Part I. Techniques for biological detection of specific citrus graft-transmissible diseases

Inoculation procedures for detection of citrus graft-transmissible pathogens (CGTPs)

Tristeza
Greening
Stubborn
Blight and related diseases
Exocortis
Cachexia
Satsuma dwarf
Tatterleaf
Infectious variegation and leaf rugose
Psorosis complex: psorosis-A, psorosis-B and ringspot
Concave gum
Impietratura
Cristacortis
Vein enation (woody gall)
Gummy bark and transmissible bud-union disorders

Inoculation procedures for detection of citrus graft-transmissible pathogens (CGTPs)

COLLECTION AND STORAGE OF INOCULUM TISSUE

Budwood is the primary inoculum tissue used for most inoculations, but bark and leaves may also be used. Budwood should not be collected during excessively hot weather because some CGTPs in the perimeter branches of field trees can be temporarily inactivated or severely suppressed by heat (Roistacher et al., 1974; Roistacher and Calavan, 1974). When the season changes and temperatures become cooler, however, the pathogen will usually return from its reservoir location in the roots or shaded parts of inner branches. An ice chest should be used for budwood storage when collecting. Clippers should be disinfected, when moving from tree to tree, by dipping or spraying in a 1 percent sodium hypochlorite solution (a one to four dilution of the 5.25 percent commercial household bleach in water). Bark samples can be placed in a small plastic tube (Figure 135 in Part II), but the tube should not be sealed. Immediately after collecting the tissue samples, they should be put into polythene bags to prevent their drying and immediately put into an ice chest. All samples should be labelled clearly at the time of collection. Upon arrival at the plant laboratory, they should be put directly into a refrigerator at 5-6°C. Avoid freezing the inoculum. Budwood can be maintained under refrigeration for two weeks or longer but should preferably be used as soon as possible.
If a field tree is selected as a primary candidate (i.e. one whose budwood will be propagated for heat treatment or shoot-tip grafting), a budstick should be taken below or proximal to a well-developed and typical fruit. A bud propagation is then made, and the propagation held in the greenhouse. This propagation will then become the primary plant, and budsticks can be taken anywhere from this plant for initial indexing, for heat-treatment, for shoot-tip grafting, or for use as positive control tissue to test the effectiveness of the heat-treated or shoot-tip grafted plant.

**Inoculation methods**

The most frequently used method for inoculating indicator plants for the detection of most CGTPs is by "bud"-graft inoculation. The term "bud"-graft includes buds with "eyes", stem pieces without "eyes" (sometimes called blind buds), and also chip-buds. These are illustrated in Figure 127 in Part II. There are other inoculation techniques, most of which are also given and illustrated in Part II. These include side grafts, approach grafts, root grafts, fruit grafts, leaf-piece grafts or leaf-disc grafts.

Mechanical transmission from citrus to citrus or from citrus to herbaceous plants is done by knife or razor-blade. The blade is first slashed through the inoculum tissue, and then a single slash is made in the stem of the receptor plant. This procedure is repeated ten to 25 times per plant. The slashed area of the receptor plant is then wrapped with budding tape. Citron is an excellent donor host as well as a receptor host for mechanical transmission by knife or razor-blade slash.

In general, seedlings are preferred as receptor or indicator plants. However, if propagated clonal buds derived from seedling lines are substituted for seedlings, they should be tested and compared against the seedling for their performance as indicators since their performance as budlings may be different from that of seedlings.

Table 1 (in the Introduction) gives a summary of the minimum number of recommended indicator plants and the pathogens they can detect. The recommended index temperatures and symptomatology are summarized in Table 3. The specific methods of inoculation, the suggested number of indicator plants to use' the preferred inoculum, instructions for plant growth, recommended index temperatures, time for development of the first symptoms, and symptoms are given in detail for each of the individual diseases covered in this handbook. Detailed inoculation procedures are given for each pathogen and a summary table of the recommended inoculation procedure is given at the end of each section.

Although "buds" are used as inoculum for most inoculations, other tissue and techniques, i.e. leaf, bark, root, or side grafts, should be continually tried and tested to find the most effective means of bringing out maximum symptom expression. This is especially true for any initial indexing of new diseases or diseases of unknown etiology.

Specific clonal selections used as scion propagations rather than seedlings have been found superior as indicators for indexing of certain pathogens, i.e. the cachexia, exocortis and exocortis-like citrus viroids. A vigorous rootstock such as rough or Volkamer lemon is recommended as a rootstock under the clonal bud. The forcing of clonal buds is recommended where tristeza is endemic and tristeza-susceptible indicators may show too strong a tristeza reaction, thereby masking symptoms of other pathogens. In many cases tristeza can be filtered out by inoculating trifoliate orange seedlings and using shoots of trifoliate as inoculum. A modification of this technique is to graft an indicator scion bud on a trifoliate or citrange seedling, inoculating the seedling and forcing the indicator bud. In most cases tristeza will be filtered from the new growth of the developing indicator shoot. Some isolates of tristeza can pass through trifoliate or citrange, but most do not.
When testing for the bud-union effect of citrus tristeza virus using a sweet orange scion budded on a sour orange rootstock, or for the bud-union crease of certain scions on trifoliate or citrange rootstock induced by the tatterleaf virus, propagation of the scion and inoculation of the rootstock can be done simultaneously and the sour orange or trifoliate rootstock seedling is then bent just above the scion bud to promote rapid forcing of that bud (Figures 47 and 48).

**Positive and negative controls**

It is extremely important that both positive and negative controls be incorporated in each index test. A collection of infected source plants containing mild and severe CGTPs should be developed and maintained as a "virus bank". Sweet orange has been found to be an excellent holding or reservoir plant for almost all CGTPs. These reservoir or bank plants should be periodically indexed to ensure that the pathogen is present or has not changed. It is important that the mildest CGTP sources be collected and preserved in the "virus bank", and these should be used as positive controls for each index test. These positive controls will provide the determining factor as to when an index test should be terminated. The inclusion of negative controls is also very important, and they should be generously incorporated into every index. Negative-control plants give an indication of possible environmental or insecticidal spray damage, and can show effects other than those induced by pathogens. They also act as a standard for plant-size comparison when subtle pathogens or diseases of unknown etiology may stunt index plants, but otherwise show no other leaf symptoms. However, their primary importance is to provide a normal control plant when reading for very mild leaf reactions in the inoculated plants. Thus, the presence of a new pathogen or a very mild form of a known pathogen can be detected. Although it may appear to be an extra use of seedlings, the presence of anoinoculated control in each container has been found to be very helpful for a number of reasons, but specifically for judging any possible reaction in the inoculated plants in the same container.

**Time of first symptom appearance**

The time in weeks from inoculation to appearance of the first symptoms under optimum growth and temperature conditions is given in detail in the specific section for each disease. During critical flush periods, plants should be observed daily for development of symptoms for certain CGTPs. Symptoms of psorosis, psorosis-like pathogens and concave gum oakleaf patterns may disappear from the young developing leaves as the leaves mature, and symptoms may not reappear in later flushes. Leaf-flecking symptoms are best observed during the first to third flushes of growth. Plants should be watched carefully to catch the growth at maximum unfolding of the leaves for best reading of young leaf symptoms. Different pathogens will show leaf reaction at different times. Records should be carefully maintained for the time of appearance of symptoms with a detailed description of the plant reaction.

Maintaining proper temperatures is extremely important for appearance of some symptoms. If the temperatures are kept too warm, certain "cool" temperature pathogens may not show symptoms in leaves, or show them very poorly (Roistacher et al.,1974). If temperatures are kept too cool, symptoms for diseases such as stubborn, cachexia, exocortis or certain citrus viroids, which require warm temperatures for best symptom expression, may not show or may develop poorly in indicator plants. Also, citron reservoir plants used for PAGE detection of citrus viroids may not build a high titre of viroid under cool conditions. These plants must be held at warm temperatures (Semancik, unpublished).

The liberal inclusion of mild- and severe positive controls gives a working indication of the proper time and temperature for symptom appearance. The lack of any symptom development in plants inoculated with these mild-positive controls would invalidate the index.
Vigorous growth is important for production of good leaf and stem-pitting symptoms. Stem pits are poorly produced in poor unthrifty plants.

**Checking inoculum survival**

Two to three weeks after inoculation, the wrapping tapes should be removed, the inoculum examined for survival, and the survival recorded. If tapes are cut with a knife or razor-blade, these tools should be disinfected in a 1 percent sodium hypochlorite solution between each cut. When buds are taken from mature wood of a dark-coloured budstick, or when bark inoculum is used, it is sometimes difficult to tell if the inoculum tissue is dead or alive. A small slice or cut made into the brown bark surface of the inoculum will reveal the bright green colour of living tissue beneath, thus indicating that the inoculum is alive. If both inoculum "buds" are dead, the plant should be reinoculated, or new inoculations made to another plant. Generally, if one of the two inoculum "buds" is alive, the plant need not be reinoculated provided there are sufficient replications.

**Records**

A record sheet for each index must be kept. This should include: the experiment number, date of inoculation, source of the inoculum, indicator plants used, inoculum survival rate, reading dates, and a large space reserved for notes on observations. Records should preferably include temperatures and light conditions under which indexing was done, and any use of artificial lighting.

**Indexing using field trees**

Certain indexes require a longer term for completion of the expression of the mildest symptoms. At such times the inoculated index plants growing in the plant laboratory (greenhouse) need to be set out in the field, or field trees need to be inoculated and observed. For example, in the long-term index for cachexia the mild-positive controls may show no symptoms in the greenhouse even after one year. Therefore, it is best to move the indicator plants to the field and plant them at close spacing until the mild controls show positive symptoms. Similarly, certain strains of exocortis or related citrus viroids may require a field test to show mild bark cracking on their trifoliate or Rangpur lime indicator rootstocks. The testing of sweet orange on sour orange rootstocks for the classical quick-decline tristeza reaction may also require an extended period of time for typical tristeza decline symptoms to develop. The testing for cristacortis also requires long-term observation of plants or trees in a screenhouse or in the field. These indexes should be carried out in an environment where temperatures are conducive to best symptom expression. Again, mild- and severe-positive controls should be present.

For certain diseases, trees in the field may have to be tested or inoculated to observe specific symptoms, i.e. testing for blight, or observing fruit for symptoms of impietratura. Specific field indexes are discussed in detail under each of the diseases in this handbook.

**REFERENCES**

