Cross Protection Against *Citrus tristeza virus* - a Review

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ABSTRACT. Tristeza, caused by *Citrus tristeza virus* (CTV) is now in its second century as one of the most destructive and most researched diseases of citrus. This review encompasses the early history of tristeza and its relationship to the 19th century *Phytophthora* epidemic which caused worldwide destruction of citrus then grown primarily as seedlings. The sour orange then evolved as a highly regarded and popular *Phytophthora*-tolerant rootstock. However, this combination of sweet orange, mandarin or grapefruit on the sour orange rootstock was susceptible to a new highly destructive vector-transmitted disease aptly named tristeza. There are two primary vectors for CTV: *Aphis gossypii* and the more efficient *Toxoptera citricida*. When tristeza enters a country, sour orange ultimately will disappear as the primary rootstock. All attempts at cross protection to salvage sour orange as a rootstock have failed. In the presence of the efficient vector *T. citricida*, severe stem pitting isolates of CTV evolved, which began a second wave of tristeza destruction with symptoms of severe stem pitting, small fruit size, and low yield. By searching for trees which survived, followed by extensive experimentation, protective isolates of stem pitting/small fruit CTV were found in Brazil, Argentina, Australia, South Africa, India, and Japan and recently in Peru. It may take 10 to 15 yr or longer to find and develop effective protection against stem pitting tristeza as was done by Costa and Müller in Brazil, van Vuuren in South Africa and by Bederski in Peru. The protective CTV isolates developed by Müller and Costa have proven highly effective for over 40 years in Brazil. A new technique is presented for creating protective CTV isolates by attenuating severe isolates by passage through *Passiflora gracilis* or *P. caerulea* via aphid transmission. Some of these isolates have proven highly effective in the revival of the citrus industry in Peru. The spread of *T. citricida* into Central America, the Caribbean islands, Mexico, Florida, the Madeira Islands, northern Portugal and Spain should stimulate concern for all citrus growing areas where this aphid is still not present, and argues strongly for accelerated research on cross protection. For a complete picture slide show on tristeza cross protection see EcoPort slide show #103.

With *Toxoptera citricida*, the principal vector of *Citrus tristeza virus* (CTV), currently present in northern Portugal and Spain and in Central America, Mexico, Florida and the islands of the Caribbean, its spread to the other citrus producing countries of Europe, North Africa, Texas and California is certain. The sour orange as a rootstock will ultimately disappear and new and severe CTV strains will eventually appear. Since *T. citricida*, the most efficient vector of CTV, has the ability to spread severe stem pitting strains, the only currently effective procedure to continue a citrus industry in the face of the severe stem pitting isolates is cross protection. This paper reviews the history of cross protection in citrus (one of the very few tree crops where cross protection has proven effective) and begins with the history of tristeza epidemics, the use of tristeza-tolerant rootstocks and cross protection for the survival of a citrus industry where severe and destructive stem pitting CTV isolates have emerged.

Early history and emergence of a new disease. China is probably the home of many species of citrus and also may be the original home of CTV. The probable routes of tristeza through movement of infected plants and vectors were from China to Japan, to the Philippines, India, Australia, and South Africa. CTV and its principal vector *T. citricida* became endemic in these countries. It is probable that CTV was present in California and Florida in the

1880's with the importation of satsuma mandarin trees from Japan (66). Wallace et al. (81) reported: *"Records found in South Africa indicate that 1,400 Lue Gim Gong orange trees on rough lemon rootstock were exported to the Argentine in July, 1930, followed by a further exportation of 250 navels, 500 Valencias 350 Lue Gim Gong and 100 Ruby blood on rough lemon rootstock about one year later."* It is almost certain that these trees were infected with CTV and most likely the vector *T. citricida* was present on the exported trees. Fraser and Broadbent (18), in reviewing the history of tristeza in Australia, indicated the presence of the disease and its most efficient vector *T. citricida* well before 1890 and perhaps earlier than 1870. They also indicated that there is evidence that in 1933, large shipments of citrus trees were sent from Australia to estates being developed in Argentina.

There may be a direct relationship between the great *Phytophthora* epidemics of the 19th century and the ensuing tristeza epidemics which began in the 1930's. The invention of the Wardian case or terrarium in 1827 probably led to a more effective method for shipment of plants across wide distances by specially constructed terrariums or Wardian cases aboard sailing ships (47, 86, 87, 88) (Fig.1). However, not only were plants moved from country to country, but also the soil beneath the plants. Thus, dangerous *Phytophthora* species were transported and disseminated worldwide. It is probable that the great *Phytophthora infestans* potato blight epidemic which began in 1845 was due to the introduction of this fungus in soil brought to Ireland aboard a sailing ship in a Wardian case (on display at the Smithsonian Institute, Washington D.C.). Similarly, citrus *Phytophthora* species were probably also introduced to the countries of Europe by this means, thus inducing the worldwide destruction of citrus seedling trees by *Phytophthora* beginning in 1836.

Fig. 1. A large Wardian case (terrarium) used in 1834 to transport plants on sailing ships to and from the Antipodes. Reproduced from (88).

Prior to this date, citrus worldwide were grown primarily as seedling trees. Bonavia (8) reported: *"In the Azores the trees up to 1836 were in perfect condition. Then, 200 and 300 year old trees which were producing 6,000 to 20,000 oranges began to die. On the trunks, and sometimes beneath the ground the bark opened and drops of tears of yellow gum exuded, hence the name `lagrima' from the Portuguese word for tears."* This was *Phytophthora*!

Fawcett (15) described the incredible destruction of seedlings trees worldwide by the introduction of *Phytophthora* into the soils where old and established seedling trees had been prospering for years. *"Beginning with the first recorded outbreak of commercial importance in 1832 in the Azores, it was found causing trouble in Portugal in 1845, in Hyères southern France, in 1851; in northern Italy in 1855;*

in Greece about 1860; in Messina, Sicily, in 1863; in Reggio, Italy, 1864; in Palermo, Sicily, 1865; in Genoa, 1870; and in the Balearic Isles, 1871. About this same period, 1860-1870, it was reported in New South Wales, Australia. In the United States, it attracted attention in California in 1875 (30) and in Florida about 1876. It had attracted attention in South Africa by 1891. It is probably the most destructive disease which occurs in Japan at the present time and is also of general occurrence in China and the Philippines. It was the first disease to attract serious attention in Florida, and, although known there since 1876, the first serious outbreak was reported in 1879 following a year of very heavy rainfall. Between 1869 and 1880 it destroyed all the lemon trees in the islands of Paros, Euboea, Chios, and the entire Aegean Archipelago, and between 1910 and 1916 it destroyed all the citrons in eastern Crete. Trees replanted in this region are now mainly on sour or Seville orange stock."

Fig. 2. The destruction of seedling trees by *Phytophthora* led to the development of citrus trees grafted on *Phytophthora* tolerant rootstocks such as the sour orange which is still grown in Mediterranean city streets. Shown here are seedling trees of sour orange growing in Seville, Spain.

The sour orange as a rootstock. The destruction of seedling trees of citrus by *Phytophthora* led to the development of grafted trees of desired scions on specific rootstocks. The sour orange growing as seedling trees in Spain (Fig. 2) was found to be both highly tolerant to *Phytophthora* and also to be an excellent rootstock; thus it was quickly adopted as a rootstock throughout the Mediterranean area to replace seedling trees destroyed by *Phytophthora.* It was later adopted in North and South America as the primary rootstock (3, 4). The sour orange was tried as a rootstock in Australia prior to 1870 (15, 18) and it attracted attention in South Africa (43).

Fig. 3. A 9-yr-old sweet orange on sour orange rootstock showing poor growth in South Africa in 1900. This budded tree had been imported from California and all imported varieties of sweet orange, mandarins or lemons budded to sour orange rootstock showed poor growth or died in a relatively short time.

Its failure in both countries was thought to be due to incompatibility, but was in fact due to tristeza. Tristeza as a disease was not known at that time and its prime vector *T. citricida* was probably well established in both Australia and South Africa. When sweet orange was put on sour orange rootstock in South Africa under an instruction from the Cape Agricultural Department in 1896, the trees would not grow and they ultimately died (43, 89). Also, all varieties of sweet orange, mandarins or lemons budded to sour orange died in a relatively short time (43) (Fig. 3).

Death of citrus on the sour orange rootstock. The first report of decline and death of sweet orange trees on sour orange rootstock in Argentina in 1930 was by Zeman (92). Bitancourt (7) reported on a similar death of trees on sour orange rootstock in Brazil occurring in 1937. Toxopeus (69) suggested that the incompatibility of sweet orange on sour orange rootstock was due to a substance which was formed in the sweet orange canopy and when transported across the bud union was lethal to the sour orange rootstock. The combination of sweet orange budded to sour orange as a rootstock failed in all parts of Java and symptoms were identical to the failure reported for this combination in South Africa. Yet, he cited that this combination of sweet on sour was highly successful in so many other countries.

Webber (89), visited South Africa and observed that the sour orange as a rootstock failed everywhere in the country. He reported that the failure was not due to off-type sour orange rootstock seedlings, incompatibility, soil or climate differences and he was the first to postulate a theory suggesting this new disease was vector-transmitted and caused by a virus. Webber (90) concluded that: *"the disease was most likely caused by some virus that might be carried to the sweet orange by an unknown vector --- a virus not noticeable in its effect on the sweet orange but when introduced into the sour orange, is toxic to that species."* His analysis made over half a century ago was prophetic and accurate.

With the known importation of citrus from South Africa and Australia to the new

citrus plantings in South America, a new devastating disease emerged and began to ravage the citrus of Argentina and Brazil. It was appropriately named 'tristeza' by Prof. Silvio Moreira, which in Portuguese and Spanish means sadness (31). Knorr and Ducharme (22) visited Argentina in 1950 as part of a joint USDA project with Argentina and Brazil to study this new disease and they reported on the devastation observed.

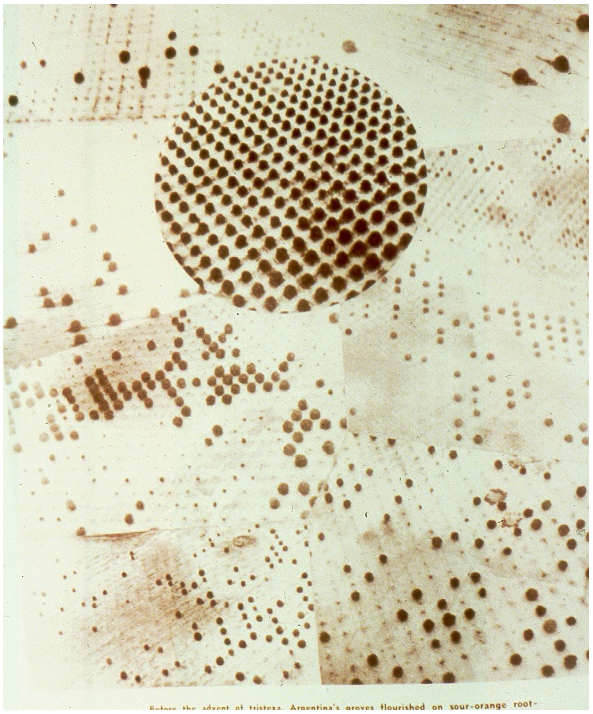

Fig. 4. Drs. Knorr and Ducharme visited Argentina in 1950 as part of a joint USDA project with Argentina and Brazil to study this new disease (tristeza) and they reported on the devastation they observed. This figure reproduced from their paper (22) and they wrote: *"Before the advent of tristeza, Argentina's groves flourished on sour orange rootstock and presented the luxuriant panorama shown in the inset, center. Today, two short decades since the introduction of infected stock from overseas, thousands of groves lie devastated, reflecting from the air the dismal aspect of a checkerboard at game's end."*

What they saw (Fig. 4) was poetically written as follows: *"When for the first time we looked down on the citrus acreage between Buenos Aires and Concordia we saw a sight, that in its desolation exceeded all anticipation. It was so calamitous, so appalling and heart-rendering, that we felt no previous account ever pictured tristeza's rapacity."*

The destruction of sweet orange on sour orange rootstock in California began in 1939. It was called `quick decline' because the trees declined very rapidly during the spring. This decline was first noted in the Covina-Azusa area of Los Angeles County in 1939 and by 1945 had increased to about 23,000 collapsed trees (64). At that time, the cause of this decline was not known. All research resources of the State were mobilized and personnel from the University of California at Riverside and the California Department of Food and Agriculture began intensified studies and surveys of this problem. Death of trees by the new `quick decline' of citrus was shown to be related to the death or necrosis of the phloem cells in the cambium tissue of the sour orange (61) (Fig. 5). This effectively girdled the tree at the bud union. With death of these phloem cells, the starch produced in the leaves of the canopy was prevented from being transported to the roots; the roots decayed and died when CTV titer was high over the cool winter months. When the weather warmed up in the spring the trees quickly declined - hence the name `quick decline'. In transmission experiments by Fawcett and Wallace (16), 100 young budlings of Valencia on sour orange rootstock were graft-inoculated with 3 buds from quick decline infected trees, and one hundred similar trees were left uninoculated After 2 yr, 86% of the inoculated trees showed decline, thus implicating a transmissible pathogen.

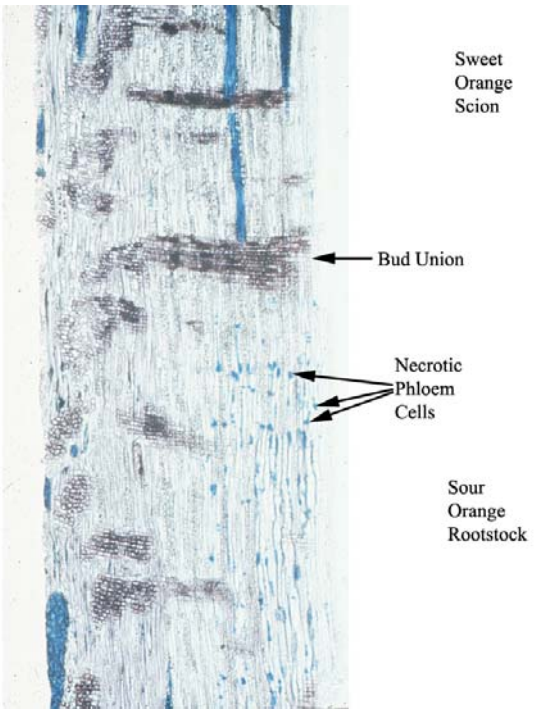

Fig. 5. Death of citrus trees by 'quick decline' was shown to be related to necrosis of the phloem cells in the cambium tissue of the sour orange rootstock (shown as blue stain below the bud union). Dr. Henry Schneider pioneered these studies on phloem necrosis (61, 62).

The vector. In Brazil, Meneghini (29) showed transmission of an apparent virus by the aphid *T. citricida*, (called *Aphis traversi* in that publication) and Costa and Grant (11) showed that a single aphid of *T. citricida* could transmit the virus. Dickson et al. (14) showed that *Aphis gossypii* was the primary vector for CTV in California even though it represented only 4% of the aphid population and he showed a transmission rate of less than 5%. Norman and Grant (39) showed that *A. spiraecola and A. gossypii* would transmit CTV in Florida and also at low rates of transmission. Yokomi and Damsteegt (91) compared the efficiency of *T. citricida* and *A. gossypii* on transmission of five severe and exotic isolates of CTV from various countries using single aphids. The results showed *T. citricida* to be 83 to 96% more efficient than *A. gossypii* in its ability to transmit CTV.

Types of tristeza. In a comprehensive three part series reviewing his lifelong experience with tristeza, McClean (26, 27, 28) described the three types of declines induced by CTV as follows: (i) The necrosis of the phloem cells in the sour orange rootstock just below the bud union causing girdle and death of trees. (ii) Seedling yellows tristeza - strains of CTV which induced a seedling yellows reaction in certain seedlings such as lemon, grapefruit or sour orange. (iii) Stem pitting tristeza which induced stem pitting in the scion or rootstocks of lime, grapefruit or sweet orange.

Rootstocks and cross protection for CTV decline on sour orange. When tristeza destroyed millions of trees on the sour orange rootstock in South America, California, Spain, and elsewhere, the fortunate fact that certain tristeza-tolerant rootstocks could be used to grow citrus successfully allowed for the replanting and rehabilitation of citrus industries worldwide. New rootstocks were tested and found to be resistant to the bud union phloem necrosis induced by CTV. Rootstocks found tolerant to CTV are the trifoliate orange and its hybrids such as the Troyer or Carrizo citrange and citrumelo. Also, rough lemon, Volkamer lemon, Rangpur lime and Cleopatra mandarin were all found tolerant to CTV and the sour orange was replaced as the primary rootstock by these tolerant rootstocks where tristeza epidemics occurred. However, without a certification and indexing program, untested scions harbored the exocortis or cachexia viroids and additional decline problems occurred (57).

The sour orange was, and still is, such a superior rootstock that attempts were made to find isolates which would cross

protect sweet orange on sour orange rootstock. A number of experiments were done worldwide in this attempt and all have failed. The dying trees on the left in Fig.6 show the failure of protected sweet orange on sour orange rootstock in Australia (65, 67, 68). In California, Wallace and Drake (82, 83, 84, 85) working with recovered shoots from seedling yellows infected plants discovered that CTV strains in these recovered shoots would provide protection of sweet orange on sour orange rootstock. Protected trees were put in the field at the University of California at Riverside. For the first 6 yr results were very promising. However, trees then began to decline. In Florida, Cohen (10), Pelosi and Powell (41) and Powell et al. (42) reported initial success in protecting Pineapple sweet orange on sour orange rootstock using a number of protective isolates. However, protection also broke down with time. In South Africa, van Vuuren et al. (71) tested 11 mild CTV isolates against exposure to natural South African field isolates of CTV. All failed in this experiment. To date out of over 100 million trees worldwide which were destroyed on sour orange rootstock by tristeza (58), no effective protective CTV isolate has emerged to be in general use. An alternative route currently under investigation is to try and create somatic hybrids of mandarin and pummelo in an attempt to develop a replacement for sour orange which has all its good attributes, but does not succumb to CTV (21).

Fig. 6. The failure of sweet orange on sour orange rootstock in Australia as observed by the senior author in 1979 (65). Note the decline of the four trees on the left on sour orange rootstock. The two healthy trees on the right are presumably on a tolerant rootstock. Apparently new severe CTV strains appeared and were highly destructive to these trees on the left.

Seedling yellows cross protection. A second type of CTV called seedling yellows induces a severe reaction in seedlings of lemon, grapefruit or sour orange. Symptoms are a severe reduction in size of the inoculated seedling plus severe chlorosis of the foliage. This symptom was first reported and shown by Fraser (17) (Fig. 7).

Fig. 7. Dr. Lillian Fraser first reported the seedling yellows reaction in seedlings of lemon (17). Lemon seedlings, when inoculated with certain severe CTV isolates, may show this seedling yellows reaction (left). The uninoculated control plant is on the right.

There is evidence that this more severe form of CTV can affect citrus scions causing debilitation and even death of the trees (44). Seedlings of Eureka lemon, grapefruit or sour orange are excellent indicator plants for the detection of seedlings yellows tristeza. Following up on the studies of Wallace and Drake (82, 83, 84, 85), Roistacher et al. (53) obtained a number of isolates of seedling yellows tristeza from recovered shoots of grapefruit, lemon and sour orange. Out of 42 isolates they collected from recovered shoots, 28 were rejected and 14 were held for further research. All of these isolates were grafted to sweet orange as holding plants in our virus bank.

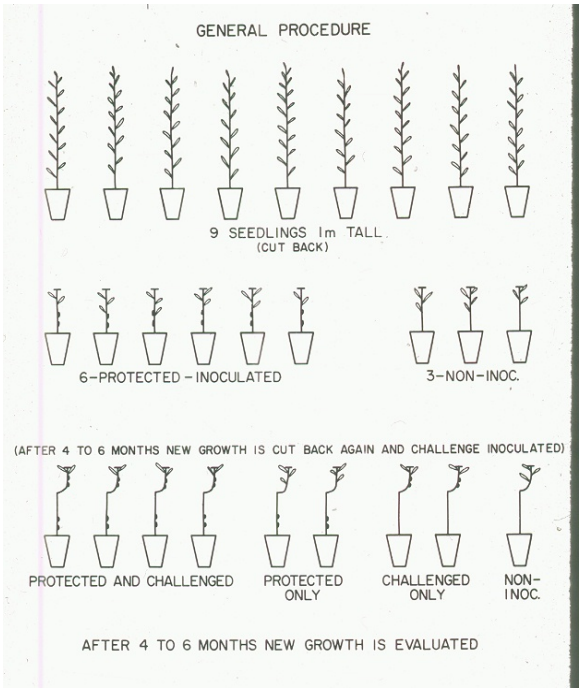

Fig. 8. The technique for evaluating cross protection in greenhouse plants: Nine seedlings of sweet orange or grapefruit are grown to about 1 m and then cut back. Six seedlings are inoculated with the protective isolate and three seedlings are left uninoculated. After about 3 mo of new growth of a single stem, four of the seedlings which had received the protective inoculum are challenged with a severe seedling yellows or stem pitting CTV isolate; the other two seedlings are left containing only the original protective isolate. Of the three seedlings so far uninoculated, two are inoculated with the seedling yellows or CTV-stem pitting source, as the positive control. One seedling remains uninoculated as the negative control. All seedlings are then cut back to force new growth. Diagram from Roistacher et al., (55). See Fig. 18 for the results.

Seedling yellows cross protection. The technique used for testing protective isolates is shown in Fig. 8 from Roistacher et al. (55). Nine seedlings of grapefruit were grown to about 1 m and cut back. Six seedlings were graft-inoculated with the protective isolate and three seedlings were left uninoculated. After about 3 mo of new growth of single stems to approximately one meter, four of the six seedlings which had received the protective inoculum were challenged with the original severe seedling yellows isolate and the other two were left as they were, containing only the original protective inoculum. Two of the three seedlings which were not previously inoculated were tissue-grafted with the original severe seedling yellows isolate as the positive control and the remaining seedling was left as the uninoculated negative control. All seedlings were then cut back to force new growth.

Eight of 14 isolates obtained from recovered shoots of seedling yellows, when tested for protection against the original seedling yellows isolates from which they were derived, showed excellent cross protection. Shown in Fig. 9 are greenhouse results for three seedling yellows protective isolates QQ, KK and TT. None of these eight successfully protective seedling yellows isolates had been field tested. It is probable, however, that under field conditions protection of sweet on sour orange would fail as did those tested by Wallace and Drake (84, 85) and Stubbs (65).

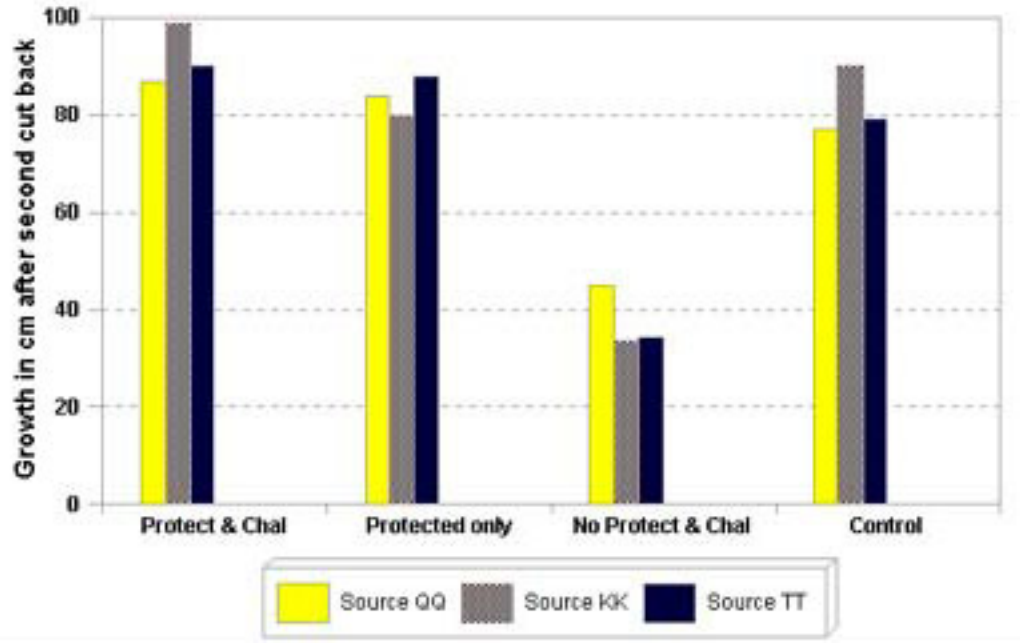

Fig. 9. Showing the successful protection for three seedling yellows tristeza (SYT) sources (QQ, KK and TT) when challenged against the original sources from which they were derived. These three protective sources were obtained from recovered shoots of grapefruit, lemon or sour orange plants which had a severe SYT reaction (53). The original source of QQ was from SYT-560, a *Citrus macroptera* tree which had SYT and ultimately died. Sources KK and TT were from a Parson's Special mandarin code SYT-565 (CRC-300) and when indexed also had severe SYT.

Stem pitting tristeza. Severe stem pitting CTV is the most serious of all types of tristeza, since it attacks both the scion and/or rootstock directly, and changing to tolerant rootstocks would not be effective. These severe isolates reduce fruit size (Figs. 10 and 11) and yield, and can debilitate the tree. Tristeza is one of the very few virus-induced diseases where mild strain cross protection has been used successfully in commercial production of various tree crops and is currently the only way a citrus industry faced with severe and destructive stem pitting CTV can survive. However, new CTV isolates will and do emerge, and research must continually develop new protective isolates against newly emerged destructive isolates. The pathological anatomy of stem pitting was illustrated and discussed in detail by Schneider (61).

Fig. 10. In the coastal valleys of Peru the primary citrus grown was the navel orange. Small fruit size as well as severe stem pitting and declining trees was prevalent in all orchards observed in 1987 by the senior author (46).

Fig. 11. This Marsh grapefruit selection was imported into the variety collection at Kutchinotsu, Japan as a virus-free budline from California. During the first years of fruiting, the trees produced typically large fruit. However, very severe CTV isolates were transmitted by *Toxoptera citricida* from nearby infected citrus into the new grapefruit trees inducing the very small sized fruit observed here.

Early history of studies on cross protection against CTV. Grant et al. (19) reported that plants invaded by the mild form of the tristeza disease were protected against the severe form and Grant and Higgins (20) were the first to demonstrate tristeza mild strain cross protection in seedlings of Mexican lime. Stubbs (65) conducted cross protection experiments over a period of 11 yr on grapefruit and sweet orange on sour orange rootstocks. However, the trees ultimately failed in field trials (Fig. 6).

Wallace and Drake (83) showed that budwood of recovered seedling yellows plants would protect trees of sweet orange on sour orange rootstock. However, as with the earlier work of Stubbs (65), this protection ultimately broke down in field trials at Riverside, California. Sasaki (60) controlled the Hassaku dwarf disease by preimmunization with mild CTV strains. Some success was shown, but protection broke down in the field after 12 yr. Balaraman and Ramakrishnan (1)

demonstrated mild strain cross protection in the small fruited lime in India using selected mild strains. However, this research was not continued. The original protected lime trees when observed by the senior author in 1992 at the Citrus Institute at Bangalore were in decline and most were dead or deteriorated.

A review of cross protection in Brazil. At the end of the 1960's many orchards of Pera sweet orange on tristeza tolerant rootstocks were in severe decline showing stem pitting, small fruit and severe stunting of trees. The situation was so severe that it was advised to replace the very popular Pera sweet orange with other orange varieties (59). An extensive search was begun by Costa and Müller to find trees of Pera sweet orange which survived the severe stem pitting problem. Müller and Costa (32) collected budwood from 70 outstanding trees of Pera sweet orange throughout São Paulo state, Brazil. They also collected surviving trees of Galego lime and grapefruit showing mild symptoms in the field. Forty five selections were given field exposure and 50 were tested in the greenhouse. Of these, 10 lime and three Pera sweet orange selections were challenged with severe CTV and evidence of cross protection was obtained. This was the first highly intensive field and greenhouse cross protection research against stem pitting tristeza. In September, 1962 an outstanding Pera tree was found showing no decline and when indexed to seedlings of the small fruited Galego lime it showed a very mild reaction. This was the first protective isolate and was named #66. Extensive search and studies for finding and testing cross protective isolates for sweet oranges and limes were reported by Costa and Müller (12) and Müller and Costa (32, 33, 35, 36) and Müller et al. (37, 38). Protective isolates were found which led to complete revival of the Pera industry in Brazil. In addition, protective isolates were found for the small fruited Galego lime in Brazil. Figs. 12 and 13 show historical pictures of protection of Pera sweet orange and Galego lime in Brazil. Costa and Müller (12) reported that some 8 million protected trees throughout Brazil had not broken down over a period of 12 yr and to this date (2008) this is still valid 40 yr after release of the original cross protective isolates.

Fig. 12. This historical picture taken in August, 1977 shows Dr. Gerd Müller standing next to a preimmunized lime tree (right) compared to a non-preimmunized lime tree (left).

Fig. 13. A Pera sweet orange on Rangpur lime rootstock preimmunized (left) and a naturally CTV-infected Pera tree (right). Dr. Gerd Müller stands between the two trees in this historical photograph taken in August, 1977.

Cross Protection in South Africa. In a review on cross protection by da Graça and van Vuuren (13), they indicated that South Africa, like Australia and Brazil before it, had established cross protection for all its budwood sources. A certification scheme was started because of losses incurred by severe CTV stem-pitting and other diseases, as well as the propagation of horticulturally inferior trees (79). Another motivation was that rough lemon, the primary rootstock being used, although tolerant to most graft-transmissible pathogens, did not induce the best fruit quality in the scion, and was unsuitable in replant situations because of its susceptibility to root pathogens. Many of the alternative CTV-tolerant rootstocks were susceptible to other viruses and viroids, so the first step was to select trees which were free of pathogens such as exocortis, cachexia and tatter leaf. Older, vigorous trees were naturally selected since they were potential sources of mild CTV isolates for cross protection. Once shoot-tip grafting was introduced to remove viruses, it became essential to inoculate all budwood sources with mild CTV. CTV-induced stem pitting in grapefruit had been noted as a serious problem back in 1941-1943 (40) (Fig. 14). In 1970 the use of mild strains in South Africa was suggested (63), but it was not until the selection of grapefruit budwood sources in 1976-77 for the Citrus Improvement Program (78), that an earnest attempt was made to initiate their use commercially.

Fig. 14. Stem pitting in the trunk of a field grapefruit tree observed in Nkwalini, South Africa in 1985. Stem pitting induced by CTV is very commonly observed in South African grapefruit trees.

A number of CTV isolates were screened initially for symptom development on Mexican lime and Marsh grapefruit in the greenhouse. Using as a measure the number of pits per square centimeter that developed on the indicator hosts, significant differences were found between potential protecting isolates (74). A field trial using Marsh grapefruit was then set out (72), and three isolates, which were rated the mildest in the greenhouse trials, provided significantly better protection against natural infection by severe strains. One of them, GFMS12, was derived from a Marsh grapefruit tree planted in 1926, (23) and which was still producing a profitable crop in 2004. The first budwood protected by GFMS12 was released in 1984 (25). Initially, GFMS12 was chosen as the universal protecting isolate for all citrus in order to protect grapefruit. However, with the finding that a Mexican lime-derived isolate, LMS6, gave the best protection for Mexican lime (73), and the later discovery that GFMS12 was not an ideal protector for Star Ruby grapefruit (74), the recommendations were changed. GFMS35, isolated from a Redblush grapefruit tree, was found to be more effective than GFMS12 for Star Ruby (74), and LMS6 is being used for lime, sweet orange and mandarin (24). However, based upon desired levels of tree health and economic return, a higher level of protection was desirable, and a search was initiated to find mild strains naturally selected in Star Ruby trees in the field. Initial results after 5 yr of field data indicate that GFMS35 and a Star Ruby-derived isolate gave the best protection (77).

One aspect of concern with cross-protecting isolates is the composition of the isolate and potential changes to this composition. Bio-indexing of single aphid-transmitted sub-isolates of GFMS12, GFMS35 and LMS6 demonstrated that these isolates were mixed infections, with some components more severe than the parental mix while other components were milder (24, 75, 76). Furthermore, SSCP analysis indicated that changes occurred to protective isolates in grapefruit in the field and with these changes there is the likelihood of super-infections of new strains via aphids (70).

An experiment was also conducted to determine whether mild isolates could offer protection against decline for sweet orange trees on sour orange rootstock in South Africa. Eleven isolates from different countries were tested, and all trees, except those inoculated with an isolate from Israel, developed quick decline within 4 yr (71). No quick decline developed when the trial was

repeated in a hot climate, but foliage on protected trees was sparse and fruits were small with thick rinds and of low economic value (van Vuuren, unpublished data). This was a likely manifestation of super infection with more severe CTV.

Severe stem pitting CTV isolate 12B in California. Seedling yellows isolates were known to be present in the variety collection at the University of California Riverside (UCR) since 1956 (80). During the IOCV pre-conference trip to Israel in 1975, serious and destructive spread of CTV in a citrus grove on sour orange rootstock in the Hibbatz Zion area was observed. When indexed, the CTV isolate that was spreading was shown to be a seedling yellows isolate (2). Upon returning to Riverside, the senior author initiated indexing tests to check for the presence and possible spread of severe CTV isolates in the UCR variety collection. Several severe isolates were found (50) and many of these isolates had high transmission rates and some showed 100% transmissibility (51). These rates were much higher than the 4 to 5% initially observed by Dickson et al. (14). Exploratory indexing was done by Calavan et al. (9) to test for severe CTV isolates in the experimental fields at UCR and especially in the variety collection. An isolate was found in a Minneola tangelo in field 12B that transmitted severe stem pitting CTV to grapefruit and sweet orange and had a transmission rate of 100% by *A. gossypii* (51, 52). The finding of this isolate led to a massive program for indexing of all trees in the UCR citrus experimental groves at Riverside as well as a search for seedling yellows and severe stem pitting isolates throughout citrus orchards in southern California (45). As a result of this finding, 20,000 trees in the UCR experimental orchards were indexed for seedling yellows and stem pitting CTV isolates. Some 262 trees were found infected with these severe CTV isolates and these were either destroyed or the more desirable selections were shoot tip grafted and heat-treated for elimination of pathogens and were replaced in the variety collection.

This 12B stem pitting CTV isolate was shown to severely affect all sweet orange seedlings or scions when they were either bud or vector inoculated. An experiment was designed to test the protective ability of 101 California isolates of CTV which induced no stem pitting in sweet orange seedlings against this severe 12B stem pitting isolate. In a replicated experiment, these isolates were challenged by both vector and buds and not one of these 101 isolates gave protection against the severe 12B isolate (56).

As a general summary of mild strain CTV cross protection, all attempts to protect sweet orange on sour orange rootstock have failed. Cross protection using attenuated strains of seedling yellows CTV has shown some promise in greenhouse tests but has not been applied in field trials. Cross protection continues to be used in South Africa, Australia and Brazil. However, there is always the likelihood of changes to the protective isolates, and super-infections of new strains are possible. This argues strongly for continued research for new protective strains. Finding new protective strains is a long term process and requires much effort is searching and field testing which could take many years of expensive research.

A new approach for developing protective isolates of CTV by passage through *Passiflora.* Once *T. citricida* enters a country, sooner or later, severe stem pitting isolates of CTV will emerge and attack scions of grapefruit, lime, tangelo or sweet orange. Currently, cross protection is the only defense available and will be needed if the industry is to survive. Approaches for finding protective isolates

are:
1) Look for mild-reacting CTV-inoculated Mexican lime index plants and challenge these with severe isolates to see if there is any cross protection (19, 20 56).
2) Use recovered shoots of seedling yellows-affected Eureka lemon, grapefruit or sour orange and use these as protective sources (53, 82, 83, 84, 85).
3) After the citrus industry is seriously debilitated, search for trees showing little or no stem pitting, small fruit or other symptoms associated with severe CTV-stem pitting. These will need to be tested in the field or laboratory and it may take 10 to 15 yr to find reliable protective isolates (5, 6, 12, 78).
4) Attenuate severe CTV isolates by passing them through *Passiflora* species by aphid transmission. This takes a relatively short time to find protective isolates (54, 55)
5) Develop CTV-resistant trees by transgenic technology.

Transmission of CTV to and from *Passiflora*. Transmission of CTV into *Passiflora* was initiated in 1980. The concept for this work came from the studies of Müller et al. (34) who showed that *Passiflora gracilis* could harbor CTV, with striking symptoms similar to that shown in Fig. 15 produced in this herbaceous host. In their paper, CTV was transmitted from infected citrus to *P. gracilis* by *T. citricida*. They also tried vector transmission of CTV to many weeds but found that only *Passiflora* could be infected. Roistacher and Bar-Joseph (52) were able to repeat this work using *A. gossypii* as the vector and they transmitted CTV from infected sweet orange to two *Passiflora* species and then vector-transmitted back from CTV-infected *Passiflora* to Mexican lime. For transmission studies, *A. gossypii* was reared on muskmelon, *Cucumis melo* L. 'PMR 45', in special rearing cages which were kept in a small glasshouse with temperatures controlled at 21 ± 3 °C. Melon leaves carrying mature and immature aphids were cut into small segments and caged with CTV-infected young sweet orange shoots or with *Passiflora* leaves and placed in a growth chamber for a 24 h acquisition feeding at 24 °C. After this feeding, small shoots of sweet orange bearing aphids were transferred to a Mexican lime receptor plant

Fig. 15. Showing the severe reaction on *Passiflora gracilis* when a severe CTV-seedling yellows isolate was introduced by the vector *Aphis gossypii*. This reaction was similar to that obtained by Müller et al. (34).

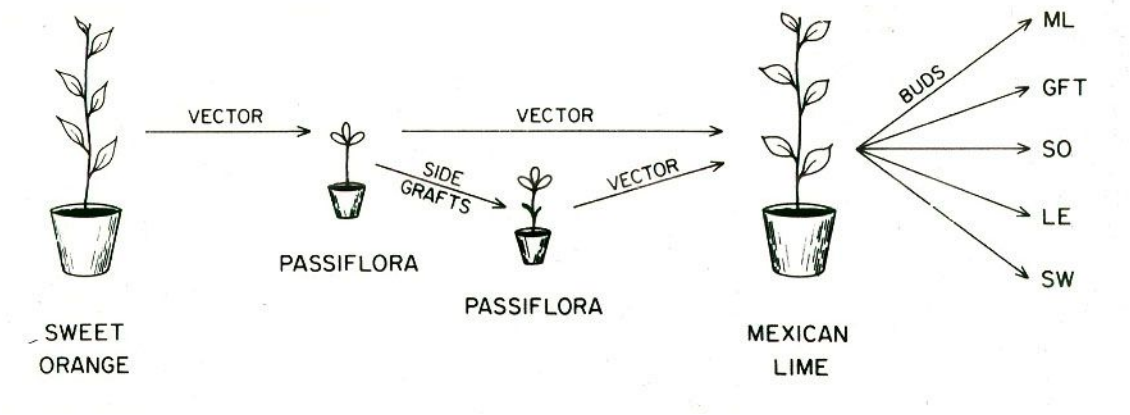

Fig. 16. A diagram showing the technique used for developing attenuated strains of CTV to be used in cross protection by passage of CTV through *Passiflora* (54). The virus is vector transmitted from CTV infected sweet orange, by *Aphis gossypii* to *Passiflora caerulea* or *P. gracilis*. The virus can then be transmitted from *Passiflora* by vector to Mexican lime or by graft-transmission to other *Passiflora* species. From Mexican lime the virus is then transmitted by bud-graft to other index plants such as Mexican lime (ML), grapefruit (GFT), sour orange (SO), lemon (LE) and sweet orange (SW). In this way attenuation of CTV isolates was observed.

and again placed in the chamber for 24 h for the infection feeding. At the end of this time all aphids present on the receptor plants were counted and then killed by spraying with 1% nicotine sulfate. Details of these procedures are diagrammed in Fig. 16 and illustrated in the EcoPort.org slide show #103 on cross protection (48).

Transmission of CTV by *A. gossypii* was attempted from leaves of infected sweet orange to five *Passiflora* spp: *P. gracilis, P. caerulea, P. incense, P. edulis* f. *flavicarpa* and *P. incarnata.* Vector transmission was also attempted from CTV-infected sweet orange to *Saponaria* spp., *Chenopodium capitatum , C. quinoa, Beta vulgaris, Trifolium repens, Cassia* sp., *Sonchus* sp., *Nicotiana tabacum* and *Catharanthus roseus. A. gossypii* readily fed on all species of *Passiflora* tested. Except for *Passiflora* there was no transmission on any of the nine herbaceous species in 170 individual tests. All plants were held in a glasshouse at 26-29/19-20°C (max. day/min. night) temperatures.

The vector-inoculated *Passiflora* plants were observed for symptoms and periodically indexed for presence of virus by ELISA. All vector-inoculated Mexican lime plants were held for 4-6 mo and observed for symptoms. If symptoms were found, sub-inoculations were made by bud-grafts from the infected Mexican lime to grapefruit, lemon, sour orange, and sweet orange seedlings to test for the presence and intensity of virus symptoms and stem pitting (52). Graft-transmissions from infected *Passiflora* plants to other *Passiflora* plants were done by side grafts. New shoots were observed for symptoms and periodically indexed for CTV by ELISA. When aphid transmissions were made to *P. gracilis*, the symptoms were so severe that plants died within months and *P. gracilis* plants could not be used as holding plants for CTV (Fig. 15). *P. caerulea* was found to be an excellent holding plant for the severest CTV isolates, with plants growing in the greenhouse for years showing small-leaf symptoms (Fig. 17).

Fig. 17. CTV-infected leaves of *Passiflora caerulea* (below) are considerably smaller than uninfected leaves (above). The presence of CTV was readily confirmed in these leaves by ELISA. CTV-infected *P. caerulea* plants will survive for many years in the greenhouse and can be used for transmission studies.

Fig. 18. Protection from a CTV isolate attenuated by passage through *Passiflora.* Left to right: four plants protected and then challenged with the original severe stem pitting isolate; two plants inoculated with the protective isolate only but not challenged; two plants inoculated with the severe challenge inoculum only (positive control); uninoculated negative control plant.

The general procedure for evaluating protection was described by Roistacher et al. (55) and is the same as described for cross protection studies for seedling yellows (Fig. 2a). The results of a typical successful protection are shown in Fig. 18.

Codes 37 and 40. These two cross protection isolates were obtained by passage of a severe stem pitting CTV through Passiflora. *A. gossypii* was the vector for transmission from a sweet orange holding plant of SY-563 into *P. caerulea* and then vector transmitted out of *P. caerulea* into seedlings of Mexican lime. The origin for isolate Code 37 was a Brazil navel from the UCR variety collection coded as SY-563 (CRC-957). It was originally imported as USDA Plant Introduction (P.I.) 37757 in 1914 and was almost certainly free of tristeza when introduced at this early date. However, when indexed, budwood from the field tree of this Brazil navel was found positive for both seedling yellows and stem pitting CTV. After passage of this isolate through *P. caerulea*, a single positive Mexican lime plant showing only mild CTV symptoms was designated as Code 37 in September 1982, and a bud-inoculation from this Mexican lime to a sweet orange holding plant was designated as Code 37A.

Code 40 was an independent source derived by vector transmission in the same way from the Brazil navel SY-563 holding plant to *P. caerulea* and vector transmitting from CTV-positive to Mexican lime in November, 1982 and was designated as Code 40. It was then sub-inoculated to a sweet orange holding plant in March 1983 and designated as Code 40A. Shown in Table 1 is the protection given by Codes 37, 37A and 40 when challenged with eight different severe grapefruit stem pitting sources. Unprotected grapefruit seedlings showed 38 to 286 pits per 100 cm^2 of surface area for five replicates whereas the seedlings protected with code 37 had from 0 to 41 pits, with many showing 0 pits for all five replicates. All sources protected with code 40 gave complete protection showing no pits for all eight severe stem pitting sources.

TABLE 1
NUMBER OF PITS PER 100 CM^2 OF STEM SURFACE AREA FOR FIVE GRAPEFRUIT SEEDLINGS PROTECTED OR UNPROTECTED BY *PASSIFLORA* CODES 37, 37A AND 40, THEN CHALLENGED WITH EIGHT SEVERE *CITRUS TRISTEZA VIRUS* STEM PITTING SOURCE

Code numbers of severe CTV challenge stem	Protective isolates			Unprotected
	Code 37	Code 37A	Code 40	
No challenge control	0	0	0	0
26	0	0	0	154
58	8	3	0	93.6
545	18	0	0	106
583	0	4	0	38
1225	0	0	0	286
7868	41	3	0	198
11118	0	0	0	66
563B	0	0	0	99

Challenge inoculum buds were removed 5 weeks after graft-inoculation
563B is the source from which *Passiflora* codes 37, 37A and 40 were all derived

TABLE 2
PROTECTION OF MADAM VINOUS SWEET ORANGE SEEDLINGS BY CODE Z-5 DERIVED BY PASSAGE OF A SEVERE CTV STEM PITTING ISOLATE THOUGH *PASSIFLORA CAERULEA*

Protected	Challenged	No.pits/100 cm^2*
Yes	Yes	1
Yes	No	1
Yes	Yes	1.061
Yes	No	0

*Total number of pits for 10 replicates averaged for 100 cm^2 of stem surface area

Fig. 19. C. N. Roistacher (left) and Klaus Bederski (right) are shown standing in front of a grapefruit tree at the Topara nursery in Peru in August, 2003. This grapefruit seedling tree came from a budstick of Code 37 which was given to Bederski in 1989 from the virus source plant at the Rubidoux facility of the University of California at Riverside and was budded to rough lemon as the rootstock. Note the large sized fruit and the vigor of the original tree. This source tree showed good protection against the severe stem pitting strains of *Citrus tristeza virus* present at the Topara nursery in Peru. Code 37A has proven to be highly successful in protection of both grapefruit and navel oranges (5, 6).

Protection by Code Z-5 derived from severe stem pitting isolate 12B. During the studies on attenuation of severe CTV by passage through *Passiflora*, all six isolates obtained were protective (53, 54 55). One such isolate, Code Z-5, derived by passage of the sweet orange stem pitting 12B (Code SY-568) isolate through *P. gracilis* by *A. gossypii* showed remarkable cross protection as shown in Table 2. After final harvest some of the remaining protected plants were again challenge-inoculated by tissue-grafts with the severe CTV stem pitting 12B inoculum. The new growth, observed 3½ mo later, was found to be totally protected. A third re-challenge gave similar results. However, after a period of 3 yr the plants protected with Code Z-5 and bud challenged with the severe isolate 12B, protection began to break down and severe stem pitting was observed in the sweet orange test plants.

Success of Codes 37 and 40 for protection of citrus in Peru. In December 1989, budsticks of Codes 37A, 37B, 37C and 40A were taken to Klaus Bederski's Topara nursery in Peru and grafted on field grown rough lemon seedlings (four per coded source). The objective was to test the long term stability of these attenuated

protective isolates against the severe challenge of the local Peruvian stem pitting CTV. Despite the risk of having exotic California CTV strains at the Topara nursery and in Peru, the perceived benefits of stopping the destruction of citrus by the severe CTV stem pitting isolates currently in Peru was felt to justify any risks involved (5, 6, 46).

The results of protection by these attenuated strains were published by Bederski et al. (5, 6). After 21 yr in the field, the protective isolates were doing well. Under the severe CTV stem pitting inoculum pressure at the Topara nursery, Code 37A proved to be the outstanding protective source for Navelina, Fukumoto Cara Cara, Lane Late and Navelate navel oranges. Code 37C also proved to be the outstanding protective source for Star Ruby, Marsh and Flame grapefruits. Code 37C was the outstanding protective source for the Oroblanco hybrid grapefruit and Code 37B proved outstanding for protection of Marsh grapefruit (Fig. 19). Code 40A was not as good in its protective abilities compared to Codes 37A and 37B. A slide show illustrating cross protection in Peru reviewing the history and development of protective isolates can be seen in the EcoPort slide show #142 (49).

Genetic engineering. Genetic engineering for developing transgenic plants immune to tristeza is a most important path for the future, but at present there are many problems. A number of workers involved in this field were asked for their views on current status of genetic engineering for citrus:

Erik Mirkov (Texas A & M University): *"Pathogen derived resistance, i.e. use of viral coat proteins as approaches to engineer resistance have met with little success for Closteroviruses in general and especially CTV. Several groups have produced hundreds of transgenic citrus trees with various CTV genes, but only a handful of these show some limited resistance, and no group has reported immunity using this approach. We are now in the process of making transgenic citrus that has both of the genes from* Poncirus *and hope to see complete resistance."*

Mikael Roose (University of California): *"There are at least three important biotech approaches to CTV control. None are yet ready for field implementation, but all are promising. All involve production of transgenic plants. If the technology were proven to work in a lab setting, which may occur within 1 yr for all three methods, then it would still have to be field tested for efficacy and transferred into the important commercial cultivars. Then we have field testing of the transgenic cultivars that would probably take about 5-6 yr to be sure that the trees are reasonably normal. Thus it seems to me that even with a large amount of funding, it would take 10-12 yr to release a resistant cultivar. An additional problem is that the transformation methods and other materials used are patented, and licenses or rights would have to be negotiated before release. Field and food safety testing of transgenics is also very expensive (millions) and it is not clear who would pay for this."*

Allan Dodds (University of California): *"In order to manage CTV by this method the following will have to happen: 1) The gene(s) chosen as resistance genes will have to be shown effective against a wide range of CTV strains. 2) The ability to transform all standard citrus types will have to be shown. 3) The ability to have similar levels of resistance after independent transformations will have to be demonstrated. 4) Each country will probably want to do the work with their own lines rather than accept improved lines from other programs. 5) Multiple varieties or lines of each citrus type will have to be*

transformed independently. This will be a most laborious exercise. 6) Horticultural evaluation of each transformed line will be needed. 7) Once a transformed line is accepted, then it will be necessary to replant entire industries. One issue will be what to do when the genetic engineers come up with something better every five years. Replant again? None of these concerns mitigate away from doing the work of making genetically modified citrus plants, but the changes will not be seen in the industry for decades.

Bill Dawson (University of Florida): *"Genetic engineering is a two-edged sword. It has huge potential to control virus diseases, but at least at this time presents marketing problems as GM food. There was much hope to use virus-induced silencing to produce resistance against CTV in citrus trees as has been done in many other plants. Unfortunately, for reasons not understood, this has not worked for CTV after expenditure of huge amounts of effort and time. At this time, probably the best bet is inserting of the CTV resistance gene from* Poncirus *into citrus."*

Richard Lee (USDA-ARS): *"Genetic engineering citrus plants for resistance to CTV offers great potential for the future, but at best, this will be realized in the long term. To be more easily accepted by the public, transformations should be made without the marker genes. Genetic engineering of virus strains for cross protection is a long term promise also. Regulatory issues will probably dictate that any "designer virus" for cross protection can be used for localized areas only; this issue of recombination of virus isolates needs to be addressed. Additionally we don't know what virus genes control expression of stem pitting or other symptoms although some preliminary information is being developed. Even with "designer viruses", there will still be the need to constantly look for new virus genotypes which may break the cross protection and monitor the efficiency of the cross protection. The issue of intellectual property rights coupled with declining budgets and the desire to get a payback from products being developed will al*so *have an impact on how widespread genetic engineered forms of resistance will be used."*

DISCUSSION AND SUMMARY

The spread of the efficient vector *T. citricida* into Central America, Florida, Mexico, the islands of the Caribbean - plus its presence in northern Portugal and Spain should stimulate concern and argue strongly for funding for research on cross protection and for exploring all techniques for developing protective strains against the severe stem pitting isolates of CTV. In this review, we have presented a brief history of tristeza showing the influence of the great *Phytophthora* epidemics in the last half of the 19th century which resulted in the use of rootstocks to replace the seedling trees then in worldwide usage. The sour orange was discovered to be a most excellent rootstock for citrus and, based on its success as a rootstock in Europe and in California, it was used in the new developing citrus industries in Brazil and Argentina. However, it failed as a rootstock in Australia and South Africa. It is important to note that *Phytophthora* still remains one of the most serious fungal diseases of citrus if susceptible rootstocks are used, and it still attacks susceptible scions, especially the sweet orange.

Then, in the 1930's a new and devastating disease struck the citrus industries in Brazil and Argentina and the disease was aptly named `tristeza'. A similar disease named quick decline developed in California and was found to be highly destructive to citrus on the sour orange

rootstock. Ultimately it was discovered to be the same disease as that in South America, and found to be caused by a virus. It killed the phloem cells in the sour orange rootstock just below the bud union thus effectively girdling the tree, which became stunted or died. Sweet orange, mandarin and grapefruit on sour orange rootstock were universally affected in the presence of CTV, and over 100 million trees worldwide were killed.

All cross protection trials for sweet orange on sour orange rootstock ultimately failed. However, certain rootstocks (the trifoliates, hybrids of the trifoliate, rough lemon, Volkamer lemon, Rangpur lime and Cleopatra mandarin) were found tolerant to CTV and the industries which were killed on the sour orange rootstock were revived.

The two primary vectors of tristeza are *T. citricida* and *A. gossypii. T. citricida* was shown to be extremely efficient in transmitting CTV and a single aphid would transmit the virus. When *T. citricida* first enters a region or country where tristeza is present, there is a rush to replant to tolerant rootstocks. However, without an indexing and certification program, citrus viroids are especially damaging, particularly where temperatures are hot. Once *T. citricida* enters a country, sooner or later, severe stem pitting isolates of CTV will appear and attack scions of grapefruit, lime or sweet orange. Some form of cross protection will be needed for the industry to survive. Protective strains of CTV are found by: a) searching for mild-reacting CTV in Mexican lime index plants and challenging these with severe isolates to see if there is cross protection; b) using recovered seedling yellows shoots of Eureka lemon or sour orange as protective sources; c) searching for trees showing little or no stem pitting, small fruit or other symptoms of CTV. This is usually done after the citrus industry is seriously debilitated. These sources of potential resistance will need to be tested in the field or laboratory and it may take 10 to 15 yr or longer to find reliable protective isolates; d) by attenuation of severe CTV isolates by passing them through *Passiflora*. This takes a relatively short time to find protective isolates; e) developing CTV resistant trees by transgenic technology. This may be a most promising path for the future but is not feasible at the present time.

To date, all attempts at cross protection for citrus on sour orange rootstocks have failed. With over 100 million trees killed, no surviving trees have ever been found to provide sources for cross protection. In California, over 100 local isolates of CTV failed to protect against the severe sweet orange stem pitting 12B isolate. In the variety collection at UCR, seedling yellows tristeza was found to be destructive and spreading. However, CTV isolates obtained from recovered shoots of seedling yellows infected grapefruit or sour orange were found to be protective in greenhouse cross protection experiments. These protective isolates were never field tested.

The most destructive type of tristeza is that which induces stem pitting, small fruit and destruction of trees. By searching for trees which survive heavy inoculum pressure, and then testing these trees in extensive experiments, protective isolates have been found in Brazil, Australia and South Africa. This took many years of searching and testing and required funding and Government and grower support.

Studies were initiated on a new approach for finding protective isolates. This was done by vector transmitting severe CTV isolates through *Passiflora* species. It was discovered that severe CTV stem pitting isolates could be attenuated after passage through *Passiflora* and these attenuated isolates had potential for cross protection. This technique is described in detail and results of successful experiments are shown.

In field trials, protective isolate Codes 37 and 40 derived by passage of severe CTV seedling yellow/stem pitting isolates through *Passiflora* have proven successful in protecting against the severe Peruvian stem pitting tristeza which had virtually destroyed the navel orange industry in Peru.

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