions such as *Pyronia veitchii*, or *Malus micromalus* if the indicator clone is cleft-grafted using a 2-3 bud scion instead of chip-budded.
- After growing for about 4 weeks, indicator shoots are cut back to about 10 cm. and completely defoliated. Rootstock sprouts should be removed periodically. (Certain indicators produce symptoms in the first flush of growth and may not require defoliation.)
- Plants are grown in a cool (about 22 C), partially shaded glasshouse for best symptom development.
- Indicators are evaluated about 6 weeks after defoliation, or when positive checks are showing distinct symptoms (see rating scale in appendix).
- Two indicator plants are inoculated for each accession being tested. Infected and healthy checks are included in each test.

Procedure For Leaflet Graft

(see: Converse 1987; Frazier 1974)

- Inoculum leaves are collected from plants to be tested and kept between moist paper towels. Inoculum leaves should be young but fully expanded. Lateral leaflets are removed and the terminal leaflet is trimmed to reduce surface area and weight.
- Two leaves on indicator plant are selected for grafting and center leaflets are removed. Lateral leaflets may be trimmed for easier handling and to help locate the grafts later.
- Inoculum leaflets are grafted onto indicator plants. A razor blade or scalpel is used to make a cut down the petiole between the two remaining leaflets of the indicator plant. Alternatively, a small slice can be made between the leaflets, after which the petiole can be separated by gently pulling apart on the two lateral leaflets. A tapered wedge is cut at the base of the prepared inoculum leaflet, and the leaflet is inserted into the cut petiole and wrapped with parafilm or latex bandage. (See illustrations on page 6-7 of Converse 1987).

- Plants should be periodically sprayed with a mist bottle until they are moved to a mist bench.
- **Important**: All ungrafted leaves (with the exception of new young growth) are removed from indicator plant. This promotes graft survival and virus transmission.
- Indicator plants are placed on mist bench. After 1 week, plants are moved to a glasshouse bench to await symptom development and plants without at least 1 surviving graft are flagged (blue pot label). These flagged plants are not recorded if results are negative, but they are recorded if results are positive.
- At least two indicator plants (total 4 grafts) are inoculated for each accession being tested. Healthy and virus infected checks must be included in each group of tests.
- Indicator plants must be grown in a cool (about 22 C.), partially shaded location and fertilized at least weekly for best symptoms.
- Indicator plants are evaluated 2-3 times per week for symptoms. Plants with questionable symptoms are flagged with a yellow pot label. Plants with definite symptoms are flagged with a red pot label. Most virus symptoms appear about 3 weeks after inoculation. At 6 weeks results are recorded (see rating scale in appendix) and plants are discarded. (some viruses such as Strawberry Pallidosis on UC-10, or Featherleaf on UC-5 may take longer than 6 weeks to produce symptoms).

Procedure for the Bottle Graft

- Inoculum shoots are collected from several branches of the plant to be tested, and are kept moist. Shoots should be about 15cm in length.
- A small test tube is filled with water and tied onto the stem of the indicator plant below the graft location. An inoculum shoot is placed into the test tube, and side-grafted to the indicator plant at a convenient point above the test tube.
- One or two grafts are made per indicator plant, with two indicator plants per sample. Test tubes are kept full of water until graft unions are formed, about 3-4 weeks.
- Inoculated indicator plants may need to be observed for up to 2 years for symptoms.

4.2. ELISA

4.2.1. Antiserum and Testing Sources (updated 10/2012)

ELISA kits, reagent sets, microbial cultures and other various services are available from the following sources. Also, see list of antiserum sources in ISHS (1983).

- AC Diagnostics
  http://www.acdiainc.com/
  phone: (479) 595-0320
  1131 W Cato Springs Road, Fayetteville, AR 72701, USA

- Agdia
  (http://www.agdia.com)
  phone: 574-264-2615
  30380 Country Road 6, Elkhart, Indiana 46514 USA

- American Type Culture Collection
  http://www.atcc.org/
  phone: 703) 365-2700
  ATCC, P.O. Box 1549, Manassas, VA 20108 USA

- Bioreba-AG (Agro-Diagnostics)
  http://www.bioreba.ch/
  Gempenstrasse 8
  CH-4008 BASEL Switzerland
  Telefon 061/35 04 55

- California Seed and Plant Labs
  http://csplabs.com/
  7877 Pleasant Grove Rd
  Elverta, CA 95626 USA
  phone: (916) 655-1581

4.2.2. Antiserum Preparation

If raw antiserum is obtained, IgG must be purified and conjugated to an appropriate enzyme before using in ELISA. (see appendix; and purification and conjugation procedures in: Hampton et. al. 1990, Clark & Adams 1977, or Converse 1984).

4.2.3. ELISA Protocol (Revised 9/3/97)

(see chart in appendix for specific antisera dilutions)

COATING

- Coat ELISA plate with antibody (=IgG) diluted in Coating Buffer according to chart. Prepare 11 ml per plate. Check pH of buffer before using. Use 100 ul octapette to fill plate.
- Incubate overnight (longer OK) in refrigerator.
- Rinse 2 times.
- (Optional) Add Blocking Solution (100 ul per well, dry milk in PBS-tween). Prepare fresh if more than 1 week old. Incubate 15-30 minutes.
- Rinse 1 time.
- Plate can now be stored in the refrigerator for several weeks.

Samples

- Collect samples. Grind in about 3 ml virus buffer. Add 100 ul per well, 2 wells per sample. Can use octapette with 2 tips. Cut off end of tips and rinse several times in two water rinses between samples. (Use special virus buffers for *Vaccinium* samples, or for apple mosaic and prunus ringspot virus - see "Modifications" below).
- Each ELISA plate should contain at least one positive check
- Incubate overnight in refrigerator
- rinse 3 times

Conjugate

- Dilute conjugate in conjugate buffer according to chart. Add 100 ul per well using octapette.
- incubate at room temperature 4 - 6 hours
- Rinse 4 times.

Substrate

- Prepare Substrate. Add 20 mg substrate tablet to 21 ml. substrate buffer or 40 mg. tablet to 42 ml substrate buffer for as needed to allow for about 10 ml per plate. Check pH of buffer before use. All glassware, plasticware, stirbars etc. that contact substrate must be very clean. A dedicated beaker and stirbar can be set aside for substrate only. Add 100 ul per well using octapette.
- Incubate at room temperature
- Record results after 1 hour or when deep yellow color develops in positive controls.
- If color is very faint, incubate longer, or store overnight in refrigerator and read results again next day.
- Read results using plate reader. Enter date etc. and blank recorder on buffer wells (or known healthy wells).

Modifications

- For grinding *Vaccinium* samples .5% nicotine (5ml per liter) must be added to the virus extract buffer (not to the conjugate buffer) to overcome the acidity of the plant tissue, or a higher pH borate buffer can be used instead.
- For ilarviruses (apple mosaic, prunus ringspot, possibly tsv) it may be helpful to add 4.5 g. DIECA (sodium diethyldithiocarbamate trihydrate)per liter of virus extract buffer.

  Volumes for 11 ml per plate
  (@100 ul/well):

  1:500 dilution = 22 ul/11 ml
  1:1000 dilution = 11 ul/11 ml

SECTION 5. Pathogen Elimination

Viruses can often be eliminated from infected plants by dissecting an apical meristem or shoot-tip from a heat-treated plant, and growing it into a new plant. Many variations of this procedure are used for different plants, but the basic procedure is the same: grow the infected plant at an elevated temperature (about 38°C) for several weeks, and generate a new plant from the apical meristem.

- All accessions indexing positive will be replaced by another plant of the same clone that indexes negative, where possible.
- If a virus free selection is not readily available, the infected clone will undergo thermotherapy and/or meristem tip culture. Resulting plants will be re-indexed. This process will be repeated until a plant is produced that indexes negative.
- Plants produced by thermotherapy or tissue culture must re-index negative after at least one natural cold dormant period, and preferably for 3 years before successful virus elimination is assumed.

- Alternative virus elimination procedures including in vitro heat therapy and in vitro chemotherapy should be considered. In vitro heat therapy can be used for *Fragaria* in particular, where production of runners is difficult using conventional heat therapy.
- Identity of plants should be verified before original plants are discarded.

5.1 Procedure for hot air treatment of trees

- Seedlings potted in clay pots to allow evaporative cooling of root zone. (Lower root temperature favors host survival.)
- Infected scion is grafted onto rootstock, Alternatively, about 5 candidate buds are chip budded onto each rootstock. Take buds from terminal portion of infected plant to increase possibility of escaping virus. When buds begin to grow, plants can be placed in heat chambers.
- If plants are not treated soon after growth commences, terminal growth can be pinched back to force more lateral shoots.
- Heat treatment same as for small fruits below.

5.2 Procedure for hot air treatment of small fruits

- Well rooted plants are established in clay pots. Shoots can be cut back to encourage lateral growth.
- Plants are placed in heat chambers and temperature is gradually increased over a period of 2 - 4 days.
- Treatment temperatures and times may vary with the ability of the host plant to survive, the method of shoot tip propagation, and the effectiveness of the treatment against the particular virus. If meristems are propagated the treatment times may be reduced. The following temperature regimes have been used successfully:

  A. Constant temperature of 38 degrees C.

  B. Alternating 4 hours 38 C, 4 hours 30 C.

  C. Alternating 8 hours 38 C, 4 hours 25 C.

- Plants should be treated for 4-8 weeks if no meristem culture will occur, however, 3 weeks is adequate for most viruses if followed by apical meristem culture using meristems <0.5 mm in length.
- Heat sensitive clones are more likely to survive alternating temperatures. The 4 hour alternation has worked very well, and has given very satisfactory virus elimination. This is the standard method now used at NCGR-Corvallis. The high temperature may need to be reduced to 35-36 degrees for *Vaccinium* or *Ribes*.
- Meristems of treated plants should be grown in tissue culture where possible. Pyrus shoot tips (about 5.0 mm) can be micrografted onto young seedling rootstock if in vitro methods are not available. Rootstock species different than the scion will aid in differentiating rootsprouts from the graft.

- Plants resulting from heat treatment must reindex negative for 3 years before successful virus elimination is assumed. Virus levels may be reduced to non-detectable levels during the first year or two following thermotherapy.

5.3 Procedure for hot water treatment of dormant propagules

- Hot water treatment will not eliminate viruses, but may be useful for sanitizing mature scionwood or canes. This procedure has not been effective at eliminating virus diseases, however may eliminating most insects, fungi, bacteria, and phytoplasmas.
- Scionwood should be at room temperature prior to heat treatment. (Gradual increase in temperature favors host survival if material has been stored in a cooler).
- Wood is immersed in a large volume of actively circulating water which is maintained at 50 C for 15 minutes.
- Material is then cooled by immersing in cold water for about 5 min.
- Surface moisture is allowed to dry before repackaging for storage.
- Date, source of material, water temperature at start and finish of treatment are recorded in record book. Material is labeled to indicate that it was heat treated.
- Material is now ready to be grafted to clean rootstock, or rooted in a mist bed.

SECTION 6. Records

Indicators are rated for symptoms on a scale of 1 - 9. A rating of 5 or greater is considered positive. Indexing results for each clone are maintained in computer files. A separate directory is maintained for each genus with several key virus indexing files for each genus. "xxx" represents the genus-code or first three letters of the genus.

xxxVIR.dbf = database file with a summary of all bioassay results, ELISA results, notes, and an overall virus 'status' for each accession which is defined below. Virus testing summaries are copied to the inventory files where they are available to the NCGR staff, and to the GRIN database in a field called 'VIRSTATUS'.

The following VIRSTATUS codes are used:

"TESTED"
Clone has been subjected to two or more assays, and no pathogens have been detected, or derived from a meristem from a heat-treated plant.

"UNTESTED"
Clone has not been tested, or very limited testing has been completed, or one or more tests were inconclusive.

"INFECTED"
Clone has tested positive for one or more viruses.

Two additional databases are maintained for each genus containing results of each specific ELISA or bioassay:

xxxLISA.dbf = database with all ELISA results. This data is summarized in the ELISA field of each xxxVIR.dbf and the summary is duplicated in the ELISA field in collection inventories and on GRIN

xxxBIO.dbf = database with **bioassay** results. This data is summarized in the BIOASSAY field of each xxxVIR.dbf and the summary is duplicated in the BIOASSAY field in collection inventories and on GRIN.

SECTION 7. Virus Collection

Positive (virus infected) controls must be included in all bioassays and ELISA tests. A collection of standard isolates of important small fruit, pome fruit, mint, hop and hazelnut viruses are maintained at the repository. With the widespread availability of virus certification programs, and with the increasing use of pathogen-free propagation material by the nursery industry, many viruses are becoming rare. Numerous fruit tree and small fruit viruses can only be stored as infected plants, and these infected plants are an important resource for plant pathologists and other researchers. Virus cultures are needed for developing new detection techniques, comparing with newly characterized pathogens, determining the susceptibility of plants and establishing the effect of these pathogens on growth and production. The NCGR-Corvallis pathologist collects, maintains, evaluates, documents and distributes the pathogen germplasm as an important aspect of the virus indexing program, and as a service to other researchers. A list of the various virus isolate collections is presented in the appendix.

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SECTION 9. Appendix

A. Virus buffer recipes.

B. Antisera inventory and dilutions for ELISA.

C. Procedure for purification and conjugation of antisera.

D. Indicator lists and result codes by genus for:
Fragaria, Pyrus, Ribes, Rubus, Vaccinium

E. Virus collections
E.1 Tree fruit pathogens.
E.2 Small fruit and other pathogens.

Appendix A - Sap Inoculation and ELISA Buffers

PBS 1X (Phosphate Buffered Saline):

	1 liter	3 liters	4 liters
NaCl	8.0 g	24.0 g	32 g
KH_2PO	0.2	0.6	0.8
Na_2HPO_4 - 7 H_2O	2.17	6.51	8.68
KCl	0.2	0.6	0.8
NaN_3	0.2	0.6	0.8

- Adjust pH to 7.4. Store at 5 degrees C.

ELISA Coating Buffer:

H_2O	1.0 liter
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

- Adjust to pH 9.6 with HCl.
- Can be stored at 5 degrees C
- Reheck pH before use.

ELISA Blocking Solution and Conjugate/Monoclonal Antibody Buffer:
(PBS-Tween + .2% dry milk)

	200 ml	500 ml
PBS	200 ml	500 ml
Tween 20	100 ul	250 ul
Powdered Dry Milk	.4 g	1 g

ELISA Plate Washing Solution:

Use .5 X PBS

ELISA Sample Grinding Buffers:
(Virus Buffer)

PBS	1 liter
Tween 20	.5 ml
Ovalbumin (egg albumin)	2 g
Polyvinylpyrrolidone (PVP mw 10,000)	20 g
Powdered Dry Milk	1 g

- PVP may take a long time to dissolve.
- Store at 5 degrees C and use within 1 month.
- For apple mosaic or prunus ringspot virus add 4.5 g. Dieca/liter (sodium diethyldithiocarbamate trihydrate)
- For extracting *Vaccinium* samples add 5 ml. Nicotine/liter An alternative buffer for *Vaccinium* is .05 M Borate Buffer pH 8.0:

.5 M Boric acid	90 ml
.5 M disodium tetraborate	10 ml
H_2O	900 ml

ELISA Substrate Buffer:

H_2O	1 liter
diethanolamine (liquid)	97 ml
NaN_3	0.2 g

- Adjust to pH 9.8 with HCl.
- Store at 5 degrees C.
- Recheck pH before each use.

Sap Inoculation Buffer:
[.05 M phosphate buffer pH 7.0 + 2% PVP (Martin & Converse 1982)]

H_2O	1	liter
Na_2HPO_4 - 7 H_2O	8.17	g
NaH_2PO_4	2.34	g
Polyvinylpyrrolidone (PVP mw 10,000)	20	g

Serum	Source	IGG Concentration	Conjugate Concentration	Status
American Hop Latent (AHLV)	Hampton 4/89	1:1000	1:1000	
Apple Chlorotic Leafspot (ACLSV)	AGDIA 1988	PRECOATED	-	POOR for pear
Apple Mosaic (=ROSE MOSAIC) (ApMV)	ATCC 254	1:500	1:500	GOOD
Apple Mosaic (ApMV)	ATCC 32	1:500	1:500	GOOD
Apple Mosaic (ApMV)	Hampton 4/89	1:500	1:500	GOOD
Apple Stem Grooving (ASGV)	AGDIA 2/89	PRECOATED		POOR-FAIR
Apple Stem Grooving (ASGV)	East Malling	-	-	POOR
Apple Stem Pitting (Pear Vein Yellows)	Hadidi 89 (from Japan)			GONE
Arabis Mosaic (ArMV)	AGDIA 8/89	1:1000	1:1000	GOOD
Arabis Mosaic (ArMV)	ATCC 192	-	-	GOOD
Arabis Mosaic (ArMV)	Casper 840	-	-	GOOD
Arabis Mosaic-S (ArMV)	East Malling	-	-	GOOD
Blueberry Leaf Mottle (BBLMV)	AGDIA 2/89	PRECOATED		GONE
Blueberry Red Ringspot	Converse 2/90			POOR
Blueberry Scorch Carlavirus	MacDonald 2/89	1:500	1:250	GONE
Blueberry Scorch Carlavirus	Martin 6/92	1:1000	1:1000	V. GOOD
Blueberry Shock Ilarvirus	Martin	1:500	1:250	GOOD
Blueberry Shoestring (BBSSV)	Ramsdell	1:1000	1:800	V.GOOD
Carnation Ringspot (CRSV)	ATCC 21a	-		UNTESTED
Cucumber Mosaic (CMV)	ATCC 242			POOR?
Fra. chiloensis ilarvirus (+ mono)	Martin 94			GOOD
Hop Latent (HLV)	Hampton 4/89	1:1000	1:1000	
Hop Mosaic	Hampton 4/89	-	-	
Mint Veinbanding	Stace-Smith 4/90	1:400	1:500	GOOD
Prunus Ringspot Fulton-G	Hampton 4/89			GOOD
Prunus Ringspot	ATCC 22	1:1000	1:800	GOOD
Raspberry Bushy Dwarf (RBDV)	AGDIA 90		1:1000	GOOD depleted
Raspberry Bushy Dwarf	Martin	1:1000	1:200	V.GOOD
Raspberry Ringspot (RRV)	Casper 979	-	-	UNTESTED
Raspberry Ringspot (RRV)	East Malling	-	-	UNTESTED
Strawberry Latent Ringspot (SLRV)	Casper 339			UNTESTED
Strawberry Latent Ringspot (SLRV)	East Malling			UNTESTED
Strawberry Mild Yellow Edge Potex	Martin 94	1:2000	mc 1:2000	V.GOOD
Tobacco Ringspot (TOBRSV)	ATCC 157	1:1000	1:1600	GOOD

Tobacco Streak (TSV)	AGDIA 2/89	-	-	UNTESTED
Tobacco Streak (TSV)	ATCC 276	1:100	1:400	FAIR-GOOD
Tobacco Streak Virus (+ mono)	Martin 94			
Tomato Black Ring (TomBRV)	Casper 289	-	-	UNTESTED
Tomato Ringspot (TomRSV)	ATCC 174	1:1000	1:400-800	GOOD, Depleted
Tomato Ringspot (TomRSV)	ATCC 239	1:1000	1:500-800	GOOD
Tomato Spotted Wilt - I (TSWV-I)	AGDIA 90	1:1000	1:1000	GOOD
Tomato Spotted Wilt - L (TSWV-L)	AGDIA 90	1:100	1:1000	GOOD

Appendix C - Procedure for purification and conjugation of antisera.

Preperation of immuno gamma globulin (IgG) - Hampton Protocol

1. Combine 1.2 ml whole antiserum with 1.2 ml distilled water.
2. Combine the 2.4 ml. antiserum-water with 2.4 ml of 36% sodium sulfate (Na_2SO_4) (3.6 g sodim sulfate dissolved in 10 ml distilled water) mix and let stand for 10 minutes.
3. Centrifuge, 22C for 15 min. at 12,000 g. Discard supernate - retain white pellet.
4. Add 10 ml of 18% sodium sulfate to pellet. Vortex.
5. Centrifuge as before, retain pellet.
6. Resuspend pellet in 1 ml PBS. Dialyze 3 times against 1 liter PBS at 4 C, one overnight.
7. Examine for visible precipitate; remove any precipitate by centrifugation (15 min.)
8. Estimate IgG concentration.
   (absorbance at 280 nm = 1.4 when IgG = 1mg/ml)

Conjugation of enzyme with IgG - Martin Protocol

Check form of Alkaline Phosphatase. May need to do the following:
Centrifuge the equivalent of 0.1 ml of alkaline phosphatase (0.5mg/ml) in a solution with $(HaH_4)_2SO_4$ for 2 to 3 minutes in a small centrifuge. Discard supernatant.
Dissolve precipitate in 0.2 ml IgG (at 1 mg/ml).

1. Combine 2 mg. alkaline phosphate (Sigma P-6774) with 1 ml. IgG (1 mg/ml).
2. Dialyze overnight against 0.06% glutaraldehyde in PBS.
   (1 ml. 25% glutaraldehyde in 416 ml buffer)
3. Dialyze against PBS to remove excess glutaraldehyde (3 changes, one overnight).
4. Add 5 mg./ ml. bovine serum albumin

optional: Dilute to 1/10 using PBS, and store in siliconized tube.

Appendix E - Virus Collections

E.1 - Tree fruit pathogen collections maintained at NCGR Corvallis.

HOST GENUS	PATHOGEN	ISOLATES
Corylus	Apple Mosaic Ilarvirus	3+
Corylus	Hazelnut Stunt ?Phytoplasma	1
Malus	Apple Blister Bark	1
Malus	Apple Chlorotic Leafspot Trichovirus	2
Malus	Apple Flat Limb	1
Malus	Apple Mosaic Ilarirus	2
Malus	Apple Rubbery Wood	4
Malus	Apple Stem Grooving Capillovirus	4
Malus	Apple Stem Pitting Virus	8
Malus	Apple Swollen Limb	1
Malus	Apple Swollen Stem (twisted twig)	1
Malus	Cherry Rasp Leaf Nepovirus (Flat Apple)	1
Malus	Genetic Variegation (non-chlorophyll mutant)	1
Mespilus	Apple Chlorotic Leafspot Trichovirus	1
Pyrus	Apple Stem Grooving Capillovirus	3+
Pyrus	Bark Disorders	2
Pyrus	Blister Canker	3
Pyrus	Concentric Fruit Ringpattern	1
Pyrus	Hardy Fruit Ring	1
Pyrus	Pear Blister Canker Viroid	2+
Pyrus	Pear Decline Phytoplasma	1
Pyrus	Pear Ringpattern Mosaic Virus (ACLSV)	4+
Pyrus	Pear Vein Yellows Virus	3+
Pyrus	Rough Bark	1
Pyrus	Seedborne Vein Yellowing	3
Pyrus	Stony Pit ?Virus	8
Sorbus	Chlorotic Leafspot ?Virus	2

(+) indicates additional infected plants in clonal germplasm collection

E.2 - Small fruit and miscellaneous other pathogens maintained at NCGR Corvallis.

HOST GENUS	PATHOGEN	ISOLATES
Fragaria	Black Concentric Fruit ring (?TSV)	8
Fragaria	Chlorotic Fleck ?Virus	1
Fragaria	Cryptic Virus (Ghost)	1
Fragaria	Fragaria Chiloensis Ilarvirus	2
Fragaria	June Yellows (gentic disorder)	3
Fragaria	Multiplier Disease Phytoplasma	2

Fragaria	Strawberry Crinkle Cytorhabdovirus	2
Fragaria	Strawberry Greenpetal Phytoplasma	1
Fragaria	Strawberry Latent-C ?Rhabdovirus	2
Fragaria	Strawberry Leafroll ?Virus	1
Fragaria	Strawberry Mild Yellow-Edge Luteovirus	3+
Fragaria	Strawberry Mild Yellow-Edge Associated ?Potexvirus	5+
Fragaria	Strawberry Mottle Virus	3
Fragaria	Strawberry Pallidosis ?Virus	5
Fragaria	Strawberry Vein Banding Caulimovirus	3
Fragaria	Strawberry Vein Banding Virus (Western)	1
Fragaria	Tobacco Streak Ilarvirus (Necrotic Shock)	2+
Fragaria	Tomato Ringspot Nepovirus	1
Humulus	American Hop Latent Virus	1+
Humulus	Apple Mosaic Ilarvirus	1+
Humulus	Hop Latent Virus	1+
Humulus	Hop Latent Viroid	1+
Humulus	Hop Latent Carlavirus	1+
Humulus	Hop Mosaic Carlavirus	1+
Kalmia	Rhododendron Necrotic Ringspot ?Potexvirus	1
Mentha	Mint Veinbanding Virus (proposed name)	3
Melissa	Lemon Balm Variegation Virus	1
*Physocarpus**	Tomato Ringspot Nepovirus	1
Ribes	Arabis Mosaic Nepovirus	3
Ribes	Black Currant Yellows	5
Ribes	Tomato Ringspot Nepovirus	2
Ribes	Vein Banding Viruses	5+
Rubus	Alpine mosaic agent (Darrow Sterility)	1
Rubus	Black Raspberry Latent Virus	1
Rubus	Blackberry Calico Carlavirus	4
Rubus	Raspberry Bushy Dwarf Virus	5+
Rubus	Raspberry Mosaic Viruses	4+
Rubus	Rubus Yellow Net Virus	1
Rubus	Strawberry Mild Yellow Edge	1
Rubus	Tobacco Streak Ilarirus	4+
Rubus	Tomato Ringspot Nepovirus	3+
Sambucus	Cherry Leafroll Nepovirus	2
Sambucus	Elderberry Latent ?Carmovirus	2
Sambucus	Elderberry Carlavirus (European)	3
Sambucus	Elderberry Carlavirus (North American)	3
Vaccinium	Blueberry Mosaic ?Virus	1
Vaccinium	Blueberry Mosaic (?genetic variegation)	1
Vaccinium	Blueberry Red Ringspot Caulimovirus	1
Vaccinium	Blueberry Scorch Carlavirus	2
Vaccinium	Blueberry Shock Ilarvirus	1
Vaccinium	Blueberry Shoestring Virus	1
Vaccinium	Cranberry Falseblossom ?Phytoplasma	1

Vaccinium	Cranberry Ringspot	?Caulimovirus	1

* local native plant - common name "Ninebark"

(+) indicates additional infected plants in clonal germplasm collection