GRRAFT-TRANSMISSIBLE DISEASES OF CITRUS
Handbook for detection and diagnosis

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INTERNATIONAL ORGANIZATION OF CITRUS VIROLOGISTS

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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The designations employed and the presentation of material in this publication do not imply the expression of any opinion whatsoever on the part of the Food and Agriculture Organization of the United Nations concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.
Chester N. Roistacher has been specializing in the detection and diagnosis of citrus virus and virus-like diseases for many years. During this time, he has developed a general philosophy on indexing that has led to the concept of the plant laboratory as a most important means of detecting several of the citrus virus and virus-like diseases.

The International Organization of Citrus Virologists, from its very inception, has had a history of international cooperation for the study, detection and ultimate elimination of citrus virus and virus-like diseases. Since its foundation over 30 years ago, one predominant principle has emerged: the problems related to citrus virology in one country, no matter how remote or distant that country may be, are similar to the problems in any other citrus-growing country. Thus, the findings of workers in one institute are applicable and helpful to workers in another. This handbook exemplifies the spirit of cooperation that has prevailed in our organization in the sharing of knowledge among citrus scientists.

The importance of the practical applications of the material of this handbook might best be appreciated by relating the story of my own meeting and personal relationship with the author. I first met Chet Roistacher in 1972 while working at the University of California in Riverside, attempting to define and improve the shoot-tip grafting method and to understand its mechanisms and principles. I had accumulated in test-tubes many hundreds of small plants. I had worked very hard during many long and late hours under a hood and using a microscope in cutting extremely small tips from their microscopic shoot apices, carefully grafting them to small, toothpick-sized seedlings, and then putting the grafted seedlings back into the test-tubes. I was now ready to transplant these many small growing plants to the soil. However, past experience with other greenhouses led me to fear that, once these small plants were removed from their sterile and secure homes in the test-tubes, they might easily be destroyed by phytophthora infection, poor soil or neglect. At this point I was introduced to Chet at the Rubidoux greenhouse, and he enthusiastically explained to me many of the concepts and principles now detailed in this handbook. Most important, he gave me hope and confidence that my many hard months of research would not be destroyed and we would have success with survival of my test-tube plants.

In truth, we achieved over 95 percent success in survival and growth of the plants which had been successfully transplanted to the UC soil mixture. We watched them grow from small, fragile plants just after the shock of
transplanting to mature, vigorous plants in the greenhouse, and ultimately to fruiting trees in the field. I became a convert to this system of plant growth, which recognized the extreme importance of sanitation, nutrient balance, a good soil mixture with balanced fertilization plus responsible care. We later went on to index all of these shoot-tip grafted plants, and I was able to see and study at first hand the importance of good plants for the detection of citrus viruses. I also came to appreciate better the concept of the plant laboratory. I copied the formulae and concepts learned at Riverside and brought them back to Spain. At my request, Chet came to Spain in 1975 to teach and to see if those principles, which worked so well at Riverside, would work equally well in Valencia. They did!

Chet has always enthusiastically shared his knowledge with his many visitors from all over the world who go to the University of California at Riverside, in his many trips to most citrus-growing areas, and with all the members of the International Organization of Citrus Virologists. At the meetings of this organization, Chet has made many important contributions not only with his formal presentations of papers but also in his long discussions with everybody on the concepts and procedures for indexing citrus virus diseases.

*Detection and diagnosis graft-transmissible diseases of citrus* is a most significant contribution to the citrus industries of all countries and for the advancement of citrus virus research. I feel it will be extensively used, both by new scientists entering this field and by scientists with wide experience in citrus virus diseases.

Finally, on behalf of the IOCV, I wish to acknowledge the wisdom and foresight of FAO in stimulating and supporting the publication of this handbook.

**Luis Navarro**  
Chairman  
International Organization of Citrus Virologists
Citrus is one of the most important commercial fruit crops grown in all continents of the world, and areas under citrus are in continuous expansion. While citrus fruit contributes to the nourishment and refreshment of the people, citrus products and by-products provide the basis for local agricultural industries, generate employment, raise income and, in many cases, constitute an important source of foreign revenue to developing and developed countries.

As with any commercial crop, citrus is subject to various pest problems. These include insects, mites, fungi, bacteria, nematodes, viruses, viroids etc. that can cause varying degrees of damage and affect the quality and quantity of the produce on the one hand and the vigour and longevity of the trees on the other. Most of these pest problems can be solved in the field through conventional control measures, i.e. cultural practices, field sanitation, chemotherapy etc. Exceptions are problems posed by virus and virus-like diseases for which the only known cure is to provide healthy (virus-free) planting material, usually within the framework of a phytosanitation programme based on diagnosis, detection and elimination of the causal agent(s) and the maintenance and distribution of healthy stocks.

Detection of virus and virus-like diseases of citrus has significantly developed over the last half-century. At present, a wide variety of indicator plants exists for the detection of almost all known virus and virus-like diseases within a reasonably short time, provided that appropriate indexing facilities are available. In addition to biological indicators, many laboratory methods, ranging from culturing of the causal agent to molecular biology-based techniques, have also been developed during the last two decades. Such rapid detection methods are now used for quarantine purposes and large-scale surveys as well as for sanitation programmes in conjunction with biological assays.

When virus and virus-like diseases have been diagnosed by any of the above methods, and no source of healthy planting material is available, the only solution is to eliminate the infection from the diseased material. To accomplish this, various methods have been developed, including the production of nucellars, thermotherapy and shoot-tip micrografting. Such methods, used singly or in combination, have proved effective in eliminating virus and virus-like diseases, thus enabling the reutilization of valuable, sanitized resources.
The first attempt to collate information on detection techniques of citrus virus diseases was made over 20 years ago, when the United States Department of Agriculture published \textit{Indexing procedures for 15 virus diseases of citrus trees}. This was prepared by the Committee on Indexing Procedures, Diagnosis and Nomenclature, established by the International Organization of Citrus Virologists (IOCV). With the continuing flow of knowledge and new findings on virus and virus-like diseases, the need for an up-to-date publication on detection techniques for citrus graft-transmissible diseases was expressed on various occasions. Such a publication was needed to assist in the detection of quarantine-significant citrus diseases before any introduction of citrus planting material; to help in conducting regular surveys to detect and avoid the potential spread of citrus vector-transmitted diseases such as tristeza, greening and stubborn; and, more important, to aid the various national and regional citrus sanitation programmes in the diagnosis and detection of citrus virus and virus-like diseases.

Realizing the importance of such a publication, FAO and IOCV cooperated in the preparation of this handbook on detection of citrus graft-transmissible pathogens. It was written somewhat in the form of a “recipe book” wherein all available knowledge on detection techniques is presented in the simplest and most straightforward way.

The handbook has been prepared by C.N. Roistacher, Emeritus Plant Pathologist of the University of California, Riverside, United States of America, who has devoted almost 40 years to the detection of citrus viruses and has played a significant role in the development of the California citrus clonal improvement programme. Other contributors to the handbook are scientists acknowledged as world authorities on the various detection techniques presented here. The author and most of the contributors are also members of the International Organization of Citrus Virologists. Without the dedication and generosity of all those who have voluntarily agreed to give their time and share their experience, this handbook would not have been written.

It is worth mentioning here that, although Part III of the handbook deals with laboratory detection techniques of citrus graft-transmissible pathogens, some of these techniques are also applicable to other viruses and virus-like pathogens of various field crops, vegetables, fruit trees and ornamentals.

As a follow-up to this handbook, and in order to make available to all interested parties the current technology on production of healthy citrus planting material, another publication on the elimination of citrus graft-transmissible pathogens is being prepared and will be published by FAO in due course.
Finally, it is hoped that this handbook will help to boost existing citrus phytosanitation programmes and will encourage the development of new citrus phytosanitation activities.

Lukas Brader
Plant Production and Protection Division
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The invaluable help of Dr Steven M. Garnsey is gratefully acknowledged in the detailed editing of all chapters, but especially for the many suggestions for improvement in the outline and content. His many hours of work in editing are sincerely appreciated.

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Special thanks are due to Dr M.M. Taher, FAO Regional Plant Protection Officer for the Near East, without whose persistence and inspiration this handbook would not have been published.

Finally, the author acknowledges the valuable assistance of Miss Brenda Jones in proofreading and typing the text.

***

This handbook is dedicated to Dr Edmond Clair Calavan, scientist, scholar and mentor, without whose inspiration, guidance and foresight many of the techniques and procedures mentioned in this handbook might not have been developed.

Dr Calavan was instrumental in developing the necessary indexing facilities for the Citrus Clonal Protection Program at the University of California at Riverside, and inspired and encouraged research to develop new methods for the detection of plant virus and virus-like pathogens. He was, and still is, a constant source of inspiration and encouragement to the author and it is fitting that this handbook be dedicated to him.

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Abbreviations

Bl
Blight

BU
Bud-union

Ca
Cachexia

cDNA
Complementary DNA

CEV
Citrus exocortis viroid

CG
Concave gum

CGTP
Citrus graft-transmissible pathogen

CIVV
Citrus infectious variegation virus

CLRv
Citrus leaf rugose virus

Cr
Cristacortis

CTLV
Citrus tatterleaf virus

CTV
Citrus tristeza virus

CV
Citrus viroid

DNA
Deoxyribonucleic acid

dPAGE
Denaturing polyacrylamide gel electrophoresis

dsRNA
Double-stranded RNA

Dweet
Dweet tangor

EDTA
Ethylenedinitrilotetraacetate

ELISA
Enzyme-linked immunosorbent assay

EM
Extraction medium and/or electron microscope

Gft
Grapefruit

Gr
Greening

IgG
Immunoglobulin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Synonym</th>
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<tbody>
<tr>
<td>Imp</td>
<td>Impietratura</td>
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<tr>
<td>ISEM</td>
<td>Immunosorbent electron microscopy</td>
</tr>
<tr>
<td>IV</td>
<td>Infectious variegation</td>
</tr>
<tr>
<td>Le</td>
<td>Lemon</td>
</tr>
<tr>
<td>Mand</td>
<td>Mandarin</td>
</tr>
<tr>
<td>MCE</td>
<td>Mercaptoethanol</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OLP</td>
<td>Oak-leaf pattern</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene-glycol</td>
</tr>
<tr>
<td>Ps</td>
<td>Psorosis</td>
</tr>
<tr>
<td>Ps/RS</td>
<td>Psorosis/ringspot</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RM</td>
<td>Re-suspension medium</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RS</td>
<td>Ringspot</td>
</tr>
<tr>
<td>RT enzyme</td>
<td>Reverse transcription enzyme</td>
</tr>
<tr>
<td>Sd</td>
<td>Satsuma dwarf</td>
</tr>
<tr>
<td>Sdlg</td>
<td>Seedling</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SSEM</td>
<td>Serologically specific electron microscopy</td>
</tr>
<tr>
<td>St</td>
<td>Stubborn</td>
</tr>
<tr>
<td>SW0</td>
<td>Sweet orange</td>
</tr>
<tr>
<td>Tang</td>
<td>Tangelo</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TL</td>
<td>Tatterleaf</td>
</tr>
<tr>
<td>Tr</td>
<td>Tristeza</td>
</tr>
<tr>
<td>Tr-SP</td>
<td>Trif</td>
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<tr>
<td>---------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Tristeza-stem pitting</td>
<td>Trifoliate</td>
</tr>
<tr>
<td>Tr-SY</td>
<td>VE</td>
</tr>
<tr>
<td>Tristeza-seedling yellows</td>
<td>Vein enation</td>
</tr>
</tbody>
</table>
This working handbook is designed to facilitate the detection of the major citrus graft-transmissible pathogens (CGTPs) and their associated diseases. Most aspects of detection are included. The primary object of this handbook is to enable users anywhere in the world to “see” or detect CGTPs, thereby assuring their elimination from citrus tissue. The ultimate objective is the elimination of these pathogens from propagative budwood and the establishment and maintenance of disease-free primary or foundation budwood source trees and nurseries to produce healthy, high-yielding citrus groves.

The importance of a good indexing programme as a means of identifying causal agents of transmissible diseases cannot be over-emphasized. A prime responsibility of those in charge of a certification programme is the prevention of the distribution of thousands or millions of trees contaminated with pathogens via propagation of infected budwood. This handbook is an aid and guide toward reaching that goal.

Indexing may be defined as any test that will consistently confirm the presence or absence of a transmissible pathogen, or identify a disease. The index test should be specific for the pathogen or disease. Inoculation of plants is the primary means by which most graft-transmissible diseases of citrus are diagnosed (Table 3). Inoculation is primarily by graft-transmission, but can also be effected by vector or mechanical transmission. Other approaches to indexing are direct observation of the pathogen in plant cells by light or electron microscopy, by microscopic observation of inclusion bodies or gum deposits within the plant cells, by antibody-antigen reaction, by analysis of pathogen-induced chemical changes, by culturing an organism, by observation of pathogen-specific nucleic acids via polyacrylamide gel electrophoresis (PAGE) or by molecular hybridization. Other indexing techniques involve detection of physiological changes, such as in water uptake or mineral content of trees as used in testing for blight.

In general, any consistent, measurable or striking change in the morphology or chemistry of a plant brought about by the direct or indirect presence of a pathogen, or the direct observation of the pathogen, its by-products or constituents, can be considered as an index.

Many diseases can be diagnosed by specific field symptoms. Where these field symptoms are diagnostic, they are presented and illustrated. However, many graft-transmissible diseases are symptomless in commercial citrus, and these can be detected only by indexing.

The majority of the citrus diseases dealt with in this handbook are detected primarily by graft-transmission to indicator plants. Proven indexes are given in Part I. Some new detection techniques are mentioned under the heading of “Miscellaneous”. These may be promising and useful but are not yet fully proved.

A listing of the indicator plants needed for a comprehensive indexing programme designed to detect the known major CGTPs reviewed in this handbook are given in Table 1. Other CGTPs not mentioned in this handbook (psorosis-like

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1 Citrus graft-transmissible pathogens (CTGPs) will be used as a general term to include the known viruses, viroids, bacteria, Spiroplasma and other non-identified virus-like causal agents of the graft-transmissible diseases of citrus.
pathogens, Dweet mottle virus, yellow vein virus and other CGTPs) can also be detected by many of the listed indicator plants.

A controlled-climate greenhouse, i.e. a plant laboratory for the production and maintenance in isolation of indicator plants, is essential to any successful programme for detection and diagnosis of these diseases. Details for the plant laboratory and its requirements, together with grafting techniques and inoculation procedures, are given in Part II.

Detailed procedures and pertinent references for laboratory methods of detection of virus and virus-like diseases are given in Part III.

### Major Graft-Transmissible Diseases of Citrus

The graft-transmissible pathogens of citrus have two general effects. Either they are destructive and cause sharp decline and death of the tree, or they do not kill the tree but cause severe stunting, loss of vigour, reduced fruit size and yield, and result in extensive long-term losses.

The major graft-transmissible diseases of citrus are presented below, based on economic impact and transmissibility:

- Diseases which induce severe loss and where vector transmission is known, i.e. tristeza, greening and stubborn, or which

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### Table 1

The minimum number of indicator plants recommended for a comprehensive indexing programme and the pathogens that can be detected

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Sdlg or scion</th>
<th>Minimum No of Plants per container</th>
<th>Variety</th>
<th>Viruses detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Primary</td>
</tr>
<tr>
<td>Lime</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Mexican, Key, etc.</td>
<td>Tr, VE</td>
</tr>
<tr>
<td>Sweet orange</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Pineapple, Madame Vinous</td>
<td>Ps/RS, CG</td>
</tr>
<tr>
<td>SwO</td>
<td>Sdlg</td>
<td>2</td>
<td>1 Madame Vinous</td>
<td>Tr-SY, Tr, SP</td>
</tr>
<tr>
<td>Mandarin</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Ponkan</td>
<td>GR</td>
</tr>
<tr>
<td>Mandarin</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Satsuma</td>
<td>SD</td>
</tr>
<tr>
<td>Tangor</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Dweet</td>
<td>CG, Cr and Imp (OLP)</td>
</tr>
<tr>
<td>Sour orange</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Standard</td>
<td>Tr-SY, VE</td>
</tr>
<tr>
<td>C. excelsa</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Kalpi lime</td>
<td>TL, Tr</td>
</tr>
<tr>
<td>Citrange</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Rusk, Troyer, Carrizo</td>
<td>TL</td>
</tr>
<tr>
<td>Citron</td>
<td>Scion</td>
<td>2</td>
<td>3 861, 60-13</td>
<td>IV, Ps/RS</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Duncan</td>
<td>Tr-SY, Tr-SP</td>
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<td></td>
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| For detection of cool-temperature pathogens

<table>
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<th>Indicators</th>
<th>Plant</th>
<th>No of Plants</th>
<th>Variety</th>
<th>Viruses detected</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lime</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Mexican, Key, etc.</td>
<td>Tr, VE</td>
</tr>
<tr>
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<td>Sdlg</td>
<td>4</td>
<td>3 Pineapple, Madame Vinous</td>
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</tr>
<tr>
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<td>Sdlg</td>
<td>2</td>
<td>1 Madame Vinous</td>
<td>Tr-SY, Tr, SP</td>
</tr>
<tr>
<td>Mandarin</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Ponkan</td>
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<tr>
<td>Mandarin</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Satsuma</td>
<td>SD</td>
</tr>
<tr>
<td>Tangor</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Dweet</td>
<td>CG, Cr and Imp (OLP)</td>
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<tr>
<td>C. excelsa</td>
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<td>3 Kalpi lime</td>
<td>TL, Tr</td>
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<tr>
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<td>Sdlg</td>
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<td>3 Rusk, Troyer, Carrizo</td>
<td>TL</td>
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<tr>
<td>Citron</td>
<td>Scion</td>
<td>2</td>
<td>3 861, 60-13</td>
<td>IV, Ps/RS</td>
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<tr>
<td>Grapefruit</td>
<td>Sdlg</td>
<td>4</td>
<td>3 Duncan</td>
<td>Tr-SY, Tr-SP</td>
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### For detection of warm-temperature pathogens

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<thead>
<tr>
<th>Indicators</th>
<th>Plant</th>
<th>No of Plants</th>
<th>Variety</th>
<th>Viruses detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citron</td>
<td>Scion</td>
<td>5</td>
<td>1 861-S-1/rough lemon stock</td>
<td>Exocortis and misc. citrus viroids</td>
</tr>
<tr>
<td>Mandarin</td>
<td>Scion</td>
<td>6</td>
<td>1 Parson's/rough stock</td>
<td>lemon Cachexia</td>
</tr>
<tr>
<td>SwO</td>
<td>Sdlg</td>
<td>5</td>
<td>1 Madame Vinous</td>
<td>Stubborn</td>
</tr>
</tbody>
</table>
are suspected to be vector-transmitted, i.e. blight and related diseases.
• Diseases which induce moderate loss, are mechanically transmitted and also bud-transmitted or vectored by unknown means, i.e. exocortis, cachexia, satsuma dwarf, tatterleaf, infectious variegation and ringspot psorosis.
• Diseases which are readily controlled by use of virus-free budwood and are primarily bud-transmitted by humans, i.e. the psorosis-A family and the oak-leaf pattern family, including concave gum, impietratura and crista cortis.
• Diseases which are vector-transmitted but of minor economic impact, such as vein enation.
• Miscellaneous diseases and those of unknown etiology, i.e. gummy bark and abnormal bud-unions.

Table 2 lists these transmissible diseases of citrus grouped by causal agent (procaryote, virus, viroid etc.) and by primary mode of transmissibility (graft, vector or mechanical).

Vector-transmitted citrus tristeza and greening rank as the most serious and destructive of the citrus diseases. Where sour orange is the predominant rootstock and appropriate vectors are present, tristeza can spread rapidly and destroy an industry. Even where tristeza becomes endemic in an area and is contained by use of other rootstocks and cross-protection techniques, some of the stem-pitting forms can superimpose, decimate and destroy the industry. In many areas of the world, where greening has been introduced, citrus as a viable crop has declined. It is most important to understand the destructive potential of these two diseases and to develop every strategy to prevent, detect and destroy the pathogens before they can become established within an area, country or region. Recently, blight and related declines have inflicted severe damage on the industries of many countries, and some diseases, such as the Misiones disease or fruta bolita in northern Argentina, have destroyed local industries.

Some of the graft and mechanically transmissible diseases of citrus such as tatterleaf, psorosis, ringspot and satsuma dwarf, are considered moderately destructive. Although they may severely limit crop production, these diseases can be readily detected in propagative budwood, are easily eliminated and their rapid spread curtailed. The mechanically transmitted viroid diseases, such as exocortis and cachexia, are destructive to certain varieties and, once introduced and established in a country, can spread relatively rapidly by mechanical means. These diseases can be readily detected by indexing and should not be permitted to enter expanding citrus industries of new or old regions.

Other citrus diseases, such as concave gum, crista cortis, impietratura, infectious variegation and vein enation, are generally less serious compared with the other diseases because they are relatively rare, do not cause severe effects or are spread only by grafting. Once eliminated from propagative budwood, they should not pose further problems.

Certain pathogens, once distributed throughout a region, are difficult to remove and may remain indefinitely with the citrus industry. Therefore, prevention by rigid quarantine should be the first priority. Detection by indexing and a programme for reducing inoculum, by either eradication or a certification programme, may have wide benefits.

**BENEFITS AND OBJECTIVES OF INDEXING**

The benefits derived from the development of an indexing programme will usually far outweigh the costs of development and maintenance of the programme. In those countries where no
organized programme of testing has been established, most old-line citrus cultivars are infected with viruses, viroids or other graft-transmissible agents. Reports from Spain, Brazil, Florida and the Foreign Budwood Importation Program in California have shown that almost all citrus was infected with one or more CGTP.

The first benefit of a comprehensive indexing programme is accurate knowledge of the pathogens that are present in citrus within the country or area. This knowledge allows formulation of sound strategies in the development of a programme to avoid pathogens in propagative budwood.

One indirect benefit of establishing a comprehensive indexing programme is its potential for early discovery of introduced pathogens that could be highly destructive to citrus should they become disseminated via vector or budwood. In this world of rapid movement of people and plants by air transportation, budwood from other countries is continually being introduced by uninformed nursery staff and growers. This budwood can readily harbour pathogens potentially destructive to citrus. A well-equipped and properly staffed diagnostic laboratory can be a first line of defence for the rapid identification of these potentially destructive pathogens. If detected early, they may be eliminated before they can spread.

Perhaps the most rewarding aspect of a comprehensive indexing programme is the

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### TABLE 2

#### A classification of the major graft-transmissible diseases of citrus

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease</th>
<th>Mode of transmission</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procaryote</td>
<td>Greening</td>
<td>x</td>
<td>Gracilicute-like bacteria</td>
</tr>
<tr>
<td></td>
<td>Stubborn</td>
<td>x</td>
<td>Spiroplasma citri</td>
</tr>
<tr>
<td>Virus and probable virus</td>
<td>Tristeza</td>
<td>x</td>
<td>Virus</td>
</tr>
<tr>
<td></td>
<td>Vein enation</td>
<td>x</td>
<td>Probable virus</td>
</tr>
<tr>
<td></td>
<td>Concave gum</td>
<td>xxx</td>
<td>Probable virus</td>
</tr>
<tr>
<td></td>
<td>Impietratura</td>
<td>xxx</td>
<td>Probable virus</td>
</tr>
<tr>
<td></td>
<td>Cristacortis</td>
<td>xxx</td>
<td>Probable virus</td>
</tr>
<tr>
<td></td>
<td>Psorosis-A</td>
<td>xxx</td>
<td>Probable virus</td>
</tr>
<tr>
<td>Viroid</td>
<td>Exocortis</td>
<td>xxx</td>
<td>Virus</td>
</tr>
<tr>
<td></td>
<td>Citrus viroid</td>
<td>xxx</td>
<td>Virus</td>
</tr>
<tr>
<td></td>
<td>Cachexia</td>
<td>xxx</td>
<td>Virus</td>
</tr>
<tr>
<td>Other and miscellaneous</td>
<td>Blight</td>
<td>x</td>
<td>Not known</td>
</tr>
<tr>
<td></td>
<td>Declinio</td>
<td>x</td>
<td>Not known</td>
</tr>
<tr>
<td></td>
<td>Fruta bolita</td>
<td>n</td>
<td>Not known</td>
</tr>
<tr>
<td></td>
<td>Gummy bark</td>
<td>xxx</td>
<td>Not known</td>
</tr>
<tr>
<td></td>
<td>Abnormal bud-union</td>
<td>xxx</td>
<td>Not known</td>
</tr>
</tbody>
</table>

1. **xxx** = The primary mode of transmission
   - **x** = Readily transmitted
   - **xx** = Secondary mode of transmission by this mode
   - **n** = Not known

2. Certain isolates psorosis-A have been shown to be mechanically transmissible.
production of pathogen-free trees derived by
shoot-tip grafting in vitro or by thermotherapy.
Trees so produced are healthy, more uniform,
higher-yielding with better coloured fruit, and
ultimately more profitable to the grower. Those
countries which have well-established indexing
and certification programmes have demonstrated
the economic benefits of such programmes to
the grower. Production of disease-free trees
requires the knowledge of which CGTPs are
present and verification of their elimination by
thermotherapy and/or shoot-tip grafting.

In summary, the ultimate objective or goal of
any indexing programme should be the
development of certified pathogen-free budwood
to improve production in the industry, and also to
have the means for detection of new, serious
pathogens.

CONCEPTS IN DETECTION OF CITRUS
GRAFT-TRANSMISSIBLE PATHOGENS
The visible diseases
Many CGTPs will induce specific symptoms in
the field tree, and many of these symptoms are
diagnostic for a specific disease. This handbook
illustrates some of these field symptoms. However,
the individual responsible for indexing
should read and study the literature relative to
each of the diseases. Although some pertinent
literature is given with each chapter in this
handbook, a study of the literature presented in
all the volumes of the Proceedings of the
International Organization of Citrus Virologists
(IOCV) gives a more comprehensive and helpful
reference guide. A historical review and
bibliography for each of 14 diseases plus shoot-
tip grafting is given by Roistacher (1988a).
Recommended publications illustrating many
of the citrus graft-transmissible diseases are:
Bové and Vogel’s *Description and illustration of
citrus virus and virus-like diseases* (1980); the
fourth volume of *The citrus industry*, containing
articles by Wallace (1978) on citrus virus and
virus-like diseases and by Calavan *et al.* (1978)
on registration, certification and indexing of
citrus trees; and the *Compendium of citrus
diseases* (Whiteside *et al.*, 1988).

Diseases which may show characteristic and
diagnostic symptoms in the field on certain
varieties are cachexia, concave gum, cristacortis,
exocortis, greening, infectious variegation,
satsuma dwarf, psorosis-A, ringspot, stubborn,
tatterleaf, tristeza and vein enation-woody gall
(Table 3). However, clear diagnostic symptoms
are not always obvious on infected trees in the
field. It is very important to realize that trees
infected with most of these same CGTPs are
symptomless in various hosts and may show
little or no diagnostic evidence of their presence
in the field. Plants showing symptoms of one
disease may also be infected with other
pathogens. Thus, these CGTPs can unknow-
ingly be spread throughout a country by
bud-propagation or by mechanical or insect
transmission. These are the “invisible diseases”
which can be detected only by indexing.

The invisible pathogens
Most of the CGTPs listed in Table 3 exist in an
invisible state in many citrus cultivars and can be
detected only by indexing. Unless identified, the
invisible pathogen can be readily spread in
propagative budwood and can be distributed en
masse within a country. The primary sources of
infected budwood usually lie within the country,
but with the current facility of air transport new,
exotic and possibly destructive strains are being
imported (often unwittingly) by nursery staff
and growers.

Here are examples of CGTPs that can be
symptomless in citrus:

- Certain isolates of citrus tristeza virus
  (severe stem-pitting and seedling-yellows
  strains) do not show symptoms in field trees
### TABLE 3

**Diagnosis of citrus graft-transmissible diseases (summary)**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Field diagnosis</th>
<th>Plant indicators</th>
<th>Temp.¹ (^{('}C)</th>
<th>Symptoms in plant indicators</th>
<th>Other diagnostic methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blight</td>
<td>Wilting, thin foliage, delayed flush, chlorosis, water sprouts, dieback (Fig. 30)</td>
<td>No seedling</td>
<td>-</td>
<td>-</td>
<td>- Water uptake* - Zinc level* - Microscopy</td>
</tr>
<tr>
<td>Bud-union crease</td>
<td>Enlarged bud-union area with crease and brown line (Figs 103,104)</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cachexia</td>
<td>Gummimg in bark and wood of tangelo, Mand., Mand. hybrids, etc. (Figs 44,45)</td>
<td>Parson's Special Mandarin/rough lemon*; Orlando tangelo sdig</td>
<td>Warm 32-40</td>
<td>Gum in bark and scion: Electrophoresis gum at bud-union and cut-back joints</td>
<td></td>
</tr>
<tr>
<td>Concave gum</td>
<td>Concavities in trunk of SwO, Mand., Tang. (Fig. 81); concentric rings of gum in limbs (Fig. 82); OLP in spring flush of growth (Fig. 83)</td>
<td>Dweet*, SwO*, Mand.</td>
<td>Cool 24-27</td>
<td>CLP</td>
<td></td>
</tr>
<tr>
<td>Crista-cortis</td>
<td>Deep pits in scion and rootstock (Figs 90 to 93); OLP in spring flush (Fig. 95)</td>
<td>Orlando tang.* for pitting; Dweet, SwO, Mand. for OLP</td>
<td>Cool 24-27</td>
<td>Pits in tangelo. OLP in Dweet, SwO, and Mand.</td>
<td></td>
</tr>
<tr>
<td>Exocortis and related viroids</td>
<td>Scaling of bark on Trif. stock (Figs 35 to 37); yellow stem blotch on Trif., Trif. hybrids and Rang. lime (Fig. 38); stunting on Trif., or Trif. hybrid stocks (Fig. 43)</td>
<td>861 -S1citron*</td>
<td>Warm 32-40</td>
<td>Electrophoresis for CEV; mild leaf epinasty petiole, tip or midvein browning for citrus viroids</td>
<td></td>
</tr>
<tr>
<td>Greening</td>
<td>Trees in decline with yellow shoots, leaf mottle, yellow veins (Fig. 14); stylar end greening (Fig. 15)</td>
<td>Mexican lime*, SwO*, Ponkan' for Asian greening</td>
<td>25-32</td>
<td>Smaller yellow leaves, leaf blotch, spotty chlorosis, zinc-like deficiencies - Vector transm. - Fluorescence* - Microscopy - Dodder transm.</td>
<td></td>
</tr>
<tr>
<td>Gummy</td>
<td>Gum pockets in bark and wood of SwO; stem pitting (Figs 100,101)</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impietratura</td>
<td>Gum spots on fruit (Figs 84 to 88); OLP in spring flush of growth (Fig. 89)</td>
<td>Fruit of Gt. for gum spots; Dweet, SwO*, Mand. for OLP</td>
<td>Cool 24-27</td>
<td>Cool spring for gum in fruit. OLP in leaves</td>
<td></td>
</tr>
<tr>
<td>Infectious variegation</td>
<td>Severe leaf crinkle with distorted, puffed or puckered leaves with or without variegation in Le., Mand., SwO or Gt. (Fig. 63)</td>
<td>Citron, Le.*</td>
<td>Cool 24-27</td>
<td>ELISA Distorted, puckered, puffed leaves; epinasty in Le. or citron. Necrotic chlorotic spots in cowpea; yellow vein clearing in kidney bean</td>
<td></td>
</tr>
<tr>
<td>Citrus leaf rugose</td>
<td>Flecking in lemon</td>
<td>Citron, Le.*</td>
<td>Cool 20-22</td>
<td>ELISA* Distorted, puckered leaves in Le. or citron. Necrotic chlorotic spots in cowpea; yellow vein clearing in kidney bean</td>
<td></td>
</tr>
<tr>
<td>Psorosis</td>
<td>Bark scaling of SwO, Mand and Gt. (Fig. 72)</td>
<td>SwO*, Mand., Dweet, Le.</td>
<td>Cool 24-27</td>
<td>Shock, young leaf patterns</td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>Field diagnosis</td>
<td>Plant indicators</td>
<td>Temp.(^1) (°C)</td>
<td>Symptoms in plant indicators</td>
<td>Other diagnostic methods</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------------------------------------------------------------------</td>
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<td>-----------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Ringspot</td>
<td>Severe mature leaf patterns (Fig. 78); patterns on fruit (Fig. 73); blisters on twigs and leaves (Fig. 74)</td>
<td>SwO(^<em>), Gft(^</em>), Chenopodium quinoa (a)</td>
<td>Cool 21-27</td>
<td>Shock, young leaf patterns in SwO. and Gft.; chlorotic local lesions in C. quinoa</td>
<td></td>
</tr>
<tr>
<td>Satsuma dwarf</td>
<td>Severe dwarfing of field trees (Fig. 51)</td>
<td>Citron, Le., Dweet, Mand.</td>
<td>Very cool 12-18</td>
<td>Distorted, puckered,uffed leaves; epinasty</td>
<td>ELISA*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White sesame(^*)</td>
<td>Cool 21-23</td>
<td>Necrotic local lesions primary leaves, necrosis, curl and epinasty in secondary stock</td>
<td></td>
</tr>
<tr>
<td>Stubborn</td>
<td>Severe stunting and compressed tree, small leaves and internodes (Figs 21,22); chlorotic leaf mottle (Fig. 23); green fruit (Fig. 24a); acorn-shaped fruit; seed abortion (Fig. 24b)</td>
<td>Madame Vinous SwO(^*)</td>
<td>Warm 32-38</td>
<td>Stunted shoots and plant; chlorotic spots near leaf tip; pointed leaf tips</td>
<td>- Culturing(^<em>) (preferred method) - ELISA</em></td>
</tr>
<tr>
<td>Tatterleaf</td>
<td>Brown ring at bud-union with Trif. or its hybrids as stock, Fluting of Trif. hybrid stocks (Figs 55 to 58)</td>
<td>Citrus excelsa, Rusk citrus(^*)</td>
<td>Cool 24-24</td>
<td>Tattered leaf margins; chlorotic spots; severe stunting</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cowpea(^*)</td>
<td>Cool 21-24</td>
<td>Brown necrotic spots in primary leaves</td>
<td></td>
</tr>
<tr>
<td>Tristeza</td>
<td>Decline and death of SwO. Gft. or Mand. on sour Or. stock; inverse pitting below bud-union on sour Or. (Figs 4,5)</td>
<td>Mexican lime(^*)</td>
<td>Cool 24-27</td>
<td>Vein clearing and cupping of leaves. Stem pitting</td>
<td>ELISA(^*) - dsRNA - PAGE - Immunodiffusion - Microscopy - Fluorescent antibodies</td>
</tr>
<tr>
<td>Tristeza stem pitting</td>
<td>Severe stem pitting in numerous cultivars - specifically Gft.; trees may be stunted, unproductive and with small fruit</td>
<td>SwO(^*) and Gft. sdigs</td>
<td>Cool 24-27</td>
<td>Stem pitting in Gft. and SwO sdigs</td>
<td></td>
</tr>
<tr>
<td>Tristeza seedling yellows</td>
<td>Severe decline and chlorosis. Severe decline and death of SwO. Gft. and Mand. on sour Or. rootstock. (Fig. 4)</td>
<td>Gft(^<em>), sour Or(^</em>) (preferred) or Le. sdigs</td>
<td>Cool 24-27</td>
<td>Yellowsin Gft. , Le., sour Or. leaves</td>
<td>dsRNA</td>
</tr>
<tr>
<td>Vein enation-woody gall</td>
<td>Galls on trunk of rough or Volkamer lemon (Figs 96,97). Vein enations on leaves of young sour orange seedling in the nursery</td>
<td>Mexican lime(^*), sour orange</td>
<td>Cool 24-27</td>
<td>Enations on small galls on veins of leaves</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) Preferred indicator or method of detection.

\(^1\) Maximum daytime temperatures.

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of mandarin cultivars. However, when these mandarins are planted near sweet orange or grapefruit trees, aphids will transmit the citrus tristeza virus from the symptomless mandarins into reactive hosts (Roistacher, 1988b).

- The citrus cachexia viroid, which induces no symptoms in grapefruit or sweet orange cultivars, can induce a severe reaction in tangelo or mandarin if infected buds are used as scions or if the viroid is mechanically transmitted to these cultivars.

- Citrus tatterleaf virus expresses no symptoms in most citrus cultivars including sweet orange, mandarin, sour lemon, Meyer lemon and grapefruit. However, if
virus-infected budwood is used as scion wood and grafted to citrange or trifoliate orange or any hybrids of trifoliate used as a rootstock, a brown bud-union crease will usually be evident, and deep pits and grooves may develop in the rootstocks, and trees of these stionic combinations will usually decline.

- Citrus vein-enation virus induces no symptoms in most mature scions and can usually be detected only by indexing. The concave gum pathogen induces no symptoms in lemon and grapefruit.
- The psorosis-A pathogen may be present for seven to 15 years without inducing symptomatic bark scaling in sweet orange.
- In some rare cases psorosis-A-infected sweet oranges may be symptomless for up to 50 years. However, progeny trees propagated with budwood from such symptomless trees will usually show bark scaling symptoms within seven to 15 years. The psorosis-A pathogen induces no symptoms in infected lemon, sour orange or many varieties of pummelo.
- Exocortis and related citrus viroids will induce no symptoms in most sweet orange, mandarin and grapefruit scion cultivars. However, when viroid-infected budwood is grafted on rootstocks of trifoliate orange, hybrids of trifoliate, Palestine sweet lime or Rangpur lime, distinct symptoms may occur on these rootstocks.

**Pathogen-free budlines**

If a control strategy is adopted based on pathogen-free budwood obtained by shoot-tip grafting in vitro with or without thermotherapy, the questions are often asked: “Why should we index? Won’t the shoot-tip grafted budwood automatically be pathogen-free?” Shoot-tip grafting and thermotherapy, though proved excellent as therapeutic methods for eliminating many pathogens from citrus budwood, will not guarantee 100 percent success in eliminating all pathogens (Calavan et al., 1972; Roistacher et al., 1976; Navarro et al., 1976). In fact, 100 percent elimination of pathogens by these techniques is the exception, and indexing is an absolute necessity if freedom from CGTPs is to be assured. Distribution of budwood after therapy, but without indexing, may result in dissemination of infected material.

Once pathogen-free budlines are obtained by therapeutic means, and foundation or mother trees are planted, these foundation-source trees must be periodically reindexed to ensure they have not been contaminated by mechanical transmission or by insect vectors. In countries where vectors are present, insect-proof screenhouses are needed to protect foundation and mother trees as well as primary increase block trees from reinfection.

**New developments in indexing**

The use of serology, especially ELISA, for the mass detection of certain pathogens is one of the most exciting and helpful developments in recent years in the field of indexing. The rapidity of the test and its accuracy for detection of some CGTPs make it a necessary part of any indexing programme. ELISA has proved useful in mapping the distribution of such viruses as tristeza, satsuma dwarf and infectious variegation over large areas, and its future is bright for the detection of other pathogens affecting citrus. It can also be used in
combination with plant indexing for detection of virus strains which are very mild-reacting in plant indicator hosts and otherwise could escape detection. As shown in Table 3, ELISA and other immuno-assay techniques are available for detection of tristeza, infectious variegation, satsuma dwarf and stubborn. It is important to realize that ELISA cannot be used as a substitute for indexing with plants in the critical evaluation for presence of pathogens because, under certain conditions, ELISA can fail to detect pathogens in low titre.

Electrophoretic techniques are being developed for detection of the viroids of exocortis and cachexia and many of the more recently discovered citrus viroids. With additional research, these electrophoretic methods may become the primary index for viroids difficult to index by other methods.

Identification of viral pathogens by nucleic acid hybridization assays is also promising for some applications, especially for detection of specific strains of a given pathogen and for viroid detection. Development of non-radioactive labels is needed to make this approach more useful for practical application.

The stubborn organism, *Spiroplasma citri*, can now be cultured, and culturing has replaced plant indexing as the primary index for this disease. This is one of the very few laboratory procedures that has replaced the plant itself for indexing of CGTPs.

Other techniques such as ISEM, molecular hybridization, dsRNA, and light microscopy with fluorescent antibodies, are useful for detection of specific pathogens and may ultimately replace the plant index for some applications. However, as shown in Tables 1 and 3, at present plants are still the primary means for the certain detection of most CGTPs, and the need for a well-equipped plant laboratory with skilled personnel is evident. Indexing based on plant indicators will always remain a valuable adjunct to other procedures, especially where absolute verification of virus freedom is essential. The concept should not be to replace all use of plant indicators but to utilize them efficiently in a total programme.

An old proverb expresses the concept of the need for plant indexing while continuing research for new and more rapid techniques:

> Be not the first to take the new astride;
> Be not the last to cast the old aside.

**INDEXING: FACILITIES, METHODS AND CONSIDERATIONS**

How does one get started in an indexing programme? This will depend on objectives. If the objective is to survey for presence or extent of infestation of a particular pathogen or disease within a region (i.e. tristeza, seedling-yellows tristeza, stem-pitting tristeza, tatterleaf or satsuma dwarf, etc.), then certain facilities and methods may be needed for this survey. The use of ELISA, if antiserum is available, would be the preferred strategy. Where antiserum is not available and plants must be used for indexing field trees over a large area, then other strategies must be used, i.e. multiple indexing of a number of trees to larger indicator plants.

If the objective is to establish a comprehensive indexing programme, the information contained in this handbook would be pointedly applicable. Of immediate concern would be the training personnel to develop and lead a certification programme, to develop a system for growing index plants, to obtain seed, to obtain positive controls etc.

The decision to develop a certification programme, including a foundation block with its supportive laboratory, must be well supported and adequately financed as a long-term project. The programme should include policies for education and publicity to inform growers and
the public of the dangers of introduced pathogens and the benefits of pathogen-free stock.

This handbook includes a number of methods that can be used to detect CGTPs and is designed to be self-explanatory. Although the methods are given in considerable detail, they should be used as guidelines. These are methods and procedures that have worked effectively for indexing at certain laboratories, but they should not be considered as absolute standards. Modifications are desirable and encouraged, and should be shared. Table 3 gives a summary of various diagnostic techniques that can be used for detecting a number of the major citrus graft-transmissible diseases. This table can be used as an aid in developing an indexing strategy.

Each country must establish its goals and priorities depending on the seriousness of the immediate problem, the size of the industry, government support and funding available. An important consideration is the selection of a suitable site for the plant laboratory and the provision of appropriate temperature controls. The minimum needs, as proposed in Tables 1 and 3, are a plant-growth structure and a workplace equipped for laboratory-oriented procedures such as ELISA, PAGE and nucleic acid hybridization.

Perhaps the most important ingredients for a successful indexing programme are the quality, education, training, dedication and integrity of the personnel responsible for the conduct of the programme. A well-trained, well-paid and dedicated person may mean the difference between success and failure of a programme. Training can be arranged with institutions that have established certification programmes. Courses in indexing similar to those presented in the past are projected for the future under the auspices of FA0 in cooperation with various research institutes. Comprehensive workshops are being planned at meetings of the International Organization of Citrus Virologists.

REFERENCES


Techniques for biological detection of specific citrus graft-transmissible diseases
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
Techniques for the biological detection of citrus graft-transmissible diseases

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, exocortex, and exocortex-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 a non-inoculated 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 oak-leaf 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 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
Techniques for biological detection of specific citrus graft-transmissible diseases 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

DESCRIPTION AND BACKGROUND

Tristeza is possibly the most destructive disease of citrus. Many millions of trees on sour orange rootstock have been destroyed in Argentina, Brazil, California, Florida and Spain. The disease continues to spread into new areas, e.g. Israel and Venezuela, destroying citrus plantings where sour orange is the predominant rootstock.

There are many strains of tristeza, causing various field symptoms on different scions and rootstocks. Isolates selected from field trees may induce a reaction only in Mexican lime. Some will cause bud-union failure of certain scions on sour orange rootstock, and others can induce severe pitting or stunting and yellows in a variety of indexed seedlings. Tristeza stem pitting can severely injure scions of grapefruit and sweet orange directly, regardless of rootstock, by inducing a severe pitting and enlarged cheesy bark in the scion, resulting in smaller fruit, loss of production and debilitation of the tree. Tristeza stem pitting also seriously affects lime and may limit production in many areas.

Many comprehensive reviews on this disease have been published, i.e. Wallace (1978), Cohen and Bové (1980), Roistacher (1981a, 1981b, 1982), and Bar-Joseph et al. (1981). Extensive slide and text reviews on many aspects of tristeza are given in Description and illustration of virus and virus-like diseases of citrus, edited by Bové and Vogel (1980), and these are highly recommended for reviewing the tristeza and tristeza seedling-yellows diseases.

The plant index is still invaluable for detection of CTV and its many isolates. Graft inoculation to indicator plants can detect tristeza when virus titre is very low. The severe seedling-yellows and stem-pitting forms of tristeza can currently be distinguished from mild forms only in plants. Garnsey et al. (1987) proposed a standardized host-range analysis for evaluating the severity of tristeza isolates by rating decline, stem pitting and seedling yellows on different hosts.

There are strains of tristeza that are difficult to detect in seedlings of Mexican lime but that can be easily detected by ELISA. For example, a California tristeza isolate (T-519) is very difficult to identify in Mexican lime indicator plants grown under temperature regimes conducive to good symptom development, but it is readily detected by ELISA. This illustrates the value of, and need for, using more than one technique in a programme for the indexing of important foundation block trees.

METHODS OF DETECTION

Method 1: field diagnosis

The iodine test. If the rootstock is sour orange and the scion sweet orange, grapefruit or mandarin, a sudden quick decline and wilting, followed by defoliation, especially during the first warm weather of spring, would suggest possible infection by tristeza (Figure 4). A simple field test can be carried out to detect starch depletion in the roots or rootstock below the bud-union. The disappearance of starch from the roots is a result of girdling owing to killing of the phloem cells at the bud-union. Tristeza-induced starch depletion generally proceeds from the outer tips of the roots back toward the trunk of the tree. The application of iodine to the exposed cut surface of a root is a rapid method of testing for starch. The following method is taken from
 Techniques for biological detection of specific citrus graft-transmissible diseases

the papers of Bitancourt (1944) and Fawcett (1945):

- Prepare a solution of potassium iodide and iodine by dissolving 1.5 g of potassium iodide plus 0.3 g of iodine in 100 cc of water. Keep in a coloured glass bottle, away from direct sunlight.

- Dig up roots (6-mm or 1/4-in diameter or smaller) from the outer margin of the tree and cut roots at an angle exposing the inner wood. Place a drop of iodine solution on the cut surface. Lack of development of a dark blue or black reaction suggests starch depletion, whereas a strong reaction indicates abundant starch.

A tree on sour orange rootstock showing quick decline or sudden dieback symptoms and with low starch in the roots would be suspect for tristeza. Verification should be made using ELISA or graft inoculation to Mexican lime seedlings, or both, or any of the diagnostic techniques mentioned in this handbook.

**Inverse stem pitting in the sour orange rootstock.**

A section of bark removed from the bud-union area of a tristeza-infected tree will usually show inverse pitting on the inner surface of the bark with corresponding pegs on the outer surface of the exposed sour orange rootstock (Figure 5). This symptom (on the sour orange rootstock) is highly diagnostic for tristeza.

**Stem pitting on scions.** Pitting is associated with, and diagnostic for, tristeza. Pits may be seen in the trunk and branches, and vary from severe deep depressions to closely spaced and small (Figures 9b and 13). When pitting is very severe, small branches will snap off readily at the new growth joints. The outer bark may be cheesy and, when peeled, stems may show very small closely spaced or varying sized pits. These can be seen in grapefruit, grapefruit hybrids, pummelo, tangelo, limes, various citrus hybrids, and sweet orange, but rarely in lemon, sour orange, trifoliate orange or mandarin. Severe pitting on grapefruit and sweet orange in the field is usually associated with chlorotic, tight, upright branch growth.

**Method 2: seedling indexing**

Seedling indexing to Mexican lime is still a very powerful tool for detection of tristeza virus. The small-fruited and somewhat seedy Mexican or West Indian lime (*Citrus aurantifolia*) has various common names such as *kaghzi* in India, *haladi* in Egypt, *doc* in Morocco, and *gallego* in Brazil. The name *key lime* is also commonly used. Seedlings of *C. excelsa*, citron, *C. macrophylla* or other citrus which show vein clearing and stem pitting can also be used as indicators. However, the Mexican lime is highly sensitive to tristeza and is the preferred indicator.

**Collection of budwood.** Collect budsticks from a minimum of four quadrants of each tree. For routine reindexing of important foundation or mother-block trees where there may be some danger of possible infection by vector transmission, collect from eight sectors of each tree.

**Inoculum tissue.** “Buds” (buds with eyes, blind buds or chip buds), leaf discs or leaf pieces can be used (see Part II). Graft two inoculum “buds” or leaf pieces, or a minimum of five or six leaf discs per plant. Since CTV is phloem-limited, it is important that inoculum tissue contain phloem and that cut surfaces of phloem tissue of donor and receptor plants are in good contact.

**Inoculation.** Place inoculum “buds” or leaf pieces in the lower part of the test seedling, removing as few leaves as possible from the lower stems. The seedling can be cut back to
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20-25 cm from the soil surface at the time of inoculation or at two to three weeks after inoculation when wrapping tapes are cut and the inoculum is observed for survival. The time to cut back is decided upon according to the specific environmental conditions in each plant laboratory. With the use of plastic wrapping tapes at the laboratory at Riverside, California, plants are usually cut back at the time of inoculation with very high survival rates of inoculum buds.

**Indicator plants.** As stated above, Mexican lime is the recommended general indicator for identification of all types of tristeza. To determine if the isolates will cause seedling yellows or stem pitting, inoculate grapefruit seedlings. These are highly sensitive to most CTV isolates, and are the preferred indicator in an initial index for stem pitting (SP-CTV) and/or seedling yellows (SY-CTV). Most seedy grapefruits can be used, and the Duncan variety has been found satisfactory as an indicator. If seedling yellows is found in the grapefruit indicator, then subinoculations can be made from the infected grapefruit to sour orange and sweet orange to determine the severity of the SY-CTV or SP-CTV isolate.

Sour orange is an excellent supplemental indicator for seedling-yellows tristeza and is equally as effective as the Eureka or Lisbon lemon (Figure 1). It is also preferred since it is highly polyembryonic and will produce 70 percent or more plantable seedlings compared with only 8 percent for the highly gametic lemons. Garnsey (unpublished) found that a clonal Eureka lemon grown as a cutting makes an excellent indicator for seedling yellows.

To determine whether an isolate will cause significant stem pitting in sweet orange, it is necessary to inoculate seedlings of a sensitive variety such as Madame Vinous. Although sour orange and lemon seedlings are excellent indicators for seedling yellows, they will rarely show stem-pitting symptoms.

Mexican lime used for general tristeza indexing and grapefruit and sour orange seedlings used for detection of severe CTV isolates can be grown three per container. Using two containers with three plants per container, two plants in each container are inoculated, leaving the third plant as a non-inoculated control. Sweet orange seedlings (Madame Vinous or Pineapple) used for detection of severe CTV-SP isolates should be grown one per container.

**Controls.** The negative control plant in each container of three is not inoculated. A minimum of one mild- and one severe-reacting tristeza isolate should be included as positive controls in every test. The severe-reacting positive control isolate can be inoculated into two plants in one container and the mild-reacting positive control isolate inoculated into a minimum of four, but preferably six to eight plants. The mildest-reacting isolate available should be used, i.e. one that induces very few leaf or stem-pitting symptoms in Mexican lime (Figures 6b and 9a).

Positive controls for seedling yellows or stem pitting should be “buds” taken from a minimum of two reactive sources with known symptomatology and should be inoculated into two grapefruit and/or sour orange seedlings per container. A strong and a mild reactive isolate are preferred. In large-scale tests for seedling yellows, two or more known reactive isolates should be used. Negative controls must always be included.

**Inoculum survival.** The wrapping tapes should be cut and removed two to three weeks after inoculation and the survival of the graft inoculum recorded. Although tristeza is not readily transmitted mechanically, other pathogens are,
thus it should be standard procedure to dip all tools prior to cutting into any plant (in a 1 percent sodium hypochlorite disinfectant solution).

Leaf-disc grafts can be evaluated one to two weeks after inoculation. If one of the two “bud” or leaf-piece grafts is alive, the plant need not be reinoculated. However, if both bud and leaf-piece grafts are dead, or if three or more of the five to six leaf-disc grafts are dead, then plants should be reinoculated or new plants inoculated.

Post-inoculation plant care. The developing young side shoots on the Mexican lime seedlings should not be trimmed for the first three flushes of growth, or for approximately eight weeks, in order to obtain the maximum number of leaves to examine for vein clearing. Occasionally only a bottom shoot will have leaves with symptoms. However, after the third growth flush, the side shoots should be trimmed and the most vigorous terminal shoot tied to a stake and trained to grow as a single shoot for later examination for stem pitting (Cachexia Figure 48). If not trimmed, Mexican lime seedlings have a tendency to develop as multiple shoots and, when grown three to a container, can quickly overcrowd the container and bench. Single shoots permit more light, thicker stem growth and better pesticide spray coverage. Trimmed plants are also easier to handle, and a single vigorous shoot produces a large single stem that can be readily peeled for critical observation of stem pitting.

Grapefruit, sour orange and sweet orange index seedlings used for seedling-yellows index tests should be trained as a single leader or shoot, starting with the first dominant emerging shoot. Seedlings of these varieties have a natural tendency to develop as single shoots, and little trimming is needed. Supplemental lighting during the winter will significantly aid the growth of lime and sour orange seedlings but may not benefit grapefruit seedling growth much.

Temperature requirements. Cool temperatures are necessary for maximum tristeza symptom expression in plants. Warm temperatures above 35°C may suppress development of vein-clearing and stem-pitting symptoms in Mexican lime seedlings (Roistacher et al., 1974). The preferred greenhouse temperatures for all tristeza and seedling-yellows indexing are 24-28°C maximum during the day and 17-21°C minimum at night. Temperature control is especially important when checking for mild isolates.

Time of symptom development. Figure 2 shows the time in weeks for the first appearance of vein-clearing symptoms in the leaves of 355 tristeza-inoculated Mexican lime seedlings. Plants were inoculated with a number of tristeza isolates over a seven-year period at Riverside, California. Within the first five weeks, 88 percent of inoculated plants developed leaf symptoms, and within eight weeks 97 percent of plants were diagnosed as positive. Figure 3 shows the time for the first appearance of seedling-yellows symptoms in grapefruit, sour orange and lemon seedlings. Over 90 percent of seedlings developed seedling-yellows symptoms within nine weeks, and some showed symptoms after as little as five weeks. This is based on observation of 1200 positive control plants inoculated with seven selected severe isolates over a five-year period.

Symptoms of tristeza

Vein clearing. The primary symptom in both the young and mature leaves of the Mexican lime is intermittent translucent vein clearing (Figures 6a, 6b and 7). The symptoms are best observed if the leaf is held overhead so that sunlight shines directly through the brighty exposed leaf. For critical reading of mild leaf-flecking symptoms, plants may have to be taken out into direct sunlight if the greenhouse is shaded.
Vein-clearing symptoms in leaves of Mexican limes can be readily detected in plants inoculated with most CTV isolates. However, vein-clearing symptoms induced by some mild-reacting isolates may be difficult to see. Only a few leaves may show an occasional mild fleck in the vein (Figure 6b). A non-inoculated control seedling in each container is most helpful in judging the vein-clearing symptoms in the leaves of inoculated plants. Mild vein-clearing symptoms do not usually persist in mature leaves. Plants should be observed frequently when new flushes are developing. The optimum period for observation is just as a leaf becomes fully expanded.

If the underside of leaves of lime or sweet orange is observed by reflected light, the veins will usually show distinct dark greenish-black, “water-soaked” areas (Figure 6c). These same areas, when viewed through sunlight, will show strong vein clearing. These “water-soaked” areas may persist in mature leaves after vein clearing is masked.

Leaf cupping. Figure 7 shows typical leaf cupping associated with tristeza in Mexican lime. Leaf-cupping symptoms are usually pronounced when plants are grown at cool temperatures and under good growth conditions, but they may not always be present. However, leafcupping is also a symptom induced in leaves of Mexican lime seedlings by the vein-enation virus in the complete absence of tristeza and, therefore, in the absence of other symptoms, leaf cupping alone may not be diagnostic for tristeza. Leaf cupping usually remains after the leaf matures or hardens. It may be pronounced when plants are infected with severe CTV isolates.

Vein corking. Very severe isolates of seedling-yellows tristeza may induce a corking on the veins of Mexican limes, sweet orange or grapefruit very similar to symptoms induced by boron deficiency. The vein corking can be mild, or severe as shown in Figure 8.

**Stem pitting.** For most CTV isolates, pitting can be observed after about eight weeks by peeling back the bark and observing the peeled stem. However, pitting is best evaluated at the end of the index test about four to six months after inoculation when the bark of the large single shoots can be completely peeled and the stems carefully observed. If the bark does not peel readily, steaming the stems in an autoclave is helpful for loosening tight bark.

Most tristeza isolates, except perhaps the very mildest, induce pitting in Mexican lime seedlings. Pits may be few (Figure 9a) or numerous (Figure 9b). The pitting symptom in the stems of Mexican lime or any other seedling indicator is highly diagnostic for tristeza. Observations of many thousands of tristeza-infected Mexican lime seedlings have indicated that stem pitting is strongly associated with vein clearing in the leaves.

Observation of stem pitting is very useful to confirm diagnosis when conditions are not optimum for leaf symptoms.

**Symptoms of seedling-yellows tristeza.** When three plants of grapefruit, sour orange or sour lemon are grown in an individual container and two are infected with seedling yellows, the symptoms are clear and dramatic as shown in Figures 10 and 11. Stunting may be severe, moderate or mild. The leaves are usually smaller, chlorotic, sometimes yellow, and may have pointed tips (Figure 12), and the shoots are compressed, thus giving the plant a stunted appearance.

**Symptoms of stem-pitting tristeza.** Figures 13a to 13d show the stem-pitting reaction in stems of grapefruit, sour orange, sweet orange and rough lemon, respectively.
Almost any seedling of any variety can be pitted by some specific tristeza isolate. Most CTV isolates, however, do not cause pitting in mandarin, sour orange, sour lemon, rough lemon or sweet orange seedlings or trees. Stem pitting can be very severe in limes, Citrus macrophylla, grapefruit, grapefruit hybrids, tangelos and certain pummelo cultivars, and may limit their use where severe isolates are endemic. Certain CTV isolates may severely pit sweet orange. The Pera orange of Brazil is particularly susceptible, and a severe stem-pitting isolate affects navel orange trees in Peru.

When grapefruit seedlings infected with seedling-yellows tristeza show severe stunting, as in Figure 10, pitting may be difficult to evaluate since infected seedlings are too small and stems too thin. Inoculations into larger seedlings may be necessary to force vigorous shoots if pitting is to be seen and judged. Some isolates of CTV will induce pitting without inducing stunting or yellows reaction in grapefruit or sweet orange (Roistacher, Dodds and Bash, 1988).

Occasionally severe CTV isolates may induce a thick “cheesy” bark, usually associated with many very fine pits. This symptom is highly diagnostic both in field trees and in inoculated plants.

**Termination**

*Tristeza reaction in Mexican lime.* When shoots reach about 1 m (approximately four to six months from inoculation), one or two of the mild-inoculated positive control plants should be harvested, the bark peeled and the peeled stems critically observed for pits. If pitting is evident in the control plants which had been inoculated with the very mildest-positive isolates, and if vein-clearing symptoms were observed and recorded in past readings in these mild-positive controls, then all plants can be harvested, peeled and examined for pits. Pitting can be recorded as none, mild, moderate or severe on a scale of 0 to 3 or 0 to 5. If no pitting is found in the mild-positive controls, the plants should be cut back, new shoots forced and the evaluation procedure repeated.

If no pits are seen in the mild-positive control plants, ELISA should be used to verify presence or absence of CTV. A modified procedure has been incorporated into the Citrus Clonal Protection Program at Riverside, California, which combines both the plant index and ELISA. "Buds" from budsticks collected from foundation block trees are first inoculated into Mexican lime seedlings.

After about eight weeks at optimum temperatures, the lime plants are trimmed to single shoots, with the exception of a side shoot which is allowed to develop. When this side shoot reaches a length of about 20 to 25 cm, it is harvested, and the bark peeled and processed for ELISA. In this manner, the tissue used for the ELISA has been grown under optimum temperature conditions in the greenhouse, and the low titre problem associated with seasonal temperature variation (Dodds *et al.*, 1987) is circumvented. This combined procedure of plant index and ELISA uses the best of both index tests to assure freedom from tristeza in prime budwood source plants.

The T-5 19 tristeza isolate, which showed little or no vein clearing or stem pitting, has consistently indexed strongly positive by ELISA.

*Seedling yellows and stem pitting.* When three plants are grown per container, most symptoms of seedling yellows can be seen fairly dramatically within ten weeks after inoculation, as indicated in Figure 3. However, the seven isolates used in this study represent selected severe-reacting seedling-yellows. Milder-reacting isolates would take somewhat longer to induce reactions. Also, as mentioned before, some stem-pitting isolates may show no yellows
reaction in grapefruit or sweet orange but may show severe pitting. Therefore, all grapefruit or sweet orange plants should be maintained until they reach about 1 m in height before harvest. When possible, known positive stem-pitting controls should be used.

The sour orange used for seedling-yellows indexing rarely shows stem pitting and need not be peeled in routine indexing. The index test can be terminated when the milder-reacting seedling-yellows positive controls show definitive symptoms. This may not be until three to four months after inoculation.

Method 3: ELISA
The use of ELISA for detection and diagnosis of tristeza is now a well-tested and proven technique and should be incorporated into any indexing programme.

It is an excellent technique for surveys and large-scale testing, for obtaining very rapid results and for verifying the presence or absence of CTV isolates that are mild reacting in indicator plants. However, at present it cannot be used to distinguish between various isolates of tristeza, nor should it be relied upon as the sole index for testing important foundation or primary budwood sources. Practical details and procedures are set out in Part III.

New methods of identifying CTV strains or isolates by use of differential hybridization techniques (Rosner, Lee and Bar-Joseph, 1986) may, in the future, provide rapid identification of certain seedling-yellows and stem-pitting isolates, which currently can be distinguished only by long-term plant index.

Method 4: microscopic detection of inclusion bodies
Inclusion bodies of CTV may be seen in sectioned tissue by light microscopy. This can permit a very rapid means of tristeza identification. Details for sectioning fresh or fixed citrus tissue for detection of CTV are given in Part III, and have been reviewed by Garnsey et al. (1980).

Miscellaneous

CTV detection by dsRNA analysis. A specific band in stained electrophoretic gel is diagnostic for tristeza (Dodds, Tamaki and Roistacher, 1983), and there is evidence that certain seedling-yellows isolates are distinguished from tristeza isolates by specific band migration locations. Details are given in Part III.

Detection of CTV by electron microscopy. CTV virus particles can be detected and identified within minutes by dicing small quantities of tissue in a specific buffer, placing it on grids and observing it in an electron microscope. This is the most rapid method of detecting and diagnosing CTV (Garnsey et al., 1980).

TRISTEZA DETECTION
Summary
Graft transmission to Mexican lime
Indicator: Mexican lime.
No. plants/test: 3 (plus 1 control in each of 2 containers).
Inoculum: “Buds”, leaf pieces, or leaf discs.
Plant growth: Allow all shoots to develop for the first three growth flushes, then prune and train as a single shoot.
Temperature: Cool: 24-27°C max. day/18-21 °C min. night.
First symptoms: 3 to 5 weeks (end of first or second growth flush).
SEEDLING YELLOWS DETECTION

Summary
Graft transmission to seedling indicators

Indicators:
Seedlings of grapefruit, sour orange.

No. plants/test:
2 (plus 1 control in each of 2 containers).

Temperature:
Cool: 24-27°C max. day/18-21°C min. night.

Plant growth:
Train as a single shoot.

First symptoms:
Within 10 weeks.

Symptoms:
Small yellow leaves, compact growth, stunted plants. Distinctly smaller and more compact than control.

STEM-PITTING DETECTION

Summary
Indicators:
Seedlings of grapefruit, and Madame Vinous or Pera sweet orange.

No. plants/test:
2 to 4 (grown 1 per container).

Temperature:
Cool: 24-27°C max. day/18-21°C min. night.

Plant growth:
Train as a single shoot.

First symptoms:
Within 4 months.

Symptoms:
Pits in grapefruit stems can be very severe. Less frequent in sweet orange stems, but pitting can be very severe with certain isolates. Thick, cheesy bark evident with some isolates in grapefruit. Seedlings of grapefruit or sweet orange may or may not show seedling yellows.

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Roistacher, C.N., Dodds, J.A. & Bash, J.A. 1988. Cross-protection against citrus tristeza seedling yellows (CTV-SY) and stem pitting (CTV-SP) viruses by protective isolates developed in greenhouse plants. In *Proc. 10th Conf. IOCV*, p. 91-100. Riverside, IOCV.


FIGURE 1
A comparison of sour orange and Eureka lemon for their reaction to seven severe seedling-yellows isolates in experiments carried out over a five year period. Note the similarity of reaction of sour orange and Eureka lemon to all seven isolates. Since sour orange seedlings are highly nucellar and uniform, and uniform nucellar seedlings of lemon are difficult to obtain, the sour orange is the preferred indicator.
FIGURE 2
The time in weeks after inoculation for the first vein-clearing symptoms to appear in leaves of Mexican lime seedlings. Graphs are based on eight tristeza isolates inoculated into 355 seedlings recorded over a seven year period. Most isolates induced vein clearing within eight weeks.
FIGURE 3
The time in weeks after inoculation for the first symptoms of seedling yellows to appear in grapefruit, sour orange and Eureka lemon. Graphs are based on seven severe seedling-yellows isolates inoculated into 1200 seedlings and recorded over a five-year period.
FIGURE 4
Quick decline of a sweet orange on sour orange rootstock caused by citrus tristeza virus (Israel). The sudden collapse and death of sweet orange, mandarin or grapefruit on sour orange rootstock is an indication of tristeza.

FIGURE 5
A section of bark cut through the bud-union of a sweet orange on sour orange rootstock showing inverse pitting in the bark of the orange. Note the small, closely spaced pits in the inner bark. The sour orange trunk would show corresponding small pegs.
FIGURE 6a
Vein-clearing symptoms in the leaf of a Mexican lime seedling (right) as viewed from the back of the leaf into direct sunlight. Control on the left.

FIGURE 6b
Mild vein-clearing flecks induced in a leaf of Mexican lime by a mild-reacting tristeza isolate.

FIGURE 6c
Symptoms due to tristeza virus, viewed on the underside of a leaf of sweet orange by reflected light. Symptoms are the dark-green-to-black broken lines in the leaf veins. These are the same areas that show up as translucent when viewed from the underside of the leaf in transmitted direct sunlight.
A handbook for detection and diagnosis of graft-transmissible diseases of citrus

FIGURE 7
Cupping on leaves of Mexican limes inoculated with tristeza virus. Note also vein clearing.

FIGURE 8
Vein-corking symptoms on leaves of Mexican lime seedlings inoculated with a very severe seedling-yellows tristeza isolate.

FIGURE 9a
A mild pit in the peeled stem of a Mexican lime seedling induced by the mild-reacting T-519 CTV isolate.

FIGURE 9b
Severe pitting in the peeled stems of Mexican lime seedlings. Most tristeza isolates will induce this type of pitting.
FIGURE 10
Seedling-yellow reaction in two Duncan grapefruit seedlings with a non-inoculated control in the centre. Note the severe stunting in the two inoculated seedlings.

FIGURE 11
Seedling-yellows reaction in two standard sour orange seedlings with a non-inoculated control in the centre. Note the stunting and yellows of the two inoculated seedlings.

FIGURE 12
Close-up of seedling-yellows reaction in a sour orange seedling showing smaller pointed leaves and yellows reaction. Control plant on left.
FIGURE 13
Stem pitting in the peeled stem of (a) grapefruit, (b) sour orange, (c) sweet orange and (d) rough lemon. Normally the sour orange, sweet lemon and rough lemon are resistant to stem pitting by many isolates of tristeza. However, this illustrates that there are tristeza isolates
DESCRIPTION AND BACKGROUND
Greening is probably the most serious and devastating of all the diseases affecting citrus. It is characterized by a chlorosis of leaves on one or more limbs, followed by twig dieback, sparse foliage, distinct yellow shoots, and fruits which do not fully colour at the stylar end and remain green; hence the name greening (Figures 14 and 15). Later the trees may show an open, sparse foliage, severe fruit drop and many small yellow shoots; in many cases the result is severe decline and death. For a detailed illustrated description of the disease, see Schwarz and Bové (1980).

The disease is caused by a gracilicute-like gram negative bacterium. There are two general types of greening: Asian and African. Asian greening has been reported as huang longbing or yellow shoot in mainland China, likubin in Taiwan Province, leaf mottle in the Philippine Islands, vein-phloem degeneration in Indonesia and citrus decline in India. They are probably all related, with origins in China. All are vector-transmitted by the psyllid Diaphorina citri. Mention was first made of yellow shoot in Chaoshan district of Fujian Province, China, in the late 1800, and it became epiphytotic in the mid-1920s. Also, the first evidence of transmission by grafting was shown in China by Lin in the late 1940s (Zhao, 1981).

African greening is a cooler-temperature disease, and its associated organism does not tolerate very hot temperatures. It was first reported from South Africa in 1929 (Oberholzer, Von Standen and Basson, 1965) as yellow branch disease. The vector associated with it is Trioza erytreae.

Greening has been reported from most countries of Central and southern Africa, Madagascar, Reunion Island, Asia, Taiwan Province, the Philippine Islands and Indonesia. It has recently been reported in the Arabian peninsula and in western Pakistan near the Afghan border. There is a distinct threat that this disease and its vectors may invade the Mediterranean region and, with its potential for destruction of citrus, this would be a most serious problem.

All species of citrus appear to be susceptible, but sweet orange, mandarin and tangelo are most affected. Laflèche and Bové (1970) reported a bacterium found in the sieve tubes of diseased trees associated with the disease. For a description of the organism see Garnier, Latrille and Bové (1976), Garnier and Bové (1983) and Garnier, Martin-Gros and Bové (1987).

Greening disease is probably not seed-transmitted and is primarily a vector-transmitted disease. Exclusion of the organism and the vectors by rigid quarantine measures is the only way of keeping this disease from infesting new areas. Aubert and Quilici (1984) achieved excellent biological control of the greening vectors by introduction of two ectoparasites into Reunion Island and virtually eliminated the vectors, thus controlling the disease.

METHODS OF DETECTION
The field symptoms of greening are usually pronounced and striking and, when combined with presence of the vector, they may be sufficient to be diagnostic. However, verification is important, especially when the disease is suspected in a new area or country. Severe stem-pitting isolates of CTV can cause “canopy” symptoms similar to greening in some cultivars.
Greening does not, however, cause the stem pitting associated with CTV. Also, specific detection procedures are needed for positive diagnosis if the organism is to be studied, or for vector transmission studies. With the exception of the fruit albedo fluorescence test or the direct observation of the organism under the electron microscope, there has been no quick or rapid index or test for accurate diagnosis. However, with the recent possible culturing of the greening organism (Garnett, 1984/85) and production of monoclonal antibodies against the bacteria-like organisms associated with the disease (Garnier et al., 1987), there are good possibilities for more rapid detection of the organism.

Although the name “greening” is used as a general term, there may be differences in symptomatology, vector transmissibility, temperature requirements and other factors among the various greening-disease isolates in different countries or locations within a country.

Method 1: Field diagnosis
As mentioned, and illustrated in Figures 14 and 15, field diagnosis is an important means of identifying greening in most areas of the world. The yellow shoots, yellow veins, mottle and zinc-like deficiency patterns in leaves are partially diagnostic. However, when combined with greening of the fruit and presence of the vector, they are highly diagnostic. Figure 14a shows a typical tree in the field affected with Asian greening. Figure 14b shows emerging yellow shoots; hence the name “yellow shoot” given to the disease in mainland China. Figure 14c shows leaves with the characteristic chlorotic leaf mottle and yellowing of midribs and veins. Figure 14d shows a single leaf with striking vein yellowing, sometimes typical of greening. Typical fruit symptoms showing stylar-end greening are shown in Figure 15. The field symptoms can be highly diagnostic, i.e. severe decline associated with yellow shoots, zinc-like leaf deficiency patterns, severe fruit drop, lopsided fruit showing early and persistent greening at the stylar end. Some of these symptoms are very similar to those of stubborn disease. In chronic stages greening-affected trees are chlorotic, show extensive dieback and are non-productive.

Figure 16 shows a drawing of the psyllids *D. citri* and *T. erytreae* with their eggs and five nymphal instar stages (Catling, 1970). The angled appearance of *D. citri* feeding on citrus leaves is illustrated in Figure 17. Figure 18 shows the very characteristic bumps on the underside of the citrus leaves where *T. erytreae* has been feeding. The mottle leaf found in sweet orange or grapefruit, as illustrated in Figures 14c and 14d, is strongly associated with both greening and stubborn diseases and should be looked for if greening is suspected.

If severe isolates of tristeza are present or nutrition is poor, field diagnosis may be difficult. This is true for the citrus-decline problem found in many parts of India, Sri Lanka and elsewhere.

Method 2: Graft transmission to indicator plants
Graft transmission of greening disease is variable. Factors that may affect the success of graft transmission are: the kind of tissue used, i.e. buds, side grafts or leaf pieces; the age of the tissue, i.e. young or mature; the indicators; and the season of the year inoculum is collected. In general, African greening appears to be more difficult to graft-transmit than Asian greening.

When tristeza is present, it is sometimes difficult to obtain good symptom expression in indicator plants due to interference. Ponkan or other mandarins may be used as indicator seedlings as a differential host to distinguish tristeza from greening, i.e. tristeza will not react in mandarin but greening will. Tristeza can be
bypassed by using trifoliate orange as a filtering host. This is done by first inoculating the infected tissue into a trifoliate seedling, cutting the seedling back and forcing new growth. When the new growth tissue matures, it can be used as inoculum and should be free of CTV.

**Inoculum tissue.** Side grafts and leaf-piece grafts are superior to bud or bark grafts for transmission and are recommended. Budwood should be collected during the cooler periods of the year. Ten budsticks and/or six young shoots with small emerging leaves are collected from around each test tree, put in plastic bags and transferred immediately to an ice chest.

**Inoculation**

**Side grafting.** The technique of side grafting is described and illustrated in Part II. Briefly, two side grafts are put into each seedling: each piece of graft tissue consists of part of a branch approximately 4-5 mm thick and 3-5 cm long. A wedge cut is made at one end of the budstick, a cut made in the seedling, and the wedge fitted into the cut. The side grafts are then securely wrapped with polythene budding tape, and a sleeve cut from a polythene bag is placed over the area above and below the grafts to create a moist chamber (Figure 138 in Part II, and also Figure 26 in Stubborn).

**Leaf grafting.** The technique of leaf-piece grafting is described and illustrated in Part II (Figures 129 and 130). A small rectangular section of leaf about 3 by 12 mm is cut from the midrib area of a young, succulent leaf and placed into a T-cut in the bark of the seedling, as for standard bud-grafting. The area is then securely wrapped with polythene tape in the same manner as with buds. Two to three leaf grafts per plant are suggested.

**Indicator plants.** Recommended indicator plants are seedlings of sweet orange and Orlando tangelo for African greening, and sweet orange or Ponkan mandarin for Asian greening. Grapefruit seedlings can also be used in the absence of severe tristeza isolates. Seedlings should be grown one per container as a single shoot to about 1 m, with a thickness of 5 to 7 mm. A minimum of five plants should be inoculated to index a given source tree; each plant may be inoculated with two side grafts, two to three leaf-piece grafts, or a side graft and two leaf-piece grafts.

Having healthy, vigorous indicator test plants free of micronutrient or other deficiency symptoms is of extreme importance for diagnosing greening disease.

**Controls.** Positive and negative control plants are essential in any indexing procedure. If possible, it would be helpful to have known positive greening-infected control plants maintained in the greenhouse, and tissue can be collected from these plants when needed for control purposes. Negative or self-inoculated control plants should always be included in each index test.

**Inoculum survival and post inoculation care**

**Side grafts.** At ten days to two weeks after inoculation, the bottom ends of the polythene sleeves are opened to permit partial drying around the side grafts. After three weeks, the polythene sleeves are removed and grafts observed for survival. The plants are then cut back to about 25 cm from the soil surface. One terminal shoot is then permitted to grow and it is trained and staked to grow as a single leader, as in Cachexia Figures 47 and 48.

**Leaf grafts.** The wrapping tape surrounding the leaf graft is cut two to three weeks after inoculation, and inoculum survival recorded. Plants are then cut back, and new growth trained
to a single shoot as for side grafting. The leaf-piece can be seen to grow within the T-cut of the grafted seedling (Figure 13 in Part II).

**Temperature requirements.** Hold indicator plants at 20-25°C for African greening and 25-32°C for Asian greening.

**Symptoms.** Symptoms in sweet orange, tangelo or mandarin will be a typical leaf mottle and chlorosis similar to that shown in Figures 14c and 14d and Stubborn Figures 23 and 29a. The shoots will be distinctly smaller, more chlorotic and with smaller leaves when compared with those of the non-inoculated or self-inoculated controls. Symptoms should appear with the first emerging shoots within eight to 12 weeks, or earlier if healthy, vigorous plants are used. Schwarz (1972) found that some strains transmitted at very low percentages. Many workers report variable transmission at different seasons, and Asian greening appears to be more readily graft-transmitted than African greening. Graft transmission by procaryotic pathogens is not so uniformly successful as with viruses or viroids, and failure to transmit by grafting does not imply absence of the disease. Other diagnostic methods should be tried.

**Method 3: Direct observation of the pathogen by sectioning and electron microscopy**

Lafléche and Bové (1970) first reported “mycoplasma”-like bodies in citrus sieve tubes. Chen, Miyakawa and Matsui (1971) observed mycoplasma-like bodies in the leaf phloem tissue from likubin-infected trees under the electron microscope. Garnier and Bové (1983) later showed these bodies to be gracilicute bacteria (Figures 19 and 20). Direct observation of the greening organism in the phloem tissue is highly diagnostic if the typical wall structure showing three layers of about 250 Å thick is present (Figure 19). This would be an excellent confirmation of the distinct visual field or greenhouse-induced symptoms associated with greening.

When collections made in the field are to be taken or shipped a long distance to a laboratory, the following technique, given by Bové and Garnier (1984), is suggested:

- Leaves and fruit are collected from suspect trees, put in a polythene bag and placed in an ice chest until they can be properly processed for shipment.
- Leaf midrib tissue is cut with a razor-blade and chopped or diced into 2-4 mm pieces. Midrib pieces from five to ten leaves are put together for each tree tested.
- The pieces are placed in a 5 ml screw-top tube filled with 2 percent glutaraldehyde in a 0.1M cacodylate (phosphate) buffer pH 7.4.
- Samples can be shipped and will keep for two weeks or longer in good condition.

Bové and Garnier (1984) report that the peduncular end of the fruit axis is rich in phloem tissue. This columella tissue can be chopped into 2-4 mm pieces and fixed in the same manner as above for shipment.

The technique used by Garnier and Bové (1983) for direct observation of the organism is as follows:

- pieces of leaf midrib tissue from leaves showing typical mottle symptoms of greening are cut or diced into 1 mm pieces, using a razor-blade;
- they are fixed in 4 percent glutaraldehyde in a 0.1M cacodylate (phosphate) buffer pH 7.5 for 6 hours;
- rinsed three times in the same buffer;
- post-fixed with 1 percent osmium tetroxide (OsO₄) in the same buffer;
- dehydrated in alcohol;
- embedded in Epon 812;
• thin-sectioned with an ultramicrotome;
• stained with lead citrate and observed in the electron microscope.

Detection of the greening organism by microscopic examination may be difficult and requires expertise and experience. It is important to look at healthy controls, and helpful to examine known positive sources. If the organism is found, it is good confirmation of symptom diagnosis. However, if not found, this does not mean it is not present, and its absence cannot be taken as negative in certification programme diagnosis.

Method 4: Detection of greening by fluorescence
Schwarz (1968) demonstrated that a component (a gentisic glucoside) present in greening-affected fruit of certain varieties could be detected by examining fruit or chromatographed extracts with ultraviolet light. This test was used for diagnosis of greening throughout Africa and Asia. The fruit albedo test is effective for sweet orange, but not so effective for mandarin or tangelo. The bark extract test is effective for sweet orange, mandarin and tangelo, but not definite for lemon, lime and pummelo. The test can be done at all seasons of the year, and is recommended for surveying and rapid confirmation of field symptoms but not recommended for certification work. The following description of the techniques is taken from Schwarz (1976):

Fruit fluorescence. The fruit of sweet orange collected from a suspect tree is halved and examined under an ultraviolet lamp (360 nm wave-length). It is important that the ultraviolet light be pure (no more than 5 percent visible light). Fluorescence will appear in fruit taken from trees affected with greening but not other pathogens, i.e. psorosis, tristeza, exocortis or cachexia.

This is a simple and effective test if sweet orange is the primary crop and if fruit is available for testing.

Bark fluorescence
• Collect three twigs 4 cm long from second- or third-year branches from different sectors of the tree.
• Strip the bark and cut or dice it into pieces 2-3 mm wide using a razor-blade. Place the diced pieces in a small vial with 5 ml water and shake for half an hour.
• Decant water extract to a small, round plastic disc fitted into a watchglass and dry in an incubator at 50°C.
• Take up the concentrate with a few drops of water and spot in quantities of x, 2x, 3x, and 4x on silica-gel TCL plates that have been pre-activated by heat at 110°C for half an hour.
• Develop plates with a chloroform:methanol mixture (9:1) and dry.
• After drying, spray plates with saturated aqueous N-borate solution and inspect under a 360-366 nm ultraviolet lamp. (The lamp must emit nearly pure [>95 percent] UV light.)

The samples from greening-affected trees show a bright purple spot at an Rf of 0.5 to 1.0 just next to the spotting point. It is important to run positive controls with every 50 or fewer samples. Depending on the concentration of the marker substance in the sample, the spot is more visible in the profile of either the low concentration (x) or the high concentration (4x).

Citrus species and varieties differ in fluorescent non-marker spots. Occasionally the sample may contain a fluorescent non-marker that interferes with the reading of the marker. In such cases, the mobile phase can be modified by increasing the methanol content from 10 to 20 percent and decreasing the chloroform concentration. This
increases the Rf of the gentisic glucoside marker and may improve readings.

**MISCELLANEOUS**

**Vector transmission**

Use of the insect vector for transmission is an excellent method for separating the greening organism from other pathogens. It should be used only if vectors are present. The definitive studies by Capoor, Rao and Viswanath (1974) should be reviewed by anyone using this technique. The following is a summary of the important conclusions reached by these workers:

- Transmission will occur only with the fourth and fifth instar or with the adult of *D. citri*. There is no transmission by the first, second or third instars.
- A 30-minute acquisition feeding period is sufficient.
- A waiting period of eight to 12 days is required before the psyllid can transmit the pathogen.
- Psyllids retain infectivity throughout their life span.
- The greening organism will not go through the egg.
- The greening organism apparently multiplies in the psyllid.

There has been no definitive study on transmission by *T. erytreae*. McClean and Oberholzer (1965) found no transmission using nymphs, and approximately 50 percent transmission when large numbers of *Trioza* (over a thousand) were used.

The following general procedure is suggested for vector transmission as a means of testing for presence of Asian greening, or separating the greening pathogen from other pathogens, and is based on the method of Capoor *et al.* (1974) for *D. citri*. It is similar to the technique illustrated for aphid transmission of tristeza (Roistacher, 1981).

**Psyllids**

For the testing of field trees, transfer 50 to 150 existing psyllids found feeding on suspect trees to indicator plants, or use artificially reared, newly emerged adults, and put them on symptomatic leaves of field trees for at least two weeks for their acquisition feeding and waiting period.

**Acquisition feeding**

Insects may be collected directly from symptomatic leaves of suspect field trees by cutting a branch containing feeding psyllids on suspect leaves and placing the cut end of the branch in water. This is then transported to the plant laboratory. Psyllids reared separately may be transferred to suspect field trees by tying young leaves containing abundant psyllids next to symptomatic leaves. Place a cage over the feeding area containing the leaves and insects and allow at least two weeks of field feeding. As the leaves of the source plant dry up, the psyllids will migrate and feed on the leaves of the suspect tree.

**Infection feeding**

After acquisition feeding, transfer the leaves containing the feeding psyllids (step 2) by tying them to the young leaves of sweet orange or Mexican lime indicator seedlings. Ensure there are at least 50 to 100 insects present. Allow an infection-feeding period of 24 hours at 20-24°C.

**Completion**

After the 24-hour infection-feeding period, spray leaves with malathion or some other pesticide to kill the insects. Place the inoculated plants in a controlled environment for symptom development (24-32°C for Asian greening).

**Controls**

Negative control plants should be exposed to
non-infective psyllids raised on healthy citrus. A few non-exposed healthy indicator plants should also be used. If available, greenhouse-grown infected plants should be used as positive controls. Psyllids should be fed on these infected plants and transferred to test seedlings in the same manner as described for testing field trees. This work should be done in a plant laboratory with excellent insect control.

GREENING DISEASE DETECTION

Summary
Graft transmission
Indicator:
Sweet orange, Orlando tangelo or Ponkan mandarin.
No. of plants/test:
5 seedlings (grown 1 per container plus controls).
Inoculum:
Side grafts or leaf pieces.
Plant growth:
Single shoot.
Temperature:
First symptoms:
Eight to 12 weeks or earlier under ideal conditions.
Symptoms:
Leaf mottle and chlorosis. Smaller shoots and leaves. Distinct and characteristic mottle in absence of tristeza or other nutrient-deficiency symptoms.

REFERENCES


Techniques for biological detection of specific citrus graft-transmissible diseases


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Fig 14a
A typical greening-affected mandarin tree in the field (New Territories, Hong Kong)

Fig 14b
Emerging, stunting yellow shoots showing severe chlorosis, typical of greening infection. Yellow shoot or huang-longbing is the name given to this disease in mainland China (Taiwan Province)

Fig 14c
A characteristic greening-affected branch showing leaves with chlorosis, mottle and yellow midribs and veins (South Africa)

Fig 14d
Close-up of a leaf from a greening affected tree showing vein yellowing and mottle (South Africa)
Fig 15
Typical greening of fruit. Two normal Valencia oranges surrounded by greening-affected fruit (South African). Note the stylar end greening and smaller fruit.

Fig 16
Drawing of adults and instars of *Diaphorina citri* and *Trioza erytreae* (Source: Catling, 1970)
Fig 17
*Diaphorina citri* feeding on a young shoot and leaves of a citrus tree. Note the angular position taken by the insect when feeding (Pakistan) (Photo: L.C. Cochran)

Fig 18
Characteristic bumps on the underside of sweet orange leaves caused by the feeding of *Trioza erytreae* (South Africa)

Fig 19
Gracilicute-like bacteria found in cells of a greening-affected citrus leaf. Note trilayered wall (China) (Photo: Ke-Chung)

Fig 20
Abundant greening organisms in the haemolymph of *Trioza erytreae* (Photo: J. Moll)
DESCRIPTION AND BACKGROUND

Stubborn is found in most countries that grow citrus under desert or semi-arid conditions. It is destructive in the warmer areas of California and Arizona and in most countries of North Africa, the Near East and the Arabian peninsula. Stubborn has been reported in Turkey, Greece, Italy, Mexico, Spain, the Sudan and Pakistan. Stubborn disease is rare in cooler climates since both the vector and organism prefer hot temperatures. It is not found in warm subtropical areas, presumably because of lack of suitable vectors.

This disease was first noticed about 1915 in navel orange trees near Redlands, California, and named “stubborn” by E.R. Waite, a budder. J.C. Perry observed that buds refused to grow properly, and “some influence was transmitted to good buds that were used in topworking” (Fawcett, Perry and Johnson, 1944). The name “acorn disease” was also used to describe the disease because of the many acorn-shaped fruit on diseased trees. A similar disease called “little leaf” was reported in Palestine by Reichert (1930), who illustrated the small shoot and leaf condition as well as the small and misshapen fruit. Fawcett et al. (1944) first showed the transmissible nature of stubborn disease. For a review of stubborn disease, see Gumpf and Calavan (1981), and for an illustrated description and background see Calavan (1980) and Wallace (1978).

A mycoplasma-like organism in the sieve tubes of stubborn-infected citrus tissue was discovered independently by Igwegbe and Calavan (1970) in California and by Laflèche and Bové (1970) in France. Both groups of workers concluded that a mycoplasma, and not a virus, was probably the cause. Fudl-Allah, Calavan and Igwegbe (1972) in California and Saglio et al. (1971) in France were able to culture a mycoplasma-like organism in liquid and solid media. The organism was described and named *Spiroplasma citri* (Saglio et al., 1973) thereby establishing a new genus of mollicute. Antisera have been prepared to cultured *S. citri* and used for detection in various assays, including ELISA.

*Spiroplasma citri*, the causal organism, is described and illustrated in detail by Bové (1980). It is a motile, helical mollicute with no cell wall and no peptidoglycan. The spiral or helical morphology and motility can be seen by phase-contrast or dark-field microscopy (Figure 25). For positive identification of the causal organism, the first test is to observe the motile spiral spiroplasmas in a drop taken from culture media and placed under a dark-field or phase-contrast microscope.

Transmission of *S. citri* in California is primarily by the beet leafhopper *Circulifer tenellus*, but also by *Scaphytopius nitridus* (Kaloostian et al., 1975, 1976). *S. citri* was shown to be spread from weed or vegetable hosts to a wide variety of weeds or vegetables by leafhoppers (Oldfield and Calavan, 1980). The weeds became infected, stunted and yellow, and when they dried up under warm or hot conditions the vectors containing *S. citri* moved from the weed hosts to citrus. Young citrus are more susceptible than older trees. Transmission is primarily from infected weeds to citrus and to a lesser degree from infected citrus to citrus. The leafhopper *Neoaliturus* (*Circulifer*) *haematoceps* is a vector of *S. citri*.
appears to be the primary vector in Corsica and in certain countries of the Near East (Bové et al., 1988).

METHODS OF DETECTION

Method 1: Field diagnosis
Stubborn-infected trees in the field appear compressed and stunted, sometimes severely so (Figure 21). At times only a portion of the tree is affected and branches show compressed growth, with smaller leaves similar to those in the young budded nursery tree in Figure 22. Leaves may show a chlorotic mottle (Figure 23), which is also characteristic of greening-infected trees. Stunted trees remain small; they rarely recover or die. The fruit does not colour properly and the stylar end retains its green colour (Figure 24a). The navel orange is the most susceptible to fruit greening. Stubborn infected fruit is usually small and distorted, and may also be acorn-shaped in appearance. Fruit of seedy varieties may have a number of considerably smaller, darker purple seeds or completely aborted seeds (Figure 24b). The fruit may have an insipid taste. The foregoing signs in combination are diagnostic for stubborn. Grapefruit, sweet orange (especially the navel orange), tangelo, mandarin, lime and pummelo are affected. Trifoliate and trifoliate hybrids, lemons and limes appear more tolerant.

During a period of intensive indexing of stubborn-infected trees in the Coachella and Central Valleys of California, many hundreds of index tests were made from symptomatic trees in the field to indicator plants in the greenhouse. The correlation of field symptoms with positive transmission was extraordinarily high, and much of the later diagnosis was made just by diagnosing symptomatic field trees (Calavan and Blue, unpublished).

However, where symptoms are found in new areas, either transmission tests to indicators or isolation and culturing of the organism should be carried out.

Method 2: Transmission to indicator plants

Inoculum tissue. The best tissues for transmitting the Spiroplasma from citrus to citrus are stem pieces 5-7 mm in diameter obtained from compressed and stunted branches, or from the very young leaves of a new flush of growth. Extensive indexing tests for stubborn and results of comparative tests indicate that best transmission is made with side grafts (Calavan, Roistacher and Christiansen, 1968). Calavan et al. (1968) also showed that the stubborn organism is poorly distributed in symptomatic trees and best index results were obtained with tissue collected during the spring months. Ten budsticks, and/or six young shoots with small emerging leaves, growing from compressed symptomatic twigs are collected per test tree. Budwood and/or young shoots are put in plastic bags and transferred immediately to an ice chest.

Inoculation

Side grafting. The technique of side grafting is described and illustrated in Part II. Briefly, two side grafts are put into each seedling; each piece of graft tissue consists of part of a branch approximately 4-5 mm thick and 3-5 cm long. A wedge cut is made at one end of the budstick, a cut made into the seedling, and the wedge fitted into the cut. The side grafts are then securely wrapped with polythene budding tape, and a sleeve cut from a polythene bag is placed over the area above and below the grafts to create a moist chamber (Figure 138 in Part II, and also Figure 26).

Leaf grafting. The technique of leaf-piece grafting is described and illustrated in Part II (Figures 129 to 131). A small rectangular section of leaf about 3 x 12 mm is cut from the midrib area of a young succulent leaf and placed into a
T-cut in the bark of the seedling, as for standard bud-grafting. The area is then securely wrapped with polythene tape in the same manner as with buds. Two to three leaf grafts per plant are suggested.

**Indicator plants.** The Madame Vinous sweet orange seedling is recommended as a superior indicator for detection of stubborn disease. One seedling should be grown per container and trained as a single shoot to about 1 m, with a thickness of 5-7 mm. A minimum of five plants should be inoculated to index a given source tree; each plant may be inoculated with two side grafts, two to three leaf-piece grafts, or a side graft and two leaf-piece grafts.

**Controls.** It is helpful to have a known positive stubborn source plant growing in a warm room in the greenhouse. Stubborn-infected Madame Vinous sweet orange source plants have been held in the warm room at the Rubidoux laboratory (32-38°C maximum day temperature) in Riverside, California, for over 15 years, and have continually shown stubborn symptoms. Both stem and leaf parts taken from these plants have consistently transmitted stubborn over this period. Negative or self-inoculated control plants should be included in each index test.

**Inoculum survival and post-inoculation care**

**Side grafts.** Ten days to two weeks after inoculation, the bottom ends of the polythene sleeves are opened to permit partial drying around the side grafts. At three weeks, the polythene sleeves are removed and grafts observed for survival. The plants are then cut back to about 25 cm from the soil surface. One terminal shoot is then permitted to grow (Figure 26), and it is trained and staked to grow as a single leader, as in Cachexia Figure 48.

**Leaf grafts.** The wrapping tape surrounding the leaf graft is cut two to three weeks after inoculation, and inoculum survival rate recorded. Plants are then cut back and new growth trained to a single shoot as for side grafting. The leaf piece can be seen to grow within the T-cut of the grafted seedling (see Figure 131 in Part II).

**Temperature requirements.** Development of symptoms requires warm or hot greenhouse temperatures. Temperatures should be maintained at 32-38°C maximum during the day and not below 27°C at night. However, care must be exercised not to exceed 40°C for any length of time. This will result in development of small, abnormal leaves.

**Symptoms.** The first symptoms are a semi-wilted appearance of the young single shoot and leaves (Figure 27). The leaves are smaller, paler and slower-growing than those of the controls. The stubborn-infected shoot remains small and stunted whereas the new-growth leaves of the control shoot are much larger and upright (Figure 29). Leaves of stubborn-infected Madame Vinous indicators will develop translucent chlorotic areas near the leaf margins, especially in the vicinity of the tips, giving them a pointed appearance. The chlorotic spots remain visible after the leaf matures (Figure 28). These leaf symptoms produced under warm greenhouse conditions are highly diagnostic for stubborn disease. As the plants continue to grow, negative control plants will develop normal vigorous shoots, but stubborn-infected plants grow very slowly and remain stunted and chlorotic, showing smaller leaves and closer internodes (Figure 29b). Leaves may turn yellow or develop strong, typical zinc-deficiency-like spotted mottle (Figures 27 and 29a). At times plants become severely stunted as in Figure 29a, or may show intermediate stunting with smaller leaves and closer internodes as shown in Figure 29b.
**Time for first symptoms and termination**
Definitive symptoms can be expected eight to 12 weeks after inoculation. Under optimal conditions, symptoms may appear earlier. If no symptoms occur in the inoculated plants within 12 weeks, but the positive control plants show good stubborn disease symptoms, the experiment can be terminated. Stubborn-infected Madame Vinous plants generally show symptoms with the first emerging shoot and will rarely show a delayed positive response in subsequent growth flushes, provided plant growth and temperature conditions are optimal.

**Method 3: Isolation and culturing of ** _Spiroplasma citri_ 
(Specific details are given in Part III)

**MISCELLANEOUS**

**Use of ELISA.** The ELISA technique has been successfully used for detection of stubborn, as reported by Saillard _et al._ (1980) and Bové _et al._ (1984). The latter showed that ELISA was as effective as culturing in detecting stubborn in Syria. Of 47 apparently stubborn-infected field trees tested, 38 were found positive by ELISA and 38 positive by culturing. When both methods were combined, 42/47 were positive. It is important to note that the trees in the field were highly symptomatic, and field observation is still an excellent means of diagnosis.

Studies by Lin and Chen (1985), using monoclonal antibodies specific for _S. citri_, have shown high specificity and ability to differentiate _Spiroplasma_ strains within the same species or sero-group. However, all of the monoclonal antibodies tested reacted with nine United States strains and one from Morocco but did not react with four strains: one from Iran, one from Algeria, and two from Israel.

Antiserum may be requested from INRA, near Bordeaux, France, or from Rutgers University, New Jersey.¹

**Immunofluorescence for detection of _S. citri_**
_S. citri_ can be detected in sections of infected tissue by fluorescent light microscopy. Rhodamine-labelled antibodies to _S. citri_ bind to antigen in the sections and are detected by use of appropriate fluorescent light source.

**STUBBORN DISEASE DETECTION**

**Summary**
Graft transmission

**Indicator:**
Madame Vinous sweet orange.

**No. of plants/test:**
5 seedlings (grown 1 per container plus controls).

**Inoculum:**
Side grafts or leaf pieces.

**Plant growth:**
Single shoot.

**Temperature:**
Warm to hot: 32-38°C max. day/27°C min. night.

**First symptoms:**
8 to 12 weeks.

**Symptoms:**
Stunted shoots with smaller internodes. Smaller leaves with translucent chlorotic spots near edges and tips.

**REFERENCES**


¹ Laboratoire de biologie cellulaire et moléculaire, INRA, 33140 Pont de la Maye, France, and Department of Plant Pathology, Cook College, Rutgers University, New Brunswick, NJ 08903, United States of America.


Reichert, I. 1930. Diseases, new to citrus, found in Palestine. *Phytopathol.*, 20: 999-1002.


Fig 21
The characteristically stunted and compressed appearance of a stubborn-infected navel orange tree. A non-infected normal tree of the same age is on the left (California).

Fig 22
A stubborn-infected young sweet orange tree in the nursery row (left) showing the small leaves and shorter internodes symptomatic for the disease in warm areas. Compare with the normal nursery tree on the right (California).

Fig 23
Chlorotic mosaic-like mottle in leaves from a stubborn-infected sweet orange tree. These are typical of leaves from stubborn or greening-infected trees.
Fig 24a
The stylar-end greening effect on fruit from a stubborn-infected navel orange tree in California. This symptom, induced by the presence of *Spiroplasma citri*, is indistinguishable from symptoms of fruit on greening-infected trees caused by Asian or South African greening (Figure 15).

Fig 24b
Seed abortion in a Valencia orange in California. The presence of small dark purple-coloured seed (right) is symptomatic for stubborn disease. Such seeds are excellent for culturing of *S. citri*. Normal seed from a normal fruit is on the left.

Fig 25
*S. citri* the causal organism of stubborn disease as seen in the dark-field microscope. Note the spiral-helical nature of the organism. Motility can be seen in the dark-field microscope (Photo: E.C. Calavan).
Techniques for biological detection of specific citrus graft-transmissible diseases

Fig 26
A seedling with two side grafts showing a single young shoot to be trained as a single leader. The side graft technique is very effective in transmitting stubborn disease.

Fig 27
The first symptoms of stubborn in a young shoot of an inoculated Madame Vinous sweet orange seedling. Note the slightly semi-wilted appearance of the young shoot and leaves.

Fig 28
Characteristic symptoms associated with infection by S. citri in leaves from a Madame Vinous sweet orange seedling showing pointed tips and translucent chlorotic spotting at the leaf margins and near the tips. These symptoms developed under warm temperatures and will persist in mature leaves. The control non-inoculated leaf is on the left.
Fig 29a
A stubborn-infected Madame Vinous seedling on the right and a control plant on the left. Note the extreme stunting and chlorosis induced by stubborn infection.

Fig 29b
Three stubborn-infected Madame Vinous plants on the left compared with three control plants on the right. Note the smaller leaves and compressed growth in the infected plants. Plants were inoculated by side grafting and held in a warm greenhouse.
DESCRIPTION AND BACKGROUND
Blight is a wilt disease primarily affecting oranges and grapefruit on various rootstocks, rough lemon being most susceptible. The disease was first described in Florida over 100 years ago. In the mid-1960s it became a very serious problem in young plantings on rough lemon and cитrange rootstocks in Florida. For a comprehensive historical background of this disease in Florida, see Smith (1974b) and Nemec (1985/86). Figure 30a shows a typical declining, blight-infected grapefruit tree in Florida, and Figure 30b shows a similar blight-infected Pera orange tree in Brazil.

The disease is also known as “sand hill decline”, “roadside decline” and “young tree decline”. Related diseases are declinamiento in Argentina, declinio in Brazil, marchitamiento repentino in Uruguay and “decline on rough lemon” in South Africa. The fruta bolita or Misiones disease in Argentina may be related. Blight and these related diseases are currently spreading in many areas of the world and, where present, are developing into a very serious problem.

No causal agent has been identified, but the disease has been transmitted by grafting roots from diseased trees to five- or six-year-old healthy trees in the field. Symptoms appear in two to four years (Tucker et al., 1984; Lee, Brlansky and Derrick, 1988). These successful transmission experiments suggest an infectious agent for blight (Figure 34).

Symptoms of blight
The earliest symptom of blight is a persistent and unseasonable wilt on one or more branches. The decline begins to spread and within a few months affects the entire tree (Swingle and Webber, 1896; Rhoads, 1936). Zinc-like deficiencies may appear in the leaves on some branches, and leaves are usually smaller. Symptoms are related to water stress, i.e. typical wilt symptoms, reduced vigour, thinning of foliage and canopy, delayed flushes of growth and bloom. In later stages, the tree will show terminal twig and small-branch dieback (Figure 30) with suckers of new growth from the main trunk. Off-colour (dull green) foliage is quite common, with occasional chlorosis and yellowing.

Blighted trees are especially conspicuous at spring flush because of the delay in formation of new growth. These tree symptoms, combined with lack of water uptake, may be sufficient to diagnose the disease. During later stages of decline, the tree shows loss of leaves and lack of new growth with some branches skeletonized. The blighted tree may stand out as smaller, with pockets of open leaf areas especially in the top and the outer side branches (Figure 34). Higher zinc levels in the wood and distinct characteristic amorphous plugs in the xylem cells (Figure 33) would give further evidence to substantiate the diagnosis.

METHODS OF DETECTION
At present, no greenhouse index using seedlings or budded trees has been developed for diagnosis of blight. However, the disease can be diagnosed by two different tests, and a positive response to both is highly diagnostic for blight. The tests involve the determination of the water uptake potential of the tree, and zinc accumulation in the xylem wood. In addition, the procedure for observing blight-associated amorphous plugs in
xylem tissue is also included as a supplementary test for diagnosis of blight.

**Method 1: Water uptake**

**Water injection test.** This test is a modification of the gravity infusion test (see below) and was developed by Lee et al. (1984) as a more rapid test. It is equally effective in measuring the potential for water intake but has the advantage of being considerably quicker. The procedure is as follows:

- Using either a brace (Figure 31b) or battery-powered cordless drill (Figure 32a) and a 1/8 in (3.2 mm) drill bit, a hole is drilled into the trunk about 3 cm deep and 25-40 cm above the bud-union.
- The newly drilled hole is freed of loose sawdust by moving the drill bit in and out of the hole, and the mouth of the hole is widened slightly to accommodate the tip of a tapered syringe.
- A 30 ml plastic disposable syringe (Pharmaseal stylex or equivalent) with a Luer tip but without a needle (Figure 32b) is filled with 30 ml water and fitted snugly into the drilled hole.
- Using one hand to hold the barrel of the syringe, maximum hand pressure is applied to the plunger with the other hand (Figure 32b). Pressure should not be so extreme as to break the plunger.
- To quantify results, the amount of water injected is recorded for a given period and converted to ml/sec.
- If feasible, a number of healthy and declining trees should be tested and compared, and enough trees should be used to give a statistical comparison.

Lee et al. (1984) reported that the amount of water injected into a healthy tree is about 0.5 to 1.0 ml per second whereas less than 0.3 ml/sec of water can be injected into a blight-affected tree. Often, very little water can be injected into a blight-infected tree and the difference between healthy and blighted trees is immediately obvious.

**Water uptake by gravity infusion.** This test, first proposed by Cohen (1974) and later modified by Lee et al. (1984), measures the ability of a tree to accept water into the xylem region. The water uptake or gravity infusion test of Cohen is as follows:

- A graduated 100 ml burette with a rubber hose attached is suspended from a branch inside the tree as shown in Figure 31a.
- A 1/4 in (6.4 mm) hole is drilled 4 in (10 cm) deep into the trunk of the tree about 25-40 cm above the bud-union. Either a brace and bit or a battery-powered hand drill can be used (Figures 31b and 32a).
- A tapered and hollow stainless-steel or aluminium connector tube (made specifically for this purpose and shown in Figure 31c) is hammered into the hole. Water is injected into the hole immediately after drilling, using a hypodermic syringe to avoid excessive introduction of air or drying.
- The rubber tubing is then placed on the metal connector and, using a hypodermic syringe and needle, water is injected through the tubing at a point very close to the connector. This forces the air out of the system and fills the burette. After injection, leakage from the small hole made by the needle in the rubber tube is avoided by sliding the rubber tube over the connector and past the needle hole.
- The water level in the burette and the current time are then recorded.
- After an interval of 12 or 24 hours, the water level and time are again recorded.
- If feasible, a number of healthy and declining trees should be tested and compared, with
enough trees used to give a statistical comparison (Lee et al., 1984).

Cohen (1974) observed that in a 24-hour period blighted trees would absorb from 0 to 45 ml of water whereas healthy trees absorbed from 200 to 500 ml of water. By experimenting with normal and healthy trees, a standard time period can be determined for the test. A tree’s very poor ability to absorb water is highly indicative of the presence of blight.

**Method 2: Zinc accumulation in the trunk wood**

The association of significantly high zinc levels with blight-affected trees was first shown by Smith (1974a). Further studies by Wutscher, Cohen and Young (1977) also showed a striking association of zinc accumulation in the xylem wood of citrus with blight. The procedure for testing zinc content of the inner wood is taken from Wutscher et al. (1977) as follows:

- Using a 1/2 in (12.5 mm) zinc-free drill bit, two holes are drilled 2.5 cm deep into opposite sides of the tree trunk about 20 cm above the bud-union. A hand brace or battery-powered drill can be used (Figures 31b and 32a).
- The first turnings of outer bark are discarded, and the wood shavings representing the outer xylem are collected in a plastic cup. Care should be taken to remove wood chips from the drill bit prior to drilling a new hole.
- The shavings are transferred to a screw-top glass vial, taken to the laboratory, the screw tops removed and the shavings dried in a 70°C draught oven for 24 hours.
- Samples of approximately 2 g dry weight, weighed to three decimal places, are incinerated in a muffle furnace (450°C) overnight.
- The ash is dissolved in 25 ml of 5 percent HCl, and the zinc content determined by

atomic absorption spectroscopy.

- If feasible, a number of healthy and declining trees should be tested and compared, using enough trees to give a statistical comparison.

Wutscher et al. (1977) found that trees with blight showed an average of 19.8 ppm zinc whereas normal trees showed an average of 3.6 ppm zinc. Zinc levels in healthy trees vary depending on grove management conditions; therefore a comparison must be made between healthy and declining trees in the same grove. Infected trees showed five times the zinc level compared with normal trees, and the differences were significant. Similarly, Smith (1974a) showed approximately the same significant differences between blight-infected and healthy trees.

**MISCELLANEOUS**

**Detection of amorphous plugs**

This diagnostic technique for detection of blight uses light microscopy, transmission electron microscopy (TEM) or scanning electron microscopy (SEM) for observation of specific occlusion bodies within the xylem (Cohen, Pelosi and Brlansky, 1983; Brlansky et al., 1984). The presence of dense amorphous plugs appears to be associated with blockage of water movement typical of blight and may be diagnostic. Brlansky, Lee and Collins (1985) have shown that the typical amorphous plugs associated with blight, as shown in Figure 33, are different in appearance from those present in the xylem of trees infected with psorosis-A, stubborn disease or Phytophthora, when viewed with the SEM. However, at times, the amorphous-type plugs associated with concave gum may resemble those associated with blight. Under a light microscope it may be difficult to separate the amorphous plugs associated with blight, concave gum or psorosis-A, as all appear solid and yellow to gold in colour. The morphology of the plugs is
different when viewed with an SEM. However, if the water uptake and zinc levels are included in the diagnosis, this presents no problem.

The procedure for observing blight-induced plugs by microscopy, as outlined below, is taken from the publications of Cohen et al. (1983) and Brlansky et al. (1984, 1985):

- A core sample of wood about 6 cm long is taken from the trunk of a citrus tree about 25-40 cm above the bud-union using a standard forestry 5 mm increment borer.
- Pieces approximately 1 cm long are cut from the core sample and fixed immediately in 3 percent glutaraldehyde solution in 0.066 M sodium-potassium phosphate buffer for a period of eight to 15 hours, and washed with the same buffer.
- For light microscopy, longitudinal and transverse sections are cut, 30-40 µm thick, using a sliding microtome and observed under the microscope for amorphous plugs (Figure 33b).

For details of SEM and TEM methods for observation of amorphous plugs see Brlansky et al. (1984, 1985).

**Detection of blight by root graft**

Blight has been shown to be a transmissible disease (Tucker et al., 1984; Lee et al., 1988). Transmission has been achieved by moving known infected trees, placing them next to six-year-old healthy trees and approach or splice grafting their roots together. Decline symptoms became evident in two to four years (Figure 34). Similarly, successful transmission has been done by grafting pieces of root from infected trees to roots of healthy trees.

At present there is no short-term plant index for detection of blight. With further development of evidence that blight can be transmitted by root graft in the field, it would be highly desirable and helpful to develop a relatively rapid plant index. Where a tree is selected as a source for propagative budwood in countries where blight is endemic, it may be desirable to monitor the source tree for at least two years after buds are obtained.

**REFERENCES**


Smith, P. F. 1974b. History of citrus blight in


Fig 30a
A typical blight-affected grapefruit tree on the left with a non-affected tree on the right, in Florida

Fig 30b
A typical blight-affected Pera sweet orange tree on Rangpur lime rootstock in Brazil. Infected tree on the left with a non-infected tree on the right
a) Graduated burette hanging from an inside limb

b) Drilling into the trunk with a hand drill. The plastic tube shown entering the tree trunk is attached to the graduated cylinder in (a). It is filled with water, and water uptake is measured from the graduated cylinder.

FIGURE 31
Apparatus used for gravity water infusion as a test of water uptake in blight-affected trees. Method of Cohen (1974)

Fig 29b
Three stubborn-infected Madame Vinous plants on the left compared with three control plants on the right. Note the smaller leaves and compressed growth in the infected plants. Plants were inoculated by side grafting and held in a warm greenhouse.
FIGURE 32
Apparatus using a hyperdermic syringe for the rapid measurement of water uptake ability of the tree (Method of Lee et al., 1984) (Photos: R.F. Lee)

a) Cordless electric drill making a small hole in truck

B) Injection procedure showing stopwatch and pressure being applied to the plunger of the syringe
FIGURE 33a
Showing the dense amorphous plugs diagnostic for blight as observed in the scanning electron microscope

FIGURE 33b
The dense amorphous plugs as seen in the light microscope (Photos: R.H. Briansky)

FIGURE 34
Field tree on the left shows symptoms of blight. This was root-grafted with roots from a five- or six-year-old blight-infected sweet orange tree in the centre. This infected source tree was transplanted between two healthy field trees. The tree on the right is the non-inoculated healthy control
DESCRIPTION AND BACKGROUND

The exocortis disease of citrus, caused by the citrus exocortis viroid (CEV), was first reported and described by Fawcett and Klotz (1948) as a bark-shelling disorder of trifoliate orange rootstock. Bento et al. (1949) reported that this disease had been known in Australia since the early 1930s as “scaly butt” and had been found to be transmissible. They recommended that budwood for propagation be taken only from older trees on trifoliate rootstock showing no scaling of the butt or trunk.

The disease is present in almost all citrus-growing regions of the world. Although many of the commercial citrus cultivars are symptomless carriers, trees may be stunted to some degree on rootstocks normally considered tolerant. In countries where trifoliate orange is the primary rootstock, bud selection over many years has avoided CEV, but other citrus viroids may be present.

Shell bark of lemon is sometimes confused with exocortis. Although CEV or a citrus viroid (CV) may be factors in enhancing shell bark symptoms, they may not be causal. Incidence of shell bark is greatly diminished by use of virus and viroid-free nucellar or shoot-tip grafted lemon bud selections.

Gummy pitting and gum pocket have been reported from Australia and South Africa and may be associated with certain citrus viroids. Symptoms are gum-impregnated pits or pockets in the trifoliate rootstock, which can be seen on the wood surface if the bark is removed. Gum-impregnated areas range from few to numerous and from small to large (Fraser and Broadbent, 1979) and are more prevalent in older trees.

As stated above the causal organism of exocortis disease is the citrus exocortis viroid (Semancik and Conejero-Tomas, 1987; Semancik, 1988). It is a low-molecular-weight RNA consisting of 371 nucleotides. It can exist as either linear or circular molecules and is highly mechanically transmissible by tools from tree to tree. CEV is transmissible into Gynura aurantiaca, petunia or tomato plants, and causes distinct and characteristic severe epinasty symptoms on leaves of these hosts. Nucleic acid extraction can be made from these hosts or from young symptomatic citron shoots and analysed by polyacrylamide gel electrophoresis (PAGE). CEV will migrate in a band on the gel and can be visualized by staining with silver or ethidium bromides.

Recent studies indicate that there are a number of citrus viroids of a molecular weight lower than CEV that can also induce symptoms in citron. Some of these viroids induce mild bark cracking in trifoliate orange (Figure 37) distinct from the severe bark shelling associated with CEV. These citrus viroids should be considered as independently transmitted and distinct pathogens. Schlemmer, Roistacher and Semancik (1985) were the first to report that citron-variable viroid reacted only in citron but not in Gynura or other herbaceous hosts in which CEV reacts and multiplies. Duran-Vila et al. (1988) reported on four additional distinct viroid groups (named citrus viroids I to IV) found in Spain and California, with a size range down to 275 nucleotides. These viroids induced specific mild reactions in citron, and were found in field trees of citrus in pure form or in various combinations. Currently there are some ten to 12 viroids in the
CV complex, some of which appear closely related, based on nucleotide number and nucleic acid hybridization assays. However, they may induce different reactions in field trees. For example, the cachexia viroid will migrate on a polyacrylamide gel and form a band (IIb) containing approximately 300 nucleotides. It very closely resembles another citrus viroid (IIa) that contains about 305 nucleotides. However, symptoms induced in citron and other citrus cultivars and rootstocks are clearly and strikingly different. In any indexing programme (citrus, grape or stone fruit) with its object of producing disease-free primary stock, it is important that all viroids be recognized and, if possible, removed from propagative budwood.

Citrus viroids are distributed primarily by the introduction and propagation of infected budwood and subsequently by mechanical transmission. The viroids are transmitted mechanically by hedging equipment, tools and knives, especially from lemon to lemon. Mechanical transmission of CEV was first demonstrated by Garnsey and Jones (1967) who showed that contaminated tools could be disinfected by a mixture of 2 percent sodium hydroxide plus 2 percent formaldehyde. Roistacher, Calavan and Blue (1969) demonstrated that low dilutions of sodium hypochlorite are readily available, less toxic and a very efficient disinfectant for CEV. Although mechanical transmission from orange, mandarin or grapefruit is less efficient than from lemon (Garnsey, 1968), once the viroid is present it will spread from tree to tree throughout an orchard over a period of time by hedging, pruning, clipping of fruit or collecting of budwood. Neither CEV nor CVs are known to be vector- or seed-transmitted, and root transmission, though possible, would be overshadowed by mechanical transmission.

All of the citrus viroids appear to be readily eliminated by shoot-tip grafting or by use of nucellar budlines. They are extremely tolerant to heat and have not been successfully eliminated from budwood by thermotherapy.

At first the diagnosis of exocortis was a long-term process in which trifoliate orange or Rangpur lime as a rootstock, under a vigorous-growing scion such as lemon, was inoculated and placed in the field. Symptoms would appear in the rootstock after two to six years or longer, depending on the severity of the viroids present. The use of citron as a rapid indicator was first proposed by Salibe and Moreira (1965), tested by Frolich et al. (1965), and modified by Calavan et al. (1964), who demonstrated rapid development of symptoms by forcing a bud of a sensitive clonal line of citron on a vigorous seedling stock. Roistacher et al. (1977) developed a citron selection via shoot-tip grafting (861-S-I), which is highly sensitive to CEV and the milder reacting CVs.

The detection of CEV and related CVs by graft-transmission and by PAGE requires the production of the highest quality plants growing under ideal conditions of nutrition and temperature. The need for an exacting type of plant laboratory (as outlined in Part II) becomes apparent when indexing for these viroid-induced diseases. Recent important developments in the detection of a whole range of new citrus viroids are reviewed by Duran-Vila et al. (1988) and techniques for PAGE analysis are given in Part III. Principles relating to these viroids may be applicable to similar viroids now being found in grapes and stone fruit.

Field symptoms range from mild bark cracking to very severe bark scaling, primarily on trifoliate and Rangpur lime rootstocks, accompanied by various degrees of stunting of the tree (see Figures 35-37 and 43). For more detailed descriptions and photographs see Weathers (1980) and Wallace (1978). Both Rangpur lime
and trifoliate orange twigs and branches may show a chlorotic stem blotching which is symptomatic (Figure 38). Citrons are highly susceptible and, when inoculated, may show bark cracking (Figure 41b), leaf epinasty and necrosis of the leaf veins (Figure 41a). Sweet limes and certain lemon varieties may show elongated bark cracks. Certain mechanically inoculated herbaceous hosts, i.e. Gynura petunia and tomato, will show a distinct leaf curl, epinasty and vein necrosis. Trees on trifoliate hybrid rootstocks can be stunted to varying degrees (Figure 43) and some of these hybrid rootstocks will show bark cracking. Trees on trifoliate rootstock affected with both exocortis and tristeza can be very severely stunted. Trifoliate orange rootstock infected with individual or combinations of CVs (excluding CEV) may show mild to moderate bark cracking (Figure 37). Occasionally CEV can induce stunted, acorn-shaped fruits on citron or Volkamer lemon (Figure 39) (Bitters, Duran-Vila and Semancik, 1987).

METHODS OF DETECTION

Method 1: Field diagnosis
If the rootstock is trifoliate orange, certain citranges or Rangpur lime, typical bark cracking as shown in Figures 35 and 36 is symptomatic and diagnostic for CEV. Citrus viroids I to IV (excluding cachexia) do not induce the severe bark cracking typical of CEV, but may cause a mild bark cracking in trifoliate orange stock (Figure 37). Twigs and branches of CEV-infected trifoliate orange or Rangpur lime may show a yellow blotch as seen in Figure 38. The use of trifoliate orange as a seedling or rootstock for long-term field indexing is no longer recommended. However, it may be useful for detecting and classifying the milder reacting CVs. Studies under way show that certain CVs will crack trifoliate orange rootstock in the field and cracking will vary, depending upon combinations of viroids present in the inoculum (Roistacher and Semancik, unpublished). Severe stunting of trees on trifoliate orange rootstock, even in the absence of bark scaling, may indicate a viroid infection.

Method 2: Citron index
The use of sensitive clonal citron selections such as 60-13, 861 or 861-S-1 budded to a vigorous rootstock such as rough or Volkamer lemon, or propagated as cuttings, is the preferred and recommended index method. It is relatively rapid, sensitive and highly diagnostic (Calavan et al., 1964; Roistacher et al., 1977). Citron seedlings may be used for detection of CEV, which causes strong symptoms. However, when indexing for the milder reacting citron viroids of groups I to IV, 861-S-1 budded to a vigorous rootstock is the recommended indicator.

Collection of budwood. In a routine index, a minimum of four budsticks should be collected, one from each quadrant of the tree. However, for periodic critical reindexing of foundation block trees, where they may have been subject to possible contamination by infected tools via mechanical transmission, eight budsticks should be collected from around the tree. In the Citrus Variety Improvement Program in California, each foundation tree is indexed once every three years for possible contamination by CEV or CVs. At times CEV infection has been discovered in only one of the eight sectors of the tree. Whenever collecting budwood, tools must be dipped or sprayed with a 1 percent sodium hypochlorite solution when going from tree to tree.

This practice is extremely important and necessary as a sanitary precaution, and should be incorporated as standard sanitary procedure in the field as well as in the plant laboratory.
**Inoculum tissue.** The best inoculum tissue for indexing is the “bud” (bud, blind bud or chip bud). A minimum of two inoculum buds are recommended per test plant. Leaf tissue should not be used. Studies by Blue et al. (1975) showed poor transmission of CEV by leaf-disc grafts.

Inoculation. The preferred indicator scion is 861-S-1. When obtained, a bud of 861-S-1 is propagated on rough lemon or other lemon type stock and maintained as a source plant. Extreme care should be taken to disinfect clippers when collecting budsticks from the source plant for use as scion indicators. A 1 percent sodium hypochlorite solution is the preferred disinfectant for CEV or other CVs (Roistacher, Calavan and Blue, 1969).

The 861-S-1 citron bud is then grafted to a vigorous seedling rootstock, such as rough or Volkamer lemon, at about 25 cm above the soil surface. (Other rootstocks should be tested for compatibility, since citron does not grow well on all rootstocks.) When wrapping the citron bud with budding tape, the “eye” of the bud may be exposed for forcing, or it can be completely wrapped, and unwrapped two or three weeks after budding.

Inoculation can be done at the same time as grafting the citron scion bud. Two inoculum “buds” are grafted anywhere below the citron bud and completely wrapped. The seedling is then bent just above the citron bud and the top portion of the seedling tied to the base of the plant to aid in the forcing of the citron bud (Nauer and Goodale, 1964). This is the same procedure used for forcing the Parson’s Special mandarin bud in the cachexia index (Cachexia Figures 47 and 48). However, if rough lemon seedlings are used as the rootstock, the seedlings can first be cut back at the time of inoculation to 25 or 30 cm above the soil surface and the citron bud inserted near the top of the cut seedling and wrapped. The inoculum buds are then inserted below and wrapped. The compatibility of citron with rough lemon is excellent and cutting back at the time of inoculation favours the forcing of the citron bud. This procedure is more convenient than bending and has been successfully used.

**Number of indicator plants.** Indicator plants should be grown one per container for CEV indexing. The milder symptoms associated with citrus viroids are best expressed and observed in plants grown one per container. A minimum of four inoculated plants is recommended for each index test, and the inoculum collected from the various sectors of the field tree to be tested should be uniformly distributed among the test plants. After inoculation, the used inoculum budwood should be refrigerated and saved. It can be used later to reinoculate any plants where grafts have failed, provided that fresh inoculum is not readily available.

**Controls.** Two positive controls are sufficient for CEV. However, the mild-reacting citrus viroids (Duran-Vila et al., 1986, 1988) require additional specific positive mild controls for their identification. The very mild-reacting citrus viroids such as CV Ila require more control plants (six or eight are recommended). The mild-reacting citrus viroids are usually found in

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1 861-S-1 citronbudwood is available from the Department of Plant Pathology or the Department of Plant Sciences, University of California, Riverside, CA 92521, United States of America.

2 It is important that these mild-reacting viroid sources be used as positive controls. Citrus viroid Code E-818 contains a single viroid RNA Ila which is used as the positive mild control in the indexing programme in the California Clonal Protection Program. Similar pure mild-reacting viroid RNA-infected budlines may be obtained from the Spanish Variety Improvement Programme.
mixtures with CEV and can be isolated from CEV by mechanical transmission or by shoot-tip grafting (Roistacher et al., 1969; Duran-Vila et al., 1988). When found, they can be preserved by transferring them into sweet orange seedlings and holding these as source plants in a “virus” bank. The specific type and identity of the viroid can be determined by PAGE (Part III).

**Inoculum survival.** After two or three weeks the budding tapes covering the inoculum “buds” plus the citron scion bud should be removed. It is extremely important that the razor-blade or knife used to cut the tape be disinfected in a 1 percent sodium hypochlorite solution when moving from plant to plant. Any dead or dying grafts should be recorded and the plant reinoculated, or a new test plant used if both inoculum grafts are dead. If the citron scion bud at the top is dead, the plant should be rebudded or a new test plant established.

**Post-inoculation care.** The forced citron scion bud should be trained to grow as a single shoot or leader, as illustrated in Figures 47 and 48. The rootstock stub should be cut close to the emerging citron shoot and clippers must be disinfected between each cut. Citron has a natural tendency to grow as a single shoot in the plant laboratory, and only occasional suckering or trimming of side shoots is needed. The young shoots or suckers should be removed by pulling them off and not rubbing them off. Exocortis can be spread from plant to plant on the hands after rubbing off sucker shoots (Roistacher et al., 1969). When the young growing shoot is approximately 10-15 cm long, it should be tied to a stake as shown in Figure 48. Standard bamboo stakes are satisfactory but should be sterilized before use to prevent contamination of the soil by pathogenic organisms. Plants should be well spaced on the bench, leaving about 400 sq cm (about 20 x 20 cm) per plant (Nauer, Holmes and Boswell, 1980). Allow the plants to reach 1 m or more. If symptoms do not show in the leaves of the mild positive controls, perhaps owing to insufficiently warm growing conditions, cut the plants back at about 10 cm above the bud-union. Retrain the new shoot to a single leader as before and once again observe new growth for symptoms.

**Temperature requirements.** The importance of maintaining warm or hot growing temperatures for maximum symptom development of CEV or CVs cannot be over-emphasized. Recommended temperatures are 32-40°C maximum during the day and 27-30°C minimum at night. Avoid temperatures above 40°C since leaves may become distorted and small (Reuther, Nauer and Roistacher, 1979). Figure 40 illustrates the importance of temperature for symptom expression in citron. Both plants were inoculated with certain citrus viroids. The plant on the left shows no symptoms and was held at normal-cool maximum day temperatures of 25-30°C, whereas the plant on the right showing symptoms was held at maximum day temperatures of 32-40°C. The inoculum was from a moderate-reacting citrus viroid.

**Time for first symptom development.** The severe symptoms associated with CEV generally appear in four to ten weeks when citrons are grown under warm conditions as single shoots. The mild symptoms associated with other citrus viroids take considerably longer, i.e. three to six months. Positive controls should be continually observed for tip browning, petiole browning, petiole wrinkle and mild leaf epinasty (Figure 42). These will occur only under proper conditions of nutrition, temperature and proper salinity balance. Non-inoculated plants to serve as negative controls are essential.
**Symptoms.** The classic leaf-epinasty symptom for CEV in citron is seen in Figure 41a. The underside of the leaves will show brown, necrotic and cracked veins, especially in the midvein. The petiole will be severely wrinkled or cracked and discoloured. The bark of the stem may be severely cracked as in Figure 41 b. Cracking may be severe to mild depending on the strain and environment.

Leaf symptoms for the milder-reacting citrus viroids are tip browning (Figure 42a), petiole wrinkle and browning (Figure 42b), midvein browning (Figure 42c), and mild leaf epinasty (Figure 42d; also Roistacher *et al.*, 1977). Petiole wrinkle may occur without browning. However, as the plant matures, browning of the petioles on the lower leaves becomes more pronounced and distinct. Leaves of the non-inoculated control plants grown with proper nutrition, temperature and watering will show no petiole wrinkle or browning (Figure 42b, right). As mentioned earlier, these symptoms are very mild and will be seen only under the best conditions of growth and temperature. They will not be seen if temperatures are too cool (Figure 40). They may not be seen in seedlings, and seedlings should not be used for detection of these milder-reacting citrus viroids (Roistacher *et al.*, 1977).

**Termination.** The index can be terminated when most or all of the mild-positive controls show clear, definitive, positive reactions. If no reaction is evident in the leaves of the mild-positive control plants after they have grown to over 1 m as single shoots, all plants should be cut back to about 10 cm above the bud-union, the temperature in the greenhouse may need to be raised, and a new citron bud forced and grown as a single shoot. The leaves on this new shoot should be observed and compared with those on negative control plants until definitive symptoms are evident.

**MISCELLANEOUS**

**Seedling index**

On occasion, seedlings of 861 citron may be used if CEV is the only viroid under index (Garnsey and Whidden, 1973); they should not be used if the milder-reacting citrus viroids are to be indexed. The procedures using seedlings are similar to those outlined in Method 2. Two to four indicator plants can be used. Positive and negative controls should be included and seedlings can be cut back at the time of inoculation. Seedlings should be grown one per container and trained as single shoots. Temperature requirements are the same. Symptoms will begin to appear in four to six weeks and within ten weeks will be similar to those shown in Figures 41a and b.

**Polyacrylamide gel electrophoresis (PAGE)**

There is much interest in PAGE techniques for identifying specific bands associated with CEV and CVs. These techniques should be used in conjunction with index plants for identification of specific viroids. There are still many parameters needing research before PAGE technology can fully replace the plant index. It is, however, an excellent tool and should be included in any comprehensive programme for indexing of citrus viroids. Details of the technique are given in Part III.

**Nucleic acid hybridization probes**

Viroids can be detected by testing for degree of hybridization between sample and labelled probes. Probes may be end-labelled purified viroid or labelled cDNA. Details of the technique are given in Part III.
DETECTION OF EXOCORTIS AND MILD-REACTING CITRON VIRIOIDS

Summary

Indicators:
861-S-1 citron/rough lemon for CEV or CVs.

No. plants/test:
4 to 6 (1 per container) for mild-reacting citrus viroids (CVs), 2 to 3 for strong-reacting CEV.

Inoculum:
“Buds” only (no leaf inoculum).

Plant growth:
Single shoot or leader, grown 1 per container.

Temperature:
Warm to hot: 32-40°C max. day/27-30°C min. night.

First symptoms:
CEV – 4 to 10 weeks
CVs – 4 to 6 months

Symptoms:
CEV – Severe leaf epinasty and bark cracking
CVs – Tip browning
- Petiole wrinkle
- Petiole browning
- Midvein browning
- Mild leaf epinasty

REFERENCES


Acorn-shaped fruit of citron induced by CEV (California). Similar acorn-shaped fruit occur on Volkamer lemon.

Temperature effect on 861-S-1 citron inoculated with a moderate-reacting mixture of citrus viroids. Plant on the left was grown at relatively cool temperatures. Plant on the right was grown at warm temperatures. Note the complete absence of symptoms in the citron branch that was held at the cooler temperature.
FIGURE 41a
Classic severe symptoms of CEV in citron showing severe epinasty of leaves.
A few normal control leaves are shown on the left

FIGURE 41b
Severe bark cracking on the stem of citron 861-S-1 indicator plant inoculated with CEV
FIGURE 42
Mild symptoms on leaves of 861-S-1 citron. These symptoms, expressed in varying degrees of severity, are diagnostic for many of the citrus viroids

a) Tip browning (control on left)

b) Petiole browning and wrinkle (control on right)

c) Midvein browning (control on left)

d) Very mild leaf epinasty. The midvein may show slight cracks at the bend
a) Five-year-old trees of Valencia orange on Troyer citrange. CEV-infected tree is on the left

b) Older trees of Valencia orange on trifoliate rootstock. CEV-infected trees are on the right (All CEV-infected trees show severe bark cracking on the trifoliate rootstock)
DESCRIPTION AND BACKGROUND
The citrus cachexia disease was named, described and first transmitted by Childs (1950). Xyloporosis, a condition affecting sweet limes, has been linked synonymously with cachexia. However, in a recent review by Roistacher (1988), cachexia is suggested as the preferred name, and xyloporosis is reserved for the specific condition or complex associated with sweet limes as originally described by Reichert and Perberger (1934).

The viroid nature of cachexia was first suggested by Roistacher et al. (1983), when many similarities between cachexia and exocortis were described, i.e. both pathogens: are highly mechanically transmissible and are readily inactivated on tools by sodium hypochlorite; cannot be eliminated from budwood by thermotherapy; are readily eliminated from microshoot tips by shoot-tip grafting in vitro; and react best in indicator hosts held under warm conditions. Cachexia is now known to be viroid-induced. It is a low-molecular-weight RNA consisting of about 300 nucleotides (Semancik, Roistacher and Duran-Vila, 1988).

The cachexia disease is found in most citrus-growing areas of the world where exocortis is found. Since most commercial citrus cultivars are symptomless carriers and the pathogen is readily transmitted in buds and highly transmitted mechanically by tools (Roistacher, Nauer and Wagner, 1980), the movement of this pathogen into new citrus-growing areas could present a problem, especially where mandarins, tangelos, tangors or C. macrophylla are grown as scions or rootstocks.

METHODS OF DETECTION
Method 1: Field diagnosis
For photographs and a description of field symptoms of cachexia, see Childs (1959,1980) and Calavan and Christiansen (1965). The diagnostic symptoms in tangelo, mandarin or C. macrophylla are phloem discoloration by gumming, undulating stem pitting or bumps and projections on the bark, which fit into depressions in the wood. Gum spots are usually prominent in the bark and are readily seen by slicing sections through the bark with a knife (Figures 44 and 45). The presence of these typical symptoms on susceptible hosts in the field can be diagnostic for the disease since very few graft-transmissible diseases of citrus show these classical symptoms, especially in tangelos, mandarins, Rangpur limes or C. macrophylla.

Method 2: Indexing Parson’s Special mandarin or Orlando tangelo
Parson’s Special mandarin forced as a scion on a vigorous rootstock is the preferred indicator for the detection of cachexia (Roistacher, Blue and Calavan, 1973; Vogel and Bové, 1976). The Parson’s Special mandarin seedling grows too slowly to be an effective seedling indicator (Roistacher, unpublished). However, if a bud from a seedling selection of Parson’s Special mandarin is grafted on to a vigorous rootstock (such as rough lemon) and forced under warm conditions, growth is vigorous and symptoms may appear at the bud-union interface between six and 12 months after inoculation.

The choice of a sensitive clonal seedling of the Parson’s Special mandarin is very important. There is variability among seedlings and some
are more sensitive than others. At the Rubidoux indexing facility, a number of Parson’s Special mandarin seedlings were tested for sensitivity to various isolates of cachexia. Seedling selections Nos 9 and 10 were found to be the most sensitive and these are currently recommended. If the Parson’s Special mandarin is obtained as seed, seedlings should be grown and observed for uniformity and vigour. All slow-growing seedlings should be discarded and about ten of the larger and more uniform selections held. These should then be tested by forcing a bud from each of the selections as scions under a rootstock that has been inoculated with mild- and severe-positive cachexia, respectively. Alternatively it may be simpler to obtain selection Nos 9 or 10 as a clonal bud line.1

Inoculated seedlings of Orlando tangelo or pathogen-free lemons or grapefruit as scions budded to Orlando tangelo as the rootstock can be used as indicators for indexing in the field. The Orlando tangelo as a seedling is preferred. The seedlings can be field-grown or preferably greenhouse-grown, “bud”-inoculated and removed to the field when convenient. The Orlando tangelo as a seedling is preferred. The procedures for indexing using Parson’s Special mandarin forced on rough lemon rootstock in a greenhouse, or using tangelo seedlings for field planting, are as follows:

Collection of budwood. Four budsticks are collected from each of four quadrants of the field tree to be tested. Ensure that the collecting tool is disinfected in a 1 percent sodium hypochlorite solution between trees. Inoculum should be labelled, secured in a polythene bag and immediately placed in an ice chest. The budwood is later stored in a refrigerator at the plant laboratory.

Indicator plants. A minimum of four but preferably six plants should be used to test each candidate or selection. “Buds” (buds, blind buds or chip buds) cut from the various collected budsticks should be distributed equally among the indicator plants. Budwood should be refrigerated for possible future use.

For the Parson’s Special index, rough lemon seedlings (or other fast-growing, vigorous seedlings) are grown one seedling per container, as a single shoot to about 1 m height. The Parson’s Special mandarin selection Nos 9 or 10 or a selected seedling line held as a reserve plant in the greenhouse is used as the scion budwood.

Controls. Mild- and severe-positive controls, plus non-inoculated or self-inoculated negative control plants, should be included for each test or experiment. A minimum of four plants should be inoculated for each control treatment. Six or eight plants should be inoculated with the mild-positive control since this will determine when the test can be terminated.

Inoculation. The Parson’s Special mandarin bud is grafted to the rough lemon (or other vigorous stock) about 20 cm above the soil surface and, when wrapping the bud, the “eye” of the bud should be exposed for forcing. A minimum of two inoculum “buds” are then grafted anywhere in the rough lemon rootstock below the Parson’s Special scion bud. Inoculum “buds” should be completely wrapped. The seedling is then bent at a point just above the Parson’s Special scion bud, and the top of the bent seedling can be tied to the base of the plant or placed under the container (Figure 47). This bending aids the rapid forcing of the scion bud. Again, knives should be disinfected in a 1 percent sodium hypochlorite

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1 These selections of Parson’s Special Nos 8 and 9 may be requested from the Department of Plant Pathology, University of California, Riverside, CA 92521, United States of America.
solution before moving to a new source of inoculum.

Tangelo seedlings are graft-inoculated with a minimum of two inoculum “buds” and these are completely wrapped. Knives should be disinfected in a 1 percent sodium hypochlorite solution before going to a new source of inoculum.

**Inoculum survival.** After two to three weeks, the budding tapes are removed from the inoculum, usually by cutting with a knife or razor-blade, which must be disinfected between plants in a 1 percent sodium hypochlorite solution. Inoculum survival is recorded and plants with dead buds should be regrafted, preferably with fresh material or with inoculum stored in the refrigerator (see methods for the collection and storage of inoculum tissue in Part II).

Where Parson’s Special mandarin is used as the scion bud, the wrapping tape may be left on until the Parson’s bud is well forced.

**Post-inoculation care**

**Parson’s Special mandarin index.** The Parson’s Special scion is trained to grow as a single shoot or leader, and is staked and tied (Figures 47 and 48). After the scion reaches about 1 m, it is cut back about 10 cm above the bud-union and a single bud near the top of the cut-back area is then forced and trained to grow as a new single leader. This new growth is then staked, tied and grown to about 1 m high (Figure 48) in the same manner as the previous shoot. This procedure of growth, cut back and regrowth is repeated until the test is complete or until plants are removed to the field for further observation.

Plants should not be crowded on the bench (Nauer, Holmes and Boswell, 1980). Adequate light is needed to maximize growth and symptom development. Each plant should have about 400 cm² (20 x 20 cm) of growing room.

**Seedling index.** After survival of inoculum is verified, the seedlings should be cut back at about 25 cm from the soil surface. The new growth should be trained to a single shoot or leader by removing all newly developed side branches. The young inoculated indicator plants are held in the greenhouse until they are large enough for transplanting to the field (about 1 m tall). The field location should be as warm as possible for maximum symptom development (symptoms may develop poorly under cool temperature conditions). The young trees should be well watered, fertilized and given good care.

Plants in the field can be close planted, preferably at about 2 m apart.

**Time for development of symptoms**

**Parson’s Special mandarin index.** Symptoms in plants inoculated with severe isolates of cachexia may appear six to nine months after inoculation. Those inoculated with mild isolates may take nine to 12 months or longer. If symptoms do not appear on plants inoculated with the mild isolates after 12 months, the plants should be moved to a warm field location and planted at intervals of 1-2 m. The young trees should be well watered and fertilized and given good care. Symptoms should be evident under the extended field index within one growing season (provided temperatures are warm or hot).

**Seedling index to tangelo in the field.** Symptoms may appear in eight to 18 months after field planting in seedlings inoculated with severe isolates. Those containing mild isolates may take two to six years, depending on the temperature and mildness of the isolate.

**Temperature requirements.** Plants under index for cachexia should be grown in as warm an environment as practical or possible. Recommended greenhouse temperatures are 32-40°C maximum by day and 27-30°C.
Techniques for biological detection of specific citrus graft-transmissible diseases

minimum at night. Symptoms induced by the cachexia viroid, similar to those induced by the citrus exocortis or other citron viroids, are best expressed in indicators under warm growing conditions.

Detection of symptoms

Parson’s Special mandarin index. After six to eight months, when plants are at maximum growth, the control plants inoculated with the mild- and severe-positive inoculum can be examined for symptoms. Three-sided rectangular cuts are made across the bud-union as shown in Figure 46. This is done at two places on opposite sides of the bud-union; the bark is lifted back and the area examined for the typical gumming symptoms diagnostic for cachexia (Figure 46). Similar cuts can be made in the regrowth area surrounding the joint where the plant had been cut back. If no symptoms are evident at the bud-union or the first joint, the bark is replaced and securely rewrapped with budding tape. Plants can be re-examined in this manner every two to three months. Early symptoms will almost always appear at the bud-union or the joint at the first cut-back area and rarely in the portion of the stem above the bud-union or in the area between cut-back joints as shown in Figure 50.

Seedling index. When the bark peels easily or is “slipping”, the mild-positive control plants are observed for symptoms. Cut three sides of a rectangle in the bark of the Orlando tangelo seedling or rootstock as shown in Figure 46. Ensure that knife blades are disinfected. Two or three such windows can be made, preferably in the lower parts of the trunk to observe symptoms. If no symptoms are seen, the bark should be replaced and wrapped with budding tape. This permits the peeled area to regraft and heal, leaving the trunk less damaged and available for more extensive bark cutting and peeling at future observation times.

Termination. When 75 percent of the mild-positive controls show definitive symptoms, the bark can be completely peeled from all of the test plants and examined for symptoms. The symptoms are then recorded and the test or experiment terminated. Symptoms on seedlings of Orlando tangelo in the field will resemble those in Figures 44 and 45. Symptoms for the Parson’s Special mandarin will be as in Figure 49a or 49b. Symptoms will almost always appear at the bud-union or at the cut-back and regrowth areas (Figures 46, 49a and 50). However, with very severe isolates, symptoms may appear throughout the stem, as shown in Figure 49b.

MISCELLANEOUS

The parameters for bringing out the maximum symptom expression in the Parson’s Special mandarin indicator scion have not been completely researched. High temperatures are definitely an important factor for inducing definitive symptoms more rapidly. Light intensity appears to be an important factor, but this has not been adequately tested. Alternating temperatures by moving the inoculated plants between hot and cool rooms has been tried with some success, but the precise parameters have not been determined (Roistacher, unpublished). Forcing the Parson’s Special mandarin scion on larger stock plants may be helpful in inducing earlier symptom expression, and using larger pieces of inoculum has been suggested as a means of enhancing symptom development. Nauer and Roistacher (1984) evaluated 63 mandarin and mandarin hybrid seedlings to find a more rapid seedling indicator for cachexia, but found none that were reactive.

The use of Parson’s Special mandarin and Orlando tangelo indicator plants as an index is a
long-term process, and there is a distinct need for a more rapid indexing procedure for detecting this disease. With the recent discovery of the viroid nature of cachexia (Semancik et al., 1988), new techniques may be developed to shorten this time period. The PAGE technique is promising for locating the viroid band and could be a relatively rapid means of confirming presence or absence of viroids after therapy. Inoculated citrons grown under warm conditions should be used as donor plants for PAGE. Mechanical inoculation of cucumber as a supplemental diagnostic technique is very rapid and should be tested and tried (Semancik et al., 1988; Duran-Vila et al., 1988). A most promising rapid future technique may be nucleic acid hybridization using cDNA probes. These techniques are illustrated in Part III.

**CACHEXIA DETECTION**

**Summary**

Graft transmission to Parson’s Special mandarin Indicator:

Parson’s Special mandarin/rough lemon.

**No. plants/test:**

4 to 8 (grown one per container; mild- and severe-positive controls should be included).

**Inoculum:**

“Buds” (buds, blind buds or chip buds).

**Plant growth:**

Single shoot grown to 1 m and repeatedly cut back.

**Temperature:**

Hot: 32-40°C day, 25-30°C night.

**First symptoms:**

Severe isolates: 6-8 months.

Mild isolates: 9-12 months or longer.

**Symptoms:**

Gum in scion just above the bud-union and also at the cut-back joint of the new growth area.

**REFERENCES**


FIGURE 44
A window in the trunk of a field of Ellendale mandarin showing severe symptoms of gumming on the bark and trunk typical of severe cachexia. Note the areas of gum in the cut-back section (Australia)

FIGURE 45
The trunk portion of a field tree of Parson’s Special mandarin as a rootstock under a grapefruit scion with the bark cut away to show the brown gum-stain. This tree was inoculated with a severe isolate of citrus cachexia viroid (Central California)
a) Tip browning (control on left)

FIGURE 46a
Parson’s Special Mandarin in a scion budded to rough lemon as the rootstock with a window cut into the bud-union showing severe symptoms in the Parson’s Special mandarin. Note that the gumming occurs only in the Parson’s Special mandarin and not in the rough lemon rootstock (greenhouse-grown Riverside, California)

FIGURE 47
Bending the rough lemon seedling just above the scion bud aids in the forcing of the Parson’s Special mandarin scion bud

FIGURE 46b
The first mild gumming symptom usually seen in an exploratory cut into the bud-union area

FIGURE 48
Growth of a Parson’s Special mandarin scion as a single shoot or leader. Note careful staking and tying
FIGURE 49
Symptom reaction on Parson’s Special mandarin after the bark is completely peeled

b) Very severe

FIGURE 50
The appearance of symptoms of gum in the Parson’s Special mandarin occurs only at the cut-back joint area, but no symptoms show in the stem above or below the area surrounding the joint
DESCRIPTION AND BACKGROUND

The family of virus diseases called satsuma dwarf is found primarily in Japan and consists of satsuma dwarf virus (SDV), navel infectious mottle virus (NIMV), citrus mosaic virus (CiMV) and natsudaidai dwarf virus (NDV). All are related serologically, are transmitted to herbaceous hosts and are caused by strains of the same virus.

The dwarfing problem of satsumas was first noted in the early 1930s in Shizuoka prefecture. Yamada and Sawamura (1952) showed it to be infectious and named it dwarf disease of satsuma, popularized to satsuma dwarf. A slide and text description of the disease is given by Tanaka (1980).

Except for its presence in Turkey (Azeri, 1973), the disease has not been reported outside Japan. It is very probable that the disease is present in mainland China, and there is no reason why the disease could not develop anywhere citrus is grown if infected budwood is introduced and cool temperatures prevail. Miyakawa (1969) found 18 species of citrus plus seven hybrids and two related genera to be susceptible when they were bud-inoculated.

Satsuma dwarf is caused by a virus 26-27 nm in diameter. The virus has been purified and characterized (Tanaka and Imada 1974). Particle size, reactivity to sesame Sesamum indicum) and citrus, and serological relationships with related viruses, i.e. NIMV, CiMV and NDV, suggest close relationships within this family (Imada, 1984). Despite the similarity of particle size and morphology of SDV and citrus infectious variegation virus (CIVV), the differential reaction to sesame and lack of serological relationship to CIVV antisera suggest that SDV is not closely related to CIVV (Imada, 1984). The satsuma dwarf family of viruses are all readily mechanically transmissible to sesame.

SDV can be readily transmitted mechanically on tools. However, the primary means of spread is by humans through propagation of infected budwood and spread through the soil (Izawa, 1966; Tanaka, Yamada and Kishi, 1971; Tanaka 1980; Koizumi et al., 1988). The soilborne nature of this virus disease of citrus may be an important aspect of its epidemiology since, once a site is infected, the disease may be permanently established. There is no evidence for above-ground vector transmission.

Koizumi et al. (1988) found SDV in a non-citrus host Chinulaurestine, a tree often used as a windbreak in satsuma orchards in Japan. Since tristeza and the severe seedling-yellows tristeza viruses are endemic in Japanese citrus, it is probable that most satsumas carry the tristeza virus. This makes it difficult to index for SDV by bud transmission to tristeza-sensitive index plants. Mechanical inoculation to sesame indicator plants is effective and can be used for indexing.

The availability of antisera for SDV enabled Kuhara et al. (1981) to use ELISA for mass screening of CiMV in field trees. They were able to test 53 000 trees and found that 38 percent were infected. In order to eliminate the threat of nationwide contamination by this virus, 17 410 field trees and 120 000 nursery trees were destroyed. ELISA is now used extensively in Japan as an index for the satsuma dwarf family of viruses.
METHODS OF DETECTION

Method 1: Field diagnosis
Trees in the field are stunted or dwarfed (Figure 51a), shoots are short and leaves are small and boat- or spoon-shaped (Figure 51 b). The boat- or spoon-shaped leaf is characteristic of the disease both in Japan and in Turkey. The malformed leaves are usually observed in the spring flush and not on other flushes of growth.

Method 2: Graft transmission to indicator plants
Since severe CTV is present in almost all satsuma trees in Japan, any budwood imported from Japan which is suspect for SDV or related viruses will also probably contain CTV. It is therefore desirable, and perhaps necessary, to eliminate the CTV in order to test for SDV, CiMV, NIMV or NDV by graft transmission. This can be done by “filtering out” the tristeza virus by inoculation into trifoliate orange seedlings. Buds from test trees or plants are first graft-inoculated into a seedling of a trifoliate orange, the seedling is then cut back and the new growth forced. If SDV is present, the leaves on the new growth will usually show psorosis-like symptoms of flecking and mottle within four to six weeks. These symptoms may not persist as the leaves mature and harden.

The use of supplemental lighting to enhance growth of trifoliate seedlings is recommended (see section on lighting in Part II). Procedure for graft transmission for detection of SDV and related viruses is as follows:

Inoculum tissue. “Buds” (buds, blind buds or chip buds) are taken from pre-inoculated trifoliate orange filter plants, or directly from suspect trees in the field.

Inoculation. A minimum of two inoculum “buds” are grafted to the lower part of the indicator seedlings. The seedling can be cut back about 20-25 cm above the soil surface at the time of inoculation, or two to three weeks after inoculation at the time inoculum survival is recorded.

Indicator plants. Natsudaidai, citron, sour lemon, Dweet tangor, mandarin or satsuma are used as seedlings. Grow three seedlings per container; inoculate two, leaving one as a negative control.’

Controls. If possible, SDV-positive tristeza-free controls should be included in each test; preferably the mildest-reacting available source plus a severe source should be used. A non-inoculated or self-inoculated negative control must always be included in every index test.

Inoculum survival. Cut the wrapping tapes two to three weeks after inoculation. Dip the razor-blade or knife in a 1 percent solution of sodium hypochlorite disinfectant when going from plant to plant.

Temperature requirements. The satsuma dwarf complex of viruses are cool-temperature-dependent for optimum symptom expression, and temperatures should be maintained as cool as possible. Night temperatures in the indexing facility should not exceed 18°C. Day temperature must not be too warm and should not exceed 26°C. A temperature of 28/23°C (maximum day/minimum night) will mask symptom expression (Tanaka et al., 1969).

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1Small quantities of satsuma seed are sometimes available from certain seedy satsumas and may be obtained by writing to Fruit Tree Research Station, MAFF, Kuchinotsu, Nagasaki-Ken 858-25, Japan; or Fruit Tree Research Station, MAFF, Okitsu, Shimizu, Shizuoka 424-02, Japan.
Symptoms. In general, the symptoms induced in indicator plants by the satsuma dwarf family of viruses (without the presence of CTV) are similar to those induced by infectious variegation (see Infectious variegation, Figures 64-67). Young leaves will show psorosis-like leaf patterns of flecking, mottle, chlorosis and perhaps some mild shock reaction within four to six weeks. The characteristic symptoms may not persist and may fade as the leaves harden. Mature leaves may show leaf curl and crinkle. Line patterns and misshapen leaves are common.

Miyakawa (1969) induced a non-persistent mottle in leaves of the young flush in almost all plants of the 27 citrus species, hybrids and related genera he tested.

Symptoms induced on leaves of satsuma seedlings under cool conditions will be typically boat- or spoon-shaped with occasional crinkle (Figures 51b and 52a). Symptoms may appear in six to eight weeks after inoculation (Figure 52b).

Method 2: Transmission to herbaceous hosts
Although cowpea and the common red kidney bean are good indicators for the satsuma dwarf family of viruses, white sesame is the preferred indicator plant. The following inoculation procedure is essentially that of Tanaka (1980):

**Inoculum tissue.** Soft young shoots under 10 cm are collected from trees or plants to be tested. Tissue from field collections should be put in polythene bags and immediately placed in an ice chest. It not advisable to collect tissue when temperatures are too warm.

**Preparation and buffers.** Sap is prepared by triturating young leaves in a cold mortar kept on ice using a two- to tenfold volume of 1/15 M Sorensen phosphate buffer solution at pH 7.0.

Inoculation. The leaves of the sesame plant are dusted with 500-mesh carborundum and rubbed with absorbent cotton or swabs dipped in the sap. After inoculation, leaves are rinsed in tap water. Five plants can be grown per container; a minimum of four are inoculated and one left as a non-inoculated control. Preferably two containers with a total of eight inoculated plants should be used per index test.

Post-inoculation care. Inoculated plants should be kept below 25°C for at least eight hours after inoculation. Greenhouse temperatures should be maintained relatively cool (20-25°C).

Time for first symptoms. Symptoms should appear within seven to 14 days, depending on temperature.

**Symptoms.** Necrotic local lesions on inoculated primary leaves (Figure 53), vein clearing, vein necrosis, curling, malformation and necrosis and spotting on secondary leaves (Figure 54).

Method 3: ELISA
Good antisera are available to SDV, and ELISA has been widely used in Japan for SDV and CiMV indexing. Specific procedures for ELISA are given in Part III. The ELISA technique was extremely helpful in the large-scale detection and eradication of citrus mosaic virus in Japan (Kuhara et al., 1981). It should not be used as the only method for the indexing of budwood from critical primary foundation trees or from shoot-tip grafted or heat-treated plants to be used for producing primary trees. However, ELISA can be used in conjunction with mechanical or graft-transmission indexes to ensure that the important foundation or mother block budwood is free of pathogens.
MISCELLANEOUS
Studies by Tanaka (1972) indicate that transmission by sap inoculation from citrus to citrus was very poor. He achieved 6/64 transmissions showing symptoms for SDV and 3/81 for NDV. The poor response to mechanical inoculation precludes this as a routine method for indexing.

SATSUMA DWARF DETECTION
Summary
- Graft transmission to citrus

Indicators:
Seedlings of Natsudaidai, citron or lemon if tristeza is not present; Dweet tangor or mandarin seedlings or satsuma/trifoliate if tristeza is present.

No. plants/test:
4 (3 plus 1 control in each of 2 containers); or 1 per container of satsuma/trifoliate.

Inoculum:
“Buds” (buds, blind buds or chip buds).

Plant growth:
Allow full flush to develop without trimming.

Temperature:
Very cool, not to exceed 26°C maximum day, 12-18°C minimum night.

First symptoms:
4 to 6 weeks.

Symptoms:
Similar to those of infectious variegation, i.e. psorosis-like leaf patterns in young growth, leaf curl and crinkle in mature leaves.

- Transmission to herbaceous hosts

Indicator:
White sesame (Sesamum indicum).

No. plants/test:
8 (3 plus 1 control in each of 2 containers of 5 plants each).

Inoculum:
Soft, young shoots.

Inoculation:
1/15M Sorensen phosphate buffer, pH 7.0. Leaf rubbed with Carborundum.

Temperature:
Cool – below 26°C.

First symptoms:
7-14 days.

Symptoms:
Primary leaves: necrotic local lesions. Secondary leaves: vein clearing, necrosis, curl, malformation.

REFERENCES


FIGURE 51
A satsuma dwarf virus-infected tree in the field (right) showing typical stunting and dwarfing (Japan)

FIGURE 51b
A branch from an infected field tree of satsuma mandarin showing typical spoon- or boat-shaped leaves (Japan)

FIGURE 52a
Symptoms on leaves from a satsuma seedling induced by graft-inoculation with buds from an infected tree in the field. Typical spoon- or boat-shaped leaves with occasional crinkle are seen (Japan)

FIGURE 52b
Graft-inoculated satsuma seedlings inoculated with SDV. Non-inoculated control plant is on the left. Note the smaller-sized plants and plants and spoon-shaped leaves. Photos taken by M. Miyakawa eight weeks after inoculation (Japan)
FIGURE 53
Necrotic local lesions on inoculated cotyledons of a sesame plant inoculated by sap containing SDV (Japan) (Photo: M. Miyakawa)

FIGURE 54
Necrosis of the upper portions of the leaves of a systemically infected sesame plant (Japan) (Photo: M. Miyakawa)
DESCRIPTION AND BACKGROUND

The tatterleaf disease of citrus, induced by the citrus tatterleaf virus (CTLV), was first described by Wallace and Drake (1962) as a transmissible disease that induced mottled and tattered leaves in *Citrus excelsa* indicator seedlings. Calavan, Christiansen and Roistacher (1963) first showed the destructive potential of this disease to citrange rootstock when tatterleaf-infected tissue was graft-inoculated to satsuma mandarin budded on Troyer citrange rootstock (Figure 56). Meyer (Beijing) lemon trees, which were first imported into the United States from Beijing (China) in 1908, were later found to contain the tatterleaf virus. Many Meyer lemon trees worldwide that originated from the 1908 introduction probably contain the virus, including many propagations and plantings of the original Beijing lemon in China (Zhang, Liang and Roistacher, 1988). The disease is endemic in mainland China and may be widespread (Zhang et al., 1988). Tatterleaf disease is widespread in Taiwan Province and in Japan and is probably present elsewhere where Meyer lemon or other infected citrus have been imported from these countries. It has been reported from South Africa in declining Shamouti orange trees on citrumelo rootstock.

Most citrus species and all commercial cultivars are symptomless carriers of the virus. Symptoms will appear as bud-union crease (Figures 55 and 56), or as a fluting and reduction of the stock as in Figure 56, where infected scions are grafted to trifoliate orange or its hybrids. When the bud-union crease is severe, the tops may shear off at the union in high winds (Figure 57). Miyakawa and Tsuji (1988) report that some isolates of CTLV do not cause bud-union crease. Trifoliate orange is immune to tatterleaf and the virus is unequally distributed in citrange.

Semancik and Weathers (1965) showed mechanical transmission of CTLV from citrus to cowpea and partially purified the virus. It was rod-shaped, 19 by 650 nm, and transmissible to 19 herbaceous hosts. Wallace and Drake (1968) suggested that two viruses were present (tatterleaf and citrange stunt) since shoots of inoculated *C. excelsa* indicator seedlings would recover after showing symptoms, and these recovered shoots contained a transmissible agent which would react in citrange but not in *C. excelsa*. Recovered *C. excelsa* shoots could be reinfected by the virus present in Meyer lemon buds and showed tatterleaf symptoms. They called the new virus “citrange stunt”. Roistacher (1981, 1988) showed that recovered shoots of *C. excelsa* graft-inoculated to symptomless carriers would eventually show the tatterleaf component. He suggested that the disease was one complex and the original name “tatterleaf” be retained to describe both diseases.

CTLV is difficult to eliminate from budwood by shoot-tip grafting but was eliminated from budwood by thermotherapy (Roistacher, 1977). Koizumi (1984) eliminated the virus from citrus tissue by combining shoot-tip grafting with thermotherapy. Recently, Navarro and co-workers (unpublished) succeeded in eliminating CTLV from citrus tissue by shoot-tip grafting *in vitro*.

The virus is readily mechanically transmitted from infected citron to citron by knife or razor cuts, and the virus can be inactivated on tools by dipping them in a 1 percent sodium hypochlorite
solution. CTLV was noted as spreading from tree to tree at the South Coast Field Station in southern California, presumably by mechanical transmission (Roistacher, unpublished). It is important that indexing for CTLV should be included in any programme for establishing primary foundation trees since many citrus species and commercial cultivars are symptomless carriers and the virus is highly mechanically transmissible. CTLV can be very destructive to citrus on trifoliate rootstock or their hybrids (Figures 55-57).

METHODS OF DETECTION

Method 1: Index to indicator plants

Collection of budwood. Collect a minimum of four budsticks from each quadrant of the tree to be indexed.

Inoculum tissue. “Buds” (buds, blind buds or chip buds).

Indicator plants. Seedlings of Rusk, Troyer or Carrizo citrange, citremon and C. excelsa are used. If tristeza is endemic, omit the C. excelsa because it is very sensitive to citrus tristeza virus, and symptoms of tatterleaf may be readily masked. Mexican lime in the absence of tristeza may show pronounced psorosis-like symptoms. Rusk citrange is more sensitive than Troyer or Carrizo citrange (Miyakawa, 1980). Zhang et al. (1988) reported that Troyer citrange was superior to Carrizo citrange as an indicator. Indicators can be used as seedlings, as a clonal budline topworked or budded to a plant containing the virus, or budded as a scion on to any inoculated seedling and forced. Grow indicator plants preferably three per container and inoculate at least four plants, two in each container, leaving the third plant as a negative control. Where Rusk or other citrange buds are to be forced as scions, grow one rootstock seedling per container. (See method described for forcing scions under “Cachexia”.)

Inoculation. Use at least two “buds” to inoculate each plant. Cut back seedlings at about 20-25 cm above the soil surface. The seedling may be cut back at the time of inoculation, or two to three weeks later when inoculum survival is recorded. If citrange is used as a forced scion, the rootstock seedling should preferably be bent just above the scion bud (Cachexia Figure 47) or cut back or topped just above the scion bud.

Controls. Both positive and negative controls should be included. Where seedlings are used and grown three per container, one of the three should be left as a negative control for each index test. Positive CTLV control buds should be inoculated into two of the three plants in one container, leaving the third plant as a negative control.

Inoculum survival. Cut the grafting tapes two to three weeks after inoculation and record “bud” survival. Reinoculate only if both inoculum “buds” are dead. Since CTLV is highly mechanically transmissible on tools, dip the razor- or knife-blade used for cutting the grafting tape in a 1 percent solution of sodium hypochlorite between plants.

Post-inoculation care. After the initial cut-back, allow seedlings or the budded Rusk citrange scions to develop all shoots without trimming. Supplemental light will enhance growth of citrange during winter months and should be incorporated in the plant laboratory as normal procedure (see Part II).

Temperature requirements. Tatterleaf is a cool-temperature virus and warm temperatures have been observed to mask symptom development.
Maintain index room temperatures at 24-30°C maximum day (or preferably cooler), and 18-21°C minimum night.

**Time for first symptoms.** Five to seven weeks in *C. excelsa* and six to eight weeks or longer in Rusk or other citrange. Occasionally, delayed reaction may result in the appearance of symptoms beyond eight weeks.

**Symptoms.** The first symptom is a mild chlorotic spotting of the leaves, usually only in the first flush of growth. Later growth will show intense leaf spotting, leaf deformation and stunting. The leaves look “tattered” as if the edges were torn in a non-uniform pattern (Figures 58 and 59). Symptoms in citrange are clear spots that persist and develop into chlorotic spots. Stems are blotched. Symptoms persist in the mature leaves. Inoculated plants of *C. excelsa* may develop “recovered” or symptomless shoots after the first or second flush of growth. These are the symptomless shoots that will induce a reaction in citrange or citremon but not in *C. excelsa* (the citrange stunt component of the tatterleaf complex) (Wallace and Drake, 1968).

**Termination.** Allow two to three growth flushes to develop (eight to 12 weeks), or wait until the positive control plants show strong and well-developed symptoms.

**Method 2: Mechanical transmission to herbaceous hosts**

The method recommended for mechanical transmission is based on the procedure of Garnsey (1974), which has consistently produced reliable index results. However, it must be remembered that sap inoculation techniques require higher concentrations of virus in the inoculum than is necessary for graft-transmission, and sensitivity is sacrificed for speed of assay.

**Inoculation procedure.** Young, succulent leaf tissue is macerated in cold neutral 0.05M potassium phosphate buffer at a ratio of 0.1 g of tissue to 1 ml of buffer. Plants are dusted with 500-mesh carborundum and leaves are inoculated with a cotton swab or fingers immersed in inoculum. Leaves are rinsed with tap water after inoculation and the plants are incubated in moderate light at 21-24°C.

**Indicator plants.** The cowpea *Vigna unguiculata* subsp. *unguiculata* (syn. *Vigna sinensis*) variety Early Ramshorn, other cowpea cultivars and the common red kidney bean can be used. *Chenopodium quinoa* has been used successfully in Taiwan Province. Grow five plants per container and inoculate four plants in each of two containers, with the fifth plant as the non-inoculated control. Bean and cowpea plants are usually ready to inoculate eight to 12 days after seed planting and under good growing conditions.

**Symptoms.** Symptoms will develop in four to six days. Symptoms in cowpea and red kidney bean are chocolate-brown necrotic lesions in the primary leaves (Figure 60). Mosaic patterns may appear in the secondary trifoliate leaves of cowpea under certain conditions. Symptoms in *C. quinoa* are chlorotic local lesions on inoculated leaves and a systemic mottle or mosaic (Figure 61). Citrus ringspot produces chlorotic local lesions in *C. quinoa* and is also systemic. Local lesions are seen in red kidney bean in Figure 62.

**MISCELLANEOUS**

Attempts are now in progress to develop good antisera to CTLV. If these become available, ELISA will be a viable option for indexing.
TATTERLEAF DETECTION

Summary

Graft transmission to citrus

Indicators:

*Citrus excelsa* and/or Rusk or other citrange or citremom as seedlings. Rusk, or other citrange buds forced on any rootstock except trifoliate orange.

No. plants/test:
8 seedlings (3 plus 1 control in each of 2 containers). Grow 1 per container for forced scion buds.

Inoculum:
“Buds” (buds, blind buds or chip buds).

Plant growth:
Allow all shoots to develop.

Temperature:
Cool: 24.27°C max. day/18-21°C min. night.

First symptoms:
5 to 7 weeks.

Symptoms:
Chlorotic mottle, leaf spotting with deformed leaves. *C. excelsa* leaves look tattered. Plants are severely stunted. Stem distortion and blotching in citrange.

Transmission to herbaceous hosts

Indicators:
Cowpea, red kidney (*Phaseolus vulgaris*) bean, *Chenopodium quinoa*.

No. plants/test:
8 plants (4 plus 1 control in each of 2 containers).

Inoculum:
Young succulent leaf tissue in 0.05 to 0.1M phosphate buffer, pH 7.0.

Inoculation:
Rub carborundum-dusted leaves with inoculum.

Temperature:
Cool (21-24°C).

First symptoms:
4 to 6 days.

Symptoms:
Chocolate-brown necrotic lesions in primary leaves of cowpea and red kidney bean; mosaic patterns may appear in secondary leaves of cowpea. Chlorotic local lesions in inoculated leaves of *C. quinoa*. A systemic mottle or mosaic in secondary growth of *C. quinoa* with leaf distortion.

REFERENCES


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FIGURE 55
Bud-union crease of satsuma mandarin on trifoliate rootstock induced by citrus tatterleaf virus (CTLV). Specimen at Kuchinotsu, Japan

FIGURE 56
Severe tatterleaf reaction induced by CTLV showing bud-union crease and fluting in the trunk of Troyer citrange rootstock with satsuma orange scion (Photo: E.C. Calavan)

FIGURE 57
Breakage and separation of a tatterleaf-infected satsuma scion from its trifoliate rootstock. Specimen at Kuchinotsu, Japan

FIGURE 58
Symptoms of tatterleaf in leaves of Rusk citrange inoculated with CTLV. Note deformed leaves with spots and blotches
Symptoms of tatterleaf in leaves of *Citrus excelsa* from a seedling inoculated with CTLV. The non-inoculated control leaf is on the left. Note the tattered, irregular-edged leaves giving the disease its name.

Symptoms of necrotic local lesions in the lesions in the primary leaves of cowpea induced by mechanical transmission with CTLV from citrus tissue.

Symptoms in secondary growth leaves of *Chenopodium quinoa* showing well-distributed spotting, chlorosis and distortion induced by mechanical leaf-rub inoculation with CTLV.

Local lesions in a leaf of red kidney bean mechanically inoculated with CTLV (Photo: S.M. Garnsey).
Infectious variegation and leaf rugose

DESCRIPTION AND BACKGROUND

The citrus infectious variegation virus (CIVV), also called citrus variegation virus (CVV), was the first citrus virus transmitted experimentally. Trabut (1913) named it “infectious chlorosis” and transmitted it to other citrus. Fawcett and Klotz (1939) described it as a new disease called “infectious variegation”. They found symptomatic lemon trees in Glendora, California, and transmitted the disease to sour orange. A similar disease found in Florida by Grant and Smith (1960) was transmitted to many other citrus species. Fawcett (1936) placed this disease in the psorosis family.

CIVV is of exceptional interest since it was the first citrus virus to be mechanically transmitted from citrus to citrus and to herbaceous hosts. In addition to its presence in the United States, the disease has been reported from many countries in the Mediterranean basin and from Australia. Crinkly leaf, once thought to be a separate virus, was shown to be a milder strain of CIVV (Majorana and Martelli, 1968). CIVV will be used to designate both mild and severe strains. CIVV is serologically related to other ilarviruses, i.e. asparagus viruses II-P and II-S, citrus leaf rugose virus, elm mottle virus and Tulare apple mosaic virus (Uyeda and Mink, 1983). It is fairly similar to the satsuma dwarf family of viruses in its morphology, particle size and reaction on certain citrus seedling indicator plants. However, there are differences in components, no evidence for serological relationship and distinct differences in mechanical transmission to herbaceous hosts.

Citrus leaf rugose virus (CLRV) was described by Garnsey (1975) as a citrus crinkly-leaf type, and found in one location in Florida. It has subsequently been found in Japan and Argentina. It induces flecking in Eureka lemon, leaf rugosity in Mexican lime and severe stunting of young Duncan grapefruit plants. It is an ilarvirus, 25-32 nm in diameter. Two strains, which differ in their effect on Duncan grapefruit, have been described. It appears to be similar to the infectious variegation group but with some distinct serological differences (Garnsey, 1975; Garnsey and Gonsalves, 1976).

A variegation which occurs on leaves of sour orange called “Petri’s variegation” was reported from Italy (Petri, 1931). Leaf symptoms are identical to those of CIVV, but Petri’s variegation is non-transmissible and is probably a result of genetic sensitivity of the local sour orange seedling to winter cold (Majorana and Scaramuzzi, 1965).

Although both Fawcett (1936) and Wallace (1957) placed crinkly-leaf and infectious variegation in the psorosis family as it is based primarily on leaf symptoms, they should be considered as separate and distinct citrus virus diseases. There are no serological relationships; CIVV will not protect plants against a challenge from psorosis-A or psorosis-B; symptomatology on leaves (specifically the leaf curl is distinct), and CIVV does not cause the scaling reaction in trunks of sweet orange, grapefruit or mandarin scions induced by psorosis-A. CIVV can be separated from mixtures with psorosis-A or other psorosis-like pathogens by mechanical transmission from citrus to citrus or from citrus to herbaceous hosts. For a complete description, slides and report on CIVV, see Desjardins and Bové (1980) and Wallace (1978).
Techniques for biological detection of specific citrus graft-transmissible diseases

Mechanical transmission of the virus from citrus to citrus by tools can be prevented by dipping tools in a 1 percent solution of sodium hypochlorite. CIVV can be transmitted at low percentages through seed (Wallace, 1968); vector transmission has not been reported. Transmission of CIVV by humans via infected budwood or by mechanical means is the primary means of spread. CIVV can be eliminated from citrus tissue by thermotherapy and by shoot-tip grafting in vitro. The purification and production of antiserum have been done for some strains of CIVV, and rapid identification by immunodiffusion techniques (Garnsey, 1975) and by ELISA (Davino and Garnsey, 1984) has been reported.

METHODS OF DETECTION

Method 1: Field diagnosis
The primary symptoms of CIVV in field trees of lemon, sweet orange, mandarin or grapefruit are seen in the leaves, which show distorted, puffed or puckered leaf segments with or without variegation (Figure 63). When present, this puckered leaf symptom is strongly suggestive of the presence of CIVV. Field symptoms of CLRV are generally inconspicuous.

Garnsey et al. (1984) showed that mild strains of CIVV may exist which induce no symptoms on leaves of field trees. Therefore indexing is imperative for any budwood selection being considered for a mother or foundation tree. Also, leaf symptoms of Petri’s variegation appear identical to those caused by CIVV on leaves of sour orange and perhaps other cultivars and, again, indexing is imperative for diagnosis. At times aphid damage may induce symptoms on leaves of field trees suggesting presence of CIVV. Indexing would readily differentiate aphid damage from virus infection.

Method 2: Indexing by graft transmission to indicator seedlings
Collection of budwood. Collect budsticks from twigs showing leaves with distinct symptoms. If no symptoms are evident in the field tree, collect four budsticks per tree, one from each quadrant.

Inoculum tissue. “Buds” (buds, blind buds or chip buds).

Inoculation. Graft a minimum of two “buds” to each indicator seedling. Seedlings can be cut back at the time of inoculation, or two to three weeks after inoculation when wrapping tapes are cut or removed and inoculum survival is recorded.

Indicator plants. Citron or lemon seedlings are the preferred indicators, and should be used for critical evaluation of mild strains. Lemon should be used for citrus leaf rugose virus. If exocortis is present, lemon or other indicators can be used to avoid interference. Dweet tangor, mandarin, sour orange or sweet orange seedlings are other options. Use two containers with three plants per container; inoculate two plants in each container, leaving the third as a non-inoculated control.

Controls. In addition to the negative controls, a mild- and a severe-positive control should be included for each index test. Avery mild Florida source, e.g. CVV-2 if available, would make an excellent mild-positive control.

Inoculum survival. After two to three weeks, cut the wrapping tapes securing the inoculum “buds” and record bud survival. It is important that the knife or razor-blade used to cut the tapes be

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dipped in a 1 percent sodium hypochlorite solution between plants.

**Post-inoculation care.** After the initial cut-back at time of inoculation, allow the new growth of all emerging shoots to develop without trimming. Ensure that plants are maintained free of insect pests. To prevent leaf damage from insecticidal spray or residue, use a water spray for pest control and, if possible, withhold insecticide spray treatment until after the first or second flush of growth. (See Part II for insect control in the greenhouse.)

**Temperature requirements.** CIVV and CLRV are cool-temperature viruses and indexing should be done at 24-30°C daytime maximum and 18-21°C night-time minimum.

**Time for appearance of first symptoms**
Symptoms for most isolates of the CIVV family should appear in the first flush of growth within four to six weeks after the initial cut-back, or more definitely in the second flush of growth six to eight weeks after the initial cut-back. Symptoms of mild isolates may take longer to appear.

**Symptoms.** Symptoms induced in citron by severe CIVV isolates are extreme leaf distortion, epinasty and a pronounced chlorotic mottle (Figure 64). The presence of exocortis viroid may cause confusion or distortion and epinasty symptoms in citron; therefore other indicators should be used. Leaves of lemon, sweet orange and sour orange will show similar leaf patterns in the early young flushes. When the flush hardens, typical puckered elevated segments may remain (Figure 65). These symptoms are similar to the field symptoms shown in Figure 63. Dweet tangor or rough lemon seedlings make excellent indicators for observation of young leaf symptoms which are similar to those induced by the psorosis-A pathogen (Figure 66).

When mild isolates such as the Florida CVV-2 are inoculated into citrons, leaves will show a mild mottle (Figure 67), some epinasty, and perhaps some mild shock in the first growth flush. Subsequent growth flushes may show a transitory mild mottle in the younger leaves which disappears in the mature leaves. An occasional leaf may show the puckered or elevated leaf symptom. Leaf flecking in lemon is the most consistent symptom for this mild strain.

Symptoms of CLRV are: variable rugosity of leaves of Mexican lime, pinpoint chlorotic flecks on expanding leaves of Eureka lemon without leaf distortion, and severe stunting and chlorosis on seedlings of Duncan grapefruit.

**Termination.** If the mild-positive control Florida CVV-2 is used, the index can be terminated when definitive positive symptoms are observed in citron or lemon, as in Figure 67. In the absence of controls, hold indicator plants for three to four months under cool, spring-like temperature conditions. If new growth leaves are free of symptoms and the severe-positive controls have all shown strong symptoms, the test can be terminated.

For critical indexing of candidate trees for inclusion in a foundation block, it is desirable to inoculate herbaceous hosts and include ELISA in the indexing procedures.

**Method 3: Inoculation to herbaceous hosts**
The mild transient symptoms induced in citrus indicator plants by the Florida isolate CVV-2 suggest that similar mild isolates could easily be missed in an indexing programme based only on graft inoculation to citrus indicator plants. Procedures are available for reliable testing for CIVV to herbaceous hosts.
A number of herbaceous plants have been reported as showing positive reaction when mechanically inoculated with CIVV-infected citrus tissue. Three indicators have been selected for their ability to detect the published strains of CIVV, i.e. the California, Florida and Italian isolates, plus the Florida leaf rugose and CCV-2 isolates.

**Inoculum tissue.** Young leaf tissue or young growing tips can be collected directly from the field tree or greenhouse propagation, put in an ice chest and taken to the laboratory for inoculation. The young leaf tissue from the field tree or from the greenhouse propagations is macerated with a chilled pestle and mortar in sufficient 0.05 M neutral potassium phosphate buffer to create a 1: 10 dilution. A known positive source of CIVV should be included in each index test, plus non-inoculated negative controls. If the very mild positive Florida isolate CVV-2 can be obtained, it should be included as a mild positive control (see footnote under Method 2).

**Indicators.** The three indicators suggested are: *Vigna unguiculata* subsp. *unguiculata* (syn. *Vigna sinensis*), Early Ramshorn or California No. 5 cowpea; *Phaseolus vulgaris*, Bountiful or red kidney bean; and *Crotalaria spectabilis*. Five seeds are sown or transplanted; four seedlings are inoculated, and one left as a non-inoculated control. A minimum of four inoculated plants per container is recommended per index variety, and preferably eight inoculated plants in two containers should be used.

**Inoculation.** Leaves of the indicator plants are dusted with 500-mesh carborundum and the sap inoculum applied with a cottonwool swab. Inoculation must be done as rapidly as possible after maceration. Plants of cowpea and bean are rub-inoculated on their primary leaves before the character leaves appear. The *Crotalaria* is inoculated at the four- to eight-leaf stage. Plants should be rinsed with water immediately following inoculation.

**Controls.** Positive and negative controls must always be included.

**Post-inoculation cure.** Temperatures should be relatively cool. A temperature range of 18-24°C is satisfactory and 20-22°C is best. Some shading after inoculation may be beneficial; symptoms in *C. spectabilis* increase with intensified sunlight. Light conditions will affect the type of local lesion produced in cowpea, which can vary from a diffuse chlorotic lesion to a well-defined necrotic lesion. Local lesions produced by CLRV in bean are small and necrotic. Holding plants under partial shading after inoculation and then transferring them to normal light should induce good symptoms in the three test indicator species.

**Time for first symptoms.** Local lesions may begin to show on cowpea or red kidney bean in two to five days with CLRV. However, CIVV will show local lesions on cowpea but not on bean. Necrotic ringspot lesions will begin to appear in four to five days in leaves of *C. spectabilis*. The brilliant chlorotic vein banding in bean leaves occurs within seven to 14 days. However, the time for appearance of symptoms will be somewhat variable depending upon temperature, light, indicator variety and severity of the isolate.

**Symptoms in cowpea.** Various symptoms will occur depending on strain, light and temperature conditions. With Florida CIVV, lesions occur in the primary (inoculated) leaves and systemic mottle may or may not occur in the secondary growth leaves. Two types of local lesions occur on the primary leaves: some are
reddish and necrotic (Figure 68), and some are chlorotic spots. Approximately 25 percent of plants may show systemic symptoms in ten to 30 days. Symptoms are yellow chlorosis of the veins, mosaic mottle, vein necrosis and leaf curl (Figure 69).

**Symptoms in red kidney bean.** Small necrotic local lesions may appear in primary leaves infected with leaf rugose virus. The systemic reaction in secondary growth leaves for all CIVV isolates is usually apparent within seven to 14 days and shows brilliant vein clearing and pronounced yellow vein banding (Figure 70). Some isolates are milder than others.

**Symptoms in C. spectabilis.** Brownish necrotic ringspot lesions appear early in the primary leaves. The spots may enlarge slightly, and vein necrosis occurs when spots come in contact with veins. Symptoms range from necrotic fleck with chlorotic halo to a necrosis of the terminal portion in eight to 12 days or longer depending on temperature, light and isolate (Figure 71).

**Method 4: ELISA**

ELISA has been used to index for CIVV from both field and greenhouse sources (Garnsey *et al.*, 1984; Davino and Garnsey, 1984). Young spring flush growth is the best source for field-collected samples. Antisera to mild and severe isolates of CIVV (CVV) react equally well to all isolates tested.

Antisera to CLRV are also available and react very well in ELISA tests. The serological cross-reaction between CLRV and CIVV (CVV) is not very strong with the polyclonal antisera available. A weak positive test may be obtained to CLRV using CVV antisera and vice versa. It is, therefore, better to test for each virus separately using homologous antisera.

**CITRUS INFECTIOUS VARIEGATION AND CITRUS LEAF RUGOSE VIRUS DETECTION**

**Summary**

- Graft transmission to citrus

**Indicators:**
- CIVV - Citron, lemon (preferred), Dweet tangor, mandarin or sour orange.
- CLRV - Mexican lime, Eureka lemon, Duncan grapefruit.

**No. plants/test:**
- 4 in individual containers (or planted 3 plus 1 control in each of two containers).

**Inoculum:**
- “Buds” (buds, blind buds or chip buds).

**Plant growth:**
- Allow full flush to develop after initial cut-back without trimming.

**Temperature:**
- Cool: 24-27°C max. day/18-21°C min. night.

**First symptoms:**
- 4 to 6 weeks.

**Symptoms:**
- CIVV – Leaf distortion, puckered segments, epinasty and chlorotic mottle. Young leaf patterns in sweet orange, sour orange and lemon. Crinkle and puckering in mature leaves.
- CLRV – Flecking in lemon, leaf puckers in Mexican lime, stunting and chlorosis in grapefruit for some strains.

- Transmission to herbaceous hosts

**Indicators:**
- Cowpea, common bean or Crotalaria spectabilis.

**No. plants/test:**
- 8 (4 plus 1 control in each of 2 containers).

**Inoculum:**
- Young citrus leaf tissue-tips, leaves macerated in 0.05 M phosphate buffer pH 7.0.

**Inoculation:**
- Leaf rubbed with carborundum.
Temperature:
Cool: 20-22°C.

First symptoms:
2 to 5 days.

Symptoms:

*Cowpea*: CIVV – Chlorotic/necrotic spots in primary leaves; systemic mottle in secondary leaves.

CLR V – Small necrotic local lesions only.

*Bean (red kidney)*: CIVV - Brilliant vein clearing and yellow vein banding in secondary leaves.

CLR V – Small necrotic local lesions only.

*C. spectabilis*: CIVV - Brown necrotic ringspot in primary leaves; halo, necrotic fleck and necrosis in secondary leaves.

REFERENCES


FIGURE 63
Symptoms of CIVV on leaves of navel orange from a tree in the field (Spain)

FIGURE 64
Symptoms on a seedling showing severe epinasty, chlorotic variegation and leaf distortion induced by inoculation with CIVV (California)

FIGURE 65
Symptoms on mature leaves of an inoculated Madame Vious sweet orange seedling showing protruberances and bumps characteristic of infection with CIVV. These symptoms are typical and similar to those induced on mandarins or rough lemon leaves

FIGURE 66
Psorosis-like symptoms in young leaves of a rough lemon seedling inoculated with CIVV

FIGURE 67
Symptoms of mild isolate CVV-2 on leaves of citron. Symptoms on lemon are quite similar (Photo: S.M. Garnsey)
FIGURE 68
Necrotic local lesions on primary leaves of cowpea infected with CIVV (Photo: S.M. Garnsey)

FIGURE 69
Chlorotic lesions on primary leaves of cowpea infected with CIVV

FIGURE 70
Symptoms on secondary growth leaves of red kidney bean infected with CIV, showing bright vein chlorosis (Photo: S.M. Garnsey)

FIGURE 71
Symptoms on leaves of C. spectabilis infected with CIVV (Photo: S.M. Garnsey)
Psorosis complex: psorosis-A, psorosis-B and ringspot

DESCRIPTION AND BACKGROUND
The psorosis disease of citrus was first observed in Florida and California in the early 1890s and named psorosis based on the Greek psora = ulcer or mange. The disease was commonly called scaly bark. It originated in the Orient and was spread worldwide by the distribution of citrus species and varieties. Fawcett (1938) first proved transmissibility by graft-inoculation with buds from infected to non-infected trees.

Fawcett (1933) designated the disease as psorosis-A to distinguish it from a more virulent form called psorosis-B. Psorosis-A has been linked with the concave gum-blind pocket and crinkly-leaf infectious variegation diseases of citrus as one complex (Fawcett and Bitancourt, 1943). However, recent evidence suggests they are all separate diseases, and their distinct differences in symptomatology and cross-protection are discussed in detail in the respective sections. The psorosis disease was comprehensively reviewed by Timmer and Beñatena (1977) and reviewed and illustrated by Roistacher (1980). Typical psorosis-A trunk lesions on a field tree are shown in Figures 72a and 72b, and internal wood staining in the cross-section of a branch is seen in Figure 72c.

The agent responsible for the psorosis disease of citrus is almost certainly a virus, though it has not as yet been purified or characterized. Derrick et al. (1988) reported that two components are associated with the citrus ringspot virus. These components can be isolated, and when mixed together are infectious. Cross-protection of the milder forms of psorosis-A against a challenge with the severe psorosis-B is diagnostic for classifying a disease as belonging to the psorosis-A complex.

The major susceptible varieties showing bark scaling are sweet orange, mandarin and grapefruit. The sour orange, sour lemon and rough lemon show no external bark symptoms. Most citrus species and cultivars are symptomless carriers of the virus. Citrus diseases that show bark lesions and also somewhat resemble psorosis-A are Rio Grande gummosis of grapefruit and leprosis of sweet orange. Indexing of budwood from suspect trees by graft-transmission to specific indicator seedlings should readily distinguish these diseases from psorosis-A.

The psorosis complex of viruses has many variants and contains isolates ranging from those which are non-mechanically transmissible to some which are, such as ringspot or the very serious and spreading Argentinian psorosis. Twenty-one isolates of psorosis-A, obtained from various field trees at the University of California citrus variety collection at Riverside, were graft-inoculated into a variety of indicator plants and tested for mechanical transmissibility from citron to citron. There was much variability in symptoms found in citron, Dweet tangor and sweet orange among the various isolates, and only two isolates transmitted mechanically. However, despite this variability, all 21 isolates in sweet orange rejected a challenge from psorosis-B, confirming their identity as psorosis-A.

The disease is spread primarily by humans via propagation of infected budwood. It has been
observed that 50-year-old sweet orange tree showing no bark lesions in the field produced progeny trees with over 60 percent psorosis bark lesions (Roistacher and Calavan, unpublished). Thus, although psorosis may remain symptomless in certain host trees, the virus can be transmitted from a symptomless host and induce symptoms in progeny trees. Mechanical, seed or root-graft transmission occurs with various psorosis types. Ringspot will move by mechanical transmission in the field (Timmer and Garnsey, 1980). Currently a severe form of psorosis is spreading fairly rapidly in Argentina and is relatively destructive (Figure 72b). The means of spread has not been determined, but vector transmission is suspected.

The first use of citrus seedlings to detect a graft-transmissible pathogen in citrus was by Wallace (1945) for detection of psorosis-A. This seedling index reduced the time required for indexing from an average of approximately 11 years, for development of bark lesions in field trees, to about six weeks for symptom development in the young leaves of sweet orange. This was a revolutionary development that pioneered this relatively rapid detection of citrus pathogens by indexing to greenhouse-grown plants.

There has been much confusion about the relationship between concave gum and psorosis diseases since both may cause leaf fleck in field trees or indicator plants. Roistacher and Calavan (1965) separate these two diseases for the following reasons:

- Psorosis-A causes a distinct bark scaling in sweet orange, mandarin or grapefruit (Figures 72a and 72b), whereas concave gum causes concavities in sweet orange or mandarin trunks and branches (Concave gum, Figure 81) and does not induce scaling. These are separate and distinct symptoms.
- A tree infected with the concave gum pathogen will usually show a series of concentric gum rings in a cross-section of the trunk or branch (Figure 82; also Cristacortis, Figure 93a), whereas trees infected with psorosis-A usually show a specific wood staining in similar sections of the trunk or branch (Figure 72c). These symptoms are very distinct.
- Many leaves on trees infected with the concave gum pathogen will show diagnostically strong oak-leaf patterns (OLP) (Figure 83; also Cristacortis, Figure 95 and Impietratura, Figure 89) in the developing leaves during the spring growth flush under cool conditions, whereas leaves on trees infected only with the psorosis-A pathogen rarely show symptoms in the spring growth flush except if co-infected with the concave gum pathogen. Leaves of trees infected with some of the more severe forms of psorosis, i.e. ringspot, may show strong patterns distinctly different from OLP (Figures 73a, 74 and 75b) in the young and mature leaves in the field, but rarely show typical OLP in the spring flush unless mixtures of pathogens are present.
- When inoculated into seedlings of sweet orange or mandarin, the concave gum pathogen induces a specific mild flecking in leaves of the early flushes, and the oak-leaf pattern usually develops in leaves of later flushes. Shock symptoms are rare. Inoculations with psorosis-A infected tissue will rarely show OLP unless in a mixture, and will usually show shock symptoms in the young flush of seedling growth under cool temperature conditions.
- Concave gum-infected sweet orange plants will not protect against a challenge from psorosis-A or psorosis-B infected tissue.
METHODS OF DETECTION

Method 1: Field diagnosis
Psorosis-A can be diagnosed in the field if the two symptoms of bark scaling and wood staining of stems are observed (Figure 72). Bark scaling alone, though usually diagnostic, should not be totally relied upon for identification. Psorosis-like bark lesions, which apparently are not associated with a graft-transmissible pathogen, have been observed in several areas. The psorosis-B and ringspot forms of psorosis may show varying fruit symptoms (as shown in Figure 73) or ringspot leaf patterns (as shown in Figures 73a and 74). These symptoms are highly diagnostic for the ringspot form of psorosis. Field trees showing bark lesion symptoms of psorosis-A in California, South Africa, the Mediterranean region, Brazil and occasionally elsewhere usually do not show leaf patterns on the young growth flushes on mature trees (Doidge, 1939; Passos, 1965; Roistacher, 1980). Thus, field observation for leaf symptoms is not recommended for diagnosis of psorosis-A.

Method 2: Seedling index
Indexing to seedlings is the principal method for the positive diagnosis for the psorosis-A complex.

Budwood collection. A minimum of four budsticks are collected from each quadrant of a candidate or test tree. If a tree in the field is selected as a prime candidate for thermotherapy or shoot-tip grafting, a budstick should be taken in the proximity of a typical fruit, and a bud propagation made from this budstick held in the greenhouse or plant laboratory. This greenhouse propagation becomes the primary plant, and budwood can be taken anywhere from this plant for indexing or for use as positive control tissue for testing the effectiveness of thermotherapy or shoot-tip grafting.

Indicator plants. The sweet orange seedling is the preferred indicator, and Pineapple, Madame Vinous or Olivelands sweet orange have been found to be superior varieties for detection of psorosis-A (Roistacher and Nauer, 1964). There are definitive varietal differences in sensitivity to psorosis-A and such varieties as Koethen, Mediterranean or Diller sweet oranges should not be used (Roistacher and Nauer, 1964). Mandarins are acceptable indicators but do not always show the shock symptoms associated with psorosis-A, and they may not be so sensitive to lesion formation in cross-protection tests. Although citron and lemon seedlings are excellent indicators for psorosis, they are sensitive to exocortis, other pathogens and tristeza, and psorosis symptoms may be masked or confused in the presence of these pathogens. If seedling-yellows tristeza is not endemic, the grapefruit seedling makes an excellent indicator for certain ringspot isolates.

At least four seedlings (two each in two containers) should be inoculated per test. These should be grown three seedlings per container; two are inoculated and one left as a non-inoculated negative control in each of the two containers.

Inoculum tissue and inoculation. A minimum of two inoculum “buds” (buds, blind buds or chip buds) per seedling are recommended. They are placed anywhere in the lower part of the seedling, leaving as many leaves in place as possible. The seedling can be cut off at about 20-25 cm from the soil surface at the time of inoculation, or two to three weeks after inoculation when tapes are cut and bud survival rate is recorded.

Controls. As indicated above, a non-inoculated negative control should be included in each container. Also, if available, at least two psorosis isolates should be included as positive controls.
in each test: one mild and the other a known standard that consistently induces shock symptoms in inoculated seedlings.

**Inoculum survival.** Two to three weeks after inoculation, the wrapping tape should be cut and removed and the inoculum “buds” examined for survival. Although most psorosis isolates are not mechanically transmissible, some are, and mechanical transmission can be prevented by disinfecting the cutting tool by dipping it into a 1 percent sodium hypochlorite solution when going from plant to plant. Any dead or dying inoculum “buds” should be recorded, and if both “buds” are dead the plant should be reinoculated or a new plant used.

**Post-inoculation care and temperatures.** The temperature during the first four weeks after inoculation is critical for symptom expression. Cool temperatures will favour the appearance of shock reactions in the young emerging shoots (Figure 75), whereas warm temperatures may inhibit shock reactions and mask leaf symptoms. Psorosis-A symptoms are best expressed at relatively cool temperatures of 24-27°C maximum day and 18-21°C minimum night. Shock and leaf-pattern symptoms may not appear if temperatures are too warm (Figure 75).

After the sweet orange seedling is cut back, the new growth should not be trimmed or suckerd but permitted to grow or flush freely. The critical period for development of shock and young leaf symptoms is during the first and second flushes of growth.

If possible, the use of insecticide spray should be avoided during this critical period of symptom development.

Supplemental light may enhance symptom development and should be used during the winter months (see section on supplemental lighting in Part II).

**Symptoms and time for development of first symptoms.** Shock symptoms may show as soon as the first shoots emerge (after three or four weeks). Symptoms of shock are first observed in young emerging shoots as a wilt-like drooping (Figure 76a) followed by the drying up of these young “shocked” shoots (Figure 76b). The leafless shoot is twisted, turns brown and necrotic and ultimately withers and dries up. It is later seen as a dried-up remnant which remains with the plant permanently (Figures 75a and 76b). Shock can be complete, i.e. all emerging shoots dry up and lose their leaves. This is especially evident when plants are inoculated with severe isolates of psorosis-A or ringspot.

More typically, shock may occur only on a few emerging shoots. The shoots that do not shock will usually show varied types of young leaf symptoms, as seen in Figure 77. These appear four to six weeks after inoculation. As the leaves mature, the symptoms may disappear from the hardened leaves. However, some isolates of psorosis and those of ringspot will show symptoms which persist in mature leaves, as illustrated in Figures 73a, 74, 75b and 78. Plants which lose all their first flush of leaves because of complete shock will usually show young leaf symptoms in the next flush of growth. The new emerging shoots of the second growth flush usually do not show shock symptoms.

Shock symptoms may not occur if temperatures are too warm. Also the intensity of symptoms will vary depending on temperature and/or the isolate. Leaf symptoms may not occur or may be different (Figure 75b) if greenhouse temperatures are too warm.

Most of the leaf symptoms will appear in the first two flushes of growth. Later flushes may or may not be symptomatic depending upon the isolate and temperature. It is advisable to observe the developing leaves of the inoculated plants critically during the first two flushes of
growth or approximately during the first eight weeks.

**Termination.** The presence of shock symptoms and young leaf symptoms in the positive controls, observed within the first eight weeks after inoculation, is sufficient to make a judgement of presence or absence of psorosis-A. If the object is only to ascertain the presence or absence of the psorosis pathogen, this eight-week period is sufficient, provided that the positive control plants inoculated with the non-heat-treated or non-shoot-tip grafted bud source are positive. If the object is to identify as fully as possible the reaction observed in the sweet orange test plants, then the three sweet orange seedlings growing in each container (two inoculated and one non-inoculated control) should be permitted to grow and be trained as single shoots. These inoculated plants will then be challenged with psorosis-B as given in Method 3.

**Method 3: Cross-protection**

To determine if the virus is psorosis-A, an inoculated sweet orange seedling is challenge-inoculated with psorosis-B lesion inoculum and observed for evidence of cross-protection.

**Challenging with inoculum of psorosis-B.** A source of psorosis-B is needed for use in cross-protection tests. This is obtained by grafting lesion inoculum, taken from trunk-bark lesions of a field tree, to a sweet orange seedling or budling (see procedure for bark grafting in Part II). In six to eight weeks, under proper temperature conditions, blister-like lesions should form on the stems (Figure 79a), and later develop on the leaves (Figure 79b). The twig lesion inoculum is then used to challenge the sweet orange index plants which had shown leaf patterns, and to determine whether they belong to the psorosis-A family.

Psorosis-B source plants should be maintained in a cool greenhouse or screenhouse and should preferably show the bark-blistcr symptoms shown in Figure 79a.

**Challenge inoculation.** Two “buds” from blister inoculum, as shown in Figure 79a, are graft-inoculated into one of the two pre-inoculated sweet orange indicator plants to be challenged, originating from Method 2. The non-inoculated control seedling is similarly inoculated, and the third plant is held as the pre-inoculated control. Plants can be cut back or left. Within eight to 12 weeks blister-like lesions should form on the challenged, non-protected control seedlings. The psorosis-B lesions usually form near the initial challenge inoculation site.

If lesions develop on the pre-inoculated and challenged test plants, then psorosis-A is not indicated. Conversely, if lesions do not develop on the pre-inoculated challenged test plants, but develop abundantly on the non-pre-inoculated challenged controls, then the virus in question is most probably related to the psorosis-A virus complex.

**Method 4: Mechanical transmission of certain psorosis isolates and citrus ringspot virus**

Citrus ringspot virus has been mechanically transmitted from citrus to citrus and from citrus to herbaceous hosts. The method given below for mechanical transmission from citrus to herbaceous hosts is based on Garnsey and Timmer (1980).

**Inoculum tissue and buffer.** The best tissue is from young leaves of recently inoculated citrus plants in the shock phase. Leaves are ground in a pre-chilled mortar and pestle with cold 0.05 M TME tris buffer at pH 8.0, plus 0.5 percent (V/V) 2-mercaptoethanol.
Inoculation. Chenopodium quinoa is grown from seed in a well-fertilized and aerated soil mix to produce vigorous succulent growth. Artificial supplemental lighting should be used during the winter months. Almost fully expanded leaves of C. quinoa are dusted with 500-mesh Carborundum and the inoculum applied with a cotton swab.

Temperature. 21-27°C.

Symptoms. Symptoms will appear in four to six days as chlorotic local lesions distributed over the leaves, as illustrated in Figure 80.

Garnsey and Timmer (1980) succeeded in mechanically transmitting Florida, Texas and California ringspot isolates, plus three California psorosis-B isolates from citrus to Chenopodium quinoa. They could not mechanically transmit any isolate showing symptoms in C. quinoa back to citrus from C. quinoa, but could transmit ringspot isolates from C. quinoa to Gomphrena globosa and then back to citrus from G. globosa.

Mechanical transmission from citrus to citrus is best carried out by knife or razor slash into the stem. Citron is an excellent host and receptor plant. A slicing cut is first made into the branch of symptomatic citron tissue and then into the stem of the receptor citron. Ten slices can be used as a standard. The sliced stem is then wrapped securely with budding tape.

Many psorosis-A isolates will not transmit mechanically from infected sweet orange or citron to other citrus or herbaceous hosts (Roistacher, unpublished). Therefore, a negative response to mechanical inoculations does not indicate freedom from virus, and does not eliminate the need for bud transmission to citrus indicator plants.

PSOROSIS-A DETECTION

Summary

- Graft transmission

Indicators:
Madame Vinous or Pineapple sweet orange seedlings.

No. of plants/test:
4 seedlings (3 plus 1 control in each of 2 containers).

Inoculum:
“Buds” (buds, blind buds or chip buds).

Plant growth:
Allow all shoots to develop after initial cut-back.

Temperature:
Cool: 24-27°C max. day/18-21°C min. night.

First symptoms:
3-4 weeks.

Symptoms:
Shock, followed by young leaf flecking and mottle in leaves of new shoots.

- Mechanical transmission

Indicator:
Chenopodium quinoa.

No. of plants/test:
8 (3 plus 1 control in each of 2 containers).

Inoculum:
Young symptomatic citrus leaf tissue-leaves or leaf tips (preferably tissue in shock).

Inoculation:
0.05 M TME tris buffer pH 8.0; leaf rubbed with Carborundum.

Temperature:
21-27°C.

First symptoms:
4 to 6 days.

Symptoms:
Chlorotic local lesions, well distributed.
REFERENCES


FIGURE 72a
Scaley bark symptoms of psorosis-A on the trunk of a sweet orange in California

FIGURE 72b
Severe scaley bark lesions of psorosis on a nucellar sweet orange tree in Concordia Argentina

FIGURE 72c
Staining of interiorwood of a branch of sweet orange infected with psorosis-A. This symptom is diagnosed for psorosis-A when found on trees showing bark lesions as in (a)
FIGURE 73a
Symptoms of ringspot psorosis on leaves and fruit of Navellina orange in Greece showing indented sunken areas on fruit

FIGURE 73b
Symptoms of ringspot on fruit of Clementina mandarin in Spain showing green spotting of fruit. Impietratura may be present

FIGURE 74
Leaf symptoms induced by ringspot psorosis inoculated to seedlings of Marsh grapefruit (California)
FIGURE 55
Bud-union crease of satsuma mandarin on trifoliate rootstock induced by citrus tatterleaf virus (CTLV). Specimen at Kuchinotsu, Japan

FIGURE 75a
The effect of temperature on symptom expression, showing shock symptoms of psorosis-A on inoculated sweet orange seedlings grown under relatively cool temperatures of 24-27°C maximum day (left) compared with no symptoms on plants grown under relatively warm temperatures of 32-38°C maximum day (right) (California)

FIGURE 75b
The effect of temperature on symptom expression, showing variation in leaf symptoms due to the effect of the above temperature regimes. The leaf on the left is from a plant grown under the cool temperature and the leaf on the right is from a plant grown under the warm temperature regime. Both plants were inoculated with the same inoculum

FIGURE 76b
Psorosis shock symptoms in inoculated young seedlings of pineapple sweet orange. The plant on the left is completely shocked. Shock remnants will remain on the plant and do not drop off
FIGURE 73a
Symptoms of ring spot psorosis on leaves and fruit of Navellina orange in Greece showing indented sunken areas on fruit

FIGURE 77b
Young leaf symptoms in Pineapple sweet orange. Mild psorosis-A symptoms in leaves of sweet Pineapple sweet orange with non-inoculated control leaf on the right

FIGURE 78a
Mature ring spot-type leaf symptoms in mature leaves of Valencia orange (Photo: L.J. Klotz)

FIGURE 78b
Mature ring spot-type leaf symptoms in mature leaves of rough lemon
FIGURE 55
Bud-union crease of satsuma mandarin on trifoliate rootstock induced by citrus tatterleaf virus (CTLV). Specimen at Kuchinotsu, Japan

FIGURE 79a
Lesion bark blisters of psorosis-B on a twig and thorn of sweet orange (above) with the non-inoculated control below

FIGURE 79b
Psorosis-B-induced, blister-like lesions on the underside of a sweet orange leaf. Similar lesions may be found on leaves of field trees infected with ringspot

FIGURE 80
Local lesions on leaves of Chenopodium quinoa induced by mechanical inoculation from ringspot or psorosis-B-infected citrus tissue (control leaves on right)
DESCRIPTION AND BACKGROUND

Concave gum and blind pocket diseases of citrus were first observed and named by a grower, J.C. Perry, in Highland, California, in the early 1930s and shown to H.S. Fawcett, who described the disease (Fawcett, 1936). Concave gum and blind pocket are probably part of the same complex with blind pocket showing sharper, deeper depressed concavities. Roistacher and Calavan (1965) separated concave gum from psorosis-A based on: leaf symptoms on field trees; leaf symptoms on inoculated indicator seedlings; trunk symptoms; internal wood staining or gumming; and cross-protection reactions. These differences are reviewed in the psorosis-A section.

The relationships of the various leaf-flecking-type diseases are discussed in detail in a review by Timmer and Beñatena (1977). Concave gum and other diseases which induce oak-leaf pattern (OLP) symptoms are found mostly in the Mediterranean countries but can also be found less frequently in most of the citrus-growing areas of the world. Concave gum is a disease that primarily induces symptoms in sweet orange, mandarins, tangors and tangelos, with many citrus cultivars retaining the pathogen as symptomless hosts. Although the pathogen has not been isolated, it is almost certainly a virus and has shown cross-protection by strains that induce mild OLP symptoms against a challenge with severe OLP-inducing strains (Roistacher and Calavan, 1965). It is readily transmitted by grafting and topworking, usually by people, but can be naturally root-grafted to adjacent trees. There is a possibility that certain related isolates may be seed-transmitted (Bridges, Youtsey and Nixon, 1965). Pollen from concave gum-infected trees, when placed under the bark of indicator plants, will transmit the disease (Vogel and Bové, 1980). Concave gum disease has not been shown to be vector or mechanically transmissible. The pathogen can be readily eliminated from infected budwood by thermotherapy or by shoot-tip grafting in vitro.

METHODS OF DETECTION

Method 1: Field diagnosis

Concave gum disease can be diagnosed in the field by three types of symptoms. The first is the presence of distinct concavities in the trunks of sweet oranges, mandarins or tangelos, as illustrated in Figure 81. For other descriptions, slides and photographs of field symptoms see Klotz (1976) and Wallace (1978). A second diagnostic indication of concave gum disease is the presence of concentric gum rings in cross-sections of large twigs and branches as shown in Figure 83 and Cristacortis, Figure 93a. This symptom is usually found in association with concavities in the trunk. Vogel (personal communication) reports that certain varieties such as Washington navel and Orlando tangelo will show good symptoms of gum rings in the wood, whereas certain sweet oranges such as Tarocco, or some mandarins such as Willow leaf, do not.

A third diagnostic field symptom, usually found in association with the first two, is the presence of OLP in nearly all of the young developing leaves in the spring flush of growth and also in other flushes developing under cool temperature conditions (Figure 83; Cristacortis, Figure 95 and Impietratura, Figure 89).
The OLP symptom is also found in association with impietratura and cristacortis diseases. When OLP is found in leaves of field trees, although indicating the presence of a disease, it is not specific for any of the three mentioned diseases. In warm or hot climates, the OLP symptom may not be apparent if the leaf flushes develop when temperatures are too warm. However, Garnsey (personal communication) reports that concavities can occur on Minneola tangelo in Florida in the absence of OLP symptoms. Certain mandarin trees will naturally develop concavities that are not associated with infection but are natural to the variety. Indexing is indicated.

**Method 2: Seedling index**
The seedling index used for detection of interveinal clearing and OLP typically associated with concave gum disease is the same for the diagnosis of all three of the OLP diseases, i.e. concave gum, cristacortis and impietratura. The general seedling index procedures for all three are as follows:

**Collection of budwood.** At least four budsticks should be collected, one from each quadrant of a candidate tree. If a tree in the field is selected as a prime candidate whose budwood will be heat-treated or shoot-tip grafted, a budstick should be taken below or proximal to a well-developed and typical fruit (see Part II).

**Inoculum tissue.** “Buds”, i.e. buds with “eyes”, blind buds or chip buds, are preferred as inoculum tissue for grafting, although other tissue can be used. A minimum of two inoculum “buds” per seedling is recommended.

**Inoculation.** Inoculum “buds” are placed anywhere in the lower part of the seedling, leaving as many leaves in place as possible. The seedling can be topped or cut at about 20-25 cm from the soil surface at the time of inoculation, or two to three weeks after inoculation when tapes are cut and bud survival is recorded.

**Indicator plants.** The Dweet tangor seedling has been found to be a most sensitive indicator variety for expressing the OLP symptom. However seedlings of certain mandarins, i.e. Dancy, Kara or King, are also excellent indicators (Roistacher, 1963). The sweet orange seedling is not as effective as Dweet tangor or certain mandarins in detecting the mild isolates of OLP-inducing pathogens, and some of these mild-reacting isolates can be missed entirely in leaves of most sweet orange seedlings (Roistacher and Nauer, 1964; Roistacher and Calavan, 1965). However, sweet orange is still a very effective indicator for expressing OLP for most isolates of the concave gum pathogen.

At least four plants, two each in two containers, should be inoculated per test. These should be grown as three seedlings per container; two are inoculated and one left as the non-inoculated control.

**Controls.** In each container of three seedlings, one should be left as a non-inoculated control. A severe- and a mild-positive control (if available) should be included in each test and inoculated respectively into a minimum of two plants in the three-plant container, with the third plant as the non-inoculated negative control.

**Inoculum survival.** Two to three weeks after inoculation, the wrapping tape should be removed to expose the inoculum for examination. Although concave gum has not been found to be mechanically transmissible, it is good sanitary procedure in a plant laboratory to disinfect any tool used for cutting into a plant by dipping it into a 1 percent sodium hypochlorite solution when going from plant to plant. Any dead or dying
inoculum “buds” should be recorded and, if both inoculum “buds” are found dead, the plant should be reinoculated using fresh inoculum if available or the original budsticks stored in the refrigerator.

**Post-inoculation care.** The new growth of the Dweet tangor or mandarin indicator seedlings should not be trimmed but allowed to flush freely. The most critical period for observing OLP is usually at the second flush of growth after inoculation, and the young leaves should be inspected frequently for symptoms during this flush period. Insecticide sprays should be avoided if possible during this critical flushing period. Interveinal flecking and OLP symptoms appear in the expanding leaves of the young developing flush, and may disappear completely as the leaves mature and harden. The milder flecking symptoms usually disappear in the hardened mature leaves.

Supplemental lighting, when applied during the winter months, will enhance OLP symptom expression. For example, at Riverside, California, at 34°N latitude, the addition of five hours of 40- to 50-foot candles of supplemental light (at the plant level) during the months of October to April induced symptoms in 207 leaves on plants of four indicator varieties compared with only 60 leaves on plants grown without light (Roistacher, 1963). In addition to the more than threefold increase in the number of leaves showing symptoms under artificial lights, there was a 32 percent increase in the total number of leaves produced (see Supplemental lighting in Part II).

**Temperature requirements.** The leaf patterns associated with concave gum and related diseases are best seen in inoculated indicator plants grown under cool temperatures similar to those for tristeza, psorosis etc. (24-30°C maximum day and 18-21°C minimum night). Under warm conditions, symptoms may not appear. Cool night temperatures and moderate day temperatures characteristic of spring are best for maximum symptom expression. Since symptoms of OLP are best seen in the leaves of growth flushes during the cooler spring or autumn periods, conditions in the plant laboratory should simulate the naturally occurring temperatures as much as possible.

**Time for development of symptoms.** The first symptoms will appear on emerging leaves within five to eight weeks, usually with the second flush of growth.

**Symptoms.** The first symptom is usually mild interveinal leaf flecking similar to that in Figure 83b. Whereas psorosis-A-type leaf flecks develop in the very early young leaves, concave gum symptoms of interveinal clearing and OLP are best observed in the more developed young leaves. OLP will most frequently develop in the larger leaves, and is best seen before the leaf hardens (Figure 83). The oak-leaf pattern may appear as a thin, translucent delineation on each side of the midvein (as in Figure 83b) or as the fully expanded, translucent imprint of an oak leaf (Figure 83a; Cristacortis, Figure 95 or Impietratura, Figure 89).

**Termination.** The index test can be concluded when leaves of both the mild and severe positive control plants show clear definitive symptoms in all of their respective inoculated plants. Experience at the Rubidoux laboratory in Riverside, California, has shown that with the proper indicator plants, which have been grown under optimum nutrition, temperature and supplemental light conditions, positive controls will usually show symptoms within an eight-week period.
If space is not a critical factor, test plants can be held for one additional flush after controls have shown symptoms.

**CONCAVE GUM (OAK-LEAF PATTERN) DETECTION**

**Summary**

**Indicators:**
Seedlings of Dweet tangor, King, Kara or Dancy mandarin, or sweet orange.

**No. plants/test:**
4 plants (2 plus 1 control in each of 2 containers).

**Inoculum:**
“Buds” (buds, blind buds, chip buds).

**Plant growth:**
After inoculation and cut-back, allow seedlings to develop full flushes. Do not trim.

**Temperature:**
Cool: 24-27°C max. day/18-21°C min. night.

**First symptoms:**
5 to 8 weeks; best in second or third flush.

**Symptoms:**
Interveinal flecking followed by oak-leaf patterns in emerging young leaves. Symptoms may fade in mature, hardened leaves.

**REFERENCES**


FIGURE 81
Concavities associated with concave gum diseases in the trunk of a sweet orange tree in the field (Spain)

FIGURE 82a
Concentric gum rings in a cross-section of a branch. This is highly symptomatic for concave gum disease when present with trunk concavities and OLP (Photo: R. Vogel)

FIGURE 82b
Concentric gum rings in a cross-section of a small twig. This is also symptomatic for concave gum disease (Photo: R. Vogel)
FIGURE 83a
Strong oak-leaf patterns in the leaves from a field tree of Valencia orange showing concavities during the spring flush of growth.

FIGURE 83b
A narrow oak-leaf pattern developed about the midvein in a leaf of Valencia orange. Note the strong interveinal flecking, usually the first symptom observed in leaves on plants inoculated with concave gum virus.
DESCRIPTION AND BACKGROUND

Symptoms of impietratura were first described by Reichert and Hellinger (1930) in Palestine. The disease was called “samrah” by the growers, and the affected trees produced fruit with gumming in the albedo of citron, grapefruit and oranges. Ruggieri (1955) named the disease “impietratura” because the fruit turned hard like a stone. For further description of the disease see Catara and Scaramuzzi (1980), Catara et al. (1977) and Papasolomontos (1969). Seasonal variation in appearance of symptoms has been noted, and strain differences reported.

The disease is prevalent in all countries of the Mediterranean basin and also in Iran, Venezuela, India and South Africa. There is no reason why the disease cannot exist anywhere citrus is grown if diseased budwood is introduced and propagated. The fruit of sweet orange, grapefruit and Volkamer lemon are highly susceptible. The disease has been found on fruit of lemon, rough lemon, bergamot, tangelo, citron and mandarin.

The pathogen is believed to be a virus but has not been isolated or characterized. It may be related to, or part of, the concave gum family that produces a distinct oak-leaf pattern (OLP) on leaves in the spring flush of growth on field trees or leaves of seedling indicators. The OLP symptom in inoculated seedlings was shown to be diagnostic for impietratura by Bar-Joseph and Loebenstein (1970). The absence of OLP symptoms in indicator plants inoculated with tissue from heat-treated or shoot-tip grafted plants should indicate elimination of the pathogen from those plants. Thus, a short-term index using seedlings may be efficient for determining presence or absence of the disease.

Transmission of the disease is primarily by humans when propagating infected budwood. It is apparently not seed-transmissible (Bar-Joseph, 1976), and there is no evidence of mechanical or vector transmission. The disease was transmitted by insertion of pollen from infected trees under the bark of Orlando tangelo test trees (Vogel and Bové, 1980).

METHODS OF DETECTION

Method 1: Field diagnosis

Certain symptoms associated with impietratura are highly diagnostic when observed in field trees. These are:

- The distinct discoloration of the rind, usually as circular spots as shown in Figures 84 and 88, and the brown gum spot found directly under the cut surface of the spot as shown in Figures 85-87. These discoloured spots are usually seen as raised bumps or protuberances on the rind.
- If the fruit is sectioned, gum spots may appear throughout the rind and albedo as shown in Figure 87.
- Appearance of many small fruit, which harden, drop to the ground and, when cut, show gumming in the rind.
- The appearance of OLPs in the emerging leaves during the first growth flush during the cooler spring months. This symptom, in association with fruit symptoms, is helpful in diagnosis.

The appearance of all four of the above symptoms is diagnostic. Possible confusion with boron deficiency can be resolved by leaf analysis. A normal tree will contain 30-200 ppm boron in the dry matter of mature leaves, whereas a tree
with boron deficiency will contain 3-25 ppm boron. In addition, the young leaves of boron-deficient trees often show yellowish translucent spots, accompanied by marked leaf distortions or deformations, and old or mature leaves may show corky, split veins and midribs.

**Method 2: Inoculation to field trees**
The fruit symptoms associated with impietratura can be transmitted by grafting budwood from infected trees on to branches of field trees and the symptoms reproduced in the developing fruit.

**Collection of budwood.** Collect budwood, if feasible, from behind a fruit showing typical symptoms.

**Inoculum tissue.** “Buds” (buds, blind buds or chip buds).

**Inoculation.** Inoculate with ten to 15 “bud” grafts into a two- to three-year-old branch of a grapefruit tree which has fruited in the past. Any grapefruit tree, preferably on rough lemon or sour orange rootstock, is a superior indicator although other cultivars can be used (Papasolomontos and Economides, 1967). Inoculate two to four branches on two trees in this manner and self-inoculate a third tree as a control.

**Time of year.** Graft inoculation should be carried out in the autumn, or as early in the spring as possible whenever the bark is slipping. If the bark is not slipping, chip buds can be used, preferably in February (see bottom bud in Figure 127 in Part II).

**Inoculum survival.** Cut the tapes covering the “buds” about six weeks after inoculation and record the “bud” survival. It is always good sanitary procedure to disinfect any tool used for cutting plant tissue in the laboratory or field by dipping it in a 1 percent sodium hypochlorite solution.

**Temperature requirements.** A cool spring followed by warm late spring and summer provides the best conditions for bringing out fruit symptoms (Bar-Joseph, 1976). This is why late autumn or early spring inoculations are preferred.

**Time for first symptoms.** Bar-Joseph and Ben-Shalom (1982) reported the development of symptoms on fruits in three to six months following inoculation. Symptoms should certainly appear by the next season. However, for possible mild-reacting isolates, observe for three full seasons in the field.

**Method 3: Inoculation to indicator seedlings**
Although OLP is generally diagnostic for the concave gum family of pathogens and is not specific for impietratura, the presence or absence of OLP in the young leaves of inoculated Dweet tangor, mandarin or sweet orange indicator seedlings may be diagnostic for the disease. This seedling index is useful for diagnosing the results of thermotherapy or shoot-tip grafting, since OLP is always associated with impietratura (Bar-Joseph and Loebenstein, 1970). See Method 2 in the concave gum section for the detection of oak-leaf patterns (Figure 89) in seedling indicators

**IMPIETRATURA DETECTION**

**Summary**

**Indicators:**
Field: grapefruit/rough lemon or sour orange rootstocks for fruit symptoms.
Seedlings: Dweet tangor, mandarin or sweet
orange for OLP

**No. plants/test:**
Field: minimum 2 trees; inoculate 4 branches per tree.
Seedlings: 4 Dweet tangor, mandarin or sweet orange (3 plus 1 control in each of 2 containers).

**Inoculum:**
“Buds” (buds, blind buds or chip buds).

**Temperature:**
Field: cool spring followed by warm late spring and summer.
Seedlings: 24-28°C max. day/18-21°C min. night.

**Plant growth:**
Seedlings: allow full flushes to develop after initial cut-back.

**First symptoms:**
Field: 3 to 6 months in the developing fruit.
Seedlings: OLP in 5 to 8 weeks.

**Symptoms:**
Field: gum in fruit rind and albedo.
Seedlings: leaf fleck and OLP.

**REFERENCES**


FIGURE 84
Circular, puffed, darker green or lighter coloured spots on the rind of a navel orange (Spain). This symptom is indicative of possible impetreture infection.

FIGURE 85
When the is sliced with a knife, gum is seen in the rind directly beneath the discoloured spots of figure 84.

FIGURE 86
Gum showing in the albedo when the rind is sliced somewhat deeper.
FIGURE 87
Impietratura-induced gumming in albedo of a sectioned young grapefruit (Spain)

FIGURE 88
Impietratura-induced spotting and gumming in the rind and albedo of Navalina oranges; note the typical green spotting on coloured fruit (Spain)

FIGURE 89
Typical leaf flecking and oak-leaf patterns associated with impietratura disease. Leaves will show this pattern on field trees in the spring flush under cool conditions. Also, this symptom on index plants is used as an indication of the presence or absence of the virus after thermotherapy or shoot-tip grafting
DESCRIPTION AND BACKGROUND
Cristacortis was first described and named by Vogel and Bové (1964). Descriptions and illustrations of cristacortis are further documented by Vogel and Bové (1968, 1980b).

Cristacortis is found primarily in the Mediterranean basin, i.e. Algeria, Corsica, Italy, Morocco, Sardinia, Spain, and probably in other parts of this region. Its presence elsewhere in the world is limited, but it could become established wherever citrus is grown if diseased budwood is imported. Susceptible varieties are sweet orange, mandarin, tangelos, tangors, grapefruit, sour orange, rough lemon, siamelo, sweet lime, and occasionally lemon. It has not been found in Troyer citrange, trifoliate orange, citron, chinotto, Citrus hystrix or Mexican lime.

The disease can be distinguished from cachexia by the type and variety of pits. In tangelo or mandarin, cristacortis pits are sharp, deep and distinct, with gum usually at the base of the pit (Figures 91, 92b and 93b). In contrast, the symptoms of cachexia disease in these varieties are undulating depressions and general gumming in the wood and bark (Cachexia, Figures 44 and 45). However, where both pathogens exist together in the same host, the individual diseases may be more difficult to diagnose except by indexing to specific sensitive indicator plants.

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When the outside trunk bark is observed, the indentations formed by the internal pits caused by cristacortis may resemble severe tristeza stem pitting. Again mixtures may exist, and indexing to specific indicators is needed to distinguish them. Cristacortis differs from concave gum by the quality of the pits, i.e. those of cristacortis being sharp and deep in contrast to the more rounded concavities and cupped deformations associated with concave gum (compare Concave gum, Figure 81 with Figures 90 and 92a).

The cristacortis pathogen has never been isolated but is presumed to be a virus. It may be related to the concave gum pathogen since both induce oak-leaf patterns (OLPs) in leaves of field trees or index plants. The OLP symptom is partially diagnostic and can be used for determining the presence or absence of the pathogen in a plant index text after heat treatment or shoot-tipgrafting. Mild, moderate and severe forms of the disease exist and the Clementine mandarin is useful for strain identification (Vogel and Bové, 1976).

The disease is transmitted primarily by humans via propagation of infected budwood or by topworking with budwood from infected trees. It is readily bud-transmitted to other citrus. Mechanical, vector and seed transmission have not been demonstrated.

Transmission has been accomplished by placing pollen from infected tree’s under the bark of indicator plants (Vogel and Bové, 1980a).

METHODS OF DETECTION
Method 1: Field diagnosis
The distinct pits in both the scion and the rootstock (Figure 90), coupled with the appearance of oak-leaf patterns in the young leaves of the field tree during the spring flush of growth (Figures 83 and 95), are highly diagnostic. However, confirmation should be made by indexing.

In the Mediterranean region, where cristacortis is prevalent, the sour orange is the predominant rootstock and will show the pitting symptoms of
cristacortis (Figure 90). In contrast, cachexia-, concave gum- and impietratura-infected trees will not show these classical pitting symptoms in the sour orange rootstock. Therefore, the presence of these deep pits in sour orange rootstock is diagnostic for cristacortis.

Figure 91 shows a typical deep pit in the trunk wood with gum at the base of the pit, and its corresponding peg in the cut section of the bark of a Navelina orange tree in Spain.

Method 2: Indexing to tangelo as a budded scion

Orlando, Webber or Williams tangelo can be used as the scion and sour orange as the rootstock. The method of testing is similar to the forcing of a scion indicator bud described in the cachexia section. Buds taken from seedlings of known pathogen-free sources of these scion indicator varieties are propagated on sour orange rootstock, which is inoculated with “bud” grafts at the same time. The tangelo scion is then forced (Figure 47). Inoculated plants are held in a screenhouse under natural conditions (preferably cool). The detailed procedure is as follows:

Collection of budwood. Budwood to be indexed should be collected from a field tree. A minimum of four budsticks should be collected from each test tree.

Inoculum tissue. A minimum of two inoculum “buds” (preferably blind buds or bark pieces) should be grafted to the sour orange rootstock. The tangelo indicator bud to be forced is inserted about 25 cm above the soil surface and the two inoculum “buds” arc grafted below the scion bud. The plant is then bent to force the tangelo bud (see procedure for forcing Parson’s Special mandarin buds in the cachexia section and Figure 47).

Number of indicator plants. A minimum of four, but preferably six or eight, indicator plants should be used to test each candidate or selection. The inoculum “buds” cut from four budsticks collected around each tree should be evenly distributed among the four to eight indicator plants. After inoculation, inoculum-budwood should be retained and refrigerated for possible future use in the event of graft failure.

Controls. If available, mild- and severe-positive controls plus non-inoculated or self-inoculated negative controls should be included for each test or experiment. A minimum of four but preferably six plants for each of the controls should be used and possibly more for the mild-positive control.1

Inoculum survival. Wrapping tapes should be removed two to three weeks after inoculation and the inoculum tissue observed for graft survival. Although cristacortis is not known to be mechanically transmissible, cutting tools should be routinely disinfected in 1 percent sodium hypochlorite solution between plants. Any dead or dying inoculum buds should be recorded and if both buds are found dead, the plants should be regrafted (or a new plant inoculated) using inoculum stored in the refrigerator.

Post-inoculation core. The tangelo budling should be trained as a single shoot and tied to a stake (Figure 48). Temperatures should be from cool to moderate.

Location. Plants should be well spaced. Vogel and Bové (1972) report that in Corsica symptoms

1Mild-positive control isolates may be requested from Corsica from the Director, Station de Recherches Agronomiques de Corse 20230 San Nicolao, Haute Corse, France.
develop best under natural outdoor conditions rather than in a greenhouse. Vogel (personal communication) prefers a screenhouse in order to enhance maximum rapid symptom development.

**Time for development of symptoms.** Symptoms in tangelo will begin to show in eight to ten months, and trees inoculated with mild strains may take from 20 to 24 months.

**Symptoms.** Symptoms will first appear on the outer bark as small depressions (Figure 92a). If the bark is cut to expose the wood underneath, deep pits can be observed (Figures 91, 92b, 93b and 94). Young trees in the screenhouse should be examined in the spring during the first flush of leaf growth for OLP symptoms (Figure 95), and these recorded. The presence of OLP can be indicative of other associated pathogens, i.e. concave gum or impietratura, and its presence is not necessarily diagnostic for cristacortis. However, when OLPs are found in association with deep pits in the scion and rootstock of the indicator plants, or with pitting in the tangelo seedling as shown in Figure 94, the disease may then be diagnosed as cristacortis. The presence of cachexia may confuse the diagnosis somewhat, but the symptomatic deep pits in both scion and rootstock or in the tangelo indicator are very characteristic and diagnostic for cristacortis and not for cachexia. Also, as shown in Figure 93, cristacortis has a very different type of symptom from that of concave gum (or cachexia) when observed in cross-section.

**Termination.** When 75 percent of the inoculated mild-positive control plants show definitive symptoms, the bark from all test plants can be completely peeled and the wood examined for evidence of pitting.

**Method 3: Observation for oak-leaf patterns in indicator seedlings**
The more rapid seedling index for detection of leaf symptoms of OLP should be used if elimination of the pathogen is the primary objective (see procedures for detecting OLP symptoms under concave gum).

**Cristacortis Detection**

**Summary**

**Indicators:**
- **Screenhouse:** Orlando, Webber or Williams tangelo as scions grafted on sour orange rootstock.
- **Seedling index:** Dweet tangor, King, Kara or Dancy mandarin or sweet orange seedlings.

**No. plants/test:**
- **Screenhouse:** 4 to 8 tangelos, 1 per container.
- **Seedling index:** 4 (3 plus 1 control in each of 2 containers).

**Inoculum:**
- “Buds” or bark pieces.

**Plant growth:**
- **Screenhouse:** train as a single shoot or leader or as a small tree.
- **Seedling index:** allow development of full flushes of growth after initial cut-back.

**Temperature:**
- **Screenhouse:** relatively cool.
- **Seedling index:** cool 24-27°C max. day/l 8-21°C min. night.

**First symptoms:**
- **Screenhouse:** in tangelos:
  - Severe – 8 to 10 months;
  - Mild – 20 to 24 months.
- **Seedling index:** OLP in 5-8 weeks.

**Symptoms:**
- **Screenhouse:** distinct sharp and deep pits in tangelo scion and sour orange stock.
- **Seedling index:** leaf fleck, OLP.


FIGURE 90
Typical pitting symptom of cristacortis in the trunk of both scion and rootstock. Scion is Clementine mandarin and rootstock is sour orange (Sicily)

FIGURE 91
A typical deep pit in the trunk, with corresponding peg in the bark of a Navelina orange tree infected with cristacortis (Spain)

FIGURE 92a
Cristacortis pits showing in the outer bark of a tangelo tree (Corsica)

FIGURE 92b
When a section is cut from the bark, the typical pits and pegs are visible in the trunk and inner bark (Photos: R. Vogel)
FIGURE 85
When the is sliced with a knife, gum is seen in the rind directly beneath the discoloured spots of figure 84

FIGURE 93a
Cross-section of a branch showing both cristacortis and concave gum. The concentric gum rings are associated with concave gum infection, and the pitting in the outer circumference indicates cristacortis

FIGURE 94
Severe pitting induced in a tangelo seedling inoculated with cristacortis-infected budwood (Specimen from R. Vogel)

FIGURE 95
Typical oak-leaf pattern in a leaf from a Dweet tangor indicator seedling. This symptom is typically associated with the cristacortis disease
FIGURE 100
Gummy bark symptoms on Jaffa orange in Syria (Photo: R. Vogel)

FIGURE 101a
Gummy bark symptoms in bark of navel orange near Tarsus, Turkey

FIGURES 101b and 101c
A section of bark taken from a gummy bark-infected navel orange near Tarsus, Turkey, showing gum pockets in the bark, and the appearance of these same gum spots (c) when the bark is sliced tangentially
FIGURE 102
Bud-union crease of Valencia on rough lemon rootstock in Florida (Photo: S.M. Garnsey)

FIGURE 103a
Transmissible bud-union abnormality of Tomango sweet orange on rough lemon rootstock in South Africa. Cuts made into the bud-union bulge show indentation and severe staining surrounding the union

FIGURE 103b
Close-up showing brown line totally around trunk

FIGURE 104
Bud-union crease of Palmer navel on trifoliate rootstock in South Africa. The condition is transmissible

FIGURE 105
Bud-union overgrowth of mandarin on Troyer citrange in the Central Valley of California. This condition is attributed to environmental conditions typical for most mandarins and tangelos in the Central Valley. Trees decline in about 15 years
DESCRIPTION AND BACKGROUND
The vein-enation disease of citrus was first described from California by Wallace and Drake in 1953 and was reported from South Africa by McClean in 1954. Fraser (1959) in Australia demonstrated transmission of a disease causing woody galls on rough lemon rootstocks. Wallace and Drake (1960, 1961) reported that the woody-gall problem found in Peru, South Africa and Australia was related to vein enation. They were able to induce galls in pre-infected rough lemon seedlings by needle puncture (Figure 97). The term “vein enation” will be used to designate both the vein-enation and woody-gall disease. The vein-enation disease has been reported or observed in Turkey, Spain and Japan, and is possibly present in China.

Vector transmission by *Toxoptera citricida*, *Myzus persicae*, and *Aphis gossypii*, and transmission by dodder (*Cuscuta subinclusa*) from citrus to citrus has been reported (Weathers and Harjung, 1964).

The vein-enation disease is believed to be caused by a virus. Hooper and Schneider (1959) reported rod-shaped particles associated with enations. but Rogers and da Graca (1986) showed round, virus-like particles measuring 22-24 nm in diameter in citrus vein-enation virus-infected tissue. A causal relationship between the particles and the disease was undetermined. There has been no report of mechanical or seed transmission. Vein enation is a cool temperature pathogen, and it is readily eliminated from citrus tissue when grown in a warm room or treated by thermotherapy (Calavan, Roistacher and Nauer, 1972; Roistacher and Calavan, 1974). Shoot-tip grafting is also effective in eliminating the pathogen from infected budwood (Roistacher and Kitto, 1977).

Weathers and Greer (1967) showed a synergistic effect between the vein-enation and yellow-vein pathogens. This synergism is the only one known with citrus graft-transmissible pathogens.

Koizumi and Sasaki (1980) demonstrated cross-protection by the vein-enation pathogen against a challenge from citrus tristeza in certain hosts but not in others.

The appearance of galls (Figure 96) on trunks or branches of field trees of rough or Volkamer lemon rootstocks suggests possible woody-gall or vein-enation disease. The economic impact of vein enation is not known. Bazan de Segura and Ferrand (1969) report that, in Peru, trees on rough lemon stock with severe galls showed decline. The disease is symptomless in most citrus hosts and is readily transmitted by aphids. Fraser and Broadbent (1979) indicate that the pathogen may not be limited to citrus but may be present in many other non-citrus species showing galls. Enation symptoms on leaves of field trees are difficult to see and are relatively rare, but can occasionally be seen on leaves of the vigorous growth of young lemon trees or young sour orange seedlings in the nursery (Calavan, personal communication). Indexing is the only certain method of diagnosis. The disease is endemic throughout the coastal areas of Peru and Japan and in the cooler areas of California, i.e. Ventura and Orange counties, but is rarely found in the warmer Central Valley or hot Imperial Valley. It has not been reported from Florida, probably because of the warmer temperatures in the Florida citrus-growing areas.
Vein-enation disease can be readily imported in introduced budwood and, because it is readily transmitted by many aphid species, could become widespread in cooler climates.

**METHODS OF DETECTION**

**Method 1: Field diagnosis**
The presence of galls (Figures 96 and 97) is somewhat diagnostic if the rootstock is rough or Volkamer lemon. Galls may be induced by insect damage or other causes and are not diagnostic by themselves. Suspect trees should be tested by indexing. Vein enations may be seen occasionally on leaves of vigorous shoots of young lemon trees or on leaves of sour orange seedlings in the nursery under cool conditions (Figure 99). The presence of typical enations is diagnostic.

**Method 2: Indexing**
Vein enation can be detected and identified only by means of indexing to plants by graft transmission. Mexican lime and sour orange seedlings are the preferred indicators.

**Collection of budwood.** A minimum of four budsticks should be collected, one from each quadrant of the field tree to be tested.

**Inoculum tissue.** Two “buds” (buds, blind buds or chip buds) are grafted into each seedling. Seedlings can be cut back to 20-25 cm from the soil surface at the time of grafting, or two to three weeks later when wrapping tapes surrounding the buds are cut and bud survival is recorded.

**Indicators and controls.** Mexican lime and sour orange seedlings are the preferred indicators. The symptoms of small galls or enations have been observed on leaves of mandarins, sweet orange, and *Citrus macrophylla*. However, they are not as well-developed as those found in the preferred indicators. If severe, tristeza isolates are present, it may be difficult to see the enations in leaves of Mexican lime or sour orange. Therefore, mandarin or sweet orange seedlings should be included in any indexing programme where tristeza is endemic.

Grow three seedlings per container; inoculate two and leave the third as a negative control. If positive controls are available, they should be included in each index.

**Inoculum survival and post-inoculum core**
The wrapping tapes should be cut or removed two to three weeks after inoculation and the grafts examined for survival. Although vein enation is not known to be mechanically transmitted, knives or cutting tools should be dipped in a 1 percent sodium hypochlorite solution.

Allow plants to flush freely without trimming side shoots for the first two to three flushes of growth (approximately eight weeks), but then trim plants to a single, dominant terminal shoot for the production of large succulent leaves.

**Temperature requirements.** Temperatures must be cool for detection of the vein enations. Cool room temperatures of 24-27°C maximum day and 18-21°C minimum night, as recommended for most cool temperature pathogens, are satisfactory for good symptom production. However, if vein enation is the only pathogen under index, temperatures can be lowered to 20-24°C maximum day and 17-20°C minimum night. Hot temperatures may not only suppress symptoms but can effectively eliminate the pathogen (Roistacher and Calavan, 1974).

**Time for symptom development.** Enations are best seen in the second flush of growth six to eight weeks after inoculation. Symptoms may appear earlier under conditions of ideal temperature and plant growth.
**Symptoms.** Enations are clearly visible on the underside of leaves as small galls or protuberances on the veins (Figures 98 and 99). When looking for symptoms, it is best to hold the containers so that light is reflected at an angle on the underside of the leaf. Enation symptoms are best seen by reflected light in clear sunlight in the morning or evening hours when the sun is at a lower angle. Enations persist on mature leaves and are generally more prominent on the larger mature leaves.

An additional symptom on the leaves of Mexican lime is leaf cupping, identical to the cupping seen on Mexican lime leaves infected with tristeza (see Figure 6).

**Termination.** Under conditions of excellent growth flushes, proper temperatures and good nutrient balance, symptoms should be evident within the first three to four flushes of growth or within 12 weeks after inoculation. If positive controls are available, these can be used as a basis of judgement. If not, then train plants as a single vigorous shoot after the second flush and observe new growth for two additional flushes, or until plants reach about 1 m in height. If, during this period, symptoms are not seen, and temperatures in the index facility were cool, the test or index can be terminated.

**VEIN-ENATION DETECTION**

**Summary**

Graft transmission

**Indicators:**

Mexican lime or sour orange seedlings.

**No. plants/test:**

4 (3 plus 1 control in each of 2 containers).

**Inoculum:**

“Buds” (buds, blind buds or chip buds).

**Plant growth:**

Allow all shoots to develop for the first two or three growth flushes, then train to a single shoot.

**Temperature:**

Cool: 24-27°C max. day/18-21°C min. night (or cooler).

**First symptoms:**

6 to 8 weeks.

**Symptoms:**

Enations or protuberances on veins on underside of leaves.

**REFERENCES**


Roistacher, C.N. & Calavan, E.C. 1974. Inactivation of five citrus viruses in plants held...


Gummy bark and transmissible bud-union disorders

DESCRIPTION AND BACKGROUND

A number of miscellaneous but graft-transmissible disorders affecting the trunk or bud-union have been described. These include:

- Gummy bark or phloem discoloration of sweet orange scions on sour orange rootstock as reported from Egypt (Nour-Eldin, 1956).
- Bud-union crease of sweet orange scions on rough lemon rootstock as reported from Egypt (Nour-Eldin, 1968).
- Bud-union crease of sweet orange scions on rough lemon rootstock as reported from Florida (Grimm, Grant and Childs, 1955; Bridges and Youtsey, 1968).
- Abnormal bud-union of sweet orange scions on rough lemon rootstock as reported from South Africa (McCLean, 1974).

The above disorders share the following characteristics:

- They are graft-transmissible.
- They are rarely present in nucellar selections, and can be avoided by use of nucellar budlines.
- Bud-union problems induce similar bud-union crease, and brown to reddish-brown lines at the interface of the sweet orange scion with the rough lemon or sour orange rootstock.
- They are probably not related to exocortis, cachexia, psorosis, tristeza or vein-enation pathogens.
- Seedling or short-term indexes are not available and have not been developed for these transmissible disorders.

In addition, a serious bud-union crease of sweet orange on rough lemon has been reported to cause a decline of this stionic combination in India (Bhutani, Bakhshi and Knorr, 1972). and bud-union crease of a number of sweet orange varieties was observed in Brazil (Salibe and Cereda, 1984) and was associated with a decline of these trees; the researchers report that comparable bud-unions on nucellar selections were normal, and trees showed no decline.

A graft-transmissible agent in kumquat induces a bud-union crease in Parson’s Special mandarin on Volkamer lemon rootstock and appears to be a new graft-transmissible disease (Vogel and Bové, 1988).

Gummy bark

The gummy bark disease was first reported as phloem discoloration of sweet orange by Nour-Eldin (1956). It was found on numerous sweet orange varieties in Egypt. This disease has since been reported in many North African and Near Eastern countries including Saudi Arabia, the Sudan, Libya, Iran, Morocco, Greece and Turkey. Trees are stunted to varying degrees, and sometimes severely reduced in size. The characteristic symptom of sweet orange scions on sour orange rootstock is shown in Figures 100 and 101. If the outer bark is carefully scraped, reddish-brown, gum-stained tissue can be seen under the bark in various degrees of severity (Figure 101). Discoloration and gumming are severe just above the bud-union and can extend to 60 cm or more above the union. If the bud-union area is scraped, a gum line may
occasionally be seen similar to that in Figure 103b.

The sour orange rootstock will not show the gummy bark symptom, but will occasionally show slight stem pitting (Nour-Eldin, 1968). In addition to this very characteristic symptom, severe stem pitting can be seen when the bark is cut away to expose the wood. This is a common symptom, and the pegs on the under surface of the bark may show gum impregnation.

Transmission has been demonstrated by bud-graft inoculation to healthy sweet orange scions on sour orange rootstocks. After about five years a reddish-brown line can be noticed at the bud-union, and the stem-pitting symptoms appear a year or two later (Nour-Eldin, 1968).

Nour-Eldin (1968) noted that when rough lemon was used as the rootstock, a bud-union crease always developed, suggesting that “gummy bark of sweet orange trees grafted on sour orange rootstock and bud-union constriction of sweet orange on rough lemon rootstock are caused by the same agent”. The constriction or ring at the bud-union of sweet orange/sour orange shows up after three or four years. He also reported (Nour-Eldin, 1980) that gummy bark specimens usually contain cachexia, exocortis and psorosis, and although evidence suggests that these diseases are not causal, no “pure” source of gummy bark has been isolated or developed.

There is an urgent need for the isolation of this disease free of other pathogens and the development of a rapid index test specific for the detection of gummy bark. Bové reported that gummy bark was widespread and a serious problem in many countries of the Near East, based on his many observations in that region. (Statement made at the 10th IOCV conference Valencia, Spain, November 1986.)

Bud-union crease of sweet orange scions on rough lemon rootstock in Florida

Grimm et al. (1955) reported and described a bud-union crease of sweet orange scions on rough lemon rootstock in Florida citrus, and suggested the possible virus nature of this problem. Bridges and Youtsey (1968), in further studies of this bud-union abnormality, showed that it was not correlated with infection by exocortis, cachexia, tristeza, psorosis or vein-ination pathogens, and also suggested its possible viral nature. They found bud-union crease on 118/130 Valencia and Pineapple sweet orange scion on rough lemon rootstock, whereas O/130 nucellar sweet orange scion selections on the same rootstock showed no bud-union crease. They concluded that “a factor responsible for the abnormality was transmitted from parent to progeny”.

Abnormal bud-union of sweet orange scions on rough lemon rootstock in South Africa

McClean (1974) reported and described a bud-union crease of sweet orange scions on rough lemon rootstocks occurring on many sweet orange varieties grown in South Africa, and considered it to be the same as a disease reported in Israel in 1937. Propagations of 45 trees representing 12 varieties all showed the bud-union crease symptom (Figure 103). Transmissions by buds from infected trees to 24 trees of four varieties induced positive symptoms in 19 trees, whereas none of the ten non-inoculated trees showed symptoms. Symptoms were evident within ten years.

A gummy bark disease was observed on sweet orange scions on rough lemon rootstock in South Africa; it appears identical to that reported in Egypt (Figure 101). There have been no studies on the transmission of this disorder.
Bud-union crease of Palmer navel orange on trifoliate rootstock in South Africa

A bud-union problem was observed with many Palmer navel orange trees grafted on trifoliate orange (Figure 104). It creates a distinct brown line and crease very similar to that induced by the citrus tatterleaf virus (see Tatterleaf, Figure 57). The cause of this brown line disease is not known, but it is transmissible and possibly virus-related.

In all of these bud-union diseases, a virus or viroid may be causal. Current indexing procedures are long term, taking from three to ten years or longer. Selection of budwood for entry into a pathogen-free foundation budwood programme may be difficult without a rapid index for these diseases. Until more rapid indexes are developed in those areas of the world where bud-union problems are suspected, buds should be carefully selected from nucellar bud-lines or from mother trees on rough lemon or trifoliate rootstocks whose many progeny trees have a history of being negative for bud-union problems. All major scion sources used in a given area should be propagated on major rootstocks in a variety-rootstock block to verify that bud-union problems (either transmissible or non-transmissible) will not occur.

Bud sources can be indexed by inoculation into a sweet orange such as a Valencia, propagated on to a rough lemon rootstock and put into the field. Trees should be well cared for and soil fertility should be of the best for rapid growth. Where tristeza is not present, sour orange should be added as an additional rootstock for the indexing of gummy bark disease. The need for a more rapid index for these bud-union problems is self-evident.

There are a number of bud-union incompatibility and over-growth problems not the result of transmissible agents. One such problem (see Figure 105) shows the severe bud-union overgrowth of a mandarin on Troyer citrange in the Central Valley of California. Numerous transmission trials (Roistacher, unpublished) have shown that this condition is not caused by a pathogen but is an incompatibility probably aggravated by warm temperatures. Mandarins are especially susceptible and begin to decline after about 15 years.

GUMM BARK AND BUD-UNION
ABNORMALITY DETECTION
Summary
Indicator:
Gummy bark: sweet orange/sour orange.
Bud-union crease: sweet orange/rough lemon.
No. trees/test:
A minimum of 4, preferably 6 or 8.
Inoculum:
“Buds”.
Plant growth:
Train as trees for field planting.
Temperature:
Not known.
First symptoms:
3 to 10 years.
Symptoms:
Gummy bark: reddish-brown gum in lightly scraped bark of sweet orange scion above the bud-union. Severe pits may be present in the sweet orange scion.
Bud-union crease: indentation at the bud-union circling the trunk with pits and gum in the crease line.

REFERENCES


Part II

Facilities and techniques for biological detection of CGTPs
THE GREENHOUSE
A greenhouse, screenhouse or a controlled environment structure is necessary for the production of index plants and for indexing. This structure need not be expensive or elaborate. It should provide lighting, heating and cooling, and be sufficiently well built to prevent insect intrusion. An entrance with two doors and a darkened vestibule between them is desirable as a preventive measure against insect invasion. The exterior can be of glass or plastic. Excellent plants can be grown in a simply designed and inexpensive wooden structure covered with heavy fibreglass and containing a good system for heating and cooling. Modern structures of aluminium framing are now available.

Three greenhouse layouts are shown in Figure 106. A number of greenhouse structures in use worldwide are shown in Figures 107a to 107j. In areas where hailstorms occur, the use of glass should be avoided but, if used, it should be shielded by wire mesh (Figure 107i). Corrugated fibreglass rather than glass is recommended where hail is a problem, and in many respects is preferable since it may be cheaper, unbreakable and easier to erect and maintain.

The size of the greenhouse will depend upon the amount of indexing and research to be carried out. There should be at least three compartments: a cool room for indexing citrus graft-transmissible pathogens (CGTPs) which are best expressed in plants grown under relatively cool temperatures of 24 or 27°C to a maximum not to exceed 30°C during the day, and 18-21°C at night; a relatively warm-temperature room primarily used for growing plants which can be held at flexible temperatures ranging from 30 to 35°C maximum day and 20 to 24°C night; and a hot room which may be used for preconditioning budwood prior to thermotherapy, and for indexing of diseases that require hot temperatures for the best symptom expression. Temperatures in this room should be maintained as warm as possible without inducing plant injury or leaf distortion. A recommended maximum day temperature is 32-40°C with minimum night temperatures of about 24-27°C.

Benches
Benches for holding plant containers can be made of wood, concrete, wire mesh, plastic or any appropriate material (Figures 109a to 109g). A satisfactory bench system in use at the Rubidoux laboratory at the University of California, Riverside, uses 2 x 6-in (5 x 15-cm) Douglas fir boards spaced about 2 cm apart. The wood is painted or sprayed with a 2 percent copper naphthenate solution (Roistacher and Baker, 1954), which acts both as a wood preservative and disinfectant (Figure 109h). Wooden benches can be placed on concrete blocks or on a metal frame or other foundation (Figures 107e, 108d, 109a and 109b) at a convenient working height, usually about 80 cm from the ground. An excellent new design in plastic bench tops is shown in Figure 109g. It is a one-piece, semi-rigid perforated top mounted on a wooden frame.

Flooring
Flooring can be of concrete with provisions for drainage. However, gravel flooring with concrete walkways is highly recommended. Gravel of 1-2 cm should be spread over the ground about 8-10 cm thick (Figures 109a and 109c). This
provides good drainage and aids in maintaining sanitation. The greenhouse should be constructed on a well-drained soil base. If this is not possible, supplementary drainage tiling should be provided during construction.

**Containers**

Plastic containers are recommended for growing citrus plants. A tapered container approximately 15 cm in diameter and 15 cm deep has been found satisfactory over many years of use at Riverside and elsewhere (see Figures 109a, 109b, 109d, 109e, 109g and 109h). Containers measuring 18-20 cm in diameter, but preferably no larger, can also be used. Such a single small lightweight container filled with the proper soil mix will grow three plants to 1 m height readily and without nutritional or other problems (Figure 109a). A relatively large number of these small containers can be placed on each bench but should be adequately spaced to avoid overcrowding (Nauer, Holmes and Boswell, 1980).

Plastic containers must be tested for their ability to withstand steam (permitting their reuse) because steaming is the preferred method of sterilization. Clay pots are not recommended as they accumulate salts, are heavier and breakable, and must be soaked and washed after each use, a process requiring hard labour.

**Temperature control**

Each room must have a recording thermograph (Figure 116). These should be periodically calibrated against two thermometers for accuracy. At the end of each week, when charts are changed, the maximum and minimum temperature readings for each day should be recorded in a special book. This provides a record for research and is also a means of noting changes which may give warning of heating or cooling unit failure or breakdown.

**Supplemental lighting**

Supplemental lighting used during winter months will enhance symptom expression in the plant laboratory. For example, at Riverside, California, at 34°N latitude, the addition of five hours of 40- to 50-foot candles of supplemental light (at the plant level) from October to April induced symptoms of oak-leaf patterns in 207 leaves on plants of four indicator varieties, compared with only 60 leaves on plants grown without lights (Roistacher, 1963). In addition to the more than threefold increase in the number of leaves showing symptoms under artificial lights, there was a 32 percent increase in the total number of leaves produced.

Recent studies have shown that growth of certain citrus seedlings could be significantly enhanced during the winter months with light from 25-watt bulbs, which induced the same growth response as light from 100-watt bulbs (placed 1 m above the bench tops). This has resulted in lower electricity bills (Roistacher and Nauer, 1985).

**Heating**

Heating can be provided by gas heaters with fans, by steam heat using radiators, or by steam pipes placed along the sides of the structure. Heat may also be distributed from gas heaters using supplementary fans blowing the heat through perforated plastic tubes (Figure 108c). Most gas heaters are placed inside the structure. However, ethylene released by faulty heaters can be very damaging to plants. Preferably, gas heaters should be placed outside the structure (Figures 108a and 108b), and the warmed air circulated inside the greenhouse by a fan that forces the air through large-diameter perforated plastic tubes, as illustrated in Figures 108c to 108e.

Catalogues of the larger companies manufacturing greenhouse structures and heating and
cooling equipment are available in many countries and should be consulted for ideas and costs. The construction of a greenhouse is best carried out through local builders, with design and equipment suggested by those in charge of indexing.

**Cooling**

There are three methods for cooling a greenhouse: introducing air from the outside when the temperature is cooler than the temperature inside the house; use of evaporative coolers if the relative humidity is low enough to make such cooling effective; and refrigeration. Other innovative methods can be used, such as a double-layered plastic bubble which acts as an insulator and through which cool (or warm) air can be forced between the sandwiched layers, as in Figure 107j. Combinations of these methods may be used for economy and efficiency.

**Air cooling**. The simplest and most economical means of cooling a greenhouse is by bringing in outside air to replace the warm air within. This is best accomplished by the use of fans (Figure 119) and thermostatic control (Figure 120). When temperatures rise, the thermostat is activated, the fans turn on and cooler air is drawn through the greenhouse. A greenhouse designed to utilize the cooling ability of outside air will save energy, money and wear on cooling equipment. Air brought in from outside must be screened or filtered to keep out insects. A protective screen at the air intake is illustrated in Figure 107d, showing a screened area covered with 32-mesh plastic screen surrounding the water evaporation cells. Figure 121 shows an air-filtering system at Riverside, containing both 32-mesh plastic screen and glass wool filters. This system also has charcoal trays to filter out air pollutants.

The thermostatic controls shown in Figure 120 are designed to control both heating and cooling. As the temperature rises inside the greenhouse, the thermostat will activate the fan (Figure 119) thus bringing outside air into and through the house, forcing out the warm air. When the temperature increases further, the thermostat switches on the pump to send water to the cooling cells to begin evaporation cooling (Figure 124).

One method of using outside air to cool a greenhouse is that of providing vents at the peak of the roof. Vents may be activated mechanically by hand or by a thermostatically-controlled motor. When the vents are opened, they permit the warmed inside air to rise and bring in the cooler outside air through filtered vents at the lower sides of the structure. There are many problems associated with this method of air cooling and, though it is present in many older installations, it cannot be recommended for a plant laboratory greenhouse.

**Evaporator coolers**. The use of evaporator coolers is recommended for most greenhouses where humidity during the summer months is low. An engineering study can calculate the cooling ability of evaporator coolers where humidity is moderate or high during the warm months. Evaporative coolers may prove uneconomical and unsound if the relative humidity is too high. However, in some areas evaporative coolers can be combined with refrigeration for efficient cooling.

Equipment and methods for cooling by evaporation are shown in Figures 122-125. Figure 122 shows a standard commercial evaporator cooler available in most countries where humidity is low and where houses and buildings are cooled.

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1 An example of an informative catalogue is produced by IBG Corporation, PO Box 100, Wheeling, IL 60090, United States of America.
by this means. Figure 123 shows the inside of this cooler with the panel removed exposing the squirrel-cage fan, water reservoir at the bottom, water pump, water dripping down from two outlets at the top, and the pads made of wood fibre or glass wool housed inside the panel door. Such units have been used at the Riverside laboratory for many years and are very efficient in their cooling ability and in screening out insects. They should be carefully serviced each year by cleaning and painting and by changing the cooling pads. A standby cooler should be available for emergency replacement, as well as spare water pumps, fan belts and drive motor.

A more efficient apparatus for cooling is shown in Figures 124 and 125. Figure 124 shows cooling cells consisting of rectangular units of specially treated cardboard placed together to form a solid block. Water is pumped from a reservoir tank shown in the lower left of Figure 124, to a trough above the cells. The water then drips down by gravity over the cardboard cells. The outside air is forced through the moistened cells by the diminished pressure induced by the fans located at the opposite end of the greenhouse (Figure 119). The operation of the fans and water pump is controlled by a thermostat as shown in Figure 120. Figure 125 illustrates a greenhouse at Lake Alfred, Florida, with this cooling system but without an insect filter screen. The cooling cells occupy the full length of the outside wall of the greenhouse.

**Refrigeration.** Refrigeration can be used to supplement evaporator coolers where the relative humidity is too high during the warmer months, where extra cooling capacity is needed as a supplement for the plants in a cool-temperature indexing room or for cooling small individual rooms in a design similar to that shown in Figure 106c. Small plastic chambers can be built inside a large glasshouse to give areas of controlled cooling using refrigeration. Figure 126 shows refrigeration units used to cool a grape-indexing building in South Africa, where electrical energy was relatively inexpensive. Refrigeration is recommended in smaller greenhouses for those compartments to be held at cooler temperatures. These units should be designed so that they are easily removable for repair and replacement, and a spare unit should be held in reserve in case of breakdown.

**SOIL MIXES FOR PLANT GROWTH**

Since most symptoms of graft-transmissible citrus diseases are seen primarily in plants as shown in Tables 1 and 3 in the Introduction, plants should be of the highest quality. Therefore, the soil mixture with its balanced supply of micro- and macronutrients is of prime importance, and much emphasis will be given to the soil mixture and plant growth in this section. The University of California (or UC) system for producing healthy container-grown plants was developed by Baker and co-workers (1957) and published as Manual 23.1 This system was based on the John Innes system of soil mixes developed in England. The original objective was to provide a rapidly growing nursery industry in California with the means of producing uniform and healthy plants. This was done by developing a soil mixture of readily available ingredients and nutrients, incorporating a rigid sanitary programme at all levels of production, including clean nursery stock, sanitary greenhouse practices and soil disinfection. The system was modified by Nauer, Roistacher and Labanauskas (1967, 1968) for growing citrus by the addition of micronutrients to the artificial mixture. Micronutrients were found to be absolutely

1 This manual, now out of print in California, has been re-issued in Australia and is available from Surrey Beatty and Sons, Chipping Norton, NSW 2170, Australia.
essential for the successful growth of most citrus cultivars.

Through strict sanitation practices, the UC system provides a means for the total prevention of soil diseases (especially *Phytophthora* species). It permits fertility control, provides for a renewable, consistent and dependable set of soil ingredients and assists in salinity control. It meets the objective in the production of uniform, healthy, vigorous citrus and herbaceous plants free of deficiency symptoms, and the production of plant growth flushes producing the clear, large young developing leaves necessary for observing many symptoms.

The modified UC system containing micronutrients for growing citrus and other herbaceous plants has been in use at Riverside for over 30 years. By practising rigid sanitation, as recommended by the system, not a single case of soil-contamination by *Phytophthora* has ever appeared in that laboratory. Since plants are the “eyes” with which we see most graft-transmissible citrus pathogens, a successful indexing programme using plants would be very difficult to maintain if *Phytophthora* were present. Many of the specific index plants recommended as seedling indicators or as rootstocks under specific indicator scions are very susceptible to *Phytophthora*. Since citrus plants take six to 12 months to reach buddable size, the destruction of these plants by soilborne diseases cannot be tolerated. Every precaution should be taken to prevent *Phytophthora* infection in the plant laboratory. One such precaution is the use of a soluble copper compound in a foam pad, or Bordeaux mixture as a powder, placed at the entrance of the greenhouse.

**The UC mix**

**Ingredients.** The basic soil mixture consists of 50 percent Canadian peat moss¹ and 50 percent fine sand, with macro- and micronutrients added to the mix (Figure 110). Canadian peat moss is recommended as the prime ingredient. A trial mixture should first be prepared using equal parts of Canadian peat moss and tine sand. Comparative tests can then be made, substituting other local or more readily available types of peat.² Canadian peat has been tested and found to be superior to other peat or sphagnum mosses in nutrient retention and chelating ability. Comparative tests can be made to find a substitute for part of the more specific Canadian peat moss. Ingredients such as redwood shavings (if available), other wood shavings complemented with extra nitrogen, other peat mosses, perlite or vermiculite can be tried. The recommended mixture of 50 percent Canadian peat plus 50 percent fine sand should always be used as the standard for comparison.

It is recommended that a fine sand or silt, with a particle size ranging from 0.05 to 0.5 mm, be used. Beach sand 0.5-1 mm in diameter or clay is not recommended. Fine sands can be found in wind-blown deposits or as the fine silt separated out as waste material from a sand-and-gravel

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¹ Information on Canadian peat moss can be obtained from Sunshine Horticultural Products, Fisons Western Corp., Mr Joe Bridge, (305) – 15015 Victoria Avenue, White Rock, British Columbia, V4B 1 G2 Canada. Tel: (604) 536-2536, (604) 536-5532. Samples of compressed bales of Canadian peat moss will be sent upon request.

² J.F. Ballester-Olmos, who is in charge of the citrus-indexing programme at IVIA, Spain, has done extensive research on many peats. In a personal communication, he suggests that only oligotrophic peats should be used. These are produced in rainwater peat bogs formed in cold regions with high rainfall, i.e. Finland, Poland, Germany, Union of Soviet Socialist Republics, Canada etc. There are two types of oligotrophic peat: blond and black. Ballester-Olmos suggests that a good-quality peat for a potting mix should be a mixture of both. Peat moss used in the Spanish indexing programme is obtained from Torfwerke Neuhaus GmbH, Postfach 3129, 2915 Saterland 1, Germany. Catalogues and information are available on request.
company processing pit. A quick and simple test for determining the presence of clay in a proposed sand source is to shake a sample of the test soil in a jar with water. If the sand settles fairly rapidly and the water remains relatively clear, it is satisfactory. If clay is present, the water will have a muddy appearance and that source should not be used.

The sand should be inert and preferably siliceous. Calcareous or limestone sands should not be used since they may affect the pH. If a good grade of silicate sand is not available, try substituting the sand fraction of the mix with vermiculite and perlite in a proportion of 1/2 peat, 1/4 vermiculite and 1/4 or 1/3 of each other ingredient. The objective is to obtain an artificial medium which is consistently reproducible, will absorb and release macro- and micronutrients, and will maintain pH of the drainage water at 5.5 to 6.5.

The ingredients can be mixed together with a shovel on a flat concrete surface. However, a small or medium-sized electric or gasoline-powered concrete mixer is the preferred mixing device. The procedure at Riverside using a medium-sized concrete mixer is as follows:

- Specific numbers of uniform, standard shovel-scoops of soil, peat and redwood shavings (or other substitutes for part of the peat moss) are counted and shovelled into the apron of the concrete mixer.

  In the apron, a weighed quantity of macronutrients, i.e. phosphate, calcium and magnesium as shown in Figure 110, is sprinkled on top of the unmixed ingredients.

  The soil ingredients plus macronutrients are then dumped into the concrete mixer and thoroughly tumbled.

  °The micronutrients, weighed and mixed together in a package, are first dissolved in water and then poured into the turning mixer.

  - A small quantity of water can be added to the soil while the mixer is turning to bring the soil mixture to a friable, moist level if the soil or peat is too dry.

  - After about 20 minutes of tumbling and mixing, the soil is emptied from the mixer into a trailer fitted with steam pipes on the bottom, as shown in Figures 111a and 111b. The trailer top is covered with a cloth tarpaulin. Containers and flats may be placed on top of the soil in the trailer before covering, or steamed separately (Figure IIIb).1

*The soil mixture is then steamed. The steaming time will depend on the quantity of steam produced, this will depend on the size and capacity of the boiler. A good criterion is to continue to steam for about 15 minutes after the steam puffs up the cover in tarpaulin. Soil thermometers placed in the corners of the trailer will determine the correct period for steaming. One minute at 100°C or 10 minutes at 83°C is usually sufficient for controlling soilborne pathogens. Any longer is a waste of energy and is unnecessary. Steaming has never been found toxic or harmful to plants grown in a UC mix using a fine siliceous sand, Canadian peat moss, redwood shavings, perlite or vermiculite.

**Fertilization.** The initial mix contains both macro- and micronutrients added during mixing. The micronutrients are tied up in the peat moss. The peat, which acts as a chelating agent, releases sufficient small amounts of micronutrients to the plant for periods of up to one or two years.

1 The trailer shown in Figures 111a and 111b is fitted with 20-mm (3/4-in) galvanized pipes with 5-mm (3/16-in) holes spaced 15-20 cm (6-8 in) apart. Pipes are spaced 15 cm (6 in) apart. Holes are located at the bottom of the pipes.
Liquid fertilizer is applied with each watering using a device which injects fertilizer in proportion to the water used. An effective, simple and very inexpensive device is a Venturi-type siphon (Figure 117). Immediately after purchase, the siphon should be calibrated. Many siphons will vary considerably from the advertised ratio of concentrate to water as printed on the instructions. Calibration is done by putting a measured amount of water (500 or 1 000 ml) into a graduated cylinder, then placing the suction end of the siphon into the cylinder and measuring the final amount of water exiting from the hose. Allow water to flow and fill a container until the 500 or 1000 ml of measured liquid is siphoned up. Then measure the water in the container and convert the results to a ratio.

Another device which injects a given quantity of liquid fertilizer into the water system at a uniform rate in direct proportion to the water flow is the Smith Measuremix proportioner (Figure 118). This is a precision instrument and highly reliable. This device has been in use at the Riverside greenhouses for 30 years with a minimum of upkeep or repair problems. The proportioner is set to deliver at a ratio of 1 to 100. However it should be calibrated in the same manner as the Venturi siphon.

A liquid fertilizer mix based on that given by Nauer et al. (1968) is: (dry) parts by weight, 9 parts NH$_4$NO$_3$ (Ammonium nitrate) + 3.75 parts Ca(NO$_3$)$_2$ (Calcium nitrate) + 2.75 parts KNO$_3$ (Potassium nitrate). (If KNO$_3$ is difficult to obtain, substitute KCl.) This fertilizer should be applied at the rate of 67.5 g of mixture to 100 l water (9 oz per 100 gallons). Calculate the amount to use and put the proper amount of fertilizer mix in the concentrate tub, add the correct amount of water and stir well. With the UC system of soil mix, fertilize with every watering. The soil should be fertilized directly after mixing as well as before and immediately after planting since the basic UC soil mix contains no nitrogen or potassium.

As a general practice, potted plants in a UC system need to be watered with enough volume periodically to flush out any accumulated salts and prevent salinity buildup. It is important that the soil not be tilled to the top of the container. A space of 2-3 cm should be left between the top of the container and the soil level. This will allow a sufficient volume of water to flush the soil in the container.

**PRODUCTION AND CARE OF INDICATOR PLANTS**

The indicator plants recommended for indexing are given in Tables 1 and 3 of the Introduction. Seedlings are recommended for indexing for most of the CGTPs. However, in some cases where seedlings are difficult to obtain, or other seedlings are readily available, a clonal propagation can be made with a bud from a selected indicator plant grafted to a vigorously growing rootstock seedling. The rootstock is then inoculated, and the indicator bud forced as a scion. This procedure has been successfully used for detection of a number of pathogens. However, a comparison should be made between the seedling and a clonal propagation of the seedling to be sure that the clonal budline will induce clear positive symptoms equal to those of inoculated seedlings. In some trials, clonal budlines have not performed as well as seedlings, but in other trials they have performed equally well.

Where tristeza is endemic, the use of clonal propagations may be necessary to filter out the
tristeza virus in order to detect or see other pathogens. For example, trifoliate or citrange may be used to filter the citrus tristeza virus so that other pathogens are not masked.

For any consistent long-range index programme, a block of seed-source trees containing the desired indicator varieties should be planted as soon as possible to obtain a reliable and consistent source of seed. In the meantime, small or large quantities of seed can be obtained from commercial outlets or small quantities can be requested from other research stations (Table 4).

Seed treatment
All fruit collected for seed extraction should be picked as high on the tree as possible, and picking up fruit from the ground should be avoided because of the danger of Phytophthora infection. After extraction, the seed should be routinely treated against possible contamination from Phytophthora by a hot-water dip for 10 minutes at 52°C (125°F), followed by a short dip in cool water to return the seed to normal temperatures (Klotz et al., 1960). In addition to the hot-water treatment, the seed should be disinfected with a fungicide to aid in preserving the seed during storage and to prevent albinism when the seedlings emerge. The commercial fungicide Thiram as a 75 percent powder can be used to dust the seed after drying, or the seed can be dipped for three minutes in a 1 percent solution of 8-hydroxy quinoline sulfate, available from most chemical supply houses. The seed is then spread out on paper or on a fine-mesh screen and allowed to air-dry. It should be turned frequently during drying. Be careful to avoid overdrying. As soon as the last moisture has disappeared and the surface appears dry, the seed should be packaged in small polythene bags. The bags should be dated, labelled and sealed with a rubber band, and then placed in a second small polythene bag with a small piece of slightly moist tissue paper placed between the bags. The second bag is also sealed with a rubber band. Seeds which have been treated, packaged and sealed in this manner and stored at refrigeration temperatures of +5° to +6°C have maintained excellent viability for as long as three years (Nauer and Carson, 1985).

Seed planting
Seed can be planted in flats or containers of wood or plastic. Redwood, if available, is ideal since it is easy to use, will not decay, lasts a long time and can be steam-sterilized. Other woods can be used but should be dipped or painted with a relatively non-toxic preservative such as copper naphthenate (Roistacher and Baker, 1954). Plastic containers with drainage holes are also quite satisfactory. They should be tested to see if they will withstand steam sterilization so that they can be reused. The sizes of the two types of redwood flats used at Riverside for growing seedlings are approximately 40 x 40 cm, and 40 x 20 cm by 14 cm deep (Figures 111 b, 112 and 113).

The sterilized soil is placed in the flat and compacted with a flat metal tamper (Figure 112). It is important that the soil be levelled at about 3 cm from the top of the flat to permit uniform distribution of water. If the soil is not level, water will settle at one corner of the flat and the other corner will usually remain dry, possibly resulting in poor seed germination in that corner. A planting board (Figure 113) made of thin Masonite or plastic with 1.5 cm holes drilled 2.5 cm apart is placed on top of the soil and the individual seed placed in each hole. The seed is then lightly pressed into the soil with a dowel (Figure 113). After seeding is complete, the planting board is removed and the seed covered with about 1 cm of soil and tamped lightly. Watering should be done by using a
TABLE 4a
Commercial nurseries that sell citrus seed and virus-free budwood. Catalogues and price lists are available on request

<table>
<thead>
<tr>
<th>Nursery/Company</th>
<th>Address</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adams Citrus Nursery Inc.</td>
<td>PO Box 1505, State Road 544 East, Haines City, FL 33840</td>
<td>United States of America</td>
</tr>
<tr>
<td>AVASA (Agrupación de Viveristas de Agrios SA)</td>
<td>Reina Doña Germana 6-10-2a, Valencia 46005, Spain</td>
<td>Spain</td>
</tr>
<tr>
<td>Thermal Plaza Nursery</td>
<td>68035-P Highway 86, Thermal, CA 92274</td>
<td>United States of America</td>
</tr>
<tr>
<td>Willets and Newcomb Inc.</td>
<td>PO Box 428, Arvin, CA 93203</td>
<td>United States of America</td>
</tr>
</tbody>
</table>

TABLE 4b
Citrus research stations where small quantities of citrus seed or virus-free budwood may be obtained

<table>
<thead>
<tr>
<th>State</th>
<th>Station/Program</th>
<th>Address</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arizona</td>
<td>Arizona Cooperative Citrus Registration-Certification Program</td>
<td>Tucson, AZ 85721, United States of America</td>
<td></td>
</tr>
<tr>
<td>California</td>
<td>Citrus Clonal Protection Program</td>
<td>Riverside, CA 92521, United States of America</td>
<td></td>
</tr>
<tr>
<td>Florida</td>
<td>Florida Citrus Budwood Registration Program</td>
<td>Winter Haven, FL 33880, United States of America</td>
<td></td>
</tr>
<tr>
<td>Texas</td>
<td>Texas A. and I. University Citrus Center</td>
<td>Weslaco, TX 78596, United States of America</td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>I.V.I.A.</td>
<td>Apartado Oficial 46071 Moncada, Valencia</td>
<td>Spain</td>
</tr>
<tr>
<td>Corsica</td>
<td>Station de Recherches Agronomiques de Corse</td>
<td>San Giuliano 20230 San Nicolao, Haute Corse</td>
<td>France</td>
</tr>
</tbody>
</table>

Soft-spray sprinkler nozzle on the hose end or a watering can with a perforated sprinkler head until the seedlings emerge. An excellent soft-spray sprinkler nozzle made of cast aluminium is the Dramm nozzle obtainable from the Dramm Company, PO Box 528, Manitowoc, WI 54220, United States of America.

Seedling trays, as illustrated in Figure 114, are a satisfactory means of growing individual seedlings. Such trays are available through greenhouse and nursery suppliers. The seeded flats or seedling trays are best kept in the warm growing room but can also be kept in the hot room if rapid forcing is desired. Most
seedlings will reach transplantable size in 11-19 weeks depending on the variety (Table 5). Sweet orange seedlings take much longer to grow, averaging 29 weeks. The seedlings must be periodically and critically examined for off-type, gametic or non-nucellar variants; these must be culled and should not be used since they can be poor indicators. By permitting seedlings to reach 8-15 cm of growth rather than transplanting when they are too small, the off-type variants are more readily detected and can be pulled from the flat.

Insect control
If possible, the laboratory should be located away from citrus groves to lower the infestation pressure from insects. Ornamental landscape plants should not be grown too close to the laboratory since they may provide a host for the growth of insects. Personnel should avoid insect-infested plants in the field before entering the greenhouse. A balanced and thorough insect-control programme is of the utmost importance. Damage to indicator plants by insects or insecticidal spray can make symptom-reading very difficult.

Any insect-control programme that reduces the amount of insecticide spraying is worthwhile. Insecticide application, although necessary, should be limited and carefully controlled. Spray damage to leaves is usually in the form of circular translucent spots and may confuse symptom readings as well as damage young growth. The presence of adequate non-inoculated control plants should verify any damage to leaves done by insects or sprays or other non-viral effects. The insect-control programme used at Riverside is as follows:

- All plants are critically examined at least once each week for any signs of insects. If insects are found, the area surrounding the infestation is treated by spot spraying with an appropriate insecticide at concentrations usually below those recommended by the manufacturer to avoid chemical damage to the tender young emerging leaves.
- At least once each week all plants are sprayed with water using a standard-pressure hose nozzle with a fine-spray attachment. The objective of this waterspray programme is to control small infestations of mites. A few undetected mites are readily washed off the leaves by this water spray and usually do not return. This is a preventive measure, and it has been found very effective in reducing the number of times the entire greenhouse must be sprayed. The combination of periodic inspection, spot spraying and waterspraying have been highly successful in keeping insect infestation under control. The importance of routine periodic preventive inspection for detecting new low-level insect infestations cannot be over-emphasized.
- The use of the two-door vestibule entryway for the critical inspections of clothing for the presence of insects is important. Green-coloured clothes should be avoided during the aphid season. Only one door at a time should be opened when bringing in or taking out plants or materials.
- When spraying with an insecticide becomes necessary, select an insecticide and a dilution that will not spot or injure plants. New insecticide sprays should first be tested on a few plants. Dilutions should be carefully calculated and double-checked to be sure they are correct. A number of effective miticides should be kept on hand and their use rotated to prevent build-up of insect resistance.

In general, aphid infestation at the Riverside greenhouse has been rare and never serious.
Mites are the major pest problem. Soft brown and other scales, whitefly, mealy bugs or thrips can be serious problems. Again, the importance of periodic inspection and good insect control cannot be overstated in the maintenance of an efficient plant laboratory.

The introduction of plant material from other areas or from the field into the greenhouse should be avoided. If the plants must be brought in, they should be carefully examined for pests, then cut back to a minimum number of leaves, given a preventive insecticidal spray and isolated in a separate location until they are shown to be free of pests.

If spraying with insecticides becomes necessary, it may be wise to consider spraying all rooms or houses simultaneously. If only one room or house is sprayed, reinfestation may readily occur from other areas not sprayed.

**TECHNIQUES FOR GRAFT-TRANSMISSION IN CITRUS**

Of prime importance in grafting techniques is a good-quality budding knife. New knives must be sharpened using a medium-grit carborundum stone so that every trace of the original V-shaped tip on the cutting edge of the new blade is removed. When the cutting edge is observed with a magnifying glass, a smooth transition from the back of the blade to the very edge should be seen. The blade should be razor-sharp. The test for a well-sharpened blade is the ability to make a single, smooth slicing cut into the budstick and detach the bud or blind bud clear of the budstick without tearing the tissue as the cut is completed. The knife should be periodically resharpened with a fine-grit oilstone using oil, and honed on a leather strap. It is well to observe a number of professional or skilled budgers to study their techniques and practise under their tutelage.

**“Bud” graft inoculation**

Figure 127 shows the three types of “bud” used for graft-transmission. The tissue illustrated at the top is a bud containing an eye. This is always used for propagation purposes but may be used for inoculation. The centre “bud” is called a
blind bud i.e. a “bud” without an eye. It is cut from the portion of the stem between the buds and is used primarily for inoculation purposes.

Budsticks are collected from the field tree to be indexed and should be placed in a plastic bag, cooled in an ice chest and refrigerated at the laboratory until used. The bud or blind bud is sliced or cut from a stem or budstick. Using a well-sharpened knife, a slice is made into the stem using a continuous slicing motion. The bud should be cut free of the stem with one smooth slicing stroke. AT-cut is then made into the stem of the receptor or index plant to be inoculated. The upper portion of the bark at the top of the T-cut is opened and the bud or blind bud inserted (similar to the illustration in Figure 130). The blind bud is an excellent inoculum tissue and is preferred since it is easier to excise from a budstick and has the advantage of not having an eye, thus avoiding growth of the inoculum bud after the wrapping is removed. It is inserted in the same manner as the bud with an eye.

The third type of bud is the chip bud (Figure 127, lower). Chip buds are used when the bark of the receptor host does not slip or open up to accept a bud or blind bud. This bud is cut from the inoculum stem by first making a cut at right angles into the inoculum stem about 1-2 mm deep, and then making a slicing cut toward the initial cut to free the bud. Similar matching cuts are made into the receptor stem, and the two pieces are matched and fitted together and then wrapped. Chip buds may be cut with or without an eye.

After insertion, the inoculum “buds” are secured to the stem by wrapping with either rubber or plastic tape as shown in Figure 128. The tape should be kept stretched and the bud should be tightly wrapped. If a bud with an eye is used for propagation, avoid covering the eye with the wrapping tape so that the bud can grow. If, however, a bud with an eye is used only for inoculation, the eye should be covered and the bud wrapped completely with the wrapping tape.

Two bud-inoculations are generally sufficient for transmission. After two to three weeks, the wrapping tape is removed by cutting the tape with a knife or razor-blade and the bud is examined to see if it is alive (survival recording). If both grafts are found dead, the same plant should be reinoculated with buds or chip buds, or another test plant inoculated. If only one graft is dead, and there are sufficient replicated test plants, reinoculation is unnecessary.

When cutting tapes for removal, ensure that the knife blade is disinfected by dipping it into a 1 percent sodium hypochlorite solution.

**Leaf-piece graft**

- A rectangular piece approximately 3-4 mm wide and 2-3 cm long is cut from a young immature leaf as shown in Figure 129.
- The cut piece is inserted into a T-cut in the receptor stem exactly as is done for buds. The point of a knife is used to push the leaf into the T-cut as shown in Figure 130. It is important that the bark separate readily or be “slipping” to permit easy entry of the leaf piece into the T-cut. Two leaf-graft inoculations per plant are recommended.
- The inserted leaf piece is then wrapped in the same manner as with buds shown in Figure 128.
- The wrapping tapes may be cut two or three weeks after inoculation. The grafts are then examined for survival, and if both inoculum grafts are dead the plant can be reinoculated (if the bark is slipping) or a new plant inoculated.

After some time, the leaf piece of a successful graft can be seen to expand and grow inside the T-cut as illustrated in Figure 131.
**Leaf disc graft**  
A paper hole-punch is used to cut a disc from the midrib section of a leaf of the inoculum source, as shown in Figure 132. The leaf should be moderately mature or mature. Very young leaves should not be used since it is difficult to punch out discs and manipulate them, and the punched discs do not match up well with holes in the receptor leaf. The punched leaf discs are placed on a piece of slightly moistened tissue paper during the inoculation procedure. A number of inoculum leaf discs may be cut at one time, and at least five discs are recommended for inoculation of each plant. The cut leaf discs should be placed on the moist paper with the top leaf surface facing up. 

- A hole is punched in each of five leaves of the index or receptor test plant using the hole-punch as shown in Figure 132. Receptor leaves should also be moderately mature or mature, but not too young. 
- A piece of adhesive tape, slightly longer than the width of the leaf, is placed on the bottom of each of the receptor leaves and pressed lightly into place. 
- The cut discs, resting face upward on the moist tissue paper, are retrieved with a dissecting needle and carefully inserted into the hole previously made in the receptor leaf, as shown in Figure 133. The midribs should be as well aligned as possible. Similarly, the disc should be carefully matched to the hole. The adhesive tape at the bottom of the leaf will hold the leaf disc in place while necessary adjustments are made.
- Another piece of adhesive tape of similar length is then placed above the leaf and firmly pressed into place. The completed graft is shown in Figure 134. 

- After one to two weeks, a determination can be made for graft survival. Dead leaf grafts will turn brown, and reinoculation is necessary if three or more of the five grafts are dead.  

**Caution.** The brand of adhesive tape selected is extremely important. Some brands are toxic to the leaf. Scotch 600-brand is non-toxic. Whichever local brand is selected, it should be examined for toxicity by making some test grafts on a number of leaves. The tape should not be left in a warm greenhouse but kept refrigerated. Warm temperatures have been shown to induce chemical changes in the tape ingredients and induce toxicity.

**Bark graft**  
- Rectangular pieces of bark are cut from the trunk of the tree to be tested or indexed. This should be done only during the months when the bark is readily slipping. The cut bark pieces are placed in a plastic vial, as illustrated in Figure 135. The vial should be designed to permit some breathing. The vials containing the bark samples should be placed in an ice chest immediately after sampling and later transferred to a refrigerator at the laboratory. Tests have shown that bark collected in this manner and refrigerated at +5-6°C remained viable for as long as one month. 
- The bark pieces are removed from the vial, placed on a moist paper towel and cut into rectangular segments 3-4 mm by about 20 mm. The cambium face of the bark should be kept in contact with the moist tissue paper (Figure 136).
- An “I” cut is made in the stem of the receptor or index test plant, and the bark is teased open slightly. The inoculum bark piece is then placed between the flaps of the
open ‘I’ cut and is held in place by the bark flaps (Figure 137, bottom).

- At least two bark grafts are made per index plant, and the inoculum is then tightly wrapped with rubber or polythene budding tape in the same manner as for bud grafts (Figure 128 and centre graft in Figure 137).

- After two to three weeks, the tape is removed by cutting it free with a disinfected knife or razor-blade, and the inoculum observed for survival. At this time, the survival or vitality of the bark tissue can be tested by gently slicing into the graft tissue just underneath the outer bark using a razor-blade or knife. Dead or dying tissue will be brown, shrivelled and sometimes loose; vital tissue will be green, yellow or white.

**Side grafting**

- Inoculum stem pieces approximately 8-15 cm long are cut from the test source tree or plant to be indexed and are matched, diameter for diameter, with the indicator test plant.

- Wedge cuts are sliced on both sides at the end of the stem piece giving the appearance of a long tapered “v”. The cutting knife should be razor-sharp, and some skill and practice are needed to make the cuts very flat, smooth and straight.

- A slicing cut is then made into the receptor indicator plant using a knife or razor-blade. The depth of the cut should be about the same as the length of the wedge cut on the inoculum stem piece.

- Using a clipper, the inoculum piece is trimmed to about 3-4 cm long, as seen in Figure 138, and inserted into the stem. Some slicing and trimming may be necessary but, with practice, well-fitting grafts can be made repeatedly.

- The joined tissue is tightly wrapped with budding tape. At least two side grafts per test plant should be done (as illustrated in Figure 26 in the section on stubborn).

- The bottom end of a polythene bag is cut to convert the bag into a sleeve. This is then placed over the plant, and secured at the top and bottom with plant ties (Figure 138, left). It is important that some leaves be left on the stem near the graft to provide moisture within the bag. The cut end of the inoculum stem pieces need not be coated with protective tar. Research tests with thousands of grafts, with and without protective tar, indicate no benefit from this practice.

- After 10 days, the bottom of the polythene sleeve is opened to permit some air into the bag. At 15-20 days, the polythene sleeve is removed and grafts are examined for survival.

- The plant is then topped or cut back about 25-30 cm above the soil surface, but above the side grafts, and a single shoot is permitted to grow (as illustrated in Figure 26 in the section on stubborn).

Many thousands of side grafts have been made at the Riverside laboratory using this procedure, with a survival rate of over 95 percent. Whenever the graft inoculum died, the reason was found in a deficiency factor in the budwood rather than in human error. Certain mandarin budwood (i.e. Dancy) collected during the spring months gave a high rate of graft failure. However, most tissues of the major citrus cultivars were found to be highly graft-compatible.

A modified side graft can be made by inserting a small twig into a larger receptor stem and using a modified T-bud procedure, thereby reducing the importance of matching diameters of inoculum and receptor host. This is done by cutting the end of the twig at a sharp angle producing a long, elliptical surface area. This is
then inserted into a T-cot made in the larger receptor plant.

REFERENCES


FIGURE 106
Three designs for the layout of plant laboratory greenhouses
a) A six-room design: each room an individual cubicle with a central walkway
b) A three-room design with a central walkway
c) Three separate small greenhouses with individual temperature control for cool, moderate and warm temperatures
FIGURE 107
Various greenhouses used worldwide for indexing

a) An inexpensive wood-and-fibreglass greenhouse at Riverside, California, with two evaporator coolers

b) The interior of the greenhouse in (a) showing the wood-and-fibreglass structure. Excellent plants were grown in this inexpensive house using a UC system of solis, iertilizers and temperature control

c) The Rubidoux laboratory at Riverside, California, has a double-door entryway and three compartments, as in Figure 106b
d) A large fibreglass and aluminium-frame greenhouse. The screened portion at the far end is made of 32-mesh plastic screen to filter the outside air as it passes through cooling cells. Two large fans at the opposite end control air movement.

f) The interior of the fibreglass greenhouse at Moncada, Spain, used for extensive indexing of citrus. There are three such houses, maintained at different temperatures. Note the central walkway, metal benches with plywood top, and cooling cells at the opposite end of the house. This house is cooled in the same manner as the structure in (d) and (e).

e) The interior of the house shown in (d). This house was not divided into compartments but was held at one temperature for large-scale testing for seedling-yellows tristeza.
g) A small aluminium-and-glass house at Riverside, California, with two internal heaters and evaporator coolers on the outside. This is an excellent type of greenhouse.

h) A small fibreglass house at Nelspruit, South Africa, used for indexing. This house is cooled by cooling cells at one end and a fan at the other.

i) Glasshouse at Catania, Italy, with top and side vents for cooling and wire mesh over the structure to prevent damage to the glass from hail.

j) A double-walled polythene greenhouse at Riverside, California. The double layer gives excellent insulation, and warm or cool air can be pumped into the space between the polythene layers for additional heating or cooling. This system is very energy-efficient.
a) A small heating unit outside the structure at Riverside, California. Heating units placed outside the greenhouse are preferable since they minimize risk of ethylene damage to plants by leakage of exhaust fumes from faulty heaters.

b) Two large heaters outside the Rubidoux Glasshouse. The warm air is carried by ducts into the upper part of the house and distributed internally by fans and plastic tubing as shown in (c), (d) and (e).

c) A large internal heating unit with a perforated plastic tube attached. Note the circular hole in the plastic tube.

d) Interior of greenhouse with large perforated plastic tube running the length of the greenhouse. The warm air is blown through the tubing and forced out through the many holes in the tubing.

e) A separate fan attached near the heater. This fan can be activated independently of the heater fan by a separate thermostat. This permits the circulation of air within the greenhouse for the distribution of latent heat residing in the soil, floors and structure, and is energy-saving.
FIGURE 109
Different types of benches used to support plant containers

a) Wooden benches and concrete block supports at Riverside, California. Note the gravel floor.

b) Another view of wood-and-concrete block benches at Riverside, California. (Trees on benches are "virus" bank source plants)

c) Wooden runners to support large containers, keeping them off the ground. Note gravel floor (Riverside).

d) Benches made of concrete at Campinas, Brazil. Note the well-spaced plants.
e) Steel and wire mesh benches, Mildura, Australia. Note concrete floor

f) A steel mesh bench on concrete blocks at the USDA greenhouse, Orlando, Florida

g) Plastic bench tops on a wooden frame set on concrete blocks in a greenhouse at Lake Alfred, Florida

h) Spraying wooden benches with copper naphthenate solution (an excellent wood preservative and disinfectant)
FIGURE 110
The ingredients and fertilizers used in the modified University of California mix for growing citrus
a) A trailer fitted with pipes perforated with holes drilled on the bottom of the pipes. Steam is conducted from the pipes through the soil. The top of the trailer is covered with a tarpaulin prior to steaming. After cooling, the soil can be taken directly from the trailer to fill containers.

b) Flats and pots can be steamed directly in the trailer. Note steam pipes at the bottom.

c) A fixed steam chamber used to steam containers. It can also be used to steam small quantities of soil.
FIGURE 112
A metal tamper used to flatten and compress soil in flats prior to seed planting. The top of the soil should be level to prevent water accumulation at any low spots.

FIGURE 113
A planting board used for the even distribution of seed. One seed is dropped into each hole and pressed gently into the soil. The seed is then covered with about 1 cm of soil and the soil is tamped lightly and levelled, using the tamper as shown in Figure 112.

FIGURE 114a
Seedling trays used for growing individual seedlings at the Citrus Research Center, Lake Alfred, Florida.

FIGURE 114b
Seedling trays with seedlings at the USDA greenhouse, Orlando, Florida. One seed is dropped into each cup and covered with soil medium or soil.

FIGURE 115
A tray and pad with disinfectant placed in front of the entrance to the greenhouse (Nelspruit, South Africa).
FIGURE 116
A recording thermograph should be kept in each room of a plant laboratory. It should be checked periodically with thermometers for accuracy, and maximum and minimum daily temperatures recorded in a separate book once a week when charts are changed.

FIGURE 117
A very inexpensive device for siphoning fertilizer from a concentrated solution in a tab into the water supply. The flowing water creates suction in the Venturi restriction and draws the fertilizer through the watering hose. This device was used for many years at the University of California laboratory prior to installation of a Smith proportioning device (Figure 118).

FIGURE 118
A Smith Measuremix proportioner. This is a precision instrument. A plunger, activated by water pressure, injects a fixed amount of fertilizer into the watering system. The amount of fertilizer injected is in proportion to water flow. This proportioner is expensive but has performed very well for many years with minimal maintenance.
FIGURE 119
A large fan at one end of a greenhouse creates negative pressure in the house and brings in the outside air through cooling pads at the opposite end.

FIGURE 120
A thermostatic control panel with four thermostats for four levels of control. Thermostats independently control fans for the introduction of outside air or for pumping water over the cooling cells. They also control heating by circulating the inside air or by turning on heaters.

FIGURE 121
A system used at the Riverside laboratory for filtering incoming air using spun-glass-and-charcoal filters.

FIGURE 122
A standard commercial evaporator cooler. This cooler is effective if humidity is low. It is relatively inexpensive and, if properly maintained by replacing pads and by periodical cleaning and painting, it is effective in cooling greenhouses. Similar units were used for over 20 years at the Riverside laboratory and were very reliable.
FIGURE 123
The same cooler as in Figure 122 but with the side panel removed to show the main squirrel cage fan, the water pump and water being pumped from the top and down over a pad of wood shavings. The outside air forced through the wet pads is cooled by evaporation.

FIGURE 124
Cooling cells consisting of specially treated cardboard units placed together to make a continuous wall. Water is pumped from a reservoir tank below ground (bottom left) to the top and drips freely down over the cells by gravity. Cooling is by evaporation.

FIGURE 125
A battery of cooling cells is at one end of a greenhouse. Cooling by evaporation is most efficient when humidity is low.

FIGURE 126
Where humidity is too high, greenhouses may have to be cooled by electric refrigeration units. This method is expensive if power rates are high. Electric refrigeration units can be used to supplement evaporator coolers where humidity is moderate or high. They can be used in rooms designated for testing cold-temperature pathogens.
Bud grafting. Showing three types of "bud" inserted into the stem of a citrus seedling and ready for wrapping. At the top is a bud containing an "eye" or the meristematic bud which can grow. In the centre is a blind "bud" containing no "eye". At the bottom is a chip bud fitted into the stem. The chip bud can be blind or can contain an eye. The one illustrated is a blind chip bud.

During wrapping, the plastic tape should be kept stretched to secure a tight wrap.

Leaf-piece grafting. Cutting a small rectangular segment from the central midrib of a young leaf to be used for a leaf graft.

Insertion of the cut leaf piece into a T-cut in the stem of a citrus seedling. "Buds" are inserted in the same manner. The point of a knife is used to slide the leaf piece or "bud" into the T cut. Only seedlings in which the bark readily slips can be used. Seedlings with tight bark cannot be used for this technique.
FIGURE 131
Showing a growing leaf piece which had been successfully grafted into the stem. The tapes are removed about three weeks after grafting. The photograph was taken about six weeks after grafting.

FIGURE 132
Leaf-punch graft technique. A hole is first punched into the leaf of the plant to be inoculated, and a similar disc is cut in a leaf of the inoculum tissue.

FIGURE 133
The inoculum disc is inserted into the receptor hole in a leaf of the indicator seedling using a dissecting needle. A piece of adhesive tape is first placed on the underside of the receptor leaf.

FIGURE 134
Another piece of adhesive tape is then placed on top of the leaf and pressed firmly. After one or two weeks the leaf disc will graft at the edges. Dead grafts will turn brown.
FIGURE 135
Bark grafting. Showing the plastic tube used for collecting bark samples. A small piece of moist tissue is placed at the bottom of the tube and the bark pieces placed inside. The top cap should not be sealed but left open to allow aeration. Tubes are put into an ice chest immediately after field collection and refrigerated at the laboratory. Bark samples collected and stored in this manner will keep for over three weeks.

FIGURE 136
The bark piece is trimmed on moist tissue paper with the cambium face downward.

FIGURE 137
The bark piece is inserted into an L-cut made in the stem of the indicator seedling. The side flaps of the L-cut will hold the bark in place as shown in the bottom graft. The bark is wrapped as shown in the centre graft.

FIGURE 138
Side grafting. A side graft stem piece (top right) showing the tapered V-cut at the end ready for insertion. A cut is made into the receptor stem, and the inoculum piece is fitted and wrapped as shown on the bottom right. It is then covered with a polythene sleeve and tied above and below with plant ties. Some leaves should be left inside to provide moisture.
Part III

Laboratory methods for detection of CGTPs
Enzyme-linked immunosorbent assay (ELISA)
for citrus pathogens

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This is a guide to the use of enzyme-linked immunosorbent assay (ELISA) for detection of citrus pathogens. It describes several common variations of ELISA. Some background information is presented to help the user understand the technique and make modifications to this highly flexible procedure for specific applications. Information on the selection of techniques, on preparation of samples for testing, and on the basic steps of the ELISA protocol is provided. The supplies, reagents and equipment needed are indicated, and some specific examples are shown.

ELISA has become a standard procedure for the detection of several citrus pathogens, especially citrus tristeza virus (CTV). Properly used, ELISA is a sensitive, accurate and rapid detection method. It is especially effective where large numbers of samples must be assayed, where results are needed rapidly, and where suitable indicator plants and/or greenhouse facilities are not available. ELISA has been developed for CTV, satsuma dwarf virus (SDV), citrus variegation virus (CVV), citrus leaf rugose virus (CLRV), citrus mosaic virus (CiMoV), Spiroplasma citri, Xanthomonas campestris pv. citri, and Phoma tracheiphilla. ELISA assays are being developed for the greening organism and for citrus tatterleaf virus.

ELISA is simple and can be carried out by most people after brief training and some practice. As with any indexing procedure, some experience is necessary to use ELISA accurately and confidently. New users should consult several of the excellent general references on ELISA (e.g. Clark and Bar-Joseph, 1984; Clark, Lister and Bar-Joseph, 1988; Sanchez-Vizcaino and Cambra Alvarez, 1987) which provide additional details on theory and application. It is very useful to visit a laboratory where ELISA is practised in order to observe the procedure and to study it under the guidance of an experienced user. Begin with a well-known system, and study the effects of adjusting reactant concentrations and test conditions.

Extensive training and background in serology and immunology are not essential to use ELISA, but it is necessary to understand some basic concepts. ELISA is a serological technique and, in common with other serological procedures, it is based on the concept that many proteins are antigenic when injected into animals and that the immunized animal will form antibodies to them. These antibodies can be obtained from the serum of the immunized animal and will react
Laboratory methods for detection of CGTPs

specifically with the antigen to which they were formed. A primary requirement to begin ELISA is a useful source of antibody to the pathogen to be detected. This, in turn, means that to obtain such antibodies an antigen specific to the pathogen must be identified and purified sufficiently to produce the needed antibodies. Antigen purification and antibody production are beyond the scope of this section, but information on these topics is contained in some of the references cited (Clark, Lister and Bar-Joseph, 1988; Van Regenmortel, 1982).

Also fundamental to ELISA is the concept that various enzymes can be bound to antibody molecules to form a conjugated molecule that has both enzymatic activity and is also serologically active. Since enzymes are highly active and can be detected at low concentrations, they are effective labels. Enzyme-labelled antibodies can be detected when they are exposed to a substrate which enzymes can change. Normally, a substrate that changes colour as a result of the enzyme action is used. The amount or rate of colour change can then be used to measure the amount of antibody present. Enzyme labels provide a sensitivity similar to that of radioactive labels and have several important advantages: they are stable, the cost is low, they are safe to use and can be used successfully without sophisticated equipment.

The enzyme label may be attached directly to the antibody used to detect the antigen in question (the detecting antibody). This is called a direct assay, of which the highly popular double antibody sandwich technique described below and illustrated in Figure 139a is a good example. The label may also be used indirectly. In this case, the detecting antibody is not labelled, but rather the label is attached to a second antibody specific to the detecting antibody. Antibodies of one species are antigenic when injected into an animal of a second species. For example, rabbit immunoglobulins can be injected into another animal such as a goat to create a goat anti-rabbit antiserum. These goat anti-rabbit antibodies are useful to detect antibodies from rabbits which were originally prepared to detect another antigen.

Indirect assays are more sensitive and also avoid the need to prepare a conjugate to each antibody used. Several forms of indirect assay are described in the following section and are also illustrated in Figures 139b and 139d. The relative advantages of direct and indirect systems are discussed in the following section.

Several other molecular interactions are frequently used in conjunction with ELISA, either to purify immunoglobulins or to amplify reactions and increase sensitivity. Protein A is a cell wall component of the bacterium Staphylococcus aureus and has the unique characteristic of binding to the immunoglobulin protein of many mammalian species. The binding site is on the Fc region of the immunoglobulin and not on the antigen binding site. Protein A is frequently used to purify antibodies by affinity chromatography. It can also be conjugated with enzymes and used in assays to detect immunoglobulins.

A second important system is the biotin/avidin system. Biotin, a small vitamin, has a very high affinity for avidin, a 68 000 molecular-weight glycoprotein. Antibodies and enzymes can be conjugated with several molecules of biotin to form a “biotinylated” molecule. Each avidin molecule has four binding sites for biotin. This multiplying interaction has been exploited in several ways to amplify the number of enzyme molecules associated with each antigen-bound antibody and thereby increase sensitivity. One example is illustrated in Figure 139c.

Another fundamental concept for ELISA is that proteins such as antibodies and virus coat proteins will adsorb strongly to the surface of
certain plastics such as polystyrene and polyvinyl-chlorides. Protein binding also occurs to some forms of cellulose nitrate. These materials are frequently referred to as “immunosorbents” or the “solid phase” in ELISA protocols. The protein binding to immunosorbent materials is not specific and is not a serological reaction such as occurs between antigen and antibody molecules. If a mixture of antibodies is exposed to an immunosorbent plastic, all will bind. Similarly, when a crude extract from a diseased plant is placed in an ELISA plate, both proteins of the pathogen and proteins of the host present in the extract will be bound.

Binding either the antibody or the antigen component of a serological system to a solid phase is very useful because the bound component can subsequently be used to probe complex mixtures of potential reactants. Only those which are serologically related will be trapped. All non-reactive components can then be removed by washing and do not interfere with subsequent steps. For example, when an extract from a virus-infected plant is placed in the wells of a microtitre plate coated with antibodies to that virus, virus antigens in the extract will be bound to the trapping antibody and all non-related proteins will be removed by the subsequent washing step.

Undesired adsorption of antibody or antigen proteins to the plastic can be avoided by using non-ionic detergents such as Tween 20 in incubating solutions or by adding an excess of a non-specific protein to block all sites not occupied by the desired serological component. For example, the buffer used to coat plates with trapping antibody does not contain Tween 20, but Tween 20 is incorporated in subsequent steps where any non-specific binding of other proteins should be avoided.

Immunoblotting procedures are not specifically discussed in this section. However, much of the information and the general concepts presented are directly applicable to immunoblotting procedures. The main differences are that the solid phase for immunoblotting is usually cellulose nitrate, the substrate used to measure presence of the antigen-antibody-enzyme complex is different, and incubation conditions may be somewhat modified.

**ELISA PROCEDURES**

Numerous variations of the ELISA procedure can be devised (Clark, Lister and Bar-Joseph, 1988; Engvall and Pesce, 1978; Jones and Torrance, 1986; Koenig and Paul, 1982; Maggio, 1980). The selection depends on the sensitivity, specificity and convenience required; the presence of interfering factors; and the type and activities of the antisera available. The basic steps for four commonly used variations of ELISA are outlined here and illustrated in Figure 139. In three of the variations, Figures 139a to 139c, the solid-phase (ELISA plate) is coated with antibody to the antigen to be detected. This antibody, identified as trapping antibody (TA), then traps its corresponding antigen (identified as V) from suspension or solution. In the fourth variation, the antigen (V) is trapped directly on the solid phase (Figure 139d) and detected with its specific antibody.

**Double antibody sandwich**

The inexperienced user should start with the double antibody sandwich (DAS) where possible. This has been the most commonly used form of ELISA for plant virus detection since its description by Clark and Adams (1977). The components of DAS are illustrated in Figure 139a. The immunosorbent surface is the wells in a plastic microtitre plate designed for ELISA as shown in Figure 140. A dilute solution of unlabelled antibody is added to the wells of the
plate, and the antibody adsorbed on the plastic becomes the trapping antibody (TA) as illustrated in Figure 139a. After washing to remove any excess antibody (Figure 154), the sample (antigen) is added as shown in Figure 142. Antigens (V in Figure 139) specific to the bound trapping antibody attach themselves to it, but other proteins remain in solution and are removed by washing. The antigen attached to the trapping antibody is detected by adding a labelled antibody (LA in Figure 139a) specific to the antigen (Figure 143). The label is the enzyme (E) previously conjugated to the antibody. When substrate specific to the enzyme is added in the final step (Figure 144) a colour develops as a result of enzyme action (Figures 145 and 146). The amount of colour and rate of development are correlated to the amount of labelled antibody bound to the antigen which had been trapped by the antibody attached to the plate.

DAS can be done with a single good quality polyclonal antiserum. The immunoglobulins present are partially purified, and one portion is saved for use as trapping antibody while another is conjugated to an enzyme. Alkaline phosphatase is commonly used as the enzyme and the conjugation can be done in the presence of dilute glutaraldehyde (Clark, Lister and Bar-Joseph, 1988). The antibodies for coating and detection do not have to come from the same source, e.g. monoclonal antibodies could be used for coating, and a polyclonal antiserum used to prepare the enzyme-labelled antibody.

**Double antibody sandwich indirect**

DAS can be converted to an indirect procedure (DAS-I), which is illustrated in Figure 139b. The first two steps are the same as in DAS. However, the antigen bound to the trapping antibody is detected by an unlabelled intermediate antibody (IA in Figure 139b) which is specific to the same antigen, but is from an animal species different from the one used to prepare the trapping antibody. For example, if the trapping antibody was prepared in rabbits, the detecting or intermediate antibody (IA in Figure 139b) could be from a mouse or a chicken. The unlabelled IA which attaches to the antigen is detected by an enzyme-labelled antibody (LA) specific to the IA. Because the IA is from a species different from the TA, the LA binds only to the IA and no non-specific binding of the LA to the TA occurs. The amount of LA is measured by adding substrate and measuring colour change as in DAS.

DAS-I ELISA involves an additional step (Figure 139b) but is more sensitive and also allows use of a commercially prepared enzyme-labelled antibody to the IA. A single LA can also be used for multiple virus detection systems. In addition, the intermediate antibody does not have to be purified and is needed in only a limited quantity. If the intermediate antibody is highly specific, e.g. most monoclonals, then a highly specific antiserum is not required for coating. The major problem is that antibodies to the same antigen must be prepared in two different animals. If the trapping and the intermediate antibodies are from the same species, the labelled antibody used to detect the intermediate antibody will also bind to the trapping antibody and result in a non-specific response.

A system has been devised to carry out DAS-I using a single antiserum (Adams and Barbara, 1982; Clark, Lister and Bar-Joseph, 1988) To do this, the antibodies are treated with the enzyme pepsin to remove the Fc portion of the molecule. The remaining F(\(\text{ab}'\))\(_2\), fragment still has the antigen binding sites and will bind to the immunosorbent, but will not bind to protein A. The F(\(\text{ab}'\))\(_2\) fragments are used as trapping “antibody” and the whole antibody is used as the intermediate antibody. Enzyme-conjugated protein A is then used instead of a labelled
antibody to detect the intermediate antibody. It does not react to the trapping “antibody” because the Fc region has been removed. The DAS-I procedure can be further modified to amplify the reaction achieved. This is commonly done using a biotin-avidin interaction where the labelled antibody is biotinylated to react with avidin molecules conjugated to multiple enzyme molecules, as illustrated in Figure 139c. Different types of amplification are possible and special kits may be purchased to perform them. Users should be aware of the possibility to increase sensitivity by amplification where the additional sensitivity is needed, but regular procedures should be tested before amplified tests are attempted.

**Plate-trapped antigen**

Another basic approach to ELISA is the plate-trapped antigen procedure (Figure 139d). The approach is to trap the antigen(V) on the plastic surface, then react the trapped antigen with an unlabelled intermediate antibody (IA) specific to it. The IA is then detected as in DAS-I using an enzyme-labelled antibody (LA) specific to the IA. This procedure, called plate-trapped antigen indirect (PTA-I) ELISA, is relatively simple and involves no advance purification of antisera or conjugate preparation if a commercially prepared enzyme-labelled antibody to the unlabelled IA is used. The PTA-I procedure is usually less sensitive than DAS or DAS-I for use with crude plant extracts, and may not be effective when antigen concentration in the sample is low. Since binding to the plate is non-specific, there is competition between the target antigen and other proteins present in the extract for the available binding sites on the plate. Plate-trapped antigen tests can be conducted as a direct assay using an enzyme-labelled antibody to the antigen, but sensitivity is even lower than for the indirect method, and the conjugate must still be prepared. Amplification procedures as described for DAS-I can also be used for the PTA-I procedure to increase sensitivity.

The specific steps and schedules for these types of ELISA are described in Schedules 1-3.

**SAMPLING**

Selection of appropriate samples for testing is critical. Although ELISA is a sensitive procedure, reliable results may not be obtained if poor samples are tested. Virus titre in citrus tissue often varies markedly, and thousandfold differences in antigen concentration can occur over a relatively short period. Virus concentrations are generally highest in young, expanding flush tissues. They decrease rapidly as tissues mature under hot-weather conditions and more slowly under cool conditions. Avoid sampling old, mature tissue during the summer months in hot climates unless preliminary testing indicates that reliable samples can be taken. If the virus or pathogen is phloem-limited, such as tristeza, greening, or stubborn, then the tissue sample collected must contain phloem tissue. Older bark tissue can be sampled if the cambium is active, but generally it is less reliable than young shoot flush, bark or young leaf midribs (Figures 148 and 149). The button area tissue and pedicel bark from fruit (Figure 149) are good phloem sources on bearing trees. Young root tips may be useful under some conditions.

A composite sample from several sites on the tree should be collected: normally three-to-five locations per tree are sampled. Increase sampling if the pathogen is irregularly distributed or when trying to monitor a recent infection.

From time to time, it is not convenient to test samples immediately after collection. Fresh tissue can normally be stored for at least seven to ten days at 4°C when kept in a plastic bag or sealed container. Tissue can also be dried over a
Laboratory methods for detection of CGTPs

desiccant (or air-dried in dry climates) and then stored over fresh desiccant. Dry samples are convenient for long-distance shipping. One convenient method for field collecting in remote locations is to place 0.25 g samples in gelatin capsules, code them, and place them in a sealed container with a desiccant. Some desiccants having a colour indicator for activity are on the market.

Samples can also be stored frozen at -20°C or below for extended periods either as unprocessed fresh tissue, or as diced tissue placed in extraction buffer and frozen in the grinding tube (Figure 150). The sample should not be ground prior to freezing because fresh extracts often lose much activity when frozen. Frozen samples should not be stored in an automatically defrosting freezer. Extracts can be stored for long periods when freeze-dried, which is a good way to store a source of consistent reference (control) samples. Always test a storage method with the specific pathogen under study in order to prove its effectiveness.

**EXTRACTION**

Numerous buffers and different additives have been used for extraction of tissue samples with different virus-host systems (Bar-Joseph and Garnsey, 1981; Clark, 1981; Clark and Bar-Joseph, 1984; Clark, Lister and Bar-Joseph, 1988; McLaughlin et al., 1981). Fortunately, extraction problems have been rare with citrus viruses, and phosphate-buffered saline (PBS) or 0.05 M Tris, pH 7.5 to 8.0 without any additives usually give good results for sandwich assays. Additives such as polyvinylpyrrolidone, EDTA and DIECA are generally unnecessary and may actually reduce reaction efficiency. Test the effect of additives before using them routinely. For plate-trapped antigen procedures, try extraction of the sample in carbonate coating buffer pH 9.6, or in 0.05M Tris, pH 8.0. Do not use Tween 20 in the extraction buffer for samples to be plate-trapped.

Normally, the ratio of buffer to sample tissue should be at least 1:10. Higher concentrations of tissue may actually reduce reaction rates and make sample preparation more difficult. Good samples of CTV-infected tissue can usually be diluted up to five hundredfold and still give strong reactions.

There are many ways to grind samples. Pestle and mortar are fine for small numbers of tender samples. Addition of an abrasive, such as fine sand or carbon dioxide, to the sample or powdering the tissue in liquid nitrogen makes grinding easier. A dispersion homogenizer (Figure 151) equipped with a 10-25 mm diameter shaft is a good choice when large numbers of samples are to be processed. A 2-10 ml sample can be rapidly ground in a test tube or centrifuge tube of suitable diameter and length with this type of homogenizer. Fibrous tissue, such as bark and leaf midribs, should be cut into short pieces (2-5 mm) prior to grinding or the shaft will become clogged with fibre and must be cleaned between samples. Two rinses of the grinder shaft in 500-1000 ml clean water are usually adequate (Figure 152). Run the homogenizer briefly in each rinse solution.

Chill samples prior to grinding to offset heating during the grinding process. It is normally not necessary to keep the sample on ice during grinding, unless unusually long grinding is required and the sample becomes warm to the touch. Frequent users of dispersion homogenizers should wear earplugs to protect their hearing, and homogenizers should be isolated.

If necessary, samples can be prepared with very minimal equipment. When virus concentration is high, extensive disruption of the sample tissue is usually unnecessary. One method is simply to place a small piece of tender tissue directly in buffer in the well of an ELISA
plate with forceps and then squeeze it to release the cell contents. Tissue can also be crushed in a small plastic bag using a mallet or smooth stone and the extract moved by pipette into the test plate.

Samples containing a lot of debris after extraction can be difficult to extract by pipette. Remedies include centrifugation of the sample to pellet the debris, or filtering the sample through a coarse filter such as cheesecloth or glass wool (Figure 153). Cutting off a portion of the tapered tip of plastic pipettes creates a wider orifice and is often quick and effective. It is frequently quicker to rinse a repeating pipettor between samples than to change tips so only a limited number of tips need to be modified.

**WASHING**

Proper washing of the plates between steps is important. The standard procedure is to wash the plate three times between each step with phosphate-buffered saline (PBS) containing 0.5 percent Tween 20 (PBST). Sodium azide is frequently included in PBST solutions as a preservative. However, it is highly poisonous and may form an explosive complex with some metals. It is unnecessary to use sodium azide in ELISA wash solutions and it should be omitted. The two most critical wash steps are after sample incubation when cross-contamination must be avoided between wells containing different samples (omit the first wash immediately if carry-over between wells occurs) and after the conjugate incubation step. If even a minor residue of unattached conjugate remains, high background readings may occur (add another wash at this point when in doubt). Various plate washers are available which can promote consistent washing operations, but a plastic squeeze bottle will work well for small volumes of plates (Figure 154). Solutions in the plate wells can be removed by aspiration to avoid contamination, but usually the plate is inverted rapidly with a quick shake of the hand and tapped firmly on clean blotting paper or paper towels.

**TEST CONDITIONS**

A wide variety of reactant concentrations and incubation times and conditions have been reported for ELISA (Clark and Bar-Joseph, 1984; Clark, Lister and Bar-Joseph, 1988; McLaughlin et al., 1981). The choice of conditions depends to some extent on the basic goals. By using high concentrations of reactants, short incubation times can be used and, if necessary, the entire ELISA procedure can be completed within two hours. Increasing the incubation time while decreasing concentration (especially of the conjugate) will conserve reactants. Reactions occur most rapidly at 30-37°C, but room temperatures (20-28°C) will give satisfactory results. Gentle shaking during incubation may improve efficiency. Many workers find it convenient to do the sample-incubation step overnight and often do this at 4-6°C.

Some experimentation will be necessary to determine optimum conditions for each situation. Schedules 1-3 give examples which should provide a good starting point. Moderate changes in times and conditions are unlikely to cause a test failure, and changes can often be made to render the schedule more convenient for the user with no loss of information. New users should certainly experiment with different schedules to find the optimum for their purpose.

One of the major variables in ELISA to be evaluated is the concentration of conjugate to use. Commercially prepared enzyme-labelled antibodies normally have a recommended working dilution (frequently between 1/1 000 and 1/2 000). Conjugates which are prepared experimentally may differ markedly and published values for other systems are of little
help. Optimum dilutions of 1/100 to 1/20 000 of the stock preparation (approximately 1 mg/ml) have been reported. The effective dilution will depend on the basic affinity of the antibody, the titre of specific and non-specific (host) antibodies, the source and activity of the enzyme used and the effectiveness of the conjugation procedure. When starting with a new or unknown batch of conjugate, test three tenfold dilutions starting at a 1/100 dilution to determine the approximate activity. Using these results, make a second test in the appropriate dilution range indicated. Normally, the objective is to obtain a strong positive reaction to a good positive sample within 20 to 60 minutes and little or no reaction to healthy extracts. If the conjugate concentration is so high that a reaction is instantly visible, a background reaction is often also observed with healthy extracts (and even with buffer controls). Reduce conjugate concentration and, if a non-specific reaction persists, adsorb the antiserum against a concentrated extract of healthy plant tissue to remove antibodies against healthy antigens. If possible, do this before purifying the IgG. Adding healthy tissue extract to the buffer used to dilute the conjugate may also reduce non-specific reactions (Clark, Lister and Bar-Joseph, 1988).

The use of appropriate controls is essential. Each plate should have at least one healthy and one known positive sample as controls. A buffer control is also useful to determine the level of background reaction to healthy extracts. Frequently, a slightly higher reading will be observed for the buffer control than for the healthy extract because proteins in the extract block exposed protein binding sites on the plastic, which may later non-specifically bind conjugate molecules. Each sample should be tested in at least two wells. A random loading pattern can be used, but paired wells are normally used for routine work. Special applications may require additional replication and random selection.

Edge effects in the plates were frequently noted when ELISA first became popular and outer wells were avoided. Plates have steadily improved and normally all wells can be used. Uniformity in new lots of plates can be checked by loading a uniform sample in all wells.

**RECORDS**

One of the major tasks of any indexing procedure is to identify samples properly during the testing process and to record results in a usable format. The identity of the sample must be maintained through the multiple steps of collection, processing, extraction and testing. It is usually convenient to give each sample a code number at the time of collection and to use this code during the test process. If samples are collected directly in the grinding vessel (usually a glass or plastic tube), labelling steps can be reduced. If the sample is collected in a container other than the grinding vessel, a transferable label is often convenient. Many ELISA plates have a coding system on the plate margins to identify individual wells, but there is no space to mark individual wells on the plate. Most workers develop a data sheet similar to the one shown as Figure 156, which is used to record the loading sequence of test samples and other pertinent data for that test, such as reactant concentration and incubation conditions. Each plate and data sheet should have a corresponding number recorded in a logbook to facilitate retrieval of information.

It is important to mark the loading pattern for each plate prior to loading samples and to arrange the samples to be loaded in the appropriate sequence. Note changes or errors that may occur during loading, and store tubes and samples under refrigeration until the testing process is complete.

Visual readings of the plate can be recorded directly on the plate data sheet. Printouts from a
plate reader (Figure 47) can also be attached to the original data form. Use of computers to store and analyse data is increasing rapidly and is convenient for long-term storage of large amounts of data. Data from the reader may also be converted into another format for further analysis and spreadsheet presentation on the computer. Permanent visual records of important tests can also be obtained by photographing individual plates on a light box or over a white background. A 35-mm transparency film is economical, and a standard exposure can be obtained if a constant light source is used.

**EVALUATION OF RESULTS**

Evaluation of ELISA results often presents some problems to the novice user. With highly specific antisera and samples with good antigen titre, results are normally very clear. When the antisera used are weak or contain some antibodies to host proteins and/or the samples have a very low antigen titre, determination of a positive result can be more difficult. Reactions can be evaluated visually with some precision if background readings for healthy controls are low. Normally, the eye can discern differences in OD$_{405}$ of 0.05 to 0.1 above a low background. A graded scale with three to five levels is useful to report the relative degree of reaction. Where greater accuracy is required, the degree of reaction can be measured by testing a diluted sample in a spectrophotometer or by reading the plate in an ELISA plate reader (Figure 147). A wide variety of plate readers are available, from simple manual models suitable for modest numbers of plates, to highly automated models capable of various levels of data analysis and storage.

Recently, emphasis has increased on measuring reaction rate rather than a single final optical density value. This eliminates some sources of error where accurate quantitative data are needed, and also allows more accurate comparison of samples with large differences in antigen concentration. Rate calculation requires several measurements of the same plate at a measured time interval, and a plate reader is essential. Some plate readers do rate calculations automatically. Expensive plate readers are not necessary until a definite need for them is identified.

The limits of reliable detection are correlated to the precision used and the number of replications. Confidence levels can be calculated statistically when in doubt. Most workers establish an arbitrary threshold value relative to the healthy control for a positive reaction, such as a reading twice the healthy control or the healthy reading plus 0.1 OD. Where reaction to healthy extracts is low (<0.05), the eye can usually consistently detect reactions 0.1 OD or higher and this becomes the effective limit for visual recording. It is best to establish a conservative threshold rating and retest all questionable samples. Some experimentation with different dilutions of known samples will help.

**PURIFICATION OF IMMUNOGLOBULINS**

Numerous procedures are now available for purification of immunoglobulins (IgG) from polyclonal antisera or for purification of monoclonal antibodies from culture fluid or ascites fluid. The easiest method is to use a commercial kit or system containing detailed instructions. Many of the kits are based on separation of the IgG component by protein A bound to a solid substrate. The IgG is subsequently eluted from the protein A by changing buffers and collected. An alternative, which is slower but inexpensive, is to precipitate the IgG from solution by use of ammonium sulphate, and then fractionate the dialysed resuspended pellet by column chromatography on a DEAE cellulose column (Figure 155).
Purified IgG solutions are normally adjusted to a concentration of 1 mg/ml (equal to an OD$_{280}$ of 1.4 when measured spectrophotometrically) and stored at 4°C in PBS containing at least 0.02 percent sodium azide. IgG preparations can also be stored in 50 percent glycerol at -20°C or freeze-dried. More detailed instructions can be found in Clark, Lister and Bar-Joseph (1988).

PREPARATION OF ENZYME-LABELLED ANTIBODIES

Conjugated molecules of antibody and enzyme can be prepared in several ways (Bar-Joseph and Garnsey, 1981; Clark and Adams, 1977; Clark and Bar-Joseph, 1984; Clark, Lister and Bar-Joseph, 1988; Engvall and Pesce, 1978) Alkaline phosphatase is the most widely used enzyme, and the single step glutaraldehyde method is commonly used to prepare alkaline phosphatase conjugates. Enzyme-labelled antibodies are formed by conjugating enzyme and antibody molecules. One way is to mix the enzyme preparation (which is purchased commercially) with purified IgG and then add glutaraldehyde to a concentration of 0.05 percent. The mixture is incubated for several hours and then dialysed. The enzyme normally comes as a salt precipitate which is centrifuged from solution and resuspended in the IgG solution. Diaalysis is also done before adding glutaraldehyde. Test this system first before experimenting with other procedures. Alkaline phosphatase is usually received as an enzyme precipitate in a salt solution. The precipitate is recovered from solution by centrifugation and about 5 mg is dissolved in 2 ml of a 1 mg/ml solution of IgG. The mixture is dialysed thoroughly to remove excess salts and then fresh glutaraldehyde is added to a final concentration of 0.05 percent. After 4-h incubation, the conjugate is dialysed three times to remove the glutaraldehyde, and stored at 4°C in PBS containing 0.04 percent sodium azide and 5 mg/ml bovine serum albumen. Use an enzyme source with a quality suitable for ELISA. Careful dialysis is very important for good results. New lots of conjugate must be tested to determine optimum dilutions for use (Sanchez-Vizcaino and Cambra Alvarez, 1987). Good-quality conjugates can normally be used at at least a l/500 dilution and dilutions as great as l/5 000 or more may be possible. See Clark, Lister and Bar-Joseph (1988) for further details and for preparation of horseradish peroxidase conjugates.

Conjugates are stable for long periods at 4°C. Freezing or freeze-drying are not recommended unless preliminary testing indicates it is possible. If freezing is necessary, add 50 percent glycerol.

BASIC SUPPLIES AND EQUIPMENT

Sources of supplies and equipment change rapidly and new equipment continues to appear. It is not possible to list all suppliers in every country. To be of help, we have indicated some possible sources for some essential supplies. It is not essential to use these specific sources, and the user may, in fact, find a more convenient and economical local source than those listed. The Laboratory buyer’s guide, available from International Scientific Communications, Inc., PO Box 870, Shelton, CT 06484, United States of America, is a useful directory of manufacturers. Basic equipment and supplies are emphasized rather than some of the more sophisticated and expansive equipment also available for huge-scale clinical work. It is assumed that most users of the latter already know the sources of supply.

A source of antiserum to the pathogen you wish to detect is essential. Both polyclonal and monoclonal antisera have been produced to a number of citrus pathogens, and more are being developed. Polyclonal antisera are frequently preferable for general detection work where discrimination of a particular isolate is either
unnecessary or undesirable. Unless the antigen used to produce the antiserum was well purified, polyclonal antisera frequently contain some antibodies to plant proteins as well as to the specific pathogen. It is advisable to check the specificity of the antiserum to be used in the initial stages to ensure acceptability. Monoclonal antibodies are generally more specific because, if properly prepared, only a single epitope of the antigen is involved. To do DAS-I assays, antibodies are needed from two animal species unless the F(ab')2 procedure is followed.

Production of high-quality antisera is frequently a time-consuming and difficult task, especially for people without experience in this process. Inexperienced users of ELISA should ordinarily try to obtain a small amount of antiserum from existing sources for their initial work. Frequently, a modest supply of antiserum can be obtained from a colleague working on citrus pathogens. Most scientists working on virus-like pathogens of citrus are members of the International Organization of Citrus Virologists (IOCV) and can be identified by writing to the Secretary-Treasurer of IOCV, c/o Department of Plant Pathology, University of California, Riverside, CA 92521, United States of America. Small amounts of purified IgG or enzyme-labelled conjugate may also be available for limited experimental tests.

Increasing access to antiserum from commercial sources and the American Type Culture Collection can be expected. Current commercial outlets of ELISA supplies for citrus pathogens include: Agdia Inc., 30380 County Road 6, Elkhart, IN 46514, United States of America; Ingenasa, Hermanos Garcia, Noblejas 41,28037 Madrid, Spain; and Sanofi Santé Animale, Z.I. de la Ballastière, BP 126, 33500 Liboume, France.

If a prepared ELISA kit or purified sources of IgG and labelled conjugates are available, ELISA tests can be done in a very simple laboratory equipped with a balance, a simple pH meter, basic glassware, a refrigerator and a supply of deionized water. The other essential items are ELISA plates, several repeating pipettes and the chemicals to prepare the necessary buffers (see below). To purify IgG and to prepare antibody-enzyme conjugates require access to a low-speed centrifuge, a UV spectrophotometer and some column chromatography equipment.

ELISA plates are available from numerous suppliers including Dynatech Laboratories, 14340 Sullyfield Circle, Chantilly, VA 22021 (United States of America), and Nunc Inc., 2000 N. Aurora Road, Naperville, IL 60540 (United States of America). Plate quality can vary. Plates that work well for sandwich assays may work less well for plate-trapped antigen assays. If possible, find a reputable local supplier and test several types of plate before buying a large supply.

Several repeating pipettes, as shown in Figures 141 and 142, are more or less essential. Fixed volume models are economical and will suffice, but adjustable models are much more convenient and can be used for many other tasks. The minimum requirement is one pipette that will measure accurately in the 1-20 µl range (to make dilutions of IgG and conjugates) and one that will operate in the 100-1 000 µl range (or 200-1 000 µl). Pipettes for the ranges of 20-200 µl and 1-5 ml are also extremely useful. Multichannel pipettes (Figures 141 and 143) that can simultaneously dispense the same volume into four to 12 wells are very useful when many plates must be loaded. All repeating pipettes use disposable plastic tips. If possible, select pipettes that can use interchangeable tips. There are numerous manufacturers of pipettes and numerous models. Consult a laboratory supply company or manufacturers such as Flow Laboratories S.A., Lugano (Switzerland) or
Rainin Instruments Co., Woburn, MA 01801 (United States of America) for current information.

Plates can be read visually, but if a large number of plates are to be done on a regular basis, a plate reader greatly speeds reading and makes evaluation of results easier. Numerous models with varying degrees of automation are available and details change rapidly. It is not necessary, and probably not advisable, to buy a reader until the user has some initial experience and knows the system will work. Before purchase, ask for a demonstration and also consult users in other laboratories for their recommendations. Large-scale users should consider readers that are computer-compatible.

See Part IV for additional information on laboratory equipment.

ELISA BUFFERS AND SOLUTIONS
A limited number of chemicals are required to make the buffers and solutions needed for ELISA, and these are shown in Table 6. Many of these should be readily available in most biological laboratories. Specific sources and catalogue numbers have not been listed, but suggestions can be obtained from other ELISA users. Sigma Chemical Co., St Louis, MO 63178 (United States of America), Boehringer Mannheim Biochemicals, Indianapolis, IN 46250 (United States of America), and Pharmacia LKB Biotechnology AB, Uppsala (Sweden), or Piscataway, NJ 08854 (United States of America), are useful general sources for chemicals, enzymes and antibodies mentioned in this section if satisfactory local suppliers cannot be located. See also the Laboratory buyer’s guide mentioned above.

The materials listed are for the alkaline phosphatase system. If horseradish peroxidase or another enzyme is used, make appropriate changes in substrate and substrate buffer.

The formulae for buffers and substrate solutions needed are shown in Table 7. Use glass-distilled or high-quality deionized water to prepare buffers and solutions. The formulae given in Table 7 are for one-litre quantities. Larger quantities of the wash buffer (PBST) than other solutions are used. The dry salts needed for PBS can be weighed in advance in units to make a convenient volume, mixed dry, and stored in sealed plastic bags until needed. A new supply of PBS can be obtained rapidly, as needed, by adding the required volume of distilled water to the weighed salts.

Use standard buffer solutions with pH values near 7.0 and 10.0 to calibrate pH meters. Store buffers (except PBST) at 4°C if possible.

SCHEDULES
Schedules are shown here for types of ELISA illustrated in Figures 139a, 139b and 139d, to provide some specific examples of reactant concentration and incubation time and conditions. The details shown are those typically used for CTV with an alkaline phosphatase enzyme-label system. No schedule is shown for the amplified form of DAS-I illustrated in Figure 139c. The preliminary steps are the same as used for DAS-I and the schedule for the enhancement steps will vary with the enhancement protocol used. Specific instructions are generally provided with the enhancement materials when these are purchased in kit form.

Normally, 200 µl of solution are added per well, but smaller volumes can be used. If NaOH is used to stop the reaction, 50 µl are added to the wells already containing substrate.

Schedule 1

Double antibody sandwich ELISA
1. Coat ELISA plates (Figure 141) with antibodies (IgG) diluted to 1-2 µg/ml in carbonate coating buffer. Incubate for 1-4 h at 2530°C and wash
TABLE 6
List of chemicals for ELISA

1. Alkaline phosphatase type VII
2. Bovine serum albumen BSA
3. DEAE cellulose
4. Diethanolamine NH(CH₂CH₂OH)₂
5. Glutaraldehyde OCH(CH₂)₃CHO
6. Hydrochloric acid HCl
7. Ovalbumen
8. 4-nitrophenyl phosphate
9. Polyvinyl pyrrolidone MW 40 000
10. Potassium chloride KCl
11. Potassium phosphate KH₂PO₄
12. Sodium azide NaN₃
13. Sodium bicarbonate NaHCO₃
14. Sodium carbonate Na₂CO₃
15. Sodium chloride NaCl
16. Sodium hydroxide NaOH
17. Sodium phosphate (dibasic) Na₂HPO₄
18. Tris(hydroxymethyl)aminomethane HCl Tris-HCl
19. Tween 20

three times with PBST (Figure 154).
2. Add sample extracts (Figure 142) prepared at a 1/10 to 1/20 dilution in extraction buffer in duplicate or triplicate wells. Incubate for 2-4 h at 30-37°C or overnight at 4-6°C. Wash thoroughly three times with PBST. Avoid cross-contamination of samples when washing.
3. Add enzyme-antibody conjugate diluted in conjugate buffer to an optimum concentration (normally between 1/500 and 1/5000) (Figure 143). Incubate 2-4 h at 37°C. Wash at least three times with PBST to remove unbound conjugate from the wells.
4. Add substrate freshly prepared at a concentration of 0.6 to 1 mg/ml in substrate buffer (10 Percent diethanolamine, pH 9.8) (Figure 144). Incubate until strong colour change develops in positive controls (normally 30-60 mm) (Figure 145) and read plates (Figure 147). Plates may be read at several intervals without stopping the reaction, so rate of reaction can be calculated, or the reaction can be stopped at an appropriate time by addition of 3M NaOH, and a single reading made. If plates are read visually, score the estimated relative strength of reaction. If read on a spectrophotometer or with a plate reader (Figure 147), record the OD₄₀₅ values.

Schedule 2
**Double antibody sandwich-indirect ELISA**
1. Coat ELISA plates (Figure 141) with antibodies (IgG) specific to the antigen to be tested. The IgG concentration should be 1-2 µg/ml in carbonate coating buffer. Incubate for 1-4 h at 25-30°C and wash three times with PBST (Figure 154).
### TABLE 7
**ELISA buffers and solutions**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
</table>
| **1. Coating buffer**        | 1.59 g $\text{Na}_2\text{CO}_3$  
2.93 g $\text{NaHCO}_3$  
0.20 g $\text{NaN}_3$  
(pH should be 9.6) |  
(pH should be 9.6) |
| **2. Phosphate buffered saline (PBS)** | 8.00 g $\text{NaCl}$  
0.20 g $\text{KH}_2\text{PO}_4$  
2.90 g $\text{Na}_2\text{HPO}_4\cdot12\text{H}_2\text{O}$  
(1.15 g anhydrous)  
0.20 g $\text{KCl}$  
(pH should be 7.2 to 7.4) |
| **3. Washing buffer**        | 1.0 litre PBS  
0.5 ml Tween 20                                                                                      |
| **4. Extraction buffer**     | 1.0 litre PBST  
20 g polyvinyl pyrrolidone, MW 40 000  
(Option -15.7 g Tris-HCl, adjust to pH 7.8 with NaOH) |
| **5. Conjugate buffer**      | 1.0 litre PBST  
20.0 g polyvinyl pyrrolidone, MW 40 000  
2.0 g ovalbumen  
0.20 g $\text{NaN}_3$ |
| **6. Substrate buffer**      | 97 ml diethanolamine  
0.2 g $\text{NaN}_3$  
(adjust pH to 9.8 by adding HCl) |
| **7. Reaction stopping solution** | 120g NaOH                                                                                           |

1. pH should be close to value. Adjust slightly if necessary.  
2. Polyvinyl pyrrolidone is not essential for extraction or conjugate buffers.

2. Add sample extracts (Figure 142) prepared at a 1/10 to 1/20 dilution in extraction buffer. Load duplicate or triplicate wells with each sample. Incubate for 2-4 h at 30-37°C or overnight at 4-6°C. Wash thoroughly three times with PBST avoiding cross-contamination of samples.  
3. Add unlabelled intermediate antibody at an appropriate dilution, normally 0.25 µg/ml or less. Incubate for 30-60 min at 30-37°C and wash plate three times with PBST.
4. Add enzyme-labelled antibody specific to the intermediate antibody, diluted according to the instructions supplied. Incubate 1-2 h at 30-37°C and wash plate carefully at least three times with PBST.

5. Add substrate freshly prepared at a 0.6 to 1 mg/ml concentration in substrate buffer (10 percent diethanolamine, pH 9.8). Incubate until strong colour change develops in positive controls (normally 30-60 min) and read plates. Plates may be read at several intervals without stopping the reaction, so rate of reaction can be calculated, or the reaction can be stopped at an appropriate time by addition of 3M NaOH and a single reading made. If plates are read visually, score estimated relative strength of reaction. If read on a spectrophotometer or plate reader, record the OD$\_405$ values.

**TROUBLE-SHOOTING**

Several types of problem may be encountered with ELISA. Some understanding of the operating principles of ELISA helps in doing some systematic trouble-shooting to identify and correct the problem. Several of the most common situations are covered here. If the suggestions given do not solve the problem encountered, seek the help of someone who has extensive experience with ELISA.

**No reaction or reaction is very slow**

The common causes are: (a) use of an incorrect buffer in one or more steps; (b) antigen was inactivated during processing or storage; (c) loss of enzyme activity in the conjugate (commonly occurs if conjugate is accidentally frozen); (d) antibody used has low affinity for test antigens or lost affinity when it was conjugated; (e) inactive substrate; or (f) a gross miscalculation when making dilutions.

**Recommendations.** Test conjugate and substrate by mixing a small amount of dilute conjugate with fresh substrate in a small beaker. If no reaction occurs, test each separately again and replace the faulty component. Test reactivity of the antibody by an alternative procedure such as immunodiffusion or microprecipitation. Check calculation of dilutions and test a freshly prepared positive control. Test other extraction buffers. Run a different virus system with the same buffers and protocols.
Colour development is non-specific
When all wells, including buffer and healthy controls, show a strong reaction, it could indicate:
(a) the antibody source for either coating or conjugate phase antigens is giving non-specific reaction (common with antisera to SDS-degraded antigens); (b) the washing was incomplete, especially after the conjugate step; (c) the coating of the plate was incomplete, or Tween 20 was left out of the PBST buffer and enzyme-labelled antibody is being adsorbed non-specifically to the plate; or (d) the substrate is contaminated or faulty.

If the buffer control is negative, but the healthy control shows a positive reaction, the antiserum used probably has a high concentration of antibodies to healthy plant antigens. The alternatives are either to absorb the antiserum with healthy plant proteins to remove the antibodies in the serum specific to the healthy plant proteins, or to prepare other antisera.

Colour development is erratic
The common causes of erratic reaction within a plate are: (a) defective plates; (b) careless performance of one or more steps, especially washing; (c) failure to mix thoroughly diluted IgG and conjugate solutions; or (d) contamination between wells.

Recommendations. Check another source of plates and review the care used in the operating procedure.

Reaction very rapid, some reaction also in healthy samples
This normally indicates that the conjugate concentration is much too high. Try several tenfold dilutions. If differentiation still fails to occur between healthy and positive samples with normal incubation periods, see recommendations above.

BIBLIOGRAPHY
Hundreds of references are available on the ELISA technique and its application to numerous plant viruses. We have listed a few here. Many of these citations contain additional literature citations.


Diagram of the components of four popular types of ELISA: a) Double antibody sandwich ELISA (DAS). The most widely used form of ELISA for plant pathogens. The wells of the ELISA plate (the solid phase or immunosorbent surface) are coated with an unlabelled antibody specific to the pathogen, which becomes the trapping antibody (TA). The antigen (V) is captured by the trapping antibody and detected by the enzyme-labelled antibodies (LA) which are normally from the same polyclonal antiserum used for trapping and detection; b) Double antibody sandwich-indirect ELISA (DAS-I). The intermediate antibody (IA) is unlabelled and must be from a different animal species to that of the coating antibody. The LA is from an antibody specific for the IA. If the F(ab')2 antibody component is used for coating, the whole unlabelled antibody from the same animal can be used as the IA and is detected with protein A conjugated to an enzyme; c) Enhanced DAS-I. This is similar to DAS-I, but the enzyme concentration on the LA is amplified by an additional treatment to increase sensitivity. Frequently, the LA is biotinylated to react to avidin-enzyme conjugates; d) Plate trapped antigen-indirect ELISA. The antigen (V) is trapped directly on the plate surface and detected by using an unlabelled antibody specific to the antigen (IA) plus an enzyme-labelled antibody (LA) specific to the IA. Enhancement as shown for DAS-I is also possible.
FIGURE 140
ELISA is normally done using plastic microtitre plates specially formulated for that purpose. Plates with 96 wells are most common, but other configurations exist. Plastic beads or strips can also be used as the solid phase (immunosorbent surface) and are convenient for small numbers of samples.

FIGURE 141
The first step in DAS or DAS-I ELISA is to coat the plate with the trapping antibody (TA in Figure 139). The trapping antibody is prepared at a concentration of 12 µg/ml in carbonate buffer and 100-200 µl are placed in each well (note use of an eight-channel pipette for fast loading) and incubated for 1-4 h. Unbound antibody is removed by washing the plate with PBST (Figure 154).

FIGURE 142
The second step in DAS and DAS-I is to load the test samples which have been previously prepared (Figures 148-153), coded and arranged in sequence for easy loading. Each sample is placed in at least two wells, and each plate contains a positive and negative control sample for reference. Each plate is marked for identification and orientation. The plates are incubated for 2-4 h at 30-37°C, or overnight at 4-6°C, and then washed with PBST to remove materials not bound specifically to the trapping antibody.

FIGURE 143
The third step in DAS is to add the enzyme-labelled conjugate diluted to a predetermined optimum concentration in PBST (In DAS-I this step is preceded by application of the intermediate antibody). Incubate 2-4 h at 37°C. Wash at least three times with PBST to remove unbound conjugate from the wells. Thorough washing is important prior to substrate addition to avoid non-specific reactions.
The fourth step is preparation and addition of the substrate solution to the test plate. Substrates should be freshly made. Colour change in the substrate will be proportional to the number of enzyme-labelled antibodies bound to the antigen present.

Once an appropriate level of reaction is reached, the reaction can be stopped by addition of 50 µl of 3M NaOH and then the plate is evaluated. Plates may be read several times during the incubation period to determine the rate of reaction. Plates may also be frozen for future evaluation.

The reaction is evaluated by determining the degree of colour change which occurred in the substrate. This may be done visually by scoring the degree of reaction (e.g. negative, week, moderate or strong). Comparisons are made with both the healthy and positive control samples.

The degree of reaction may be determined by measuring the colour change photometrically (at 405 mm for alkaline phosphatase) by use of a plate reader, which measures and reports the absorbance in each well. By reading the plate at several timed intervals, the rate of reaction can also be calculated.
Proper selection of tissue to test is highly important for success in ELISA. Virus concentration is normally highest in new flushes of growth and these should be used wherever possible.

CTV, greening and stubborn are all phloem-limited, and the sample must include phloem tissue for these pathogens. Young twig bark, leaf midribs and the button area of young fruit are excellent tissue sources for CTV. If possible, collect from several sites on the test plant and prepare a composite sample for testing.

Sample tissue is finely diced to avoid clogging the homogenizer and placed in the grinding vessel at a ratio of 1 part tissue to 10-20 parts of buffer. Samples may be stored frozen in buffer if immediate testing is not convenient.
FIGURE 151
A dispersion homogenizer is very convenient for grinding tough fibrous tissue samples such as citrus bark and leaf midrib tissue. Samples can also be ground by other methods including a pestle and mortar (adding an abrasive makes it easier).

FIGURE 152
The shaft of the homogenizer must be rinsed in clean water between samples to avoid contamination.
FIGURE 153
Extracts of citrus tissue usually contain debris which makes pipetting difficult. One way to avoid clogging the tip is to pipette through a filter. The extract can also be centrifuged or the tip of the pipette cut off to create a larger bore.

FIGURE 154
Washing is a very important part of ELISA. Each plate is washed at least three times between each step. The plate is emptied by rapidly inverting it over a sink, blotted on a clean towel, filled with wash solution (normally PBST) by a squeeze bottle or other dispenser, incubated with gentle agitation for several minutes and the cycle repeated. The final wash solution may be left in the plate until the next step is initiated.

FIGURE 155
Antibodies are immunoglobulins (IgG) and can be purified from whole antiserum by use of commercially prepared kits, or by precipitation of the serum with ammonium sulphate and chromatography of the dialysed precipitate on a small column of DEAE cellulose prepared for that purpose.
FIGURE 158
Datra sheet for recording ELISA results
Detection of plant viruses and viroids by molecular hybridization

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PRINCIPLE

A viral particle is composed of nucleic acids (ribonucleic acid = RNA or deoxyribonucleic acid = DNA) and a capsid made up of several dozen to a thousand copies of coatprotein subunit. In some cases, the virus possesses an envelope composed of viral proteins integrated in membranes deriving from the host cell. Serological techniques detect the virus by specific recognition of the coat protein by specific antibodies developed in animals against this protein. Molecular hybridization techniques detect viral nucleic acids by specific recognition of their nucleotide sequence.

Nucleic acids are long, linear polymers of nucleotide molecules. Each nucleotide is in turn composed of several elements: a nitrogen-containing base linked to a phosphate group and a sugar molecular (ribose for RNA and deoxyribose for DNA). DNA contains four different bases: Adenine (A), Guanine (G), Cytosine (C) and Thymine (T). In the case of RNA, Thymine is replaced by Uracil (U), the three other bases being the same.

DNA is usually found in a double-stranded configuration, i.e. two chains of DNA associate through specific base pairing (A pairs with T and C pairs with G). Base pairing is extremely specific and creates non-covalent hydrogen bonds that unite the molecules associated in this way. RNA is most commonly found in a single-stranded configuration but, like DNA, it possesses the capacity to form double-stranded structures through A-U and G-C pairing.

The specific pairing of the bases composing nucleic acids constitutes the basis for the formation of hybrids (double-stranded structure) between complementary molecules and, thus, for the use of molecular hybridization as a diagnostic technique.

Nucleic acid molecules differ from one another in the order and sequence of alignment of their nucleotides (= nucleotide sequence). The presence of two molecules of complementary sequences will lead to the formation of double-stranded hybrids under suitable conditions. For example, TCGGCGTAT will pair with AGCCGCTA to make a DNA-DNA hybrid.

A probe used for virus detection in molecular hybridization experiments is a single-stranded nucleic acid molecule prepared from a viral nucleic acid, single-stranded with a nucleotide sequence complementary to that of the target viral RNA molecule.

Thus a DNA probe with the sequence: TCGGCGTAT will specifically detect RNA and DNA molecules with the respective sequences AGCCGCAUA and AGCCGCTA. An RNA probe with the same specificity would be: UCGGCGUUAU.

The molecular hybridization detection system presented here is based on a solid support
hybridization, the samples being permanently immobilized on a nitrocellulose membrane (Figure 157). We will describe the technique using the two most frequently used types of probe:

- complementary DNA probes cloned in a plasmid vector;
- in vitro transcribed complementary RNA probes prepared from complementary DNA cloned into special purpose transcription plasmid vectors.

The probes can be labelled either radioactively or by incorporation of a non-radioactive marker such as biotin. The techniques for the determination of the probe-specific activity are described in Appendix 1.

EXPERIMENTAL SET-UP

Schematically the technique can be divided into five steps:

- Probe labelling. This is achieved by incorporation of a labelled (radioactive or biotinylated) nucleotide triphosphate precursor during in vitro reactions (nick translation for DNA probes or transcription for RNA probes).
- Sample preparation. The immobilization of the nucleic acids present in the plant extract on a nitrocellulose membrane by baking it for two hours at 80°C under vacuum.
- Hybridization. The labelled probe will form double-stranded structures under suitable conditions with the target nucleic acid immobilized on the membrane.
- Washing(s). Non-hybridized probe molecules are removed by successive washings of the membrane under stringent conditions.
- Hybrid detection. For radioactive probes, this is achieved by contact of an X-ray film with the membrane (autoradiography), usually for 24 hours. In the case of biotinylated probes, three additional steps are required (Figure 158):

1. Incubation in the presence of streptavidin which reacts specifically with the biotin molecules fixed on the probe;
2. Incubation in the presence of a biotinylated enzyme which will be trapped by the streptavidin already retained on the membrane;
3. Enzymatic reaction that results in the formation of a coloured product at the fixing point of the complex probe biotin-streptavidin-biotinylated enzyme.

EXPERIMENTAL PROTOCOL

Probe labelling

**DNA probes.** Purified recombinant plasmid DNA is labelled (by incorporation of either $^{32}$P sCTP, biotinylated dUTP or dCTP) by the technique of nick translation, using one of the several commercially available kits (e.g. BRL, Amersham).  

**RNA probes.** After linearization of the purified recombinant plasmid downstream of the viral cDNA with a suitable restriction endonuclease, labelled RNA is produced by in vitro transcription using one of several commercially available kits (e.g. Promega, Biotec, Boehringer).  

Incorporation of either $^{32}$P or biotin-labelled CTP is usually carried out.

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1 BRL, Gibco BRL France, 14 rue des Osiers, BP 7050, 95051 Cergy Pontoise Cedex, France; Amersham Corp., 2636 S. Clearbrook Dr., Arlington Heights, IL, United States of America; Promega Corp., 2600 S. Fish Hatchery Road, Madison, WI 5371-5305, United States of America; Biotechnie France S.A., Soaris 139, 94524 Rungis Cedex, France; Boehringer Mannheim GmbH, Passettrasse 64, A-1201, Vienna, Austria.
Sample preparation

Many different plant samples can be used, consisting of leaves, stems, tubers, barks or fruits. It should be stressed that there is no standard protocol and that each protocol should be optimized for a given host/virus combinant. We will present here the technique we have developed for the detection of Plum Pox Virus, with additional advice on detection of other pathogens when appropriate.

Sample grinding. One gram of plant sample is ground in 4 ml of grinding buffer (Appendix 2) using a pestle and mortar (or other methods such as electric press or Polytron homogenizer when available). It is extremely important to use a buffer that will optimize the signal to noise ratio. The extract is then clarified by centrifugation for 10 min at 10 000 rpm. The samples can, if necessary, be deproteinized by including one volume of a 1:1 mixture of water-saturated phenol and chloroform during the grinding. This step is optional for the use of radioactive probes but necessary when using biotinylated probes.

Sample denaturation. If required, the nucleic acids contained in the supernatant are then denatured to ensure good binding of the nitrocellulose and availability of the sequences for hybridization. This step is important for the detection of viroids but often of no detriment in the case of most viruses. In a small microcentrifuge tube, 50 µl of sample are added to 50 µl of formaldehyde denaturation buffer (Appendix 2). The mixture is then incubated for 60 min at 60°C (the length of this incubation should be reduced for viruses). At this point, samples are ready for spotting on the membrane. They can also be stored for up to several months at -20°C. We have found that concentration of the nucleic acids present in the extract by ethanol precipitation is detrimental since it usually increases the non-specific background reactions and is therefore not recommended.

Nitrocellulose membrane preparation. Soaking of the membrane in a high-salt solution is required for proper binding of nucleic acids in the samples. The membrane is first soaked for 2 min in pure distilled water and then equilibrated for 10 min in 20XSSC buffer (Appendix 2).

Sample application and fixation. Twenty µl of sample are applied to the nitrocellulose membrane using a BRL “Hybri-dot” apparatus (Figures 167-169). Alternatively, 3-5 µl of sample can be applied directly (using a micropipette) to nitrocellulose that has been air-dried after soaking in 20XSSC. The membrane is then dried at room temperature and baked for a further 2 h at 80°C under vacuum to ensure stable binding of the nucleic acids to the nitrocellulose membrane. This can conveniently be achieved by using an electrophoresis slab gel drier or a vacuum oven. At this point, the membranes can be directly processed or sealed in a plastic bag and stored (at 4°C or -20°C) for up to several months.

Hybridization reaction

Pre-hybridization. In order to prevent non-specific binding of the probe to the membranes, they are pre-incubated in the hybridization mixture (pre-hybridization). The membranes are sealed in a plastic bag in the presence of 1 ml of hybridization buffer (Appendix 2) for each 10 cm² of membrane, taking care to avoid trapping any air bubbles. The bag is then incubated for 2-3 h in a water bath at 42°C.

Probe denaturation This step is included to remove any secondary structure of the probe and is especially important for DNA probes which are essentially double-stranded after the labelling reaction. A suitable quantity of probe is placed in
Laboratory methods for detection of CGTPs

a small disposable tube and incubated for 10 minutes (DNA probe) or 3 min (RNA probe) at 100°C in a bath of boiling water and then quickly chilled by placing the tube in an ice-bucket.

**Hybridization.** The pre-hybridization buffer is discarded and replaced by the hybridization buffer to which the denatured probe has been added. Use approximately 1 ml of buffer containing $1 \times 10^6$ cpm/ml of radioactive probe or 200 ng/ml of biotinylated probe per 15 cm$^2$ of membrane (see Appendix 1 for probe specific activity determination). The plastic bag is then resealed and incubated in a water-bath overnight at 50°C.

**Washing(s)**

After hybridization is completed, the membrane is removed from the plastic bag and washed in a small plastic tray.

**DNA probes.** Wash at room temperature for 5 min in three changes of Washing Buffer A, then proceed with two 15-min washes at 50°C in Washing Buffer B (see Appendix 2 for buffer composition).

**RNA probes.** Four 20-min washes at 60°C in Washing Buffer C (see Appendix 2 for buffer composition).

After the washes, the nitrocellulose membranes should be air-dried at room temperature.

**Hybrid detection**

**Radioactive probes.** An X-ray film (Kodak XAR or equivalent) is exposed to the membrane for 24 hours at -70°C using intensifying screens. After autoradiography, the film is developed using Kodak LX 24 developer and Ilford Hypam fixer. Within the limits of linearity of the response of the film, the intensity of the spots is proportional to the concentration of viral RNA present on the membrane. No non-specific signal should be obtained with healthy plant controls.

**Biotinylated probes.** The composition of the buffers is given in Appendix 2. Several commercially available kits can be used for the detection of biotinylated probes (e.g. BRL).

The membranes are first soaked for 1 min at room temperature in Buffer 1, then for 20 min at 42°C in Buffer 2 to saturate the protein fixing sites on the membrane. They are then dried and baked for 10-20 min at 80°C under vacuum.

Following the treatment, the membranes are rehydrated for 10 min in Buffer 2 and then incubated on a Petri dish in the streptavidin solution: 6 µl of a 1-µg/ml streptavidin solution diluted in 3 ml of Buffer 1. Incubate for 10 min at room temperature shaking occasionally.

The membranes are then washed well with at least three changes of buffer for 3 min each time. Incubate on a Petri dish with 3 ml of Buffer 1 containing 3 µl of a solution of biotinylated polymers of alkaline phosphatase (polyAP) at 1 mg/ml. Incubate for 10 min at room temperature with occasional shaking.

Wash abundantly with two changes of Buffer 1 and then with two changes of Buffer 3. The developing solution should be prepared at the last moment in the following way: add 33 µl of the Nitro-blue Tetrizolium solution to 7.5 ml of Buffer 3. Mix thoroughly, then add 25 µl of the 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution mix. Incubate the membrane in this solution in a sealed plastic bag protected from light.

Maximum colour development is usually achieved within 4 h. To stop the development, simply wash the membrane in 20 mM Tris-HCl pH 7.5,5 mMEDTA. The dried membranes can then be stored for several months in the dark to preserve the colour.
Appendix 1

DETERMINATION OF THE PROBE-SPECIFIC ACTIVITY

Following the labelling reaction, the radioactive DNA probe (1 µg) is precipitated with ethanol, freed from the unincorporated labelled nucleotides by several 70 percent ethanol washes, dried and finally taken up in 100 µl of sterilized distilled water. Two µl of the probe are then mixed with 3 ml of a 10 percent trichloroacetic acid (TCA) solution along with 10 µl of 3 mg/ml calf thymus DNA used as a carrier. The mixture is left for 30 min at 0°C and then filtered through a Whatman GF/C glassfibre filter. The filter is rinsed with 20 ml of a 5 percent TCA solution and then with 5 ml of ethanol before being dried. The radioactivity retained on the filter is then determined by liquid scintillation counting. The specific activity is given by:

Specific activity (cpm/ µg) = cpm x 100/2

Besides determining the specific activity of the probe, this technique can also help to calculate how much of the probe should be added to the hybridization reaction. Radioactive RNA probes can be counted in the same way.

For biotinylated probes, the result of the labelling reaction can be estimated by spotting dilutions of the probe on a membrane and comparing with a standard of known activity provided in the labelling kit.
Appendix 2
BUFFERS

Grinding buffer for viroids (GPS)
200 mM glycine
100 mM Na₂HPO₄
600 mM NaCl
1% SDS (sodium dodecyl sulphate)
Adjust pH to 9.5, autoclave 20 min at 120°C then add:
0.1% DIECA (Na-diethyldithiocarbamate)
0.1 mM DTT (dithiothreitol)

Grinding buffer for viruses
50 mM Na-citrate, pH 8.3
2% PVP (polyvinylpyrrolidone)
Autoclave 20 min at 120°C then add:
1 mM EDTA
20 mM DIECA

Phenol/chloroform mixture
1 vol. water-saturated phenol
1 vol. chloroform/pentanol (24/1)

20XSSC buffer
3 m NaCl
0.3 M Na-citrate
pH adjusted to 7.0

Formaldehyde denaturation buffer
5XSSC
25 mM Na₂HPO₄
5x Denhart (0.1% each of bovine serum albumin (BSA), Ficoll and PVP 360)
50% deionized formamide
200 µg/ml of denatured calf thymus DNA

DNA probes hybridization buffer
4 vol. DNA probes pre-hybridization buffer
1 vol. 50% dextran sulphate

RNA probes pre-hybridization and hybridization buffer
50% formamide
50 mM phosphate buffer pH 6.5
5XSSC
0.1% SDS
1 mM EDTA
0.05% Ficoll
0.05% PVP 360
200 µg/ml of denatured salmon sperm DNA

**Washing buffer A**
2XSSC
0.1% SDS

**Washing buffer B**
0.2XSSC
0.1% SDS

**Washing buffer C**
0.1XSSC
0.1% SDS

**Biotinylated probes development buffers**

**Buffer 1**
100 mM Tris-HCl pH 7.5
100 mM NaCl
2 mM MgCl₂
0.05% Triton X100

**Buffer 2**
Buffer 1 plus 3% BSA

**Buffer 3**
100 mM Tris-HCl pH 9.5
100 mM NaCl

**Commercial source of chemicals**

BSA
Sigma No. A6793

PVP 360
Sigma No. P5288

Ficoll 400
Sigma No. F4377

Salmon sperm DNA
Sigma No. D1626

DTT
Sigma No. D9779

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1 Sigma Chemical Company Inc., PO Box 14508, St Louis, MO 63178, United States of America; Merck Schuchardt & Co., Eduard Buchner Strasse, D-8011 Hohenbrunn, Germany.
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<td>DNA detection system</td>
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Hybridization on nitrocellulose (nc) membrane (left) infected sample containing normal plant cell RNAs and viral RNA (the target sequence to which the RNA probe hybridizes). Because of this hybridization the labelled probe will be retained on the membrane (right) Healthy sample containing only normal plant cell RNAs. The probe cannot hybridize with any sequence: it will not be retained on the membrane and will be eliminated upon washing.
FIGURE 158
Schematic representation of the system used for the detection of biotinylated probes
FIGURE 159
Plant sample consisting of leaves, bark, roots, tubers, fruits

FIGURE 160
Plant sap is extracted using an electric press

FIGURE 161
A drop of sap is added to a grinding buffer contained in the microcentrifuge tube

FIGURE 162
Sap and grinding buffer are mixed using a vortex

FIGURE 163
Low-speed centrifugation (10,000 rpm for 10 min) is carried out to pellet the plant cell debris and to separate the phases if a phenol deproteinization step is included

FIGURE 164
For viroids, the supernatants are supplemented with formaldehyde and incubated for 60 min at 60°C (omit this step for most viruses)
In the meantime, one nitrocellulose membrane and three sheets of Whatman 3MM paper are cut, soaked in water and equilibrated in 20XSCC buffer.

Twenty microlitres of extract are spotted on the membrane.

One 3MM filter and the nitrocellulose are placed in the BRL Hybri-dot system (or equivalent).

The membrane is taken out of the blotting apparatus and air-dried.
The pre-hybridization buffer is added to the plastic bag and the bag is completely sealed.

Pre-hybridization is carried out by incubating the bags for 2-4 hours in a water-bath under suitable conditions.
The probe is denatured for a few minutes in a bath of boiling water.

The bag is cut open, the pre-hybridization buffer discarded and replaced by the hybridization buffer containing the denatured probe. The bag is then resealed.

Hybridization is carried out by incubating the bag overnight at the desired temperature in a water-bath.

After hybridization, the membrane is removed from the bag and washed in several changes of washing buffer.

After washing, the membrane is air-dried.
FIGURES 182 and 183
An X-ray film is exposed to the membrane

FIGURE 184
After autoradiography is completed, the X-ray film is developed and fixed
Viroid purification and characterization

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HOST PLANTS FOR PURIFICATION AND BIOASSAY

Indicator plants used for viroid purification and bioassay commonly respond with some form of stunting reaction which may be accompanied by leaf symptoms of rugosity, epinasty, mottling and chlorotic spotting, and vein browning. However, since viroid replication may occur in the absence of any discernible symptoms, all inoculated species should be extracted and analysed for viroid content. Plant species suspected of containing viroids can be analysed directly, provided that the extraction conditions necessary to obtain a quality nucleic acid preparation are determined.

In viroid transmission studies, seedlings which in most cases are viroid-free, or vegetatively propagated plant sources which have been made viroid-free by shoot-tip culturing, are preferred host plants. Woody plant species, such as citron and grapevine, can be inoculated by slashing the stem with a razor moistened with inoculum. With more succulent species, such as tomato, a needle or fine Pasteur pipette is used to puncture the hypocotyl of a very young plant at the point where a drop of inoculum has been applied.

Major indicator hosts
- Chrysanthemum (Chrysanthemum morifolium cv. Bonnie Jean) (see Brierly, 1953)
- Citron (Citrus medica cv. Etrog) (see Calavan et al., 1964)
- Cucumber (Cucumis sativus cv. Suyo) (see Sasaki and Shikata, 1977; Van Dorst and Peters, 1974)
- Gynura (Gynura aurantiaca) (see Weathers and Greer, 1968)
- Petunia (Petunia hybrid a) (see Weathers et al., 1967)
- Tomato (Lycopersicon esculentum cv. Rutgers) (see Raymer et al., 1964)

TISSUE EXTRACTION AND PURIFICATION

Ultimate success in detecting viroids as discrete bands on polyacrylamide gels is dependent upon the quality of the nucleic acid preparations obtained from infected tissues. High concentrations of phenolic and acidic compounds can seriously interfere with the recovery of all nucleic acid species. Therefore, the composition of the extraction medium must be custom-made to the particular tissue under investigation to assure the consistency of factors such as the maintenance of a pH of about 6.5 to 9.0, the presence of appropriate additives such as polyvinylpyrrolidone to neutralize the effects of polyphenols, and adequate concentration of antioxidants.

Two protocols commonly used for the extraction of a tissue will be presented. The procedures differ basically in the manner in which the aqueous phase from the initial phenol-extraction step is treated. Concentration by ethanol precipitation is employed with tissue extracts from plants such as citron and tomato.
from which good nucleic acid preparations can routinely be recovered. “Trapping” of nucleic acids, including viroid RNA, on CF-11 cellulose has been customized for use with direct extraction of grapevine tissue or others from which nucleic acids are difficult to recover.

Even though the primary focus of the procedures presented here is the analysis of citrus, the alternative approach as indicated for “grapevines” should be employed if nucleic acid preparations are either difficult to obtain or are of poor quality. The designation of the procedures for “grapevines” simply indicates the plant tissues for which the technique was developed and does not imply an exclusive application. To date, grapevine tissues have been the most challenging for recovery of nucleic acid preparations of high quality and adequate quantity for analysis of viroid content. Therefore, the information developed for this tissue may become valuable for the analysis of particular citrus species.

Unless one can demonstrate the recovery of a typical profile of host nucleic acids, it is difficult to evaluate the relative concentration or even the very presence of viroid molecules. Therefore, it is a good practice to inspect the presence and relative concentration of particularly the 4s and 5S RNA components of 2M LiCl soluble nucleic acids following electrophoresis in the native polyacrylamide gels.

### Materials
- Infected tissue: fresh tip tissue which is actively growing and collected at least two to six weeks post-inoculation of herbaceous hosts and two to six months post-inoculation of woody species is preferred. If tissue is to be collected and stored for extraction at a later time, it should be powdered in liquid nitrogen and held at -20°C.

* Extraction medium (EM-1) for citrus species and herbaceous plants:
  - Buffer (0.4 M Tris-HCl, pH 8.9)
  - SDS (sodium dodecyl sulfate), 1%
  - EDTA (ethylenedinitrilotetraacetate), 5 mM, pH 7.0
  - MCE (mercaptoethanol), 4%

* Extraction medium (EM-2) for grapevines and plants containing a high concentration of phenols and acidic compounds:
  - Buffer (0.5 M Na₂SO₃)
  - SDS (sodium dodecyl sulfate), 1%

* Resuspension medium (RM): TKM buffer:
  - Tris 10 mM
  - KCl 10 mM
  - MgCl₂ 0.1 mM
  - Adjust to pH 7.4 with HCl

* LiCl, 4 M

* PVP (polyvinylpyrrolidone) 20% (4X stock)

* Sodium acetate, 3 M, pH 5.5

* Phenol (water-saturated) adjusted to pH 7 with 1 N NaOH

* Ethanol, 95-100%

* CF-11 fibrous cellulose powder (Whatman)

* STE buffer 10X stock (1.0 M NaCl, 0.50 M Tris-HCl, pH 7.2, 10 mM EDTA)

* Dialysis tubing

* Homogenizer: Virtis, high speed (50 000 rpm); “food” blender, low speed (15 000 rpm)

**Note:** Yield of viroid can be significantly affected by the method of homogenization.

* Centrifuge, low speed (5 000-10 000 rpm or 6 000-12 000 g), refrigerated

* Magnetic stirrer at 4°C

### Extraction of citrus and most herbaceous species
1. Grind tissue in pre-cooled EM-1 and phenol in a proportion equal to 1 g tissue: 1 ml EM-1:3 ml phenol in an ice-bath if possible.

**Note:** Phenol can cause severe burns: therefore, protection for hands (disposable gloves) as well as a plastic barrier should be employed to guard
against accidents during homogenization.
2. Transfer to centrifuge tubes or bottles and centrifuge for 20 min at 7 000-12 000 g.
3. A clear but pigmented aqueous layer is found over a solid interface of plant debris and a lower heavily pigmented phenol phase. The plant debris may also form a pellet below a liquid bilayer between the aqueous and phenol phases.
4. Remove aqueous layer and add a l/10 volume of 3 M sodium acetate, pH 5.5 and a minimum of three volumes of 95-100 percent ethanol. Hold at -20°C for 30 min or an indefinite period.
5. Centrifuge for 20 min at 7 000-12 000 g.
6. Discard supematant and drain pellets containing nucleic acids until excess ethanol is removed.
7. Cover pellets with a minimum volume of RM (1-10 ml/5-100 g fresh weight tissue) and resuspend with agitation.
8. Transfer slurry to dialysis tubing.
   Note: At this point the solution may appear quite turbid and particulate, but should clear considerably with dialysis.
9. Dialyse with rapid stirring on a magnetic stirrer at 4°C overnight against 1 L of RM.
10. Remove sample from dialysis tubing to centrifuge tubes and add one volume of 4 M LiCl. Hold at 4°C for 4 h or overnight.
11. Centrifuge for 20 min at 7 000-12 000 g.
12. Retain supematant containing LiCl soluble nucleic acids (mainly DNA, 4S and 5S RNA, dsRNAs, and viroids). Discard pellet of LiCl insoluble nucleic acids (mainly ribosomal RNA).
13. Add a minimum of three volumes of 95-100 percent ethanol and hold at -20°C for 30 min or overnight.
14. Centrifuge for 20 min at 7 000-12 000 g.
15. Decant and drain ethanol from pellets and dry in vacuo.
16. Resuspend pellets in an appropriate volume of RM (100 µl/5g fresh weight of tissue).
17. Store at -20° to -80°C.

Note: These preparations are sufficiently purified for routine viroid detection procedures and infectivity tests. However, the quality of the analysis will be markedly improved by farther processing by cellulose chromatography.

**Extraction of grapevines and tissues from which it is difficult to recover nucleic acids**
1. Grind tissue as indicated above substituting EM-2.
2. Centrifuge as above.
3. Note as above.
4. Remove aqueous phase from above interface and lower phenol layer.
5. Make solution to 35 percent ethanol and 1X STE with stirring. Add dry CF-11 cellulose powder (1 g/5g fresh-weight tissue extract). Stir for 2 h or overnight at room temperature.
6. Collect cellulose by centrifuging at 7 000g for 10 min.
7. Discard supematant and wash cellulose pellet with a solution of 30 percent ethanol in 1X STE buffer with agitation.
9. Repeat washing procedure with 30 percent ethanol-STE solution two or three times until all traces of pigmented materials have been removed from the wash solution.
10. With the cellulose in 30 percent ethanol-STE, form a chromatography column and continue to wash cellulose with three to four void volumes of 30 percent ethanol-STE.
11. Elute bound nucleic acids by eluting with two to three void volumes of STE buffer, collecting the eluant in a serial manner and not as a single batch.
12. Add 10 percent volume of 3M sodium acetate, pH 5.5, and a minimum of three volumes of ethanol. Let solution stand at -20°C for 30 min or longer as convenient.
13. Collect precipitated nucleic acids by centrifugation at 12 000 g for 20 min.
14. Discard supernatant and allow pellet to drain until reasonably dry.
15. Resuspend pellet in minimum amount of RM buffer.
16. Add one volume of 4M LiCl and let stand at 4°C for 4 h or overnight.
17. Centrifuge at 12 000 g for 20 min. Retain supernatant of 2M LiCl soluble nucleic acids.
18. Add a minimum of three volumes of ethanol to the supernatant and let stand at -20°C for 30 min or longer.
19. Collect precipitated nucleic acids by centrifugation at 12 000 g for 20 min.
20. Discard supernatant, drain liquid from pellet and dry in vacuo.
21. Resuspend pellet in a minimum volume of RM buffer usually in the range of 100 µl/5-10 g fresh weight of tissue.
22. Store samples as aliquotes at -20° to -80°C prior to analysis by polyacrylamide gel electrophoresis or infectivity.

**(Duran-Vila et al., 1986; Semancik et al., 1975; 1987. )**

**CF-11 CELLULOSE CHROMATOGRAPHY**

This technique can be utilized routinely as a preparative procedure for the removal of DNA and other pigmented components of viroid containing LiCl soluble nucleic acid preparations. The property of selective binding of viroid RNA at specific ethanol concentrations can also be exploited in recovering viroids from tissue extracts, as was demonstrated in the “trapping” procedure presented in the previous section.

More recently, an analytical approach to CF-11 cellulose chromatography has been introduced (Semancik, 1986) to characterize different viroid RNAs by serial elution with an ethanol gradient. This procedure can be utilized to remove contaminating host RNAs from viroid preparations as well as to separate individual viroids with selective elution by different ethanol concentrations.

**Materials**

- CF-11 cellulose powder, fibrous (Whatman)
- STE buffer (0.1 M NaCl, 1 mM EDTA, 0.05 M Tris-HCl, pH 7.2)
- 95-100 percent ethanol
- syringe barrels (disposable) or chromatography columns
- GF/C glass microfibre filter discs (Whatman 2.4 cm)

**Preparative chromatography**

1. An aqueous sample containing nucleic acids is made to 35 percent ethanol in STE buffer.
2. Apply the solution to a CF-11 cellulose column which has been equilibrated with 35 percent ethanol-STE.

**Notes:**

(a) The amount of cellulose used is dependent upon the amount of nucleic acid in the preparation. A proportion of 1-10 g cellulose/5-100 g fresh-weight extraction is usually adequate.

(b) A “trapping” procedure can also be employed with dry cellulose added directly to the aqueous phase from a phenol extraction made to 35 percent ethanol-STE.

3. Wash the cellulose with sufficient 30 percent ethanol-STE to remove all traces of colour from the cellulose or with a volume equivalent to at least four to six column void volumes.
4. Elute the nucleic acids retained on the column with two to four void volume equivalents of STE (0 percent ethanol).
5. Precipitate nucleic acids with addition of 1/10 volume of 3 M sodium acetate, pH 5.5 and at least three volumes of 95-100 percent ethanol and hold at -20°C for 30 min or longer.
6. Centrifuge for 20 min at 12 000 g.
7. Dry pellet in vacuo and resuspend in TKM buffer (resuspension medium from extraction procedure).
Analytical chromatography
1. Nucleic acid sample from the extraction procedure or preferably after pre-treatment on a preparative CF-11 column is made to 35 percent ethanol-STE.
2. Apply to a chromatography column containing an adequate amount of CF-11 cellulose equilibrated with 35 percent ethanol-STE.
3. Wash column with 35 percent ethanol-STE (four to six column void volumes).
4. Elute with 25 percent ethanol-STE, collecting two to four column void volumes. Retain eluant.
5. Wash column with 25 percent ethanol-STE (four to six column void volumes).
6. Elute with 20 percent ethanol-STE, collecting two to four column void volumes. Retain eluant.
7. Continue alternating wash and elution cycles with either a progressively reduced ethanol concentration, such as in 5 percent increments, or a decreasing linear ethanol gradient to a final elution in STE buffer.
8. Precipitate nucleic acids with addition of 1/10 volume of 3 M sodium acetate, pH 5.5, and at least three volumes of 95-100 percent ethanol and hold at -20°C for 30 min or longer.
9. Centrifuge for 20 min at 12 000 g.
10. Dry pellet in vacuo and resuspend in RM buffer.
11. Analyse by sequential PAGE under native and denaturing conditions. (Barber, 1986; Duran-Vila et al., 1986; Franklin, 1966; Semancik, 1986.)

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)
Optimum resolution of viroid RNA is obtained by a sequential gel electrophoresis procedure involving migration of the sample into a standard gel (5 percent PAGE), followed by excision of a piece of the gel which is then placed in contact with a second, denaturing gel (dPAGE) containing 8 M urea. This procedure exploits the unique properties of the single-stranded closed circular structure of the viroid for the separation of a distinct band. Placement of the excised gel piece in contact with the top (Semancik and Harper, 1984) or the bottom (Schumacher et al., 1983) of the denaturing gel and migrating to the anode will produce similar results.

The discontinuous pH dPAGE (Rivera-Bustamante et al., 1986) with the gel cast at pH 6.5 (TAE buffer), but migrated in a pH 8.3 running buffer (TBE buffer), enhances the separation between the circular and linear molecular forms of the viroid. In addition, the background of host nucleic acids is reduced, which aids in the recovery of pure viroid preparations for physical characterization and hybridization analysis.

Verification for the detection of a suspected viroid can be made by a PAGE analysis sequence involving:

- non-denaturing 5 percent PAGE: excise a strip of the gel in the “viroid zone” as defined by citrus exocortis viroid (CEV) and avocado sun blotch viroid (ASV, ASBV);
- dPAGE (pH 6.5): excise any slowly migrating band suspected of being a viroid circular form;
- dPAGE (pH 8.3): resolution of two distinct bands containing the viroid circular form and the linear molecular form, generated from circles during electrophoresis.

Staining with ethidium bromide to visualize nucleic acid bands is necessary when gels are to be subjected to a second electrophoresis and/or when biologically-active viroid is to be recovered. Increased sensitivity of detection can be achieved with silver nitrate staining. However, this procedure renders the viroid inactivated and immobilized in the gel.
Laboratory methods for detection of CGTPs

Materials

- Electrophoresis chamber and casting apparatus: glass plates, spacers, clamps, sample-well comb. Variations in size are available through commercial sources or can easily be custom fabricated.
- Power supply (100 mA, 1000 volt or greater capacity)
- Ultraviolet transilluminator
- Polaroid camera

Stock solutions for 5 percent gels:

**Stock A:**
- Acrylamide 30.00 g
- Bisacrylamide 0.75 g
Dissolve in distilled water, bring to 100 ml and filter.

**Stock B:**
- Tetramethylethylenediamine (TEMED)
2 ml to 100 ml with distilled water

**Stock C:**
- Tris 120 mM
- Sodium acetate.3H₂O 60 mM
- Sodium EDTA 3 mM
Dissolve in distilled water, adjust to pH 7.2 with glacial acetic acid.

**Note:** This solution is equal to 0.3X Stock D, therefore it can also be made by diluting 30 ml of Stock D to 100 ml with distilled water.

**Stock D:**
- TAE buffer, pH 7.2 (10X)
- Tris 120 mM
- Sodium acetate.3H₂O 60 mM
- Sodium EDTA 3 mM
Dissolve in distilled water, adjust to pH 6.5 with glacial acetic acid.

**Note:** Since Stock D is a 10X concentration, it should be diluted before use as a running buffer.

**Stock E:**
- Ammonium persulfate (10 percent)
2.5 g in 25 ml H₂O prepared fresh (weekly)

**Stock F:**
- TAE buffer, pH 6.5

**Tris** 120mM
- Sodium acetate.3H₂O 60mM
- Sodium EDTA 3mM
Dissolve in distilled water, adjust to pH 6.5 with glacial acetic acid.

**Denaturing gel buffer (TBE, pH 8.3 10X) stock**
- Tris 225 mM
- Boric acid 225 mM
- Sodium EDTA 5 mM
Dissolve in distilled water, no adjustment of pH should be necessary. Dilute tenfold for use as a running buffer (1X).

- Urea
- Glycerol (60%)

**Migration tracking dyes:**
- Bromophenol blue 0.3% in 60% glycerol
- Xylene cyan01 0.3% in 60% glycerol

**Ethidium bromide stock staining solution**
(5 mg/ml) (30 µl/200 ml H₂O for staining gels)

**Silver staining solutions:**
- Ethanol (50%) + acetic acid (10%)  
- Ethanol (10%) + acetic acid (10%)
- Silver nitrate (12 mM)
- Potassium hydroxide (0.75 M) + formolaldehyde (0.28%)
- Sodium carbonate (0.07 M)

Native 5% PAGE

1. Assemble glass form to receive polymerization solution.
2. Mix contents of two beakers containing the following solutions in the indicated amounts or similar proportions:

**Beaker 1**
- 12.0 ml distilled water
- 10.0 ml Stock C
- 2.4 ml Stock B

**Beaker 2**
- 5.0 ml Stock A
- 0.48 ml ammonium persulfate
3. Fill form, place sample-well comb, and let stand for 30 min.
4. Withdraw sample-well comb and lower spacer. Attach to chamber and fill electrode reservoirs with 1/10 dilution of Stock D.
5. Mix samples with about 1/4 volume of glycerol and load into wells with fine-tip Pasteur pipettes. Load outermost wells with mixture of tracking dyes.
6. Apply constant current, 54 mA, at 4°C for 2.5-3 h or until bromophenol blue dye has migrated about 8 cm and xylene cyanol has reached about 4 cm.

Note: The xylene cyanol is a useful marker for CEV since the migration of both molecules is very similar in native conditions.
7. Remove gel from the chamber and form. Soak with gentle agitation in the ethidium bromide staining solution for 10 min.
8. View the gel directly over a UV transillumination source. Cut horizontal strip as defined by “viroid zone” (CEV-ASV) or smaller, depending upon viroid, and transfer to denaturing gel.

**Denaturing PAGE (pH 6.5)**

1. Assemble glass form for polymerization solution.
2. After the urea in beaker 1 is dissolved, mix rapidly the contents of two beakers containing:
   
   **Beaker 1**
   
   14.4 g urea
   7.0 ml H₂O
   3.0 ml Stock F (TAE pH 6.5)
   5.0 ml Stock A dissolved on low heat
   
   **Beaker 2**
   
   2.5 ml Stock B
   0.5 ml ammonium persulfate
3. Immediately fill form, leaving a flat surface with sufficient space for the excised native gel piece, and allow to stand for a minimum of 1 h.
4. Remove lower spacer and attach to chamber. Do not add buffer or any liquid to the gel surface until immediately prior to use.
5. After section has been removed from native gel, fill electrode reservoirs and cover top surface of gel with denaturing gel buffer (TBE, pH 8.3, IX).
6. Float excised section on to the top of the denaturing gel, making as close a contact as possible.
7. Add a few drops of xylene cyanol-glycerol mix next to the outer edges of gel strip.
8. Apply constant current, 15 mA, at 24°C for about 4 h or until the xylene cyanol tracking has migrated to within 0.5 cm of the bottom of the gel.
9. Remove gel from form and stain with either:
   
   - ethidium bromide:
     
     a) for additional dPAGE (pH 8.3 gel) to confirm circular and linear forms;
     
     b) for elution of viroid bands for infectivity or for use as templates for cDNA probes;
   
   or:
   
   - silver nitrate for maximum sensitivity of detection.

**Denaturing PAGE (pH 6.3)**

1. Follow the same set-up and running procedure as presented above.
2. Mix rapidly the contents of two beakers containing:

   **Beaker 1**
   
   14.4 g urea
   7.0 ml H₂O
   3.0 ml TBE buffer pH 8.3 10X
   5.0 ml Stock A dissolved on low heat
   
   **Beaker 2**
   
   2.5 ml Stock B 0.5 ml ammonium persulfate
3. Stain completed gel with either ethidium bromide as before or silver nitrate.
Silver staining

1. Gel can be stained with silver directly or following ethidium bromide staining without additional treatment.
2. Soak gel at room temperature in solution of 50% ethanol + 10% acetic acid for at least 1 h with gentle shaking. Overnight soaking can sometimes improve the background.
3. Soak gel at room temperature in solution of 10% ethanol + 1% acetic acid for 1 h with gentle shaking.
4. Soak in solution of 12 mM AgNO₃ for 1 h with gentle shaking.
5. Rinse thoroughly (three times) with distilled H₂O.
6. Rinse rapidly with small volume of developer solution (0.75 M KOH + 0.28% HCHO) and discard solution.
7. Add fresh developer solution (100-200 ml) and observe until bands appear, usually within 20 min.
8. Add excess distilled water and allow gel to expand. This process reduces the background and improves the quality of photographs.
9. Developing reaction can be stopped with 0.07 M Na₂CO₃.
10. Photograph gel over a light-box using Polaroid film. (Iglei, 1983; Morris and Wright, 1975; Rivera-Bustamente et al., 1986; Schumacher et al., 1983; Semancik and Harper, 1984.)

INFECTIVITY OF NUCLEIC ACID FRACTIONS AND VIROID MOLECULES

The infectivity of a viroid-containing sample can be influenced by the quality of the preparation. In many cases, viroid transmission by highly purified preparations can be more difficult than by a more complex, less purified preparation. This may result, in part, from the presence of host nucleic acids which may function to protect the viroid molecule from inactivation. Therefore, a sample such as a 2 M LiCl soluble fraction may be valuable to demonstrate the transmission properties and host range of suspected viroid-containing preparations.

Nevertheless, an essential proof for the detection of a viroid is the transmissibility of the putative viroid-like molecule. This can be provided by the recovery of the unique, transmissible viroid structure, the single-stranded circular RNA molecule, in highly purified form followed by transmission to a host plant.

Electra-elution of the circular forms of viroids, as detected in denaturing PAGE by ethidium bromide staining, has proved to be a highly efficient procedure for the recovery of biologically-active, pure viroid.

Materials

- PAGE gel piece containing the viroid
- Electra-elution buffer (EB) (1/50 dilution of Stock D in PAGE procedure):
  - 8.0 mM Tris
  - 4.0 mM sodium acetate
  - 0.2 mM EDTA
  - adjusted to pH 7.2 with acetic acid
- Electra-elution apparatus:
  a) A chamber can be constructed to accommodate a piece of dialysis tubing filled with EB into which the gel piece has been introduced. When placed in an electrical field the viroid will migrate from the gel but be retained in the liquid phase inside the tubing.
  b) A commercial apparatus (Unidirectional Electroelutor Model UEA) which does not require the dialysis tubing containment procedure is available from International Biotechnologies, Inc.

¹International Biotechnologies, Inc., PO Box 9558, New Haven, CT 06535, United States of America.
. Power supply (25 mA, 250 volt)
. 3 M sodium acetate, pH 5.5
. Ethanol

**Electro-elution**

1. Prepare gel piece to be eluted within dialysis tubing or according to IBI instructions.
2. Apply about 125 constant voltage for 30 min at room temperature.
3. Withdraw buffer sample containing eluted viroid, add 1/10 volume of 3 M sodium acetate, pH 5.5, plus at least three volumes of ethanol and hold at -20°C for 30 min or longer.

   **Note:** The gel piece can be checked for incomplete elution of viroid by restaining with ethidium bromide and viewing over a UV transilluminator. If viroid still remains in the gel piece, the elution procedure can be repeated.

4. Centrifuge sample at 12 000 g for 20 min. Pellets may be extremely small or invisible. Nevertheless, sufficient viroid to be detected by PAGE and silver staining or infectivity can be recovered many times.
5. Dry decanted centrifuge tubes in vacuo and resuspend pellets in appropriate volume of TKM buffer (RM) or desired medium.

**REFERENCES**


FIGURE 185
Diagram of the procedure for tissue extraction and purification
FIGURE 186
Diagram of the procedure for CF-11 cellulose chromatography
FIGURE 187
Diagram of the procedure for polyacrylamide gel electrophoresis
(PAGE)
FIGURE 188
Diagram of the procedure for denaturing polyacrylamide gel electrophoresis (dPAGE)
FIGURE 189
Selection of citrus tissue for extraction

FIGURE 190
Selection of grapevine tissue for extraction

FIGURE 191
Components of extraction medium used for citrus

FIGURE 192
Components used in purification and concentration of nucleic acids

FIGURE 193
Components for resuspension of nucleic acid pellets

FIGURE 194
Components of extraction medium used for grapevines
FIGURE 195
Low-speed refrigerated centrifuge

FIGURE 196
Virtis high-speed homogenizer

FIGURE 197
Apparatus used to agitate CF-11 cellulose for "trapping" of nucleic acids

FIGURE 198
Apparatus used for preparative or analytical cellulose chromatography

FIGURE 199
Apparatus used for polyacrylamide gel electrophoresis
FIGURE 200
Polaroid photography apparatus and transluminator with ultraviolet light source for visualizing nucleic acid bands stained with ethidium bromide

FIGURE 201
Polaroid photography apparatus and visible light source for observing nucleic acid bands stained with silver

FIGURE 202
As for Figure 201 (with room lights off)

FIGURE 203
Silver-stained denaturing polyacrylamide gel as seen through the polaroid viewer
Immunosorbent electron microscopy (ISEM) and antibody coating

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The principle of immunosorbent electron microscopy (ISEM) is the selective trapping of plant viruses on to electron microscope grids precoated with a specific antiserum. This technique has been described in a number of papers and review articles (Derrick, 1973; Milne and Luisoni, 1977; Garnsey et al., 1979; Roberts and Harrison, 1979; Van Regenmortel, 1982; Milne and Lesemann, 1984) to which the reader is referred for comprehensive information.

ISEM may be combined with antibody coating (often referred to as “decoration”), a procedure whereby virus particles trapped on the microscope grid are exposed to the homologous antiserum, thus becoming visibly covered with antibody molecules.

The consensus is that ISEM is highly reliable (there are virtually no false positives), as sensitive as ELISA, fast (results can often be obtained within one or two hours), and operationally simple (it requires tools and reagents readily available in most laboratories).

Unfortunately, ISEM requires an electron microscope and is not suitable for large-scale routine testing. Specimens for ISEM, however, can readily be prepared in laboratories with no electron microscope facilities and then be shipped for observation (even over long distances) to properly equipped institutions.

BASIC TOOLS AND REAGENTS
The following are required (see Figures 204-206):

- porcelain mortars 6 cm in diameter or smaller, or glass microscope slides and glass rods;
- carborundum powder (600-mesh), or quartz sand;
- bench centrifuge with relative glass or plastic conical tubes;
- fine straight-point tweezers;
- Petri dishes 9 cm in diameter;
- bars of dental wax, silicone-treated paper, or parafilm;
- Pasteur pipettes;
- electron microscope grids (400 mesh) covered with carbon film;
- protectants:
  - 2.5 to 5% aqueous solution of nicotine
  - 2% aqueous polyvinylpyrrolidone (PVP)
  - 1% aqueous polyethylene glycol (PEG), MW 6000-7500;
  - phosphate buffer 0.1M, pH 7.0
- stock solution (1M):
  (a) 136.09 g of KH$_2$PO$_4$ in distilled water to 1 litre
  (b) 268.077 g of Na$_2$HPO$_4$ in distilled water to 1 litre
  Mix 3.86 ml of solution (a) with 6.14 ml of solution (b) and dilute tenfold.
- distilled water;
Laboratory methods for detection of CGTPs

staining solutions:  
- 1-2% uranyl acetate in distilled water, pH not adjusted;  
- 2% sodium or potassium phosphotungstate in distilled water, adjusted to pH 7 with NaOH or KOH;  
- appropriate antiserum.

PREPARATION OF TISSUE EXTRACTS
Extracts may be prepared from tissues of different organs of field- or greenhouse-grown plants (leaves, roots, bark, dormant or breaking buds) or vectors (insects, nematodes). Plant tissues (usually 100-200 mg) are ground in a mortar in the presence of carborundum powder or quartz sand and 0.3-0.5 ml of phosphate buffer or, especially with grapevine and stone fruits, one of the above protectants (nicotine, PVP, PEG). When a smooth paste is obtained, 0.3-0.5 ml of buffer are added and the sample is ground again. The slurry is transferred to a centrifuge tube and centrifuged (1500-2000 g). The supernatant fluid is collected and used.

If a centrifuge is not available, tissue extracts can be further diluted with phosphate buffer to 1:15-1:20 with respect to tissue weight, and used as such.

Insect and nematode vectors are crushed with a glass rod on a glass slide in a droplet of buffer or protectant. A droplet of buffer is then added and the extract is used as such.

ANTISERUM DILUTIONS
The purpose of using an antiserum is twofold: coating EM grids for trapping virus particles and “decorating” virus particles by attachment of antibody molecules to the antigenic sites of the particles. Crude antisera are perfectly suitable for both uses, provided that they are properly diluted.

- Coating of grids: dilute antiserum near or above its end point (usually 1:1000 to 1:5000) with buffer.
- *Decoration of virus: dilute antiserum to 1:10-1:100 with buffer.

(Use of freshly diluted antisera is advisable.)

PRECOATING OF EM GRIDS
In certain cases, precoating of EM grids with protein A, a bacterial wall protein that binds specifically to the basal part (Fc portion) of antibody molecules, can be advantageous. Protein A allows trapping of more virus particles owing to the richer antibody layer on the grid. It also allows the use of undiluted, low-titre (1:8-1:16) antisera which would not be suitable after high dilution as required by ordinary ISEM. Protein A is diluted in phosphate buffer at a final concentration of 10-100 µg/ml, a drop is placed on the grid for 5 min at room temperature, and the excess is rinsed off before exposure to antiserum.

ANTISERUM COATING OF EM GRIDS
Drops of diluted antiserum (1:1000-1:5000) are placed on dental wax or other hydrophobic supports (parafilm strips, silicone-treated paper) in a plastic Petri dish containing moist filter paper (moist chamber). A freshly prepared carbon-coated grid is gently placed, film-down, on top of each antiserum drop and floated for 5-10 min at room temperature. Grids are then removed with tweezers and rinsed.

RINSING THE EM GRIDS
Throughout the ISEM procedure, grids must be carefully rinsed to obtain clean preparations. Buffer rinse is used after protein A precoating, antiserum coating and incubation of the grid with tissue extract. Distilled water rinse is used after second antibody coating (decoration of virus particles), before negative staining, for uranyl acetate precipitates in presence of phosphate ions, or at neutral pH.
Two rinsing procedures can be utilized:

- grids are floated on drops of buffer or distilled water, as appropriate, for 5-10 min;
- grids are retained in tweezers, held vertically and rinsed with 25-30 drops of buffer or water from a Pasteur pipette held close to the grid.

NEGATIVE STAINING

Negative stain can be applied with either system used for rinsing, i.e. floating grids on small drops of the staining solution for 30 sec to 1 min, or applying the stain dropwise (five drops) with a Pasteur pipette.

SUMMARY OF THE PROCEDURE

- Prepare tissue extracts, place drops of extract in a moist chamber on a hydrophobic support (Figure 208).
- Float antiserum-coated grids film-down, one on each drop of extract (Figure 208). Incubate at room temperature or in the cold (4°C) for 6-8 h.
- Rinse with 25-30 drops of phosphate buffer from a Pasteur pipette (Figure 210). Drain with filter paper.
- Place drops of antiserum diluted 1: 10-l: 100 in the moist chamber on a hydrophobic support (Figure 211).
- Float grids on antiserum drops for 10-15 min at room temperature (Figure 211). Rinse with 25-30 drops of distilled water from a Pasteur pipette (Figure 212).
- Apply negative stain dropwise (five drops) from a Pasteur pipette (Figure 213). Remove excess with filter paper.
- Grids ready for observation (Figure 214).
- Observe with the electron microscope and read the results.

REFERENCES


Uranyl acetate (1-2 percent solution in distilled water), tenfold and thousandfold dilutions of antiserum for "decorating" and "trapping" virus particles on the EM grid respectively.

FIGURE 206

Extraction and rinsing media: phosphate buffer (PO₄) distilled water (H₂O), and 2.5 percent aqueous nicotine.

FIGURE 205

Basic tools for use with immune electron microscopy From left to right: dental wax bars, Petri dish, carbon-coated electron microscope grids, glass rods and slides, straight-point tweezers, Pasteur pipettes, porcelain mortar.

FIGURE 204

Plant organs commonly used for preparation of extracts: leaves, bark, roots, buds.

FIGURE 207
FIGURE 208
Drops of plant extract, obtained by grinding tissues on a mortar, on which antibody-coated EM grids are being floated for particle "trapping" (upper wax bar). Lower wax bar supports drops of phosphate buffer on which EM grids are being floated for rinsing.

FIGURE 209
Petri dish with a dental wax bar on which EM grids are being floated on drops of a thousandfold dilution of antiserum for antibody coating.

FIGURE 210
Rinsing EM grids with phosphate buffer applied dropwise.

FIGURE 211
EM grids being floated on drops of tenfold diluted antiserum for particle "decoration"
FIGURE 212
Rinsing EM grids with distilled water applied dropwise

FIGURE 213
Staining EM grids with uranyl acetate applied dropwise

FIGURE 214
Petri dish with EM grids ready for observation
Isolation and analysis of CTV dsRNA from citrus bark

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Peel green bark from twigs, use 2 g for each sample. Grind to a fine powder in liquid nitrogen. Transfer to a tube, seal and store at -20°C if it is not possible to proceed immediately. Will keep well for years if frozen.

Add 4 ml of STE buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA pH 6.8), 6 ml of STE buffer-saturated phenol, and 0.6 ml of 10% sodium dodecyl sulfate to bark powder. Shake at room temperature for 30 min.

Centrifuge (8000 g for 15 min), withdraw clear upper layer to a tube and adjust to a final volume of 10 ml with STE buffer. Add 2.1 ml of 95% ethanol and mix (= sample, in 16.5% ethanol). Use room temperature for this and the following steps.

Make a slurry of 1 g of Whatman CF-11 cellulose powder in STE buffered 16.5% ethanol, and dispense into a 10-ml plastic syringe barrel which has been plugged with a disk of Miracloth (or glass wool) and is held in a test-tube rack. Allow column to settle.

Pour sample through column, and discard the eluate into a pan in which rack is standing.

Pour 10 x 5 ml of STE buffer (ethanol-free) through the column.

Discard first 2 ml. Collect and retain the remainder (= dsRNA solution).

Add 2 volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate pH 5.5 to dsRNA solution, store at 4°C for 1 h or overnight, and then centrifuge (8000 g for 30 min).

Resuspend pellet in 1 ml of STE buffer, add 2 volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate pH 5.5 to dsRNA solution, store at 4°C for 1 h or overnight, and then centrifuge (8000 g for 20 min).

Resuspend pellet in 20-100 µl of electrophoresis buffer + glycerol (10%). Use for electrophoresis immediately, or store at -20°C.

Electrophorese 5-20 µl of dsRNA for 3 h at 50-60 mA/gel in electrophoresis buffer (0.004 M Tris, 0.02 M sodium acetate, 1 mM EDTA, pH 7.8), on a 6% polyacrylamide gel cast in a small vertical slab gel apparatus (83 x 63 x 1.5 mm).

Stain electrophoresed gels in ethidium bromide (25-50 ng/ml) in electrophoresis buffer for 15 min, de-stain in water for 1-5 min, photograph on a transilluminator (260 nm) using prolonged exposures. (Polaroid type 57 film, 30 sec to 2 min, f8, red wrattan 23A and yellow wrattan 9 filters.)

Comments. Do up to ten columns (for one electrophoresis run) at a time. Two cycles of chromatography and/or RNase digestion in 1.5 M NaCl and/or DNAse digestion will improve purity but are usually not needed for routine analysis of CTV dsRNA patterns. Always include non-inoculated controls.
A more detailed laboratory manual can be obtained by writing to J.A. Dodds. See also Dodds *et al.*, 1987, *Phytopathol.*, 77: 442-447 and the section on Viroid purification and characterization in this handbook for further details on cellulose chromatography and gel electrophoresis.
Isolation and culture of *Spiroplasma citri*\(^1\)

**ISOLATION OF S. CITRI FROM FRUIT ASEPTICALLY WITHOUT FILTRATION**

*S. citri* can be isolated from seeds or fleshy tissue in small fruits from stubborn suspect trees. The procedure is as follows:

- Wash the fruit in a 1% sodium hypochlorite solution or in soap and water, and dry in the open air or with a sterile towel.
- First flame the equator of the fruit, then cut into the fruit to an approximate depth of 1.25 cm completely around the equator.
- Twist the two halves of the fruit apart.
- Pick out visible aborted seeds with sterile tweezers and place them in a tube of 10 ml of media (use one seed per tube to avoid excessive acid).
- Plug the tubes and incubate at 30-32°C.
- Look for colour change as shown in Figure 215 within 7-14 days.
- Examine a drop from each tube showing a colour change under a dark-field microscope for the presence of spiroplasma. In an actively growing culture the number of small helical organisms is high.

Similar aseptic techniques are possible throughout the year using albedo tissue (preferably from immature fruits) and other phloem-containing tissues such as the columella, provided adequate surface sterilization is possible. Ten to 20 ml of media may be needed to reduce the effect of inhibitors.

**ISOLATION FROM PLANT TISSUES USING FILTRATION**

This is the method of choice for most young leaves, stems and other specimens:

- If the sample is dirty, wash away as much dirt as possible in running water.
- Soak the specimen in 1% sodium hypochlorite for five minutes.
- Rinse in sterile distilled water.
- Place specimen in about 5 ml of medium in a sterile flat dish (such as one-half of a Petri dish) and chop with a sterile razor-blade. This step can also be applied to aborted seed or columella tissue.
- After five minutes or more, filter the medium containing the chopped tissue through a 0.45 µm filter, using slight suction. If penicillin-resistant bacteria are present, finer filters of 0.2 or 0.22 µm may be needed.
- Transfer aseptically 1 ml of the filtrate to about 10 ml of medium; repeat to make duplicate tubes. If filtrate is limited, use 0.3-3 ml of medium. It is best to make more than one dilution because of inhibitors.
- Incubate tubes at 30-32°C until a colour change is noted. If the organism is present, colour will change in seven to 14 days, and tubes should be held for three to four weeks. Always have known positive controls for comparison.
- Whenever colour change is noted, examine a drop from each tube under a dark-field microscope for the presence of spiroplasmas.
- Transfer cultures promptly to avoid deterioration and store or lyophilize as desired.

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\(^1\)Source: E.C. Calavan (1980).
The medium is a modification of that of Saglio et al. (1971) and Fudl-Allah, Calavan and Igwegbe (1972). The yeast extract is omitted (Igwegbe, 1978). The foetal bovine serum may give better results than horse serum, and the serum should be mycoplasma screened by the manufacturer.

Distilled water 780 ml
PPLO broth 21 g
Fructose 1 g
Glucose 1 g
Sucrose 10 g
Sorbitol 50 g
Tryptone 1 g
Phenol red (1 mg/ml) 10 ml
Foetal bovine (or horse) serum 100 ml
Penicillin G (25mg/ml) 25 ml

The pH of this medium is about 7.6 and the colour a pale red. The presence of S. citri or any other contaminating organism will turn the colour to amber or yellow (Figure 215). Other special-medium formulae and modifications are given by Calavan (1980) and Lee and Davis (1984).

REFERENCES


FIGURE 215
Diagnosis for stubborn disease in culture medium. The presence of Spiroplasma citri causes a pH change and turns the phenol red indicator dye from reddish-orange (right) to amber-yellow (left). Confirmation should be made by taking a drop of liquid from the tube on the left, and observing it under a dark-field microscope.
Detection of citrus tristeza virus inclusion bodies using azure A staining and in situ immunofluorescence

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INTRODUCTION
Cytopathic intracellular structures, referred to as inclusion bodies, are produced in the phloem and associated cells of citrus trees infected with citrus tristeza virus (CTV) (Schneider, 1959). These structures are diagnostic of CTV infection and can be observed in infected tissues with a light microscope after staining with azure A (a thiazin dye) or treatment with a fluorescent labelled antibody specific for CTV (in situ immunofluorescence). The following will describe the use of both techniques for the detection of CTV inclusion bodies.

AZURE A STAINING
The azure A technique, described by Christie and Edwardson (1977) for the detection of inclusion bodies produced by plant viruses, has been used to diagnose virus infections and is one of 49 criteria used for classifying groups of plant viruses. A procedure for detecting inclusions produced by CTV in citrus tissues was described by Garnsey et al. (1980). The method described here is a modification of that procedure.

Tissue selection
Tissue should be selected from a young flush of growth that is fully expanded, but not hardened. Petiole, midvein, or young stem tissue (bark) should be excised using a single-edged razor-blade. The young stem bark tissue should be peeled away from the xylem. This is not necessary for the petiole or midvein tissues. Petiole tissue, especially around the abscission zone of the leaf, is preferred since it provides a marker (the abscission zone) for reference. Previous work (Brlnsky, Lee and Garnsey, 1988) has shown that preferred hosts for inclusion body formation include Mexican lime, Citrus hystrix, C. excelsa and sweet orange.

Sectioning
Both types of tissue must be thin-sectioned prior to staining. Freehand sections can be obtained by placing the tissue into a slit made into a piece of expanded polystyrene or a pith-wood stick and cutting sections with a razor-blade at a 45° angle to the long axis of the tissue. Thin sections are preferred since thick sections are difficult to de-stain and view.

Cryostat (frozen) sections are made by freezing a piece of petiole or young stem tissue on a cutting block or stub in either a drop of distilled water or in an embedding compound such as Tissue Tek or OCT compound and putting this into a cryostat or freezing microtome to freeze. After freezing, thin sections of tissue are cut either longitudinally or transversely. Transverse
sections of petioles or midveins are easier to handle and very thin sections are easily obtained. Sections 20-30 µm thick can usually be obtained with a cryostat. Using either a pair of fine-tipped forceps or a small fine-tipped brush, the sections are put into a small glass staining dish containing distilled water or phosphate buffered saline (PBS)(0.01 M phosphate buffer+0.15 M NaCl). Prior to staining, the water or PBS is removed.

**Staining**

A staining solution is made just prior to use by adding one drop of a solution of 0.05 percent azure A in ethylene glycol monomethyl ether (2-methoxyethylacetate) (prepared previously) to 9 drops of 0.2 M Na₂HPO₄. This staining solution is added to the sections, and the dish with sections is incubated for five to 10 minutes at room temperature. After staining, the solution is removed with a Pasteur pipette and the sections in the dish are de-stained by adding 95 percent ethanol. The sections are de-stained for five minutes and then the ethanol is removed and replaced with ethylene glycol monoethyl ether acetate (2-methoxyethyl acetate). After a few minutes in this solution the sections are removed using thin forceps, and mounted in Euparal on a glass slide and covered with a cover slip.

**Viewing**

The mounted sections are viewed in a compound light microscope using transmitted light. The area of the phloem is observed for the presence or absence within the cells of purple-stained bodies (Figure 216).

**IN SITU IMMUNOFLUORESCENCE**

Immunofluorescence has previously been used to detect the presence of plant viruses and their inclusion bodies (Nagaraj and Black, 1961; Brlansky et al., 1982). This technique uses an antibody to the virus in question coupled either directly with a molecule that fluoresces under UV light or indirectly with another antibody that is labelled with the fluorescent molecule. Brlansky, Lee and Garnsey (1988) described a technique for the detection of citrus tristeza inclusion bodies in sections of tissues infected with CTV. The following is a description of that technique.

**Tissue selection**

The selection of tissue is the same as that described above for azure A staining.

**Sectioning**

Sectioning of tissues for this procedure is the same as described above for azure A staining. Thin sections of tissue are preferred since the antibody specific for CTV must penetrate the cells.

**Antiserum**

The antibody specific for CTV must have the IgG (immunoglobulin G) portion separated and used for this technique. The method preferred is the protein A-Sepharose affinity column procedure of Miller and Stone (1978). The concentration of IgG is estimated spectrophotometrically using E 0.1 percent 1 cm = 1.4 (at 280 nm). The anti-CTV IgG and the normal rabbit serum are conjugated to either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), according to the procedure of Blakeslee and Baines (1976) and Brlansky et al. (1982).

**Treatment of tissue sections**

Tissue sections are removed from PBS and immediately placed in drops of the primary anti-CTV IgG diluted 1:20 with PBS. After an incubation of 1 h at room temperature or 30 minutes at 37°C, the sections are washed by flooding the dish containing the sections with
PBS. After a 10- to 20-minute wash, the sections are mounted on microscope slides in “Aqua Mount” and covered with a cover slip.

**Viewing**

The sections are observed with a fluorescence microscope in the 560-590nm wavelength range for TRITC fluorochrome or in the 380-420 nm wavelength range for FITC. Positive samples should have large fluorescing structures in the phloem while controls or negative samples should have no fluorescing structures associated with the phloem (Figure 217). Fluorescing inclusions may be photographed using Kodak Technical Pan 2415 black-and-white film. For colour slides, Ektachrome colour-slide film, ASA 400, is recommended.

** SOURCES OF SUPPLY **

Azure A – (A37730) Pfaltz and Bauer, 126-02 Northern Blvd, Flushing, NY 11368, United States of America

Euparal – Carolina Biological Supply, Burlington, NC27215, United States of America

Tissue Tek II – Fisher Scientific, 7 11 Forbes Ave., Pittsburgh, PA 15219, United States of America

Forceps No. 5, stainless steel – any scientific equipment supply company

Aqua Mount Mounting Medium – Lerner Laboratories, New Haven, CT 06513, United States of America

Tetramethylrhodamine isothiocyanate (BBL brand) – Fisher Scientific

Fluorocein isothiocyanate – Fisher Scientific

Conjugated antisera-Organon Teknika-Cappel, One Technology Court, Malvern, PA 19355, United States of America.

**REFERENCES**


FIGURE 216
Azure A-stained citrus tristeza inclusion bodies in citrus tissue as viewed through a compound light microscope. The purple-stained inclusion bodies are clearly visible in the area of the phloem (250X)

FIGURE 217
Citrus tristeza inclusion bodies in the phloem cells of citrus tissue. The tissue was treated in anti-CTV IgG conjugated with a fluorescein microscope. Inclusion bodies fluoresce and are highly visible (250X)
The importance of the plant laboratory cannot be overemphasized. The ability to grow indicator plants that can be effectively used to detect the presence of viruses is fundamental to the success of a clean stock programme. The availability of a laboratory and basic laboratory equipment for certain diagnostic procedures is also important. When a virus-detection laboratory is being established, the problem soon arises as to what equipment is needed, where it can be bought, and the cost. In this section, the basic laboratory equipment and the specialized equipment needed for three commonly used diagnostic procedures (ELISA, electrophoresis and culturing) will be given, with the current price range in the United States of America.\footnote{Specific and more detailed information on equipment requirements are given under many of the procedures in Part III of this handbook.}

Much of the following equipment may be obtained from local suppliers. As trade names may vary, the basic requirements are given rather than specific brands and model numbers. Each year the worldwide \textit{Laboratory buyer’s guide} is published for purchasers of laboratory equipment, chemicals and reagents. It includes product listings, a manufacturers’directory, and a list of laboratory dealers around the world. The \textit{Laboratory buyer’s guide} may be obtained by sending US$25 to International Scientific Communications Inc., 30 Controls Dr., PO Box 870, Shelton, CT 06484-0870, United States of America (Telephone (203)926-9300, Telex 964292, FAX (203)926-9310).

In addition to the more specialized equipment needed for some of the diagnostic procedures, there are a few basic requirements for any laboratory.

\textbf{Size}

A minimum area of about 55 m$^2$ is needed for a diagnostic laboratory. Thought needs to be given to designing effective work areas so that equipment and items needed for a particular procedure are conveniently located. With a proper arrangement of work areas, up to four persons can work effectively in a laboratory of this size. There should be a fume hood available, plus a sink for dishwashing, and cabinets for storage of glassware, equipment and reagents.

\textbf{Electrical power}

There needs to be a stable source of electrical power. If the power source is subject to interruptions, or may be not working for long periods, a generator should be provided to power important equipment and to permit work to continue. Most equipment manufactured in the United States of America for use here is 60 Hz (cycles per second). It is important that power...
sources and equipment be able to handle the cycle sequence in the country of use.

**Air conditioning**
Electronic equipment and instruments depending upon optical filters and diffraction gratings for operation, such as spectrophotometers and ELISA plate readers, are susceptible to high temperatures and high humidity. Fungi can ruin filters and gratings essential to the operation of spectrophotometers, and high humidity will cause corrosion of even solid-state electronic circuitry.

**Deionized water supply**
Costs for this vary depending on local water quality; usually a mixed bed rosin filter with a resistance meter is adequate. Culturing of microorganisms requires very high-quality water and a still may be needed.

**Glassware**
Although a single piece of glassware represents a small part of the total cost of equipping a laboratory, the availability of sufficient glassware is fundamental for effective operation of a diagnostic laboratory. A complete set of volumetric flasks and graduated cylinders needs to be available, as well as flasks, beakers, Petri dishes, test-tubes, centrifuge tubes, test-tube racks, ice containers, spatulas, and containers for buffers and solutions. If 500 samples are to be run for ELISA, there need to be 500 tubes with racks to hold the samples. Allow US$2 000 to $3 000 for glassware to equip a diagnostic laboratory.

**pH meter**
It should be capable of reading to 0.1 pH unit, with reference standards so that the instrument can be calibrated and tested. Select an electrode that will measure pH of Tris buffers. Prices start at about $100 for a handheld unit and rise depending on the features selected.

**Refrigerator**
Needed for storage of reagents, chemicals, and seed at 4°C, the refrigerator should be large enough to hold racks of ELISA tubes until the ELISA test is complete. A refrigerator with sliding glass doors is desirable. If large enough, it can be used to run native gels for PAGE with the power supply located outside. Similarly, small centrifuges can be run inside a larger refrigerator and observed through the glass doors.

**Freezer**
A freezer is needed for storage of reagents, chemicals, and samples at -2°C and to produce ice needed for use in ice-baths. Select a freezer which does not have an automatic defrost cycle: the heating cycles of an automatic defrost unit will cause a more rapid breakdown of reagents and nucleic acid preparations stored in the unit.

**Balance**
Its sensitivity should be at least 0.01 g. A top-loading electronic balance with dual range sensitivity of 0.01 to 120 g and 0.1 to 1 200 g ranges is ideal. Prices start at about $1400.

**Means of sterilizing equipment**
An autoclave is useful for sterilizing equipment, glassware and reagents used for many diagnostic procedures and it is absolutely essential for culturing. A large pressure-cooker will serve this purpose and is relatively inexpensive, while a small automated autoclave (prices begin at about $3 000) is convenient if much culturing is to be done. For sterilization of glassware, a glassware oven will be suitable.

**Centrifuge**
Ideally it should be a refrigerated model with a
rotor capable of holding 50-ml centrifuge tubes and adapters to accommodate smaller volume tubes. Prices of such a centrifuge start at about $10,000. At the simplest, a clinical centrifuge with interchangeable rotors to accommodate different sizes of centrifuge tubes will suffice in many procedures (at a cost beginning at about $1,000). If the centrifuge is small enough, it can be operated in the bottom of a refrigerator for cooling. Often changes must be made to established protocols because of limitations in the ability to carry out the centrifugation steps. In some instances the sample must be divided into two tubes because the total volume specified will not fit into one tube, or the centrifuge time must be increased to account for the reduced g force as specified in the protocol.

**Magnetic stirrer and hot plate**
They can be purchased as separate units at costs starting at about $125 each or as a combined unit at costs starting at about $300.

**SPECIALIZED EQUIPMENT NEEDED FOR ELISA**
Antisera and/or conjugates needed for ELISA are commonly available from type-culture collections, commercial sources or fellow research scientists. A low-speed centrifuge, UV-visible spectrophotometer and simple chromatography are needed if IgG and conjugates are to be prepared. Other equipment needed for ELISA is:

**Repeating pipette**
A repeating pipette which allows multiple pipettings with good accuracy is essential. At the minimum, a fixed volume (200 µl) pipette is required (cost about $125-250). It is highly desirable to have a set of three adjustable pipettes (0-20 µl, 20-200 µl, and 200-1,000 µl) at a cost of about $125-250 each. If much ELISA is to be performed, a multichannel adjustable (50-250 µl) pipette should be considered at costs beginning at about $500. Microcapillary tubes or Drummond pipettes can be used to measure small volumes of IgG and conjugate if the 0-20 µl pipette is not available.

**Grinding equipment**
Although samples may be homogenized with pestle and mortar, a mechanical device is desirable if large numbers of samples are to be assayed. Dispersion homogenizers with a generator shaft of 15 to 25 mm diameter are commonly used, e.g. Polytron, Tissumizer, VirTis, or Tissu-Tearor in a price range of $700-2,500. Rollers and stomacher devices work for some applications.

**Evaluation of results**
ELISA results can be estimated visually, but it is difficult to determine weak reactions, especially when there is a background. Photometric measurements must be made to obtain quantitative data. Aliquots of the reaction can be diluted in water and the absorbance read in a regular spectrophotometer. Manually operated ELISA plate readers, available from about $4,000 upwards, can quickly and accurately read an ELISA plate. If ELISA is to be performed on a sizeable scale, consideration should be given to purchasing an automated ELISA plate reader with an RS 232 port linked to a personal computer. Costs for such a system begin at about $20,000.

**SPECIALIZED EQUIPMENT NEEDED FOR ELECTROPHORESIS**
**Electrophoresis apparatus**
Electrophoresis is often used to diagnose viroids or for the analysis of dsRNAs to detect the presence of viruses. Electrophoresis for these procedures is most commonly performed on vertical slab gels although tube gel
electrophoresis can also be used. There are currently many models of both vertical slab and to be gel apparatus sold commercially, with prices starting at about $225 for a small apparatus to $1 500 for larger units. When the apparatus is being selected, its potential use should be taken into account. The same electrophoresis apparatus can also be used for SDS polyacrylamide gel electrophoresis for protein analyses, also non-denaturing gels for isozyme analysis. The larger units will cost more initially, will need more reagents because of the larger volume and will require longer run times, but they offer better resolution. The small units offer speed and lower cost, but often lack a high degree of resolution. For most diagnostic applications, the small unit is satisfactory. An alternative to purchasing a commercial unit is to custom-make a unit from Perspex. The most expensive component is the platinum wire needed for the electrode. This can be bought from an electron microscopy supply catalogue or an electronics shop.

**Power supply**
A power supply is essential for electrophoresis. For versatility, the power supply should be capable of running at constant volts (0-500 V range or greater) or at constant current (0-400 mA range or greater). Cost begins at about $900 and increases depending on the model and features desired. Again, be aware of the cycles per second (Hz) on purchased equipment and be sure it will be compatible with the cycles per second in your country.

**Vacuum pump and dessicator**
These are often used to de-gas acrylamide solutions before pouring gels. In addition, nucleic acid preparations are usually dried in vacuum after being collected as ethanol-precipitated pallets before being used for electrophoresis. A dessicator costs from about $50 depending on size. Hand vacuum pumps are available at costs beginning at about $30; water aspirators can also be used. An electric vacuum pump, which offers more versatility, costs from about $250 upwards.

**Visualization of samples on gels**
The usual method used to detect nucleic acids (such as viroids and/or dsRNAs) on gels after electrophoresis is by staining with ethidium bromide, then viewing over an ultraviolet (UV) transilluminator. The nucleic acids fluoresce, and the resultant diagnostic bands on the gel can be visualized and photographed. Visualization by this method allows subsequent manipulations of the nucleic acids, such as infectivity assays, electrophoresis on denaturing gels, and preparation of probes (Part III). A UV transilluminator ‘with 302 nm wavelength is recommended for use with ethidium bromide staining as handheld short-wave UV lights do not have enough light intensity to visualize any but the strongest of gel bands. Prices for a UV transilluminator start at about $1 100 and increase in price as the filter size increases.

An alternative to viewing nucleic acids by fluorescence over UV light is to silver-stain the gels (Part III). Silver-staining is as sensitive or more sensitive than ethidium bromide staining and eliminates the need for a UV transilluminator. However, silver-staining inactivates the nucleic acid and immobilizes it in the gel, which does not permit subsequent manipulation of the nucleic acid.

Documentation of the gel requires a means of photographing the gel. AUV-1 filter between the camera and the UV light source is commonly used. Polaroid cameras are routinely used. Instant cameras, with a hood which fits over the gel on the UV transilluminator and a fixed focal length, are now available with prices beginning at about $400. More elaborate Polaroid set-ups begin at about $4 000. To minimize investment and film
costs, a SLR 35-mm camera with macro-focus lens and auto exposure can be used with black-and-white film such as Kodak Contrast Process Pan or equivalent. After the gel has been photographed, the film can be processed immediately before moving the gel (usually 15-20 min of darkroom time) to verify that the gel bands have photographed well and that the focus is satisfactory.

**SPECIALIZED EQUIPMENT FOR CULTURING**
Culturing is often used to verify the presence of citrus stubborn and other disorders caused by harmful procaryotes such as citrus greening.

**Autoclave**
An autoclave is essential for culture work. This can be as simple as a large pressure-cooker or, if a lot of culture work is to be done, an automated autoclave may justify the additional cost.

**Transfer hood**
It is desirable to have a transfer hood which is equipped with filtered air and a flame to sterilize transfer loops. In more arid climates, an open work space in a room with no air movement can be lined with wet paper towels, and the surface sterilized with 70 percent alcohol and used for culture work. This will increase the number of contaminations occurring, and will not be satisfactory at all in hot, humid climates.

**Incubators**
These need to be available. Shaking capability can be easily obtained by placing small shakers in incubator cabinets. Costs of small shakers start at about $300.

**Microscopes**
A light microscope with phase contrast and good optics is needed to verify the presence of spiroplasmas and harmful procaryotes from culture. Costs for a suitable microscope begin at about $2000 and increase as quality increases. A stereoscope is also needed as an aid to culture work and also for general use and shoot-tip grafting. Costs for a stereoscope begin at about $1200.
**Glossary**

**Acorn-shaped fruit**
Fruit showing a sharp difference in size between the top and bottom portions thus resembling an acorn. In cross-section, the albedo is thicker at the stem end and thinner at the stylar end. Fruit on stubborn- or exocortis-infected trees will sometimes show this acorn-shaped appearance (see Figure 39).

**Acquisition feeding**
The feeding period during which the insect ingests sap containing the pathogen.

**Alkaline phosphatase**
An enzyme which hydrolyses certain phosphate-containing compounds under alkaline conditions; commonly obtained from calf intestine mucosa.

**Amorphous plugs**
Microscopic inclusions found in the trunk xylem tissue of blight-affected trees. These inclusions are uniformly dense and without structure (see Figure 33).

**Antibody**
A protein formed in blood serum in response to stimulation by an antigen. Antibodies are specific for their respective antigens, and antigens and antibodies are mutually attracted.

**Antibody-antigen**
The reaction or attraction formed when reaction antigens meet their corresponding antibodies or vice versa. This strong attraction is the basis of all immunodetection systems.

**Antigen**
A substance, often a virus or bacteria, which stimulates production of antibodies in an animal. Specifically, it is the corresponding molecule to the antibody in a serological test.

**Autoradiography**
The technique or process of making a picture revealing the presence of radioactive material, the film being laid directly on the object to be tested. This is frequently used for detection of radioactivity following hybridization, by exposing filter paper to sensitive X-ray film.

**Bark graft**
One of a number of types of grafts used in the transmission of graft-transmissible pathogens. A piece of bark is cut from the host scion or rootstock and grafted to an indicator plant (see Figures 135-137).

**Biotin**
One of the B-vitamin complex. It is widely distributed in plant and animal tissue, is water soluble and binds strongly to a glycoprotein named avidin.

Biotin derivatives of deoxyribonucleotides are incorporated into probe DNA by nick translation (see Part III). After hybridization the biotin can then be detected using streptavidinfluorescein complexes. The streptavidin binds to the biotin.

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*Note:* Some of the technical terms defined are from Oliver, S.G. & Ward, J.M. *Dictionary of genetic engineering* Cambridge University Press. Appreciation is also expressed to M. Bar-Joseph, J.A. Dodds, S.M. Garnsey, J.V. Leary and J.S. Semancik for their assistance in glossary definitions, and acknowledgement is made to J.A. Dodds for reviewing and editing this glossary.
by one of the strongest known biological interactions.

The enzyme (usually peroxidase or alkaline phosphatase) is then reacted with its substrate which gives a coloured product; fluorescein is detected by fluorescence under light of certain wavelength.

**Biotinylated**
A compound to which a small vitamin, biotin, has been attached. Antibodies, enzymes and nucleic acids can be labelled with biotin

**Biotinylated enzyme**
An enzyme coupled chemically to biotin

**Biotinylated probe**
A DNA probe in which certain bases were modified by chemical coupling of biotin

**Blind bud**
Not a true bud, but a section cut from that portion of the stem between buds containing no “eye” and used primarily for graft inoculations (see Eye, and Figure 127)

**Blister inoculum**
Inoculum tissue for graft transmission of psorosis-B used primarily in cross-protection studies. The tissue contains the blisters associated with the severe "B" form of psorosis (see Figure 79a)

**Blot**
As a verb, this means to transfer DNA, RNA or protein to an immobilizing matrix such as DMB-paper, nitrocellulose or nylon membranes. As a noun, it usually refers to the autoradiograph produced during the Southern or Northern blotting procedure

**BRL Hybri-dot**
A commercially available kit for applying small volumes of extracts to a membrane for testing the presence of viruses or viroids by hybridization

**Bud-graft inoculation**
A bud, blind bud or chip bud cut from a stem of the plant or tree to be indexed and grafted to an indicator plant or tree

**Bud-union crease**
A line, ridge or fold, usually discoloured as brown, yellowish-brown or reddish-brown, and formed at the bud-union. It is readily observed when the outer bark is removed (see Figures 56 and 102-104). Some bud-union creases are caused by pathogens and others by incompatibility of rootstocks and scions

**Canopy**
As in a tree canopy. The outline or total area of a tree, indicating its size

**cDNA**
Complementary DNA. The DNA complement of an RNA sequence. It is synthesized by the enzyme RNA-primed DNA polymerase or reverse transcriptase. The single-stranded DNA product of this enzyme (the reverse transcript) may be converted into the double-stranded form by DNA-primed DNA polymerase, and inserted into a suitable vector to make a cDNA clone. cDNA cloning is commonly used to achieve the expression of mammalian genes in bacteria or yeast

**cDNA probe**
A radioactive specific DNA sequence used to detect complementary sequences of RNA or DNA (see cDNA and Probe)
**Certification programme**
A programme developed by a country, state, university or research centre for ensuring that selected budwood distributed to the growers is free of graft-transmissible pathogens and the fruit true-to-type. These pathogen-free certified trees are usually registered, and budwood issued from these mother or foundation-block trees can be used to produce additional buds in an increase block for the development of certified trees.

**CF-11**
A fibrous, graded, cellulose powder sold by the Whatman Company.

**Cheesy bark**
Enlarged, swollen and softened bark tissue which, when cut into with a knife, resembles cheese. This type of cheesy bark is usually associated with the presence of severe CTV.

**Chip bud**
A piece of bark tissue used for graft inoculation. It is used when the bark of receptor indicator plants does not slip or open up to accept a bud or blind bud (see description in Part II under Grafting techniques, and also Figure 127).

**Chromatography**
The separation of mixtures of chemicals, compounds, proteins, macro-molecules etc. into their constituents or components by preferential adsorption by a solid such as a column of cellulose, or by filter paper or by gel.

**Clonal**
A budline derived from a single parent source by propagation from that source.

**Columella**
The internal, central, soft white column-like axis of the citrus fruit.

**Complementary**
See cDNA. A nucleic acid sequence is said to be complementary to another if it is able to form a perfect hydrogen-bonded duplex with it, according to the Watson-Crick rules of base pairing. A viral genomic ssRNA is complementary to “negative sense” ssRNA from which it is transcribed.

**Conjugated molecule**
The temporary or permanent union, fusion or binding of two substances. When used in ELISA, it is the combining of antibody and enzyme proteins to form an enzyme-labelled antibody conjugate. The enzyme can then be detected colorimetrically, and the colour produced will give a fairly precise picture of the amount of virus present.

**DEAE cellulose column**
A plastic or glass tube open at the top and fitted with a stopcock on the bottom. It contains diethylaminoethyl cellulose.

**Denature**
So to modify (a protein) by heat, acid, or alkali that it retains its primary structure but no longer has all its original properties.

**Deproteination**
To remove and separate proteins from other macromolecules from samples to be tested by hybridization. This is normally achieved by phenol extraction or by treating with a protein-digesting enzyme.

**Dialysis**
A procedure using a membrane to separate various components in solution in accordance with their ability to pass through the membrane.
Dicing
Cutting of tissue into small segments using a sharp knife or razor-blade. In the ELISA technique, leaf or bark segments are diced or cut up prior to grinding.

DNA
Deoxyribonucleic acid. Any of a class of nucleic acids that contain deoxyribose, found chiefly in the nucleus of cells, and that functions in the transference of genetic characteristics and in the synthesis of protein.

DNA probe
A probe for detection of specific nucleic acid segments (see Probe).

Dot-blot
A procedure which is used to determine the presence and concentration of a particular RNA or DNA species. Different concentrations of the non-radioactive nucleic acids are denatured and applied as a dot to nitrocellulose paper or other support matrix. This is then hybridized with the radioactive complementary probe under study. After autoradiography, the intensities of the radioactive images formed are quantified and compared to a series to determine the concentration of the non-radioactive molecule.

dPAGE
Denaturing polyacrylamide gel electrophoresis. Electrophoresis in a gel formed from polyacrylamide in the presence of a chemical agent such as urea (8M) or heat. It functions to minimize the effects of secondary and tertiary structure of the molecule on electrophoretic mobility.

Electra-blotting
The electrophoretic transfer of macromolecules (DNA, RNA or protein) from a gel in which they have been separated, to a support matrix such as a nitrocellulose or a charged nylon sheet. An alternative to the capillary transfer usually used in techniques such as Southern and Northern blotting.

Electra-blot membrane
A solid charged medium on to which a molecule is fixed as a result of electrophoresis from a source medium. This can be nitrocellulose or a charged nylon matrix.

Electro-elution
Removal of adsorbed material from an adsorbent by use of an electric field; or, recovery of a charged molecular species by electrophoretic migration from a source medium such as a polyacrylamide gel to a liquid medium in which concentration of the species can be accomplished.

Electrophoretic techniques
Techniques which separate components suspended in a fluid media or gel by the influence of an electric field.

Electro-transfer
The movement of a charged molecule from one medium to a second by migration in an electric field.

ELISA
Enzyme linked immunosorbent assay. Two antibody preparations are commonly used in ELISA. The primary antibody binds the antigen which is itself bound by the second antibody. The second antibody is linked to any enzyme whose activity is easily monitored, i.e. by colour change. The extent of enzymatic reaction is then a quantitative indication of the amount of trapped by the primary antibody.
**Elution**
Removal by dissolving, such as the removal of adsorbed material from an adsorbent by solvents

**Enation**
A small protuberance or growth found on a leaf, induced by insect bites or caused by the vein-enation virus. Very distinct enations can be seen on the veins of leaves of Mexican lime infected with vein-enation virus (see Figure 98)

**Enzyme-labelled antibody**
See Conjugated molecule and ELISA

**Epinasty**
An increase or decrease in growth of the upper or lower leaf surface or vein which causes the leaf to bend downward. Mild leaf epinasty is shown in an exocortis-infected citron leaf in Figure 42d, and severe epinasty in leaves of citron in Figure 41a

**Ethidium bromide**
An intercalating agent which allows the ready detection of double-stranded nucleic acid molecules in agarose gels. The nucleic acid/ethidium bromide complex fluoresces brightly when exposed to ultraviolet(W) light (ethidium bromide is highly carcinogenic)

**Eye (of a bud)**
The protruding meristematic portion of a bud which later enlarges and grows into a young shoot or flower

**Flecking**
As in leaf flecking. Usually a lighter translucent spot or small patch on leaves. Flecking in young or mature leaves is symptomatic for a number of graft-transmissible pathogens but can also be induced by environmental conditions or by the inherent genetic make-up of the plant

**Flush**
The new, young and fresh growth of shoots and leaves

**Foundation tree**
In a certification programme, the foundation tree is the primary tree derived from budwood which has been specially selected, shoot-tip grafted and/or heat-treated, and which has been indexed and certified as virus-free and also true-to-typo. It will become the primary source tree for all future progeny trees. A foundation tree can be synonymous with a mother tree or mother-block tree

**Gametic**
A gametic seedling is one which was formed by sexual union and derived from seed containing the chromosomes of both parents. This is in contrast to the nucellar seedling containing only the chromosomes from the mother tree (see Nucellar)

**Gel**
The inert matrix used for electrophoretic separation of nucleic acids or proteins. Agarose gels are used for separation of DNA; agarose or polyacrylamide for RNA, and polyacrylamide for proteins

**Grocilicute-like**
In reference to the greening organism, gracilicute is a division in the order of bacteria comprising organisms having a Gram-negative, membranous cell wall containing peptidoglycan

**Graft-transmission**
The transmission of a virus or other pathogen(s) by grafting tissue from the suspect host to an indicator plant
**Gravity infusion**
Used for testing the water uptake within a tree for the diagnosis of blight. It is the flow of water from a burette or other container suspended from a branch. The water is permitted to flow into the tree by gravity via a rubber or plastic tube (see Figure 31).

**Hybridization**
The formation of stable duplexes between complementary nucleotide sequences via Watson-Crick base pairing. The efficiency of hybridization is a test of sequence similarity. DNA-DNA, DNA-RNA, RNA-RNA hybrids may be formed. An alternate use of the word comes from classical genetics, and particularly plant breeding. Here hybridization means the formation of a novel diploid organism either by normal sexual processes or protoplast fusion.

**Immunoassay**
An assay system which detects proteins using an antibody specific to that protein. A positive result is seen as a precipitate of an antibody-protein complex. The antibody can be linked to a radioactive atom or to an enzyme which catalyses an easily monitored reaction (see ELISA).

**Immunoblotting**
A procedure whereby either the antigen or antibody molecules are bound to a protein-binding substrate, such as cellulose nitrate, and then exposed to the complementary antigen or antibody. The antigen-antibody complex which forms on the membrane is detected by an appropriately labelled antibody.

**Immunodiffusion**
A procedure where antibody and/or antigen molecules are allowed to migrate through an inert medium. A visible precipitate forms at the zone where related antigen and antibody molecules meet in a suitable concentration and react.

**Immunofluorescence**
The result of detection of antigens, often within tissues, by use of an antibody to which a fluorescent material is attached.

**Immunoglobulin**
A blood serum protein which functions as an antibody, commonly, a gamma globulin.

**Immunosorbents**
A material which can adsorb serologically active molecules (antigens or antibodies). Cellulose nitrate and some plastics, such as certain polystyrenes, are good immunosorbents. Adsorbed molecules typically retain serological functions.

**Inclusion bodies**
Cytopathic intracellular structures referred to as inclusion bodies are found in virus-infected plants. They contain virus particles, other proteins or structures specific to the virus and/or formed as a result of virus infection.

**Indexing**
Any test which will consistently confirm the presence (or absence) of a transmissible pathogen, or identify a disease. The index test should be specific for the pathogen or disease.

**Indicator plant**
A plant used to test or index for the presence of a transmissible pathogen. The inoculated indicator plant will usually show very specific symptoms, thus permitting the diagnosis of a particular disease.
**Infection feeding**
In contrast to acquisition feeding where the pathogen is ingested by the insect, infection feeding is secondary or the follow-up feeding where the pathogen is injected into the host plant by the insect.

**Inoculation**
The process of infecting an indicator plant, usually by graft, mechanical or vector transmission.

**Inoculum tissue**
Tissue which contains the transmissible pathogen or pathogens.

**Insipid (taste)**
A dull, flat, savourless taste when fruit of stubborn-infected trees are tasted. This insipid taste is helpful in diagnosing possible stubborn infection when associated with other diagnostic symptoms.

**Intermediate antibody**
An antibody used in the second step of an indirect ELISA assay. The intermediate antibody reacts to the antigen bound to the plate but is not labelled. The intermediate antibody is detected by using another antibody which is labelled and is specific only for the intermediate antibody.

**Interveinal clearing (or flecking)**
The development of a clearing, flecking or lighter colour between leaf veins is usually associated with the presence of the concave gum or psorosis pathogen (see Flecking and Figure 83b).

**Inverse stem-pitting**
A type of pitting usually associated with citrus tristeza virus reaction of sweet orange, mandarin or grapefruit scions on sour orange rootstock. When a small segment of the sour orange bark is removed just below the bud-union, it will show numerous small pits on the inner surface of the bark, with corresponding needle-like pegs on the opposite wood surface of the trunk.

**Invisible pathogen**
This refers to graft-transmissible pathogens which are present in citrus trees but which induce little or no symptoms in these trees. However, when budwood from these symptomless carriers is propagated or inoculated into a susceptible citrus species, symptoms can occur on the susceptible host (see Symptomless carrier).

**Leaf-disc graft**
A graft using round discs cut from leaves with a paper punch. The discs are matched to holes punched into leaves of an indicator plant and secured using adhesive tape (see Figures 132-134).

**Leaf-piece graft**
A graft using as inoculum a small section cut from a young leaf of the test plant to be indexed. The leaf piece is then inserted into the stem of a receptor indicator plant in the same manner as a bud-graft and secured with wrapping tape.

**Linearization**
The conversion of a nucleic acid that is normally circular into a linear form of the molecule. This is done by cutting the circular form at a single site.

**Loading**
As in loading ELISA plates. In ELISA, it is the process of adding a given amount of sample, buffer or any substance to the wells of an ELISA plate or gel apparatus.

**Mechanical transmission**
Transfer or transmission of graft-transmissible
pathogens by means other than grafting and not involving vectors. This can be done by knife cut, razor slash, hand or cotton rubbing of sap on leaves using carborundum powder, or by any other non-grafting method.

**Molecular hybridization**
See Hybridization

**Molecular probe**
see Probe

**Mollicutes**
In reference to mycoplasma-like organisms. One of the four divisions of the kingdom Procaryotae characterised by having no cell wall or peptidoglycan

**Monoclonal antibodies**
An antibody preparation which contains only a single type of antibody molecule. Monoclonal antibodies are produced naturally by myeloma cells. A myeloma is a tumour of the immune system. A clone of cells producing any single antibody may be prepared by fusing normal lymphocyte cells with myeloma cells to produce a hybridoma

**Mother trees or mother-block trees**
Similar or synonymous with foundation or foundation-block trees (see Foundation tree)

**Negative stain**
An electron-dense solution used to provide contrast around virus particles viewed on a transmission electron microscope.

**Nick translation**
A procedure to insert radioactive or other tagged bases in a DNA probe. It is a process whereby damaged dsDNA molecules are repaired with nucleotides, some of which are radioactive. It is a good way to repair a probe

**Nitrocel ludose membrane**
(CEL lulose nitrate) A nitrated derivative of cellulose which is made into membrane filters of defined porosity, e.g. 0.45 µm, 0.22 µm. These filters have a variety of uses in molecular biology, particularly in nucleic acid hybridization experiments. In the Southern and Northern blotting procedures, DNA and RNA, respectively, are transferred from an agarose gel to a nitrocellulose filter. Some centrifuge tubes are made of nitrocellulose; they are readily punctured with a hypodermic needle, and are frequently used for sucrose gradient

**Northern blot, Northern transfer**
A procedure analogus to Southern transfer but, in this case, RNA not DNA is transferred or “blotted” from a gel to a suitable binding matrix such as a nitrocellulose sheet. Single-stranded RNA is separated according to size by electrophoresis through an agarose or polyacrylamide gel; the RNA is then blotted directly on to the support matrix with no denaturation. RNA fixed to the supporting matrix can then be hybridised with a radioactive single-stranded DNA or RNA probe

**Nucellar (seedling)**
Referring to citrus, it is the seedling formed from the nucellus tissue surrounding the embryo. Nucellar seedlings resemble the parent plant and are quite uniform. However, a nucellar seedling or tree derived from a nucellar seedling is not identical to the parent tree, and may have many characteristics which are different. The nucellar process was used primarily to bypass citrus pathogens, and in particular exocortis
Nucleic acid
A DNA or RNA molecule which can be single-stranded or double-stranded

Nucleotide sequence
(See Sequencing.) It is the order (or sequence) of alignment of nucleotides of nucleic acid molecules

OLP
Oak-leaf pattern. A translucent pattern similar to the outline of an oak leaf, and induced in leaves of indicator plants or trees infected with the concave-gum family of viruses, which include concave gum, impiertatura and cristacortis (see Figures 83a, 89 and 95)

PAGE
Polyacrylamide gel electrophoresis (see Polyacrylamide gels). A method for separating nucleic acid or protein molecules according to their molecular size. The molecules migrate through the inert gel matrix under the influence of an electric field. In the case of protein PAGE, detergents such as sodium dodecyl sulfate are often added to ensure that all molecules have uniform charge. Secondary structure can often lead to the anomalous migration of a molecule. It is common, therefore, to denature protein samples by boiling them prior to PAGE. In the case of nucleic acids, denaturing formamide, urea, or methyl mercuric hydroxide are often incorporated into the gel itself, which may also be run at a high temperature. PAGE is used to separate the products of DNA-sequencing reactions and the gels employed are highly denaturing since molecules differing in size by a single nucleotide must be resolved

Pegs
The needle-like growths or pinpoint projections observed on the wood or bark. These may be symptomatic for certain diseases of citrus. Pegs usually have corresponding pits on the opposite bark or wood surface

Peptidoglycan
Polysaccharide chains covalently crosslinked by peptide chains. The presence or absence of peptidoglycan in the cell walls of bacteria is used to distinguish gracilicute-like organisms (which contain peptidoglycans) from mycoplasma-like organisms (which have no peptidoglycans)

Plant laboratory
A sophisticated greenhouse designed and used primarily for indexing

Plate-trapped antigen
Antigen which is adsorbed directly on the ELISA plate without use of a trapping antibody. For example, virus particles can be trapped to the surface of ELISA plates from extracts of infected tissue added to the wells of the plate. Other proteins, however, are also adsorbed

Polyacrylamide gels
Often referred to, incorrectly, as acrylamide gels. These gels are made by cross-linking acrylamide with N,N'-methylene-bis-acrylamide. Polyacrylamide gels are used for the electrophoretic separation of proteins and also RNA molecules. DNA molecules usually have too high a molecular weight to migrate far in polyacrylamide. Polyacrylamide beads are also used as molecular sieves in gel chromatography and are marketed under the brand name “Nio-gel”

Polyclonal antiserum
Antiserum harvested from the blood of immunized animals. Polyclonal antisera contain a mixture of antibodies to the various antigenic molecules present in the material used to
immunize the animal. In contrast, monoclonal antisera contain only a single antibody.

**Polyembryonic**
The production of one or more embryos from one seed. Nucellar seed may produce a number of seedlings from a single seed and this is sometimes called polyembryonic. In a true sense, it should be called multiple-sprouted nucellar seedlings.

**Potting mix**
A mixture of ingredients used as an artificial soil medium for container growth of plants.

**Primary leaves**
The first emerging leaves from a germinating seed. These leaves may be the cotyledons or may differ in shape from the secondary leaves.

**Primer**
A low-molecular-weight species which promotes a reaction, such as an oligonucleotide which binds to a template permitting a copy of the template to be further synthesised.

**Probe**
As a noun, probe is a specific DNA or RNA sequence which has been radioactively labelled to a high specific activity. Probes are used to detect complementary sequences by hybridisation techniques such as Southern or Northern blotting or colony hybridisation. The verb to probe is to hybridize in order to detect a specific gene or transcript, e.g. “We probed our bank with labelled viral RNA to detect clones containing viral DNA sequences.”

**Probe denaturization**
Treating the probe under conditions that will separate its nucleic acid strands and enable their subsequent hybridization with the target molecules to be tested.

**Probe labelling**
A variety of procedures that permit the labelling of probes with detectable tags.

**Procaryote**
Bacteria-like organisms in the kingdom Procaryotae that have no organized nucleus and are surrounded by a nuclear membrane. Recent taxonomic literature suggests four divisions in the kingdom according to the presence of peptidoglycan and the type of cell wall. Both the greening organism and Spiroplasma citri are procaryote organisms; one a gracilicute-like organism and the other a mollicute.

**Protein A**
A protein with a high affinity for antibody gamma globulins.

**Protuberance**
As protuberances found in the rind of an impietratura-infected fruit. These are raised bumps or blister-like emergences on the rind, and when cut they will usually show gum underneath. The surface of protuberances of impietratura-infected fruit is often a shade of colour different from the rest of the rind (see Figure 84).

**Puckered**
In reference to leaves infected with infectious variegation virus. The surface of the leaves appears drawn together into wrinkles and irregular folds, with puffed areas.

**Radioactive probe**
A nucleic acid that has been made radioactive by one of several techniques (e.g. nick translation) and is to be used to detect a complementary nucleic acid sequence.
**Recombinant plasmid**
A bacterial plasmid DNA containing an insert of DNA from a non-related source, e.g. a plasmid containing an insert of viral cDNA. It is created by recombinant DNA technology.

**Restricted endonuclease**
Enzymes which recognize and cut double-stranded DNA at specific sites determined by the sequence of bases at that site.

**Reverse transcription**
The enzyme which accomplishes the enzyme synthesis of a copy DNA from an RNA template in the presence of a primer and nucleotide triphosphates under appropriate conditions.

**Rf (replicative form)**
The intercellular form of viral nucleic acid which is active in replication, e.g. M13 phase particles contain a single-stranded DNA circle while the Rf of the same molecule is double-stranded.

**Ringspot**
Circular, ring-like translucent or yellow spots on leaves (Figure 78a) or round spots on fruit (Figure 73). Ringspot is also the name of a psorosis-like disease which induces these symptoms on citrus leaves and fruit.

**RM**
Resuspension medium (see TKM buffer).

**RNA**
Ribonucleic acid. The alternative reservoir of genetic information to DNA. Viruses have single-stranded or double-stranded RNA genomes. In organisms, RNA is transcribed from DNA and is essential for the expression of the genetic information contained within the DNA. RNA differs from DNA in having ribose instead of deoxyribose as the sugar moiety in its nucleotides, and in having uracil instead of thymine as one of its two pyrimidine bases. RNA, but not DNA, may be degraded by alkaline hydrolysis.

**Rugose**
As in leaf mgosity, meaning rough or wrinkled, with puffed areas on the upper side of the leaf (see Puckered).

**Scaly bark**
The term used for a condition of the bark in psorosis-infected sweet orange, grapefruit or mandarin scions or rootstocks. The bark is scaled and exfoliated (see Figures 72a and 72b).

**Scaly butt**
A term used in Australia indicating exfoliation or scaling of the bark of the trifoliate orange rootstock due to infection with the citrus exocortis viroid.

**Secondary leaves**
In contrast to the primary leaves of a germinating seed, the secondary leaves are the permanent type of leaves with a fixed morphology. Many seedlings produce both primary and secondary leaves, but many have just one type.

**Seed abortion**
A term used to describe a condition of seed found in stubborn-infected fruit. The seed is not truly aborted, but appears smaller and has a darker purple or brown colour (see Figure 24b).

**Seedling index**
Indexing for transmissible pathogens in citrus done primarily using seedlings of sensitive varieties.

**Seedling yellows**
A yellowing and severe stunting of young inoculated seedlings of sour orange, lemon or...
grapefruit associated with infection by severe citrus tristeza virus isolates

**Sequencing**
The determination of the order of nucleotides in a DNA or RNA molecule, or that of amino-acids in a polypeptide chain

**Sequencing gel**
A long, polyacrylamide slab gel which has sufficient resolving power to separate single-stranded fragments of DNA or RNA which differ in length by only a single nucleotide. Electrophoresis is carried out at high voltage and with the gel in a vertical position. Urea is usually included in the gel mixture as a denaturing agent. This prevents internal base pairing within the single-stranded molecules and ensures that their relative speed of migration is solely dependent on their length

**Shock**
As in the shock symptom of psorosis-A-infected seedlings. After a seedling is inoculated and cut back, the first growth of a young shoot may show withering and bending, with loss of leaves. Ultimately the young shoot will dry up and turn dark brown. This is the shock symptom of psorosis-A (see Figures 75a and 76)

**Shoot-tip grafting**
A micro-grafting procedure. The meristematic growing tip (the meristem plus one to three leaf primordia) is excised by cutting the very young tip using the cutting edge of a razor-blade mounted in a special handle. This decapitated tip is then grafted to a very young seedling with the aid of a binocular microscope. The grafted plant is then grown in test-tubes usually *in vitro* and later transplanted or grafted to produce a shoot-tip grafted plant or tree

**Side grafting**
A method of inoculation which uses a piece of stem 4-5 mm thick and 3-4 cm long. A wedge cut is made at the end of this stem piece; a cut is then made into the seedling to be indexed and the wedge end is fitted into the cut portion of the stem. The side graft is then wrapped. This type of graft is preferred when indexing for stubborn and greening diseases (see Figure 138)

**Side shoot**
A lateral branch or shoot. The Mexican lime seedling normally produces many side or lateral seedling which produces few or none

**Single leader**
In reference to the training of the young growing stem of a seedling or budded plant, to grow as one shoot. This one shoot is usually secured or tied to a stake with plant ties (see Figures 47 and 48)

**Sleeve**
As in a polythene sleeve enclosing side grafts. A polythene bag has the bottom removed or cut out to resemble the open-ended cut sleeve of a shirt or coat. The sleeve is placed over a recently grafted plant and tied at the top and bottom to ensure a moist chamber for the side grafts

**Slipping**
As in bark slipping. A condition where the bark readily separates from the wood of the plant to be grafted. The plant is said to be “slipping” during the budding operation when a cut is made into the bark and it readily separates or opens up from the wood of the stem

**Slot-blot**
See Dot-blot
Southern blot, Southern transfer
A technique which combines the resolving power of agarose gel electrophoresis with the sensitivity of nucleic acid hybridization. DNA fragments separated in an agarose gel are denatured in situ and then blotted or transferred, usually by capillary action, from the gel to a nitrocellulose sheet, or other binding matrix placed directly on top of the gel. Single-stranded DNA binds to the nitrocellulose and is then available for hybridization with labelled P\textsuperscript{32} or by colour change, in the case of a biotinylated probe. A very sensitive and powerful technique, it is often described as “blotting”

Stake
A thin stick made of bamboo, wood or metal which is forced into the soil of a container-grown plant and used to support the growing plant. The plant is secured to the stake by affixing special plant ties (see Figure 48)

STE buffer
Sodium chloride, Tris and EDTA (see Part III). Used in purification of nucleic acids including dsRNA and viroids

Streptavidin
A microbial protein which binds biotin. It is preferred to avidin because of its more specific binding (see Biotin). In molecular hybridization, streptavidin reacts specifically with the biotin molecules fixed on the probe

Stub
As in a rootstock stub. The short projecting portion of the stem which remains after the rootstock is severed from the growing scion. The stub is that small portion above the scion which should be trimmed flush with the scion at a later time

Stylar end
The bottom portion of a hanging fruit

Substrate
The substance acted upon by an enzyme. Usually this substance contains a chemical which, when acted upon by an enzyme, will change colour and the colour can be easily seen and measured

Symptomless carrier
Refers to a tree or plant which contains a graft-transmissible pathogen but shows no symptoms. Examples of a symptomless carrier would be a sweet orange scion on a sour orange rootstock containing tatterleaf virus and/or the exocortis viroid (see Invisible pathogen)

Synergism
The joint action of agents such as two viruses acting together and inducing a more intense symptom in an indicator plant than by either agent acting alone

TAE buffer
Tris, sodium acetate, 3H\textsubscript{2}O and sodium EDTA

Tattered
Ripped, torn or shredded in an non-uniform pattern. The irregular leaf pattern associated with tatterleaf virus suggests an irregularly torn leaf

Template
The molecule which is acted upon or copied, as in the production of cDNA probes by reverse transcriptase

Template primer
See Primer and Template

Thermotherapy
Treatment of budwood or plants by heat to effectively eliminate internal pathogens
**TKM buffer**
A buffer with Tris, KCl and MgCl₂

**TME**
As in TME Tris buffer. Tris, MgCl₂ and EDTA

**Trapping antibodies**
Antibodies used to coat the wells of ELISA plates (the first layer in sandwich assay procedures). Antibodies adsorbed to the solid surface of the plate trap related antigens from the sample extracts placed in the plate for testing

**Triturating**
Rubbing or grinding, as with tissue ground with pestle and mortar

**Undulating depressions**
As in symptoms induced by the cachexia viroid on the trunks of susceptible varieties. The depressions are not deep and sharp but rather have soft bends and undulations (see Figure 44)

**Vein corking**
A thick, cork-like growth on leaf veins induced by severe isolates of CTV or by boron deficiency (see Figure 8)

**Viroid**
A small molecular RNA, transmissible in plants, without an extracellular protein component or translation capacity and which can be pathogenic. It is composed of naked, single-stranded low-molecular RNA (MW 80 000–130 000) which utilizes only host components for its replication. They exist in solution as rod-like structures arranged in a series of short base-paired and non-base-paired regions

**Virus**
Viruses are macro-molecular transmissible agents capable of causing diseases in plants and animals. They are small enough to pass through a millipore filter of 0.2 microns. They have been considered to be either living organisms or simply a molecular complex of nucleic acids and proteins capable of multiplication in living cells. Viruses are characterized by a core of nucleic acids with a genome less than $3 \times 10^8$ daltons in weight surrounded by a protein coat which can induce formation of antibodies