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CITRUS BLIGHT AND OTHER DISEASES OF RECALCITRANT ETIOLOGY*

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■ **Abstract** Several economically important diseases of unknown or recently determined cause are reviewed. Citrus blight (CB), first described over 100 years ago, was shown in 1984 to be transmitted by root-graft inoculations; the cause remains unknown and is controversial. Based on graft transmission, it is considered to be an infectious agent by some; others suggest that the cause of CB is abiotic. Citrus variegated chlorosis, although probably long present in Argentina, where it was considered to be a variant of CB, was identified as a specific disease and shown to be caused by a strain of *Xylella fastidiosa* after if reached epidemic levels in Brazil in 1987. Citrus psorosis, described in 1933 as the first virus disease of citrus, is perhaps one of the last to be characterized. In 1988, it was shown to be caused by a very unusual virus. The cause of lettuce big vein appears to be a viruslike agent that is transmitted by a soilborne fungus. Double-stranded RNAs were associated with the disease, suggesting it may be caused by an unidentified RNA virus. Rio Grande gummosis, dry rot root, peach tree short life, and some replant diseases may be diseases of complex etiology. Various microorganisms have been isolated from trees with these diseases, but the diseases may be attributable in part to environmental factors. Determination of the cause of these diseases of complex etiology has proven difficult, in part, because they affect only mature trees.

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INTRODUCTION

Plant pathologists have made remarkable progress in the identification, characterization, detection, and development of control strategies for many pathogens. Progress in plant pathology has been closely linked to developments in biological methods. New and improved techniques in culture and manipulation of microorganisms—microscopy, centrifugation, electrophoresis, protein and nucleic acid characterization, immunology or genetics—are constantly being adopted for identification of "new" pathogens, characterization of known pathogens, studies in host-parasite interactions, and for disease control.

Fungi, bacteria, viruses, and nematodes were identified as plant pathogens late in the nineteenth or early twentieth century. The causal agents of many other diseases that were unknown or considered to be viruslike have been identified more recently. Modern techniques led to the discovery of phytoplasmas, fastidious bacteria, viroids, and even a few protozoans as the cause of some systemic diseases.

There may well be some additional "new" plant pathogens, but it has proven difficult to work on an unknown disease without developing a preconceived idea as to the causal agent. Such thinking led to years of fruitless research on aster yellows "virus," found to be caused by a phytoplasma; Pierce's disease "virus," found to be caused by a fastidious bacterium; and potato spindle tuber "virus," found to be caused by a viroid. The solution to difficult diseases has often required innovative approaches, thinking "outside of the box," and a goodly amount of luck.

In spite of many successes and the powerful techniques now available, there remain some important diseases of unknown or uncertain cause. For this review, several diseases of unknown or recently determined cause were selected; some are on citrus, and much of the discussion of these diseases comes from our experience. A few difficult diseases of other crops were chosen based on their continued ability to humble plant pathologists armed with modern techniques.

Many diseases of unknown cause are considered to be viruslike based on failures to associate a bacterium, fungus, or nematode with the disease and, in some cases, on being graft transmissible. Most viruses and viruslike agents are not transmitted through seed, which prevents direct transmission of these agents to the next generation. In contrast, when plants that are propagated vegetatively become infected with virus and viruslike agents, future generations are also infected. Since citrus is a perennial, and since almost all citrus is propagated vegetatively by budding, it is an ideal host for virus and viruslike agents, some of which have probably been in citrus for over 100 years. In addition, the distribution of budwood throughout the world has contributed to the wide distribution of bud-transmitted pathogens in citrus.

The degree of difficulty in detection and identification of a plant pathogen varies from relatively easy and straightforward to extremely difficult. Many pathogens are readily transmitted by mechanical inoculation and induce definitive symptoms within a few days or weeks and therefore are readily identified. Difficulties can arise when the pathogen can be transmitted only by grafting or by a vector. These problems may be compounded by uneven distribution of the pathogen in the plant, by the absence of definitive symptoms, or by the existence of two or more agents that are causing the disease. In addition, extreme difficulties can be encountered in the identification of the cause of some diseases of woody perennials, where incubation periods of several years may be required to reproduce symptoms or when symptoms occur only on fruit-bearing plants. In such cases, plants inoculated with candidate pathogens must be maintained and protected from natural infections for several years, or sufficient replications and controls must be included to account for natural spread of the pathogen that occurs during the life of the experiment.

The *Compendium of Citrus Diseases* (129) describes 33 virus and viruslike diseases and 3 diseases of unknown or uncertain cause. Of these 36 diseases, 21 are of unknown cause including concave gum, cristacortis, impietratura, brittle twig yellows, fatal yellows disease, fovea, gum pocket, gummy pitting or gummy bark, leaf curl, leathery leaf, Milam lemon stem pitting, mosaic, Nagami kumquat disease, rubbery wood, yellow vein, blight, Rio Grande gummosis, and rumple of lemons. In addition, there are many budunion abnormalities of citrus that have been shown to be graft transmitted and appear to be due to unidentified infectious agents (129). We are concerned here with five somewhat unusual and economically important citrus diseases: citrus blight (CB), citrus variegated chlorosis (CVC), citrus psorosis (CP), Rio Grande gummosis of citrus (RGG), and dry root rot of citrus (DRR). Discussions of lettuce big vein (LBV), peach tree short life (PTSL), and replant diseases of apple are included to illustrate that difficult diseases are certainly not limited to citrus.

CITRUS BLIGHT

Citrus blight was described over 100 years ago (111). The cause remains unknown, and the disease has characteristics that seem to make it a “perfect” disease of unknown cause. Blight can be transmitted by grafting only through roots with some

difficulty (suggesting uneven distribution of the pathogen in the roots), with an incubation period of at least 2 years; it appears to spread by an unknown vector; and symptoms, which occur only on bearing trees, are similar to several other disorders of citrus. Moreover, light and electron microscopic examinations of tissue from trees with CB have failed to associate any pathogens with the disease. Blight is frequently a problem when citrus is grown in humid areas, and has not been reported in drier climates such as California or the Mediterranean. The occurrence and severity of CB increases in warmer climates. In Brazil, CB was reported to be more common in groves in the warmer regions of the state of Sao Paulo and less prevalent in the cooler citrus-growing areas (105). In Venezuela, with year-round warm weather, some trees with CB died 6 to 8 months after the first symptoms were observed (90, 124). In more temperate climates, such as Florida, trees with CB seldom die and remain alive for many years in a nonproductive state. An estimated 650,000 trees are lost each year to CB in Florida (120). In the state of Sao Paulo, which is the principal citrus region in Brazil, a recent survey by Fundecitrus (a grower-funded foundation) estimated that 10 million trees are lost to CB each year.

History of CB

The lack of definitive diagnostic procedures before the 1970s precludes us from establishing the historical importance of CB. Publications in 1896 (111), 1936 (100), and 1968 (27) indicated that much of what was then considered CB was a decline of relatively young trees on sour orange (*Citrus aurantium* L.) or sweet orange rootstocks (*C. sinensis* (L.) Osb.), now known to be two of the most CB-resistant rootstocks (22). Trees on sour or sweet orange usually do not have symptoms of CB before they are 25 years old. There is no doubt that CB has been in Florida for many years; some of the symptoms described in earlier reports are consistent with CB, whereas others are similar to CTV-induced decline of trees on sour orange rootstock. Declines described on sweet orange seedling trees or trees budded on sweet orange rootstock may have been due to root rot. There were descriptions of "new and emerging" declines of trees on rough lemon in 1936 (100) and 1968 (27) that were considered different from what was then called CB. The descriptions of these "new declines" are identical to what is now known as CB.

Beginning in the 1960s and increasing in the 1970s, trees on rough lemon (*C. jambhiri* Lush.) began to decline at an alarming rate in Florida (11, 22). Similar observations were made in Brazil where the major rootstock is Rangpur lime (*C. limonia* Osb.), which is very susceptible to CB. Before 1970, there were no reports of CB in Brazil, but in 1979 a disease called "declinio" was reported as having been observed in many orchards in the last 10 years and was becoming economically important in the state of Sao Paulo (104). Diagnostic tests indicated that CB and declinio are probably the same disease (7, 104). The increased incidence of CB in Florida and Brazil in the 1960s and 1970s led to renewed research efforts on CB, and the decline was given several new names including rough lemon decline, sand hill decline, roadside decline, and young tree decline (YTD).

Reports from the 1970s usually use YTD, which was descriptive of trees on rough lemon declining after about 8 years. The name CB has historical precedence and remains in use today, even though the disease is not a "blight," and "decline" is more descriptive of the symptoms.

Some groves remain in Florida that were planted in the 1920s through the 1950s on rough lemon that have lost very few trees to CB (35), whereas other older groves were decimated by CB. A correlation was observed between the development of CB and replanting trees propagated from registered scion trees in existing old groves (11). Trees planted more recently on rough lemon in Florida invariably begin to develop CB after about 5 years, and tree losses continue at rates up to 10% per year. Numerous suggestions have been made on why CB developed into a severe problem, starting in the 1960s. The more popular "theories" include use of different seed sources, use of clonal budwood (35), use of herbicides, a decrease in tillage, decreases in the use of sulfur as an insecticide, increases in the use of lime in some groves, and excessive fertilization (10).

Diagnostic Tests For CB

Trees with CB show a general decline somewhat similar to drought stress. The first visible symptom is lack of growth. Frequently, symptoms will develop first in one sector of a tree. Infected parts of the tree appear unthrifty, with a dull-green cast. As the disease progresses, the canopy thins and produces small fruit. Infected trees have off-season flush and bloom patterns and frequently produce trunk and root sprouts. The symptoms of CB do not readily distinguish it from the various other declines of citrus, and several diagnostic tests have been developed to identify trees with CB. Affected trees frequently show zinc deficiency in leaves, indicating an interference with the translocation of zinc, but zinc deficiency symptoms are not diagnostic for CB. However, the zinc content of the wood and bark of blighted trees is significantly higher than that in healthy trees or in those affected with other disorders from the same grove (109, 130). Zinc analyses can be useful in identifying trees with CB. The drought-like symptoms of trees with CB suggest problems with water transport. The xylem of blighted trees is plugged, resulting in low water transport (28). A syringe injection test, which measures the quantity of water that can be injected into the trunk of trees in a given period of time is useful in diagnosis (75). Amorphous plugs in the xylem vessels of trees with CB, rarely seen in healthy trees, have been associated with reduced water flow. Microscopic examination of xylem for these amorphous plugs (12, 25, 30) revealed cells considerably smaller in CB-affected trees compared to healthy trees (125), but this has not been used for diagnosis.

Blighted trees contain several pathogenesis-related proteins (40), referred to as blight-associated proteins (BAPs). Two of these proteins, p12 and p35, have been partially characterized and are discussed in a later section. Serological tests for CB based on p12 are useful in distinguishing trees with blight from other diseases (5, 36) that do not contain BAPS, such as citrus tristeza virus (CTV), CVC, root

rot, citrus psorosis, murcott collapse, citrus nematode, exocortis, drought stress, and salt stress. p12 is observed at low levels in some young, nonbearing trees, but increases dramatically in young trees inoculated by root grafting. In addition, assays for p12 can detect presymptomatic trees in a grove, but the protein is very unevenly distributed in presymptomatic trees. A leaf from one branch may be positive while leaves from other branches are negative (GA Barthe & KS Derrick, unpublished observations). Likewise, symptoms of CB frequently occur in only one sector of a tree.

In summary, the characteristics of trees with CB include general decline and wilt symptoms, elevated zinc levels in wood and bark, reduced water flow, the presence of amorphous plugs in the xylem, and the presence of BAPs.

Is the Cause of CB Biotic or Abiotic?

Prior to 1984, repeated attempts to transmit or propagate CB by bud grafting or to reproduce the disease by reconstituting blighted trees from root sprouts and buds from diseased trees failed (25, 110, 132). These observations and repeated failures to associate an infectious agent with CB led to suggestions that the cause of the disease is abiotic. The pH of the soil, soil type, and rates of fertilization have been suggested as possible causes (10, 132). However, CB occurs on trees on a variety of soil types, and the distribution of affected trees in groves suggests an infectious disease (3).

The first trees to develop CB in a grove are usually randomly distributed, but a few unusual cases of nonrandom distributions have been reported (29). Blight frequently spreads to trees surrounding affected trees in the same row and in adjacent rows (56), but also to trees at some distance from those affected, providing additional foci for disease spread. Paradoxically, in any grove where CB is found, some trees will remain healthy for many years despite their proximity to trees with CB. Blight spreads slowly, usually at rates of less than 10% per year, and there are several reports showing the rate of spread is linear (24, 79, 134). Because many abiotic diseases have linear rates of spread, these observations have reinforced suggestions that the cause of CB is not infectious. In Brazil, where CB is very severe, the spread of the disease was observed to be linear in a grove where diseased trees were removed annually and replaced by healthy trees. In another grove where trees affected with CB were not removed, the disease spread was exponential (7, 72). The cause of CB will undoubtedly be debated until it is resolved unequivocally, but the repeated demonstrations that the symptoms and characteristics associated with CB can all be reproduced by root-graft inoculations (80, 103, 114, 123) certainly suggest the disease is caused by a systemic infectious agent.

Experimental Transmission of CB

In the first successful root-graft transmission of CB (123), infected trees were identified and transplanted using a tree spade between eight pairs of healthy sweet orange trees. Eight roots from the infected trees were grafted to roots of one of

the healthy trees; the other tree served as a nongrafted control. Within 30 months, seven of the eight grafted trees were showing moderate decline and were positive in diagnostic tests for CB. The nongrafted control trees remained healthy. Subsequently, several additional successful root-graft transmission experiments were reported (80, 103), including those where root pieces taken from trees with CB were used to infect mature healthy trees (114). However, attempts to transmit CB by root grafting have also failed. In a well-replicated experiment to demonstrate the transmission of CB using root pieces, the incidence of the disease after 10 years (based on symptoms and p12 analysis) was similar in inoculated, mock-inoculated, and uninoculated trees in the grove (LABC Vasconcellos & WS Castle, unpublished results).

Following transmission of CB by root grafting, there was an additional effort to transmit the disease by above-ground graft inoculations (2). Limb and root grafts were established and maintained between healthy and trees with symptoms of CB. All of the root-grafted receptor trees were positive in the p12 assay for CB (36) by year 5 and showed visible symptoms of blight after year 6. In contrast, none of the limb-grafted receptor trees had symptoms of blight or had become positive for p12 after 6 years. Thus, the only experimental transmission of CB is by root-graft inoculations, suggesting the incitant is restricted to the roots. This is supported by observations that rooted limbs from p12-positive trees with CB produce p12-negative plants (131). In addition, buds and limbs from p12-positive trees become p12 negative after grafting and growing out (GA Barthe, M Bar-Joseph & KS Derrick, unpublished information).

There is evidence that the cause of CB is unevenly distributed in the roots of affected trees. In repeated experiments to transmit CB to potted seedlings in the greenhouse by graft inoculation using single root pieces from trees with CB, less than 5% of the inoculated plants expressed p12 (KS Derrick, unpublished observations). Moreover, root sprouts are frequently produced by trees on rough lemon rootstock, but only about 5% of the root sprouts produced by symptomatic, p12-positive trees are positive for p12 (GA Barthe, LJ Marais & KS Derrick, unpublished observations). Observations that the cause of CB appears to be restricted to roots, where it is unevenly distributed, suggest that the pathogen produces a metabolite that is translocated to induce wilting, slow growth, disruption of zinc movement, plugging of xylem, and BAPs throughout the affected tree.

Movement of CB

How CB is introduced to a grove and how it subsequently spreads within the grove are not known. Some in-grove spread of CB may be due to natural root grafts. Natural root grafts between trees are frequently observed when roots are collected for analysis, and glyphosate toxicity symptoms are frequently seen on trees adjacent to stumps of removed trees treated with glyphosate (120). The distribution of blight in some groves suggests spread by an aerial vector (3), and

the incidence of the disease was decreased by supplemental insecticide sprays (1). Agents in the soil (86, 87) such as *Fusarium solani*, a soilborne fungus, have been proposed as the cause of the disease. However, these possibilities were diminished after *F. solani* was shown to be a ubiquitous, secondary colonizer of senescent citrus roots (57, 58), and CB was not transmitted by placing soil and roots from diseased trees around healthy trees (115).

The initial random distribution of CB in affected groves is consistent with an agent transmitted at low levels through seed, perhaps a few infected seed per thousand. A study on the effect of seed and budwood source on CB (92) reported no differences between using CB-affected and healthy trees as seed or budwood sources. However, the limited number of replications would not have allowed low levels of seed transmission to be detected. A further study on the effect of clonal propagation of seed and bud sources on the incidence of CB appeared to eliminate both as possible sources of initial infection. Four thousand trees produced by shoot-tip grafting and clonal multiplication of both the scion and rootstock were compared in replicated trials with 4000 standard seed- and budwood-propagated trees. After 7 years, 18 of the standard trees and 16 of the clonal trees had symptoms of CB and were randomly distributed in the grove (DPH Tucker, MR Burke & KS Derrick, unpublished information).

Looking for the Cause of CB

Prior to the discoveries that phytoplasmas, systemic bacteria, and viroids cause plant diseases, pathogens that moved systemically in plants were often assumed to be viruses. Uncharacterized pathogens that are graft transmitted are now usually referred to as being viruslike. The first observation of CB in Florida over 100 years ago predates an understanding of any of the viruslike agents that infect plants. As viruses and viruslike agents were characterized and methods developed for their detection, experiments were done to determine if any of these agents were associated with CB.

There have been reports associating xylem-limited bacteria (XLB) with CB (46, 47, 62–67, 71, 96) and that tetracycline treatments reversed the symptoms of the disease (122). However, studies using electron microscopy failed to associate XLB with CB (13), and additional experiments with tetracycline did not show a consistent remission of symptoms (76, 77, 119). In an earlier study (97), phytoplasmas were not observed in electron microscopic examinations of phloem of leaves, fibrous roots, or bark from CB-affected trees. Bacteria were not found in numerous samples of xylem fluid from roots of blighted and healthy trees examined by electron microscopy. However, large numbers of unusual filamentous structures were found in xylem fluid from blighted trees (41). The nature and function of these filaments are unknown, but they do not contain proteins based on analysis by SDS-PAGE (KS Derrick, unpublished observations). When viewed with an electron microscope they appear somewhat similar to fibrous amyloid proteins associated with prions (44).

The reports associating *X. fastidiosa* and in particular the Pierce's disease strain of the bacterium (62, 64) with CB have always been questioned in Brazil. Pierce's disease has not been reported in Brazil, and the general consensus among Brazilian plant pathologists is that it is not present there. Prior to the discovery of citrus variegated chlorosis (CVC; discussed in detail in the next section), the only disease caused by *X. fastidiosa* reported in Brazil was plum leaf scald (70). First reported in the delta of the Parana River in Argentina in 1935, this disease apparently spread into southern Brazil in 1975. Following the discovery of CVC in Brazil, many citrus groves were surveyed for the presence of *X. fastidiosa* using serological (73) and PCR (6) techniques. In these surveys, *X. fastidiosa* was found in constant association with CVC and was not associated with CB. Moreover, in Florida, *X. fastidiosa* was rarely detected by PCR in citrus trees and was not associated with CB (6; KS Derrick, unpublished information).

Rootstocks and CB

Blight is a disease of producing citrus trees; symptoms have never been observed on trees less than about four years old. All rootstock-scion combinations and seedlings appear to be susceptible to CB, but the incidence of the disease varies with rootstock (22). Rough lemon, Rangpur lime, trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and Carrizo citrange (*C. sinensis* (L.) Osb. X *P. trifoliata* (L.) Raf.) rootstocks are very susceptible, and trees on these rootstocks may develop symptoms after about 5 years. Sweet orange, sour orange and Cleopatra mandarin (*C. reshni* Hort. ex Tan.) rootstocks are less susceptible to CB; with these rootstocks, symptoms usually occur on trees over l5 years old. Apparently, no rootstocks are tolerant to CB; affected trees show severe symptoms regardless of the rootstock.

Before the 1970s, the Florida industry relied on just two major rootstocks, rough lemon and sour orange (22). Rough lemon was favored by juice producers, for its high production, and sour orange was used by fresh fruit growers, for its higher fruit quality but lower production. The spread of isolates of CTV that induce decline on trees on sour orange rootstock has dramatically decreased the use of this rootstock in Florida. In 1986, approximately 31% of the registered nursery inventory was on sour orange; by 1996, this proportion had dropped to 0.7% (R Muraro, personal communication). Sweet orange is probably the most blight-resistant rootstock. Trees on this rootstock are usually over 30 years old before any decline due to CB is observed. Unfortunately, sweet orange is very susceptible to Phytophthora root rot. Cleopatra mandarin is a low-yielding rootstock, but produces good quality fruit and is widely used by fresh fruit growers in Florida. There has been added interest in this rootstock due to its reported resistance to CB. Trees on Cleopatra mandarin usually have little CB until about 15 years of age, at which time they often begin to decline rapidly.

Most trees planted in recent years in Florida are on CB susceptible-rootstocks. As the use of rough lemon declined in the 1970s due to CB, it was replaced

by Carrizo citrange, which is now suffering severe losses to CB. More recently, the use of Swingle citrumelo [*C. paradisi* Macf. X *P. trifoliata* (L.) Raf.] has increased, but it is also susceptible to CB, with a few trees as young as eight years old showing decline. Considerable research is under way to develop improved rootstocks resistant to CB, Phytophthora and CTV, to genetically engineer citrus for resistance to CTV using CTV genes or CTV-resistance genes from trifoliate orange, and to develop strategies to control CB.

CB-Associated Proteins

Blighted trees contain several blight-associated proteins (BAP) not found in healthy trees nor in trees declining from other disorders (40). Two of the BAPs (p12 and p35) have been partially characterized (18). p35 is constitutively expressed in leaves of healthy citrus, but accumulates in xylem of trees with CB. p35 was found to be similar to a β-1,3-glucanase by N-terminal sequence determination and serology. p12 is specifically expressed in leaves of trees with CB, but not in leaves from producing healthy citrus or trees affected with other disorders.

The N-terminal amino acid sequence of p12 is unique (18), and its gene, isolated from a cDNA library from roots of a tree with CB, encodes for an 11.8-kD protein with an N-terminal hydrophobic signal peptide (Gene Bank accession AF015782). The protein predicted by the ORF is 49% similar and 31% identical to an expansin from *Arabidopsis thaliana* (At-EXP2 accession U30481). Expansins are a highly conserved, multigene family of proteins that are associated with cell wall loosening during growth (106). Known expansins are approximately three times larger than p12 and are bound to cell walls, whereas p12 is soluble. A recent BLAST of the p12 protein sequence revealed a strong match to a hypothetical protein from *A. thaliana* (AHP; accession number AAD08935). The translated AHP has 126 animo acids, compared to 131 for p12 and 254 for the *Arabidopsis* expansin. The six cysteine residues of p12 and AHP align, and the two proteins have comparable putative signal peptides, a finding that appears to confirm the expectation that plants other than citrus have genes for p12-type proteins. Research is in progress to determine whether the AHP and p12 represent a new class of pathogenesis-related proteins that have expansin activity.

CITRUS VARIEGATED CHLOROSIS

The possible association of *X. fastidiosa* with CB has been closely examined. *X. fastidiosa* was rarely isolated from citrus, and it appeared that if it was causing CB, the titer of the bacterium was usually below detection levels. In contrast, a "new" disease, CVC, observed in Brazil in the 1980s, was found to be caused by a strain of *X. fastidiosa* (23, 61). The bacterium can be readily detected in infected citrus by several procedures (48, 73, 82), and PCR procedures have been developed that distinguish the CVC bacterium from other strains of *X. fastidiosa* (6, 93).

Since its initial observation, CVC has been spread rapidly by sharpshooter vectors and graft propagations with infected budwood. Because the bacterium is restricted to xylem, transmission through budwood is low but nevertheless sufficient for widespread distribution of the disease. CVC is now a major threat to the Brazilian citrus industry and is considered to be potentially more devastating than CB (74). CVC has not been found outside of South America, but probably existed in Argentina for many years, where it was called fruta bolita and erroneously considered to be a variant of CB (107). There are some similarities between CVC and CB; both diseases cause wilt symptoms due to xylem disfunction and show zinc deficiency symptoms in leaves. Consistent with the symptoms of many diseases caused by *X. fastidiosa*, there are severe necrotic lesions on leaves of trees with CVC that are not seen on trees with CB. Assays of CVC-affected trees for p12 are negative (73), except when then they also have CB, a common occurrence in Brazil (MJG Beretta, unpublished observations).

The incidence of CVC can be dramatically reduced by removal of symptomatic young trees and the affected branches of older trees (9). Procedures for control of CVC, based on pruning or removal of symptomatic trees along with insect and weed control, have been developed that are widely used in Brazil (V Rodas, unpublished information). Thus, infected citrus appears to be a major source of inoculum for in-grove spread of CVC by sharpshooters. In contrast, many strains of *X. fastidiosa* have wide natural host ranges, and alternate hosts may serve as a major source of inoculum for affected crop plants (96). Whether there are any alternate hosts that make a significant contribution to the epidemiology of CVC is yet to be established; no weed hosts have been reported. However, a strain of *X. fastidiosa* similar to the CVC strain has been associated with a disease of coffee in Brazil (8).

CITRUS PSOROSIS

In 1933, Fawcett (45) observed flecking in young leaves of trees with psorosis (bark scaling) and suggested that the disease was caused by a virus. At that time, graft-transmissible diseases were, in general, considered to be caused by viruses. Citrus psorosis virus (CPV) was the first virus disease of citrus to be described. Subsequently, a number of graft-transmissible pathogens of citrus that cause bark scaling and/or chlorotic patterns on young leaves were placed in a loosely defined group of psorosis-like diseases including psorosis A, psorosis B, ringspot, concave gum, cristacortis, impietratura, crinkly leaf, and infectious variegation (102, 113). Citrus ringspot virus (CRSV) was described in 1968 (127). The leaf and bark symptoms caused by psorosis A and B and CRSV are similar. They were traditionally considered to be different diseases based on symptom severity (102, 113, 116), but are now known to be caused by strains of the virus associated with psorosis in 1988 (31). Ringspot and psorosis were at one time more or less synonymous; indeed, the first isolate of CPV to be characterized was called CRSV. It has been

recommended that the use of "ringspot" to describe psorosis be discontinued (102), which would reduce confusion, inasmuch as at least two other diseases of citrus have been named ringspot, both unrelated to psorosis, one from India (16) and another from Spain (85). Concave gum, cristacortis, and impietratura continue to be graft-transmissible diseases of unknown cause, but crinkly leaf and infectious variegation have been shown to be caused by distinct ilarviruses (129).

The association of foliar symptoms with CPV led to the development of procedures for biological indexing on indicator plants for selection of budwood source trees free of the disease. The introduction of budwood certification schemes for citrus have proven very dependable for CPV in some countries. Trees with psorosis do not develop bark scaling for several years, and before procedures were introduced for identifying CPV-free budwood, psorosis was a major disease of citrus worldwide (102). There are no known vectors of CPV, and in many parts of the world the primary means of spread of the virus is through infected budwood. Thus, using CPV-free budwood is an excellent control strategy in these areas. However, in Argentina, Brazil, Uruguay, Texas, and India, CPV appears to have an unknown aerial vector (102) resulting in spread of the virus and considerable tree loss.

Identification of CPV

Characterization of CPV was an enigma for many years, and little was known about the virus until 1986 (37–39). The virus has a wide experimental host range (117), and some isolates can be transmitted mechanically and induce local lesions on *Chenopodium quinoa* (53, 54). Considerable effort was made to characterize CPV prior to 1985. Isolates were available that had been passed through single lesions on *C. quinoa* and back to citrus. Procedures had been developed for virus extraction and concentration of infectivity by differential centrifugation, but all infectivity appeared to be lost following sucrose gradient centrifugation. No virus particles could be detected by examination of partially purified preparations by electron microscopy, which prompted suggestions that the virus was probably a labile, isometric particle (SM Garnsey, D Gonsalves & RF Lee, unpublished information). CPV was partially characterized after finding that the infectivity is separated into at least two components by sucrose density gradient centrifugation (38, 39). Virus particles were observed following production of an antiserum to partially purified virus using serologically specific electron microscopy (SSEM). The particles, filamentous spirals of two distinct lengths, appeared to be novel and were named spiroviruses (42). The particles could only be seen by using a positive stain (37); particles given a negative stain appeared as indistinct thin threads (KS Derrick, unpublished observations). In a report (51) published in l994, threads of 3 to 4 nm in diameter were observed in electron microscope examinations of negatively stained CPV preparations. The authors stated that these threads with novel filamentous morphology are collapsed double-stranded forms of nucleocapsid-like, highly flexuous open circles. The authors suggested further that the 10-nm diameter linear, spiral particles reported in 1988 (37) are a somewhat

misleading form of a more fundamental particle. In contrast, it has been argued that the 10-nm diameter particles, detected by positive staining, are probably the native form (4); they have a more distinct morphology than the 3- to 4-nm threads, which may be produced by disruption of the native particles by the high ionic concentrations associated with negative staining. Disruption of the CPV particles by negative staining may account for failures to detect the particles in leaf-dip and partially purified preparations using negative staining that were described in earlier unpublished studies.

Properties of CPV

CPV is a multicomponent ssRNA virus with a capsid protein of 48 kD. The ssRNA viral genome is in short (300–500 nm) and long (l500–2500 nm) filamentous particles (36–38). A sedimentable dsRNA that cosediments with long particles is also associated with CPV (43). The sequence of the 48-kd coat protein gene was determined and does not match any known sequence. A single, negative-sense ORF that encodes for the capsid protein is associated with the short particle (4).

Detection Methods for CPV

There is considerable variation in the symptoms induced by different CPV isolates (31, 102, 117) and there is serological diversity among isolates (42). Indexing of citrus budwood for psorosis is still done by graft inoculation of citrus indicator plants and observation of leaf symptoms, which can be transitory and very mild. Furthermore, some isolates apparently do not induce leaf symptoms (91). Bioindexing for psorosis is expensive and time-consuming and obviously fails for any isolates that do not induce leaf symptoms. Serological or other nonbiological indexes are needed for psorosis. Serological (42), riboprobe, and RT-PCR (4, 52) based detection methods for some isolates of CPV have been developed, procedures that readily detect known isolates under greenhouse conditions. Due to low titer, uneven distribution of virus and the considerable diversity of isolates, biological indexing will remain the preferred method for CPV detection. In addition, there are unknown graft-transmissible agents in citrus that induce bark scaling that are not associated with CPV (GA Barthe & KS Derrick, unpublished observations).

LETTUCE BIG VEIN

Lettuce big vein (LBV) is a disease that probably has a single causal agent, but progress in understanding its etiology has been slow. The causal agent, like that of CB, seems to be localized in roots, but the agent is readily transmissible. With modern molecular tools and the availability of rapid assays, LBV would seem to be relatively easy to address. Nevertheless, it has continued to baffle many experienced, capable investigators.

Lettuce big vein was first reported in the United States in 1934 (69), but has been found subsequently in Europe, New Zealand, and many other producing areas (68). The disease was assumed to be caused by a virus due to the lack of association with any fungus or bacterium, and the presence of symptoms resembling those caused by virus diseases. Nevertheless, the LBV agent was not transmitted mechanically, by aphids or through seed (98, 112).

Little progress was made on the etiology or control of LBV for many years following its discovery. In the late 1950s, soil transmission of the disease was demonstrated, and the disease was associated with the soilborne, obligately parasitic fungus *Olpidium* (50, 59). Campbell & Grogan (20) demonstrated that *Olpidium brassicae* was the vector of the LBV agent. Filtration techniques that removed zoospores of *O. brassicae* from soil suspensions also removed infectivity. Cultures of *O. brassicae* free of the LBV agent were established and could be used in transmission experiments. Thus, an essential tool for further study of the disease was developed.

Campbell and coworkers (21) graft-transmitted the disease to healthy lettuce using crowns and roots of affected plants, thereby demonstrating that the disease was caused by a systemic agent. An association of LBV with tobacco necrosis virus (TNV) has been reported (49, 50, 133). However, TNV is readily transmissible mechanically and no virus was consistently transmitted mechanically from lettuce with big vein symptoms. Campbell & Grogan (20) only rarely found TNV in association with field-grown plants affected by big vein. Even when TNV was present in an initial culture, it was lost on repeated transfer of the LBV agent with *O. brassicae*. Thus, TNV was discounted as a causal agent of the disease.

New techniques in virology for purification and detection of viruses have been applied to the study of the LBV agent, albeit with mixed success. The presumed viral nature of the LBV agent was supported by studies of Campbell (19), who demonstrated that ribavarin, an antiviral product, suppressed big vein symptoms in lettuce. Mirkov & Dodds (83) demonstrated that certain dsRNAs were associated with LBV. They found that the dsRNAs were recovered consistently from roots but not from the tops of LBV-affected plants. Graft-inoculated LBV-affected plants that were free of *Olpidium* still carried the dsRNAs. Cultures of *Olpidium*, free of the LBV-agent, acquired and transmitted the dsRNAs to healthy plants. Consistent with the conclusions of previous investigators about TNV, they found that the dsRNAs from big vein-affected plants did not hybridize with TNV, and TNV was not detected in LBV-affected plants in the field. A rod-shaped virus was observed by electron microscopy in preparations from LBV-affected plants, but it was also found in many healthy plants. Thus, the search for the causal agent appeared to have been narrowed to an RNA virus, although the nature of that virus was still not clear except that it was probably not TNV.

Lettuce big vein has now been associated with tobacco stunt virus (TSV). However, the virus has not been directly transmitted from LBV-affected plants to healthy lettuce. Huijerts and coworkers (68) consistently transmitted an agent from LBV-affected lettuce which caused local lesions on *Chenopodium quinoa* and leaf

curling and stunting on *Nicotiana occidentalis*. To demonstrate the relationship to big vein, *N. occidentalis* plants were mechanically inoculated from LBV-affected lettuce and rooted cuttings were prepared from those plants. Virus-free cultures of *O. brassicae* were then used to transmit the agent from *N. occidentalis* to healthy lettuce, and big-vein symptoms were reproduced. The virus in *N. occidentalis* resembled tobacco stunt in particle morphology, mechanical transmissibility, instability in sap, and its symptoms of *N. occidentalis*. Vetten and coworkers (126) also associated LBV with a rod-shaped virus.

Although the indications are that LBV is caused by TSV, the evidence is still indirect. The absence of obvious virus particles in symptomatic tissue and the difficulty of transmission from such tissue leave doubt about the true cause. More recently developed serological and molecular techniques have not been utilized to confirm or refute the hypothesis that big vein is caused by TSV. Much more remains to be done, but the characteristics of the system make it difficult to study.

DISEASES OF COMPLEX ETIOLOGY

Diseases already described such as CB, psorosis, and LBV probably have single causal agents but continue to pose a challenge for investigators. However, disorders and diseases that are caused by more than one agent may prove to be even more difficult to completely resolve. Many such disorders are partially attributable or complicated by the effects of environmental and edaphic factors. It is often not possible to completely reproduce the disorder experimentally. Thus, Koch's postulates, the standard of proof in pathology, often cannot be completely fulfilled. However, this is still a reasonable goal, and hypotheses as to the causal agents of these disorders that cannot, at least partially, meet these tests should be discarded.

In this section, we present some examples of diseases of complex etiology, which have been resolved to some extent. Thus, the approaches used can be of some value in understanding these kinds of diseases. We cite other less well-studied diseases in which there is no good indication that one of the many causal agent(s) that have been suggested is the correct one.

Peach Tree Short Life

In dealing with diseases that do not appear to be caused by a single agent, it is first essential to define a syndrome. Little progress was made on peach tree short life (PTSL) until this was done. Numerous ill-defined disorders of peach were observed that were called peach tree decline, peach replant problem, and peach tree survival (101). These were complicated by other known factors such as freeze injury, bacterial canker caused by a pseudomonad, and Cytospora canker. In the early 1970s researchers agreed to use the term PTSL for a specific identifiable syndrome. Trees affected by PTSL usually died to the ground in late winter or

early spring. Roots did not die and often resprouted. The immediate cause of canopy death was freeze injury and/or bacterial canker. The disorder was much more common on old peach soils rather than on new land.

Once the condition was better defined and diagnosis of a specific condition was feasible, the conditions affecting it could be investigated. Fall pruning was determined to significantly increase the disease incidence (33, 88): the trees' dormancy was disturbed, making them susceptible to freeze damage following warm periods in winter. PTSL was much more common on acid soils than on soils with a pH above 6. Soil fumigation of old peach soils reduced disease incidence (88, 135). Some rootstocks were more susceptible to the problem than others (60, 101). Nyczepir and coworkers (89) demonstrated the role of *Criconemella xenoplax* in the disease. In the absence of this nematode, PTSL nearly disappears, which explains the effect of soil fumigation and rootstock on the disease. Despite its common occurrence on PTSL-affected trees, bacterial canker could not be consistently associated with the disease, nor could Prunus necrotic ringspot virus (99).

While the PTSL problem has not been completely resolved, the syndrome and its causes are much better understood. The primary predisposing factor is high populations of *Criconemella*. However, trees do not usually die unless the dormancy is disturbed by high temperatures in winter or by early pruning (99). Other edaphic factors that are involved are not completely understood. However, PTSL can be regulated by controlling nematodes by soil fumigation, nematicides and/or resistant rootstocks, by liming soils to pH 6 or above, and by delaying pruning to late winter or early spring.

Replant Diseases

Replant diseases occur when young, healthy nursery trees are planted on old orchard sites, a phenomenon noted on various tree fruit crops for over 200 years. Usually, newly planted trees grow very poorly, are stunted, and have small leaves and short internodes. Root systems may be reduced, fibrous roots may be decayed, and if trees live, they are slow to come into production. Replant problems do not usually represent a single disease, and again with these types of problems, it is important to define a specific syndrome prior to initiation of etiological studies.

Replant problems have been attributed to numerous edaphic factors such as inappropriate pH, nutritional problems, poor soil structure, contamination by heavy metals, and by poor drainage. Although these problems may be contributing factors or even the sole cause of poor growth of young trees, typical replant problems usually have a biological basis (121). Fumigation of soils usually eliminates the problem and allows trees to grow normally (78).

While most replant diseases are biologically based, the importance of the role of different organisms varies with the situation. On apples, both specific and nonspecific replant problems have been described (78). Nonspecific replant disease not only affects trees of the same tree crop originally planted, but also of related tree crops. Its distribution is patchy in the field and it is usually associated with

populations of plant parasitic nematodes. Specific replant problems affect only the tree fruit crop that was originally planted on the site. All trees on a site tend to be uniformly affected by the disorder.

Various approaches have been used in determining the causal agents associated with different replant problems. Treatment with broad-spectrum fumigants or steam can be used to determine whether the problem has a biological basis. Subsequently, treatments with specific pesticides such as nematicides, fungicides of various specificities, bactericides, or antibiotics can be useful in elucidating the nature of the causal agent. Surveys and sampling of soil in orchards with and without the replant problem can help ascertain which pathogens are associated with the problem and which are widespread. Suspected organisms can be added to sterile soil alone or in various combinations to determine which are the primary factors. When these approaches were used with apple replant disease in Washington, bacteria were eliminated as a causal agent since plants did not respond to chloramphenicol treatment (81). *Pratylenchus* was eliminated because populations were low and stunted trees did not respond to nematicide treatment. Stunted trees responded to treatment with metalaxyl and difenconazole. Based on surveys and pathogenicity tests, the disease was attributed to *Cylindrocarpon, Rhizoctonia, Pythium, and Phytophthora* spp. The species most responsible for the problem appeared to vary with the site.

Not all apple replant diseases are due to fungi, however. In the northeastern USA, the primary factor may be nematodes, alone or in combination with plant pathogenic fungi (78). In some cases, actinomycete-like organisms may also be involved (128). Thus, in each tree crop and in each location, the primary pathogens must be determined before adequate control measures can be developed. It is not always possible to totally account for the growth reduction in all cases, but the problem can be alleviated by addressing the most important ones.

Rio Grande Gummosis

In some cases, the causal agent(s) is simply not known despite many attempts to identify it. Rio Grande gummosis (RGG) of citrus is one such disease. With this problem, gum exudation from the trunk and scaffold branches is profuse. Wood is often stained orange or pink and has large gum pockets. The disease is usually associated with rots of sapwood that follow freeze damage, mechanical injury, or weakening from shading. It occurs most commonly in the Caribbean Basin.

Many fungi such as *Lasiodiplodia theobromae* and wood-rotting basidiomycetes can be isolated from wood of affected trees. Many of the symptoms of this disease can be reproduced by inoculation with these organisms (34). However, since these symptoms are nonspecific, it is still uncertain whether these are the primary or sole cause of the disease. Childs (26) associated the disease with high levels of chlorides in the soil and with the use of fertilizers containing chlorides. The disease has also been associated with the presence of a psorosis-like agent (94). Psorosis produces gumming in the trunk like RGG but also produces bark

scaling. However, psorosis is caused by a virus and is graft-transmissible. It probably does not cause RGG but can be a complicating factor in diagnosis.

Resolving the causal agent in this case will be difficult. First, the disease affects only bearing trees so any study requires several years. Second, it is difficult to know that one has truly healthy trees free of the problem to study. Thus, initial investigations have to rely on survey information to develop consistent associations before experimental studies can begin.

Dry Root Rot–Sudden Death

This is another disorder of citrus that does not appear to have a single cause and may not be attributable to an organism. Trees affected by dry root rot have blackened, dead lateral roots. Scaffold roots usually have a brown discoloration, which may extend up into the crown area in the late stages. Often trees appear healthy until the root system is nearly entirely rotted, and then they collapse and die suddenly.

Many microorganisms can be isolated from the margins of discolored wood including *Fusarium solani* and *Coprinus micaceus* (15, 57, 108). The symptoms of dry root rot can be reproduced by inoculation with *F. solani* but only when the trees are severely stressed by water logging, excess ammonia fertilizer, or repeated pruning. In the field, dry root rot–sudden death is associated with soils with high clay content, excessive moisture and poor aeration, or with mechanical injury to roots. Often it is possible to identify the cultural factors associated with the problem and reduce disease incidence by improving drainage or soil porosity. However, the disorder may also appear where there are no obvious cultural factors to help explain its occurrence.

Disease problems of this nature are very difficult to address and often require many years of field studies. Advances in genetics or physiology are unlikely to be useful in addressing these problems, although improved diagnostic techniques may be helpful.

CONCLUDING REMARKS

Much of the excitement in plant pathology is now in genomics, transgenics, and studies of host/parasite interaction at the molecular level. Many young scientists probably have little appreciation of the effort it took to identify the pathogen that they are now studying with molecular methods. Detection, identification, and characterization of pathogens, once the main business of plant pathology, is now often considered passé. But, there are probably diseases of unknown or uncertain cause affecting most economic crops, some of which are very serious. There may be some completely unknown organisms that cause some of these diseases. Pursuit of these remaining infectious agents can be exciting, but requires a certain mindset and patient funding, usually not readily available. Becoming a “latter-day microbe hunter” is not recommended for those seeking tenure or frequent

gratification. However, tenured plant pathologists looking for excitement or a change of pace might consider working on a disease of unknown cause. It will take you back to your roots.

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