

## SILVER ANNIVERSARY

### Twenty-Five Years of Outstanding Progress in Citrus Virus Research Marks IOCV's Silver Anniversary

S. M. Garnsey

**ABSTRACT.** Remarkable progress has been made on important citrus virus and viruslike pathogens in the past 25 years, much of it through outstanding efforts of IOCV members. The causal agents of greening, stubborn, exocortis, and tristeza have been characterized. Identification and characterization of the pathogens causing these major diseases have greatly improved control strategies and advanced basic knowledge on viroids, spiroplasmas and fastidious bacteria as plant pathogens. A number of new virus and viruslike diseases have been described, as well as new variants of known ones. Detection procedures for many viruses have been improved by use of better indicator plants and by new transmission, purification and serology techniques. Shoot-tip grafting and heat therapy have accelerated production of virus-free scion sources of major varieties and facilitated budwood certification. Isolates of tristeza and exocortis have been used beneficially for cross protection and tree dwarfing, respectively. Many challenges remain, but the nine conferences of IOCV and eight volumes of IOCV Proceedings have created a strong framework for worldwide communications and cooperation to continue research.

The 9th Conference of the International Organization of Citrus Virologists (IOCV) marks its 25th anniversary. The past 25 years have witnessed dramatic progress in characterization and control of citrus virus and viruslike diseases (CVVLD) and much of this progress can be credited to IOCV and to its members.

As we enter the next quarter century of IOCV activity, it is appropriate to examine how we have improved our knowledge and understanding of CVVLD and have utilized this knowledge to control these diseases. At the same time, it is a good opportunity to recognize voids in our knowledge and needs for new research.

Much of the extensive progress made on CVVLD problems can be credited to the exchange of information and ideas and the development of joint research efforts fostered by IOCV. The eight previous IOCV conferences brought scientists together from many

different regions and countries, and with the associated pre- and/or postconference activities have taken IOCV delegates to at least 15 different countries (fig. 1). Formal exchange of information has occurred through presentation of Proceedings. The eight IOCV Proceedings published to date contain 439 papers and 2228 pages.

Study programs and tours associated with each conference have provided delegates invaluable opportunities to study disease conditions in different areas. These firsthand observations augment information in formal papers, and have clarified many previous misconceptions or misunderstandings. Delegates have also observed research experiments and facilities at each meeting site, and also have interacted with the scientific support staff in each location.

Personal interchange also has been fostered by IOCV, and many of us first became acquainted at IOCV conferences. These acquaint-

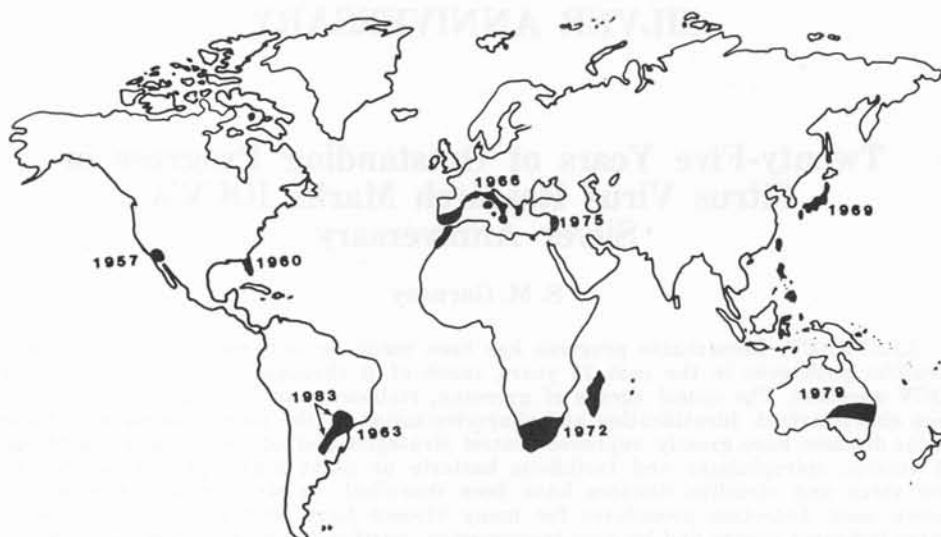


Fig. 1. Sites for the first nine IOCV conferences and associated conference programs are shown by shaded area and date.

ances have often blossomed into permanent friendships and collaborative research efforts which span the globe. The benefits from these associations are intangible and often difficult to document, but surely are a valuable legacy of the first 25 years of IOCV and one desired by the organizer of the first IOCV conference and our first chairman, Dr. J. M. Wallace.

This paper is not a comprehensive summation of all work on virus and viruslike problems over the past 25 years, but highlights some of the most significant accomplishments especially pertinent to IOCV and its members.

#### CHARACTERIZATION OF CITRUS VIRUS AND VIRUSLIKE PATHOGENS

When the first Conference was held in 1957, numerous citrus diseases had been ascribed to virus or viruslike pathogens, but the nature of the causal agents was unknown. Viral etiology was assumed based on symptoms, the infectious nature of the diseases, and lack of association with bacterial or fungal pathogens. No citrus virus had been sap transmitted experimentally

and none had been visualized in the electron microscope.

Outstanding progress has been made in the past 25 years. Some landmark contributions included visualization of the particle of citrus tristeza virus (CTV), discovery and culture of the spiroplasma causing stubborn disease, discovery of the procaryote causing greening disease, and discovery that exocortis is caused by a viroid. These pioneer advances reflect unusual insights, perseverance, and hard work under difficult circumstances. Characterization of citrus pathogens often has come through the accumulated efforts of several workers over an extended period. For example, scientists in several laboratories in different countries have contributed to characterization of the CTV particle, production of CTV-specific antisera, development of rapid, sensitive detection techniques and new approaches to CTV control. The 25-year progress in the mechanical transmission, electron microscopy, purification and serology of 16 citrus virus and viruslike pathogens is summarized in Table 1. Although extensive progress can be seen with

TABLE 1  
PROGRESS IN THE CHARACTERIZATION OF SOME CAUSAL AGENTS OF  
VIRUS AND VIRUSLIKE DISEASES OF CITRUS

Disease	Mech. trans.		E.M.		Purification		Serology	
	1957	1982	1957	1982	1957	1982	1957	1982
Tristeza	—	yes	—	yes	—	yes	—	yes
Stubborn	—	—	—	yes	—	yes*	—	yes
Greening	—	?	—	yes	—	—	—	—
Exocortis	—	yes	—	yes	—	yes	—	—
Infection variegation	—	yes	—	yes	—	yes	—	yes
Crinkly leaf	—	yes	—	yes	—	yes	—	yes
Leaf rugose†	—	yes	—	yes	—	yes	—	yes
Satsuma Dwarf	—	yes	—	yes	—	yes	—	yes
Tatterleaf†	—	yes	—	yes	—	?	—	—
Ringspot†	—	yes	—	—	—	—	—	—
Psorosis	—	yes	—	—	—	—	—	—
Xyloporosis	—	yes	—	—	—	—	—	—
Impietratura	—	—	—	—	—	—	—	—
Cristacortis†	—	—	—	—	—	—	—	—
Vein enation	—	—	—	yes	—	—	—	—
Leprosis	—	—	—	yes	—	—	—	—

\*Cultured.

†Not described in 1957.

a number of pathogens, little progress has been made with some others including two of those recognized earliest, psorosis and xyloporosis.

### TRANSMISSION

Significant new information on natural spread of CVVLD has also been developed in the past 25 years. By 1957, the aphid transmission of CTV was well recognized, but little was known about the natural spread of other pathogens. Since then two psyllid vectors of greening, several leaf-hopper vectors of stubborn, transmission of CEV on knife blades, and seed transmission of psorosis in trifoliolate orange and citranges have been discovered.

Progress on transmission has also come from input by a number of individuals over a period of time. Team efforts and international cooperation are especially evident for the work on greening and stubborn. Improved knowledge of pathogen-vector relationships and of pathogen host range have contributed markedly to develop-

ment of control strategies for stubborn and greening.

Man still remains a prime vector of CVVLD, especially over long distances, and many serious disease outbreaks can be traced to importation and spread by subsequent propagation and/or vectors.

### HOST INTERACTION

During the past 25 years, our understanding of pathogen-host interactions with CVVLD has greatly improved. Prior to 1957, classic cytological studies had revealed some effects of CTV on citrus hosts, and strain differences in pathogenicity have been observed for several viruses, including CTV.

Most of our progress in studying host-pathogen interactions, however, is based on our recent ability to obtain pure cultures of individual pathogens. Early work with mixed infections often resulted in confusion about the specific association of disease symptoms and a pathogen. Several forms of greening disease were initially as-

sociated with CTV infection because affected plants were doubly infected, and some relationships in the psorosis group established by cross-protection tests were later shown to be erroneous because of contamination. Subsequently, mechanical transmission, purification, vector transmission, differential host resistance, and differential responses to therapy techniques have all been useful singly or in combination to obtain pure cultures of many CVVLD. Lack of pure cultures still hampers identification of specific symptoms and relationships with the psorosis complex, cristacortis, and impietratura, among others.

Studies on mechanical transmission, purification, and serology have improved our ability to quantify virus accumulation within the host and to determine pathogen distribution. We have learned that virus replication is a dynamic process, and that virus titer can fluctuate greatly according to host species, tissue age and environmental conditions. Early workers concluded that citrus viruses occurred only in low concentrations within citrus hosts. Subsequently, we have learned that even some tissue-restricted viruses such as CTV can occur in relatively high concentration, and several citrus viruses are routinely purified directly from citrus.

Although citrus plants are systemically affected by stubborn, by greening, and by various viruses, the distribution of the pathogens may be highly irregular. It often is difficult to recover or transmit a given pathogen from substantial portions of an infected plant. The pathogens causing stubborn, greening, and CTV are all strongly phloem-associated. The tatterleaf-citrange stunt virus (TL-CSV) and citrus ringspot virus (CRSV), often invade and infect only por-

tions of a host in an irregular pattern.

The propagation of commercial citrus trees as grafted combinations creates a special site for viral reaction—the graft union. As classically demonstrated with CTV, a rootstock variety and a scion variety each separately tolerant of CTV may form a highly CTV-intolerant combination when grafted together. A number of interesting stock/scion-associated problems have been observed which have not been well characterized, but some are apparently due to infectious agents.

The interaction of citrus viruses with their hosts on a molecular level has not been well studied. By-products of infection have been analyzed for diagnosis, but more direct studies are lacking. The single-stranded RNAs of some citrus viruses apparently function as messenger RNAs to alter certain cell functions. Some symptoms, however, apparently are indirect responses to pathogen action. A unique situation that awaits clarification is how the small RNA molecule of citrus exocortis viroid (CEV) functions to direct its replication and cause symptoms.

## DETECTION

**Citrus indicators.** In 1957, identification of citrus virus and viruslike diseases was based entirely on symptoms in field trees or graft-inoculated citrus indicators, and the discovery that CTV infections could be diagnosed accurately and relatively rapidly on Mexican lime indicators had been the major advance. Since 1957, further improvement in utilization of citrus indicators has occurred. An outstanding example is the Etrog citron test for CEV. Sensitive indicators have been described for a number of CVVLD; however,

in some cases symptoms are not highly specific or rapidly formed. Symptoms in the wood do not appear rapidly, and diagnosis of a specific pathogen in field trees with multiple infections by graft inoculation to citrus indicators is still difficult. Citrus hosts remain valuable for characterizing severity of strains of a given pathogen.

The need for healthy, vigorous plants and appropriate growing conditions has been recognized. Mild temperature conditions favor detection of many viruses, but warm temperatures are required for CEV, stubborn, and xyloporosis. Careful selection of inoculum tissue has improved detection of some irregularly distributed pathogens such as stubborn, greening, and citrus ringspot. Novel grafting techniques have been developed to improve transmission.

**Noncitrus indicators.** As information on the mechanical transmissibility of citrus viruses has increased, indexing procedures based on mechanical (sap) inoculation to herbaceous plants have been detected in noncitrus hosts by mechanical inoculation. These procedures are now commonly used for research and for some routine indexing. Careful selection of inoculum, appropriate plants, and good conditions are required. When these conditions are met, indexing periods can be reduced from months to a few days. Less time is needed to grow indicator plants, and viruses not detected by currently used citrus indicators may be revealed.

**Biochemical tests.** Several indexing procedures are based on identification of specific marker substances produced as a result of CVVLD infection. The most successful has probably been the fluorescent marker test for greening based on presence of a gentisoyl glucoside. Difficulties in proving specificity of these procedures plus

equipment and technique requirements have limited general applications.

**Microscopy.** Light microscopy of cytological changes induced by CTV infection was used prior to 1957 for diagnosis. Light microscopy has been used more recently to identify viral-specific inclusion bodies, and to locate procaryotic pathogens. Availability of fluorescent-labeled antibodies has also enhanced detection of specific pathogens by light microscopy.

The observation of unusual filamentous particles in extracts from CTV-infected plants by electron microscopy (E.M.) led to the use of E.M. for diagnosis of CTV. A more sensitive modification, serologically (immuno) specific E.M. has made E.M. more attractive for detection of viruses to which specific antisera have been prepared. Microscopy is the most rapid detection procedure for single samples of some pathogens (table 2).

**Serology.** The greatest advances in detection of CVVLD have occurred through application of serology. Immunodiffusion procedures were developed for citrus leaf rugose virus (CLRV), Citrus variegation virus (CVV), and CTV, but required good sources of tissue for reliable results and sizable quantities of antisera for extensive use. Application of the enzyme-linked immunosorbent assay (ELISA) procedures to CTV detection provided a sensitive and efficient procedure, well adapted to large-scale use. In the past 5 years, ELISA has been used extensively in many countries for CTV detection and ELISA has been used also for stubborn, CVV, CLRV, satsuma dwarf virus, and citrus mosaic virus.

Development of serological procedures resulted from research on characterization and purification. In turn, serological assays provided

TABLE 2  
MINIMUM TIME AND SPECIFICITY OF INDEXING PROCEDURES  
FOR CITRUS VIRUS AND VIRUSLIKE DISEASES

Technique	Time	Specificity
Graft-inoculated citrus indicators	1-3 months	low
Sap-inoculated herbaceous plants	3-10 days	low
Serology	1-12 hours	high
Light and electron microscopy	10-60 minutes	moderate*
Nucleic acid analysis	12-48 hours	high

\*Microscopy combined with a serological probe for antigen is highly specific.

new insights on virus-host interactions, virus replication, and virus distribution. Procedures such as ELISA have also facilitated more comprehensive epidemiological studies.

**Culturing.** The causal agent of stubborn, *Spiroglasma citri* Saglio, *et al.*, is readily cultured in liquid media, and isolation from tissues such as seed provides a useful indexing technique. The pathogen can be visualized directly by phase contrast or dark-field microscopy after several days' incubation. In contrast, graft inoculation to indicators takes several months and ELISA of tissue extracts is restricted by low concentration of the pathogen.

**Nucleic acid analysis.** Recently, CEV infection has been detected by isolation of the viroid molecule using chromatography and gel electrophoresis. Analysis of ds-RNA species from tissue extracts is also promising for rapid detection of ss-RNA citrus viruses and possibly even for identification of specific strains. Recently, cDNAs for CEV and CTV RNAs have been cloned and sensitive nucleic acid hybridization techniques are being developed for indexing purposes. Table 2 summarizes some of the improvements in detection of CVVLD.

## CONTROL

Most research on CVVLD is designed directly or indirectly to solve disease problems. We charac-

terize pathogens, study pathogen-host interactions, develop improved identification procedures, and investigate means of spread, all to provide better tools for controlling diseases. Some control procedures, such as rootstock selection, have developed empirically but, in most cases, some knowledge of the disease and its causal agent has been essential to develop suitable controls. As our knowledge of CVVLD has increased over the past 25 years, the basis for controlling CVVLD knowledgeably has increased remarkably.

Virus-free, horticulturally desirable propagating sources can be rapidly developed and maintained virus-free by the use of shoot-tip grafting, heat therapy, and rapid indexing methods. These procedures facilitate use of budwood certification for controlling spread of CVVLD by propagation and also facilitate safe transfer of germplasm between citrus regions under quarantine. Eliminating spread of CEV and other pathogens as contaminants, and avoiding seed transmission of psorosis in certain rootstocks has also improved maintenance of virus-free propagating sources.

Once the procaryotic nature of the stubborn and greening pathogens was determined, chemotherapy procedures were tested. Injection with antibiotics has increased production and improved the condition of greening-affected trees, even though a complete cure

is usually not achieved. Chemotherapy of budwood has also been effective to produce pathogen-free bud sources.

Greening and stubborn still pose serious disease control problems where inoculum and vectors are abundant. Acceptable host resistance or tolerance is lacking in horticulturally desirable scion varieties, and alternate hosts serve as inoculum reservoirs for stubborn. Knowledge that the vectors are not highly efficient has been exploited to suppress or control greening. Biological control of the psyllid vectors has been effective in Reunion, and combined use of greening-free budwood, removal of infected trees, and insecticides has been promising in the Philippines. Growing citrus in areas climatologically unsuitable to either the vectors or the pathogen has also been utilized.

Cross protection has been used extensively in Brazil to combat aphid-vectored, severe stem-pitting forms of CTV which have threatened commercial production of many citrus varieties including Pera sweet orange. Deliberate use of propagating sources infected with mild CTV isolates which protect against natural infection by more severe isolates is under test in many other areas.

Some isolates of CEV may also prove beneficial horticulturally to control tree size. Some mild isolates of CEV cause varying degrees of stunting without bark scaling and do not adversely affect fruit quality or general tree condition. When trees deliberately stunted with CEV are spaced appropriately, yields per hectare are comparable to normal trees, and harvesting and pruning costs are reduced.

Breeding for new tolerant or resistant citrus varieties has continued, but progress has not been rapid because of the combinations

of disease and horticultural considerations which must be considered. Use of quantitative serological assays has recently enhanced the search for CTV-resistant germplasm.

## NEW AND CONTINUING PROBLEMS

While progress has been made in studying and controlling numerous CVVLD, the net total progress has been reduced by the continued discovery of new CVVLD.

Tatterleaf-citrange stunt, citrus ringspot, citrus leaf rugose, cristicortis, citrus leaf curl, citrus mosaic, navel infectious mottling, natsudaikai dwarf, dweet mottle, Australian citrus dieback, gummy pitting, multiple sprouting, transmissible budunion creases, stem-pitting problems in Milam lemon and some mandarin hybrids, and at least one mechanically transmissible, latent virus are among viruslike diseases uncovered since 1957. More are likely to be uncovered. Blight and blightlike decline diseases of unknown etiology (declinio, declinamiento, marchitamiento repentino) have become very serious in several regions, including Florida, Brazil and Argentina.

In addition, some existing CVVLD have become more serious. Isolates of CTV which cause severe stem pitting in sweet orange have appeared in several areas and pose a serious hazard to citrus production unless eradicated or controlled. Natural spread of a psorosislike pathogen in Argentina and limited natural spread of a citrus ringspot in Texas indicate that budwood certification may not adequately control those diseases.

Greening, stubborn, and CTV have become serious problems in several new areas following introduction and subsequent natural spread. The potential for introduc-

tion and spread of CVVLD into new areas presents a constant hazard.

The effects of many of the CVVLD on commercial stock/scion combinations has never been studied, and development and commercial propagation of new hybrids or other new periplasm sources have created the possibility of additional new budunion-associated problems.

### THE NEXT 25 YEARS

The future in a rapidly changing technological society is always difficult to predict. However, it seems safe to predict that CVVLD will remain serious concerns to citrus production, and that new, unanticipated problems will arise. The struggle to control these problems will require continuous effort and support, and the needs that gave birth to IOCV will continue to exist.

Characterization of pathogens will continue. Hopefully, the green-ing agent will be cultured soon and studied in detail. The causal agents of the psorosis complex and xyloporosis should be characterized with additional effort and application of new technology.

Detection procedures are sure to improve. Expanded application of labeled antibodies for CVVLD detection seems certain, and further development of monoclonal anti-

bodies should provide stable sources of highly specific antibodies which may also be strain specific.

Rapid, accurate strain differentiation without need for host assay seems increasingly possible with rapid advances in nucleic acid characterization, and sequencing techniques. Use of labeled, highly specific nucleic acid probes should prove useful for specific strain identification.

Storage and movement of citrus germplasm in virus-free condition through tissue-culturing procedures will allow safer, more rapid exchange of valuable germplasm, and genetic engineering procedures may enhance development of disease-resistant cultivars.

As the etiology and epidemiology of different CVVLD become better understood, more sophisticated control procedures will evolve. Multicomponent or combination approaches may increasingly provide answers to complex problems. For example, various combinations of host tolerance, vector control, inoculum suppression, and cross protection may provide control not achievable by a single approach.

The need for international exchange of information, ideas and cooperation is certain to continue and increase. This need is one that IOCV can and should continue to fulfill.