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TRISTEZA VIRUS STRAINS

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INTRODUCTION

Tristeza virus strains and strain mixtures are identified at present largely on the basis of visible differences in growth and in degrees of symptom expression on infected test plants, and on the reproducibility of these visible differences by repeated transmissions to comparable test plants.

A tristeza virus strain that caused mild symptoms and another that caused severe symptoms were demonstrated by Grant and Costa (*9*) in Brazil. Both strains were transmitted by the same vector, *Toxoptera citricidus* (Kirk.). The differential behavior of various rootstock-scion combinations three years after inoculation with mild and severe strains of tristeza virus has been described by Costa, Grant, and Moreira (*4*). The freedom of these sources of tristeza virus from viruses causing psorosis, xyloporosis, and exocortis was indicated by the fact that no symptoms of these diseases were present on symptom-expressing species or rootstock combinations (*10*) inoculated with tristeza virus by means of aphids.

On the basis of differences in degree of symptoms on West Indian lime plants, Hughes and Lister (*12*) recognized two strains of the virus causing lime dieback in the Gold Coast, and McClean (*13*) recognized two strains of the causal agent of stem pitting in South Africa. Similarity of the causal agents of lime dieback in the Gold Coast and of stem pitting in South Africa and tristeza in Brazil (*2*) was indicated by transmissibility by the same aphid vector and by production of comparable symptoms on several citrus species and rootstock-scion combinations.

In Australia, Fraser (*6*) made bud collections from tristeza-infected field trees, and when she budded these on seedling test plants of Eureka lemon, sour orange, and grapefruit, obtained evidence of a qualitative difference in the virus content of different citrus species. The virus complex occurring naturally in orchard trees of several varieties of sweet orange and mandarin caused severe stunting and yellowing of the test plants. However, the virus in trees of Eureka lemon, sour orange, and grapefruit caused no leaf symptoms or only occasional vein flecking in the spring growth and apparently did not stunt the test plants. Fraser concluded that the virus causing seedling yellows is distinct from that responsible for what she designated the "tristeza—stem pitting complex," although she never found seedling-yellows virus occurring separately from the latter.

In South Africa, McClean and van der Plank (*14*) studied the seedling-yellows reaction in relation to tristeza and agreed with Fraser (*6*) in regard to symptomatology, host range, and the consistent presence of the "stem pitting component" in mixture with seedling-yellows component. McClean and van der Plank are of the opinion that tristeza is caused by a virus complex and that seedling-yellows virus is sometimes a part of that complex. Wallace (*17*) found that Meyer lemon, some satsuma varieties, and other miscellaneous noncommercial citrus introductions in California are carriers

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of a strain or mixture of strains of tristeza virus that causes the seedling-yellows reaction described by Fraser (*6*). From his preliminary studies Wallace agreed with McClean and van der Plank that seedling yellows is not caused by a virus distinct from the tristeza virus.

Olson (*16*) studied the reactions to mild and severe strains of tristeza virus in Texas and reported that a mild strain from a Meyer lemon tree inhibited the development of severe symptoms in lime plants subsequently bud-inoculated with the virus from Sueoka satsuma.

Grant and Higgins (*11*) reported that tristeza virus from selected sources in Florida caused very mild, mild, or severe stunting, vein and veinlet clearing in the leaves, and pitting of the stems beneath the bark of Key lime test plants. Lineal branch growth of inoculated Key lime plants was proportional to the severity of other symptoms induced by the tristeza virus employed. Repeated selections and transmissions by leaf pieces from a mildly affected source plant indicated that the mild tristeza virus in Florida mutates readily or is a mixture of strains, some that consistently cause stem pitting and others that cause few or no stem pits on Key lime plants. Cross-protection tests with virus from selected source plants resulted in varying degrees of protective effects (*11*). The presence of virus strain mixtures in a given plant appeared to be related to the failure of any one strain to become thoroughly systemic, thus affording susceptible sites for the development of other strains.

It is the purpose of this report to describe further studies of tristeza virus strain mixtures and to discuss the results. The sources of tristeza virus T_0 (very mild), T_1 (mild), and T_3 (severe), the methods of transmission, and the care of test plants are the same as previously described (*11*). All inoculated and control plants illustrated were cut back to a single 6-inch main stem, leaving only the three uppermost leaves at the time of inoculation.

RANGE IN SYMPTOM EXPRESSION IN PLANTS INFECTED WITH TRISTEZA VIRUS

Typical differences in growth of Key lime plants following inoculation with leaf material from the T_3 and T_1 sources of tristeza virus are represented by the plants shown in figure 1. Key lime plants inoculated with T_3 virus showed vein clearing in the young leaves soon after inoculation. This was followed by yellowing and cupping of the leaves. In nine months these plants made about 26 per cent as much branch growth as the control plants, and they had many pits and striations in the stems under the bark. Comparable Key lime plants inoculated with leaf pieces from the T_1 virus source showed scattered vein clearing in young leaves. On subsequent growth under summer conditions in the greenhouse there were fewer leaf symptoms. In nine months these plants made about 70 per cent as much branch growth as the control plants and had relatively few stem pits.

Responses of sour orange and Eureka lemon plants to inoculation with the $T_3$and T_1 sources of tristeza virus are represented by the plants shown in figure 2. The plants inoculated with the T_3 virus were stunted, and their terminal leaves were yellow. The plants inoculated with the T_1 virus were shorter than the control plants but did not show the yellowing noted on the T_3 virus-inoculated plants.

It has been reported (*11*) that the aphid-transmitted T_1 tristeza virus was modified by leaf-piece selections and serial transmissions so that it produced both very mild and severe tristeza symptoms on Key lime plants. Comparative differences in the growth of five plants inoculated with leaf pieces from the third serial selection for mildest symptoms, and of five plants inoculated at the same time with leaf pieces from the fifth serial selection for severest symptoms, are shown in figure 3. Records on total branch length 178 days after inoculation showed that plants in group *A* with mildest

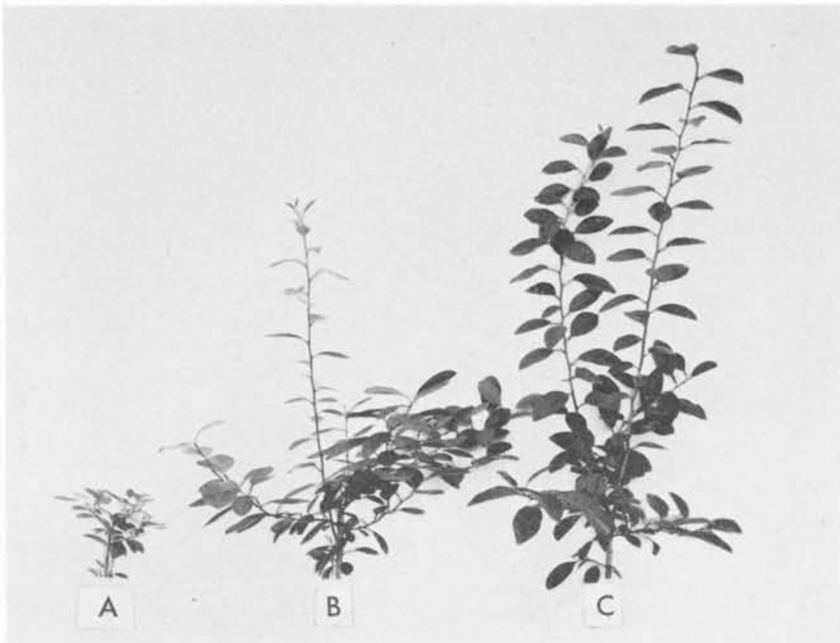

Fig. 1. Representative Key lime plants nine months after being cut to 6-inch single stems and inoculated with leaf pieces containing tristeza virus: A) T_3 (severe); B) T_1 (mild); C) comparable non-inoculated control plant.

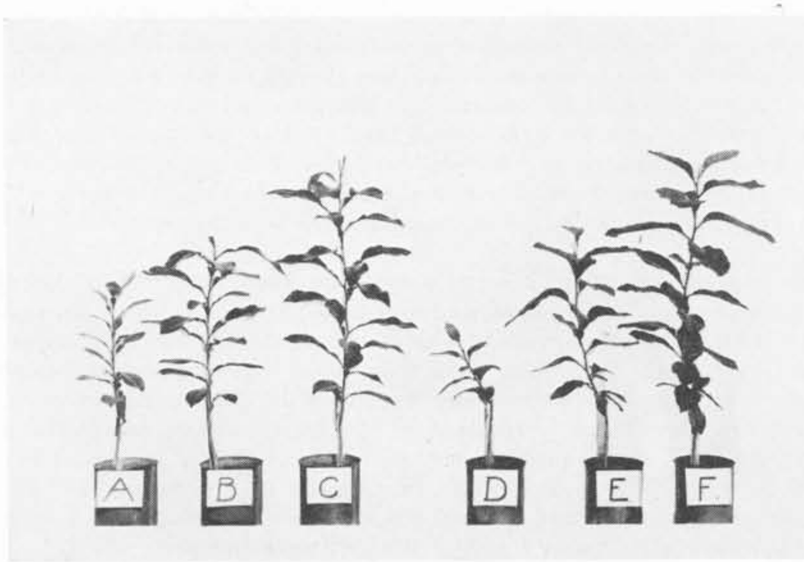

Fig. 2. Representative sour orange plants (A, B, C) and Eureka lemon plants (D, E, F) four months after inoculation with leaf pieces containing tristeza virus: A and D) T_3 (severe); B and E) T_1 (mild); C and F) comparable noninoculated control plants.

Fig. 3. Comparative differences in growth of Key lime plants 178 days after inoculation with leaf pieces from a single original source plant infected with aphid-transmitted T_1 tristeza virus. A) five plants inoculated with leaf pieces from the third serial selection for mildest symptoms; B) five plants inoculated with leaf pieces from the fifth serial selection for severest symptoms.

symptoms averaged 185 cm, and those in group *B* with severest symptoms averaged 94 cm. The total length of branch growth of the healthy control plants (not shown in fig. 3) averaged 213 cm. Since the serial T_1 leaf-piece inoculum originated from a single Key lime source plant infected as a result of virus transmission by *Aphis spiraecola* Patch, which acquired the virus from a single field tree (*15*), it must be presumed either that the aphids transmitted a mixture of strains or that the virus mutates readily.

By leaf-tissue transmissions to Key limes from the T_3 virus source that usually produces severe tristeza symptoms, it has also been possible to obtain an occasional plant that shows only mild symptoms. These results indicate also that tristeza virus exists as mixtures of strains which can be separated by leaf-piece transmission to Key lime plants.

In tests of cross protection, it was reported that where simultaneous inoculations were made with a mild and a severe source of tristeza virus, the plants showed severe symptoms (*11*). Plants inoculated first with virus from the T_0 (very mild) virus source and then, four months later, with virus from the T_3 (severe) source subsequently showed great variation in leaf-symptom intensity on the different branches. This suggested that these doubly inoculated plants had an erratic and unequal distribution of the virus from the T_3 source (*11*). To test this concept I selected five leaves from each of two of these doubly inoculated plants. Some leaves were young and succulent and others were old; some had vein clearing and others had no symptoms. A piece from each leaf was used to inoculate a Key lime plant. All ten plants were cut back to a single main stem and to uniform height at the time of inoculation. Their subsequent development in a ten-month period is shown in figure 4.

One plant (fig. 4, *A*) inoculated with a young leaf piece produced symptoms typical of infection with T_3 tristeza virus. Another plant (fig. 4, *E*), also inoculated with a

Fig. 4. Key lime plants ten months after inoculation with single leaf pieces from source plants doubly inoculated, first with T_0 (very mild) tristeza virus, and then, four months later, with T_3 (severe) tristeza virus (two months before leaf-piece transfer). A) plant showing stunted growth typical of infection with the T_3 tristeza virus. B, C, D) plants showing gradation in growth indicative of infection with varying proportions of T_3 and T_0 tristeza strains. E) plant with good growth typical of infection with T_0 tristeza strain. The average total branch length per plant was as follows: A, 76 cm; B, 171 cm; C, 232 cm; D, 348 cm; and E, 424 cm. The average numbers of stem pits per 10 cm of stem for the various plants were as follows: A, 100; B, 100; C, 22; D, 12; and E, 0.

young leaf piece, was typical of infection with T_0 virus. Eight plants (fig. 4, *B*, *C*, *D*), each inoculated with a single young or old leaf piece, some with and some without vein-clearing symptoms, produced a wide range in branch growth and leaf symptoms, thus indicating that these plants were infected with varying proportions of very mild and severe tristeza virus. The gradation in range from severe to very mild tristeza symptoms on the Key lime plants obtained from the doubly inoculated source plants provides evidence that in a two-month period, distribution of the challenging T_3 virus in the doubly inoculated source plants was unequal.

MODIFICATION OF TRISTEZA SYMPTOMS BY VARIOUS PROCEDURES

Passage of Virus Through Host Plants. The T_3 (severe) tristeza virus was used to inoculate five Duncan grapefruit seedlings. After two months, a piece of a mature terminal leaf from each grapefruit plant was used to inoculate a Key lime test plant. Subsequent growth of the five inoculated lime plants and of a representative healthy control plant in a two-month period is shown in figure 5. One plant (fig. 5, *A*) was stunted and had vein-cleared, cupped yellow terminal leaves characteristic of infection with T_3 virus. Four plants (group *B*) had milder symptoms but had not made as good growth as the healthy control plants. These results confirm the tendency previously noted in Australia (*6*) and South Africa (*14*), that transmissions from grapefruit, Eureka lemon, and sour orange trees produced milder symptoms than similar transmissions from sweet orange and mandarin trees.

That a particular plant may influence the development of a tristeza virus strain complex was also indicated by results in Florida (*11*), when serial selections were made to

Fig. 5. Effect of passage of T_3 (severe) tristeza virus through grapefruit seedlings. A) Key lime plant showing characteristic symptoms of T_3 infection two months after inoculation from one T_3-infected grapefruit seedling. B) milder symptoms on four lime seedlings inoculated, respectively, from four T_3-infected grapefruit seedlings. C) comparable noninoculated control plant.

obtain strains that differed in their ability to cause stem pitting. It was noted that in a second selection for increasing stem pits an off-type Kalpi lime plant had 9 to 40 times as many pits as the other three typical Kalpi plants in the same series. These results suggested that dominance of the strain or strains inducing stem pitting may be influenced by physiological or genetical differences in the inoculated plants, since, once established, the high level of stem pitting was reproduced in other plants by leaf-tissue transfers (*11*).

Repeated Removal of Branches. Key lime plants showing very mild symptoms characteristic of infection from the T_0 (very mild) source are not weakened appreciably more than healthy plants when they are subjected to the complete removal of all branches three times in the course of a year. There was no evidence of any appreciable change in the very mild virus symptoms on either the leaves or the stems. With the T_1 (mild) tristeza virus source it was demonstrated that by serial leaf-piece selection from infected plants, one strain could be obtained that produced very mild symptoms and one that produced strong symptoms (fig. 3). Repeated cutting back of Kalpi and Key lime plants infected with the T_1 source that produced mildest symptoms did not appreciably change the symptoms, but, with one exception, repeated cutting back of the plants with strongest symptoms did reduce both leaf symptoms and stem pitting. This indicated that in the ordinary nucellar seedlings of Kalpi and Key lime the very mild virus tended to predominate when branches were repeatedly removed. The one exception was the off-type Kalpi lime previously mentioned as having 9 to 40 times as many stem pits as the other plants in the same series. In this plant the average number of stem pits per 10 cm of stem increased with the 3-times-repeated removal of branches in the course of a year.

Heat Treatment. It has been reported (*11*, *15*) that growth of Key lime plants infected with mild tristeza virus during hot summer weather in the greenhouse showed few vein-cleared leaves and less stem pitting than did growth made under cooler weather conditions. These observed reactions led to the building of a heat chamber for exposure of infected plants. The results (*7*) showed that some young branch growth that developed during continued exposure to high temperatures was free of tristeza virus even though old leaves and stem tissues of treated plants were not. The suppression of symptoms on tristeza-virus-infected Mexican lime seedlings by heat treatment was in-

Fig. 6. Selected Key lime plants showing range in tristeza symptoms in heat-treatment tests: A) typical nontreated plant infected with T_3 tristeza virus; B) comparable plant after 161 days in the heat chamber; C-F) indicator plants graft-inoculated at different intervals with tissues from heat-treated plants similar to that shown in pot B; C) plant with severe virus predominating; D) plant with severe and mild viruses in approximately equal mixture; E) plant with mild virus predominating; F) plant free of virus.

dependently demonstrated by Desjardins *et al.* (*5*). Further heat-treatment experiments by Grant (*8*) showed that the tristeza virus was present in practically all young branch growth that developed on infected plants during the early part of their exposure period in the heat chamber. With continued exposure of the infected plants, virus inactivation appeared to be faster in young tissues than in old, and virus distribution in the plants was gradually reduced. Exposure to temperatures of 98° to 104° F for 86 to 100 days was sufficient to inactivate the virus in some of the young and old tissues tested.

Inoculations made with tissues from tristeza-infected lime plants exposed to insufficient heat to rid all tissues of virus resulted in subculture plants that showed variable severity of symptom expression as illustrated in figure 6. On the basis of differences in growth and symptoms observed on the subculture plants, it was evident that the T_3 (severe) strain of tristeza virus had been modified by heat treatment. The results suggested that a wide range in the proportional amounts of mild and severe strains might be expected from different parts of some subculture plants. In order to test this, a T_3 subculture plant that appeared to contain a predominance of mild strain but had some vein clearing suggesting the presence of a severe strain was selected from the heat-treatment tests. Leaf-piece transfers were made to healthy Key lime plants. The results are illustrated in figure 7. The range in symptoms in these Key lime plants indicated that the source plant contained varying mixtures of tristeza virus strains.

The results of the heat-treatment tests indicated that the virus or strain mixtures causing yellowing and stunting and severe symptoms are more readily altered by heat treatment than are those responsible for milder symptom expressions.

DISCUSSION

It is generally recognized that the tristeza virus is distinct from those of psorosis, xyloporosis, and exocortis of citrus. The tristeza virus has been variously discussed as composed of mild and severe strains (*4, 9, 12, 13, 16*); as composed of two distinct viruses, tristeza and seedling yellows (*6*); as composed of a complex with a stem-pitting component and a seedling-yellows component (*14, 17*); and as composed of very mild, mild, and severe strains that may occur in mixtures (*11*). It is agreed that all of the component parts of the causal agent are transmitted by the insect vector

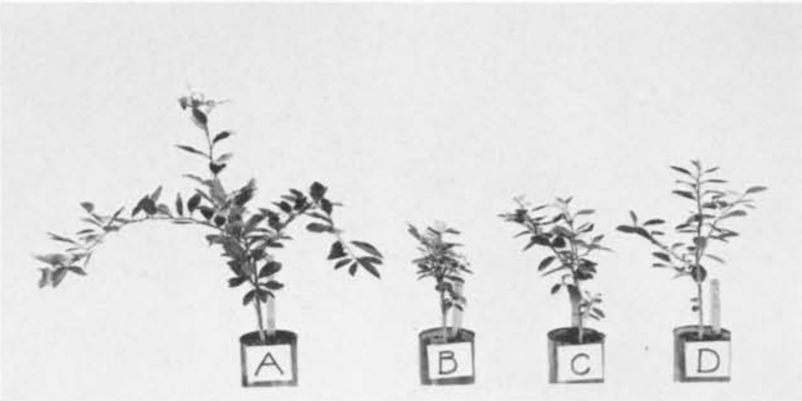

Fig. 7. A) Key lime plant 129 days after graft-inoculation from a T_3 (severe) tristeza virus source plant which had been held in a heat chamber for 25 days. The growth of plant A suggested a predominance of mild tristeza virus, but there were definite vein-cleared leaf symptoms indicative of severe tristeza virus. B, C, and D) Key lime subculture plants 63 days after grafting with selected leaf pieces from plant A; B and C) plants graft-inoculated from vein-cleared pieces of leaf; D) plant infected from a piece of a leaf that showed no symptoms. Tristeza symptoms ranged from those of plant B (relatively severe) to those of plant D (relatively mild), thus indicating that plant A was infected with varying proportions of very mild and severe tristeza viruses.

Toxoptera citricidus. As yet, the yellows component has not been obtained as a separate transmissible entity. However, it has been possible to obtain mild strains or components which cause some vein clearing in leaves and stem-pitting symptoms on lime test plants but do not cause the yellows symptoms on Eureka lemon and sour orange test plants (*6, 14, 17*). Many plant viruses studied extensively appear to exist in nature as a complex of related strains. Until better proof is available, the concept that tristeza is caused by virus strains and strain mixtures is accepted by the writer.

The results of studies described in this paper show not only that there are tristeza virus strain mixtures but that the strains may be transmitted in different proportional mixtures. This occurred in subcultures from plants in which the strain mixtures had been experimentally induced, as well as in subcultures from selected plants from the heat-treatment tests, in which the factors causing severe symptoms had been reduced or inactivated and those causing mild or very mild symptoms predominated. An analysis of the methods used and the results obtained suggests that transmissions made by stem grafts and buds tend to insure a large and perhaps more uniform supply of virus strain or strain mixture, whereas those made by small leaf pieces may have less virus and offer greater opportunity for detection of different strains or different proportional mixtures of strains. Results of leaf-piece transmissions also indicate that the virus strains do not necessarily multiply at the same rate or become distributed in the same proportions throughout infected plants. The results presented also show that a citrus species such as grapefruit may influence the rate of multiplication and distribution of different virus strains. Recognition of the existence of different strains and varying proportional mixtures of the virus strains aids understanding of the difficulties encountered in attempting to isolate specific strains.

The very mild virus strain that causes very slight vein-clearing and stem-pitting symptoms appears to be the most stable strain. It resisted heat treatment longer than the severe strain did. It remained unchanged after branches were removed from infected plants three times in the course of a year. It predominated during warm summer weather in greenhouse plants infected with mild strain. The very mild strain appears to be a specific entity.

The mild strain has not been obtained as an entity free of the very mild strain, but it causes a marked increase in the leaf and stem symptoms on Key lime plants that can be reproduced by transmission. At the same time it does not cause the severe symptoms of leaf yellowing and cupping, vein clearing, abundant stem pits and striations, or the drastic stunting caused by the severe virus strain.

The severe strain has some factor not present in either the very mild or the mild strains. This is indicated not only by the more severe symptoms produced on Key lime plants but also by the marked stunting and yellowing of Eureka lemon and sour orange plants inoculated with the severe strain, and by the lack of these symptoms on similar plants inoculated with the mild strain. Whether the severe strain is a single strain that mutates readily has not been demonstrated, but it has been shown by heat treatment of severe-strain-infected plants and by passage of the severe strain through grapefruit that the mild strain and proportional mixtures of mild and severe strains can be obtained from the severe strain.

There appear to be very distinct differences between the very mild, the mild, and the severe strains of tristeza virus. At the same time the mild strain and the severe strain appear to be composed of factors additional to those included in the very mild strain. For the purpose of applying this concept to results of present studies, let us assign the letter A to the factor which causes very mild symptoms, A + B to that which causes mild symptoms, and A + B + C to that which causes severe symptoms.

When mixtures of A and A + B + C were made by inoculating Key lime plants first with A and four months later with A + B + C, the symptoms produced in a subsequent two-month period suggested an unequal distribution of A + B + C in relation to A (*11*). Subculture Key lime plants infected by means of leaf-tissue transfers from the doubly infected plants developed symptoms that ranged from typical A to typical A + B + C, with a majority of the plants showing intermediate degrees of symptoms indicating that A, A + B, and A + B + C were present in varying proportional amounts.

The results from heat treatment of source plants with A + B + C indicated that C was altered or dispersed in a shorter heat-exposure period than was A + B. Subcultures made from source plants exposed for 10 to 20 days in the heat chamber often showed symptoms characteristic of A + B + C, but some showed symptoms indicative of the presence of different proportional mixtures of A + B and C. Subcultures from source plants exposed for longer periods produced symptoms on some plants characteristic of A or A + B only. These mild symptoms were reproduced by leaf-tissue transfers to healthy Key limes with no evidence of the presence of C. Some of the subcultures from source plants exposed for 86 to 100 days or more in the heat chamber showed no symptoms and thus indicated that A + B as well as C had been eliminated from the inoculum source tissue.

The reactions of Eureka lemon and sour orange have been used to distinguish a seedling-yellows virus (*6*) or yellows component (*14*) of tristeza. In the present tests, when A was used to inoculate Eureka lemon and sour orange, no recognized symptoms were produced. A + B in these hosts produced initial slight stunting, followed by recovery. A + B + C produced very marked stunting and yellowing in Eureka lemon plants and appreciable stunting, some yellowing, flecking, and nutrient deficiency symptoms in sour orange plants. The symptoms on sour orange were not as severe as those which occurred after inoculation of sour orange plants with severe tristeza virus in Brazil.

The results of studies with the severe tristeza virus in Brazil (*1*) showed that there were great differences in citrus species in relation to their tolerance to infection. For example, following three successive inoculations with colonies of 100 to 300 viruliferous aphids per plant, 100 per cent of the sweet orange plants, only 15 per cent of the sour orange plants, and none of the trifoliate orange plants became infected. In several tests reported (*1, 3*), tissue grafting was much more effective than aphids as a means of inoculating sour orange plants. Once infected, however, the sour orange plants showed severe symptoms of yellowing, and some plants died. When aphids were used for inoculation, it was found (*1, 3*) that small seedlings of sour orange, Eureka lemon, and grapefruit were easier to infect than nursery-size plants.

With this basic information and our more recent knowledge of tristeza virus strains and strain mixtures, an estimate may be made of what occurs under field conditions. Sweet oranges are readily infected by the insect vector and are a suitable medium for the development of factors A or B or C, alone or in any combinations. Sour oranges are much more difficult to infect by means of aphids, are a much poorer medium for development of A + B + C, and may have a differential preference for A or B over C. Grapefruit varieties appear to be intermediate between sweet orange and sour orange in reaction and, on the basis of present studies and as indicated by the results obtained by Fraser (*6*) and by McClean and van der Plank (*14*), they appear to favor A or B over C.

There are many variables under field conditions which may account for differences in symptom expression even on trees of sweet orange on sour orange rootstock. First, the aphid vectors vary in number, in efficiency, and probably in the proportional amounts of A, B, or C that they acquire and transmit from various source plants. Citrus varieties vary in relation to ease of infection and as media for the multiplication of A, B, or C, or proportional combinations of these. The age or size of the tree and the number of inoculations as a result of repeated infestations of viruliferous aphids may also influence symptom expression. The results of present studies suggest that temperatures may also have an influence on seasonal fluctuation of proportional mixtures of the virus strains. It is evident that a wide variety of plant reactions and tristeza virus strains or strain combinations, as well as mixtures with other viruses, can occur in citrus trees in the field.

SUMMARY

1. The existence of strains of tristeza virus as evidenced by differences in amount of stunting, leaf yellowing, vein clearing, and stem pitting on Key lime plants has been extensively demonstrated by repeated transmissions to Key lime and by differential reactions on sour orange and Eureka lemon plants.

2. Further evidence has been obtained that the virus culture producing severe symptoms is a mixture of strains. Cultures producing mild and very mild symptoms on Key lime were obtained from the severe symptom culture (*a*) by transmissions from plants exposed to temperatures high enough to inactivate part of the complex, and (*b*) by passage through grapefruit seedlings, which are more favorable to mild strains.

3. Cultures which produce only very mild or mild symptoms have, by means of leaf-piece selections and transmissions, produced cultures with stronger degrees of symptom expression but never to the degree characteristic of the severe tristeza virus reactions on plants of Key lime, sour orange, or Eureka lemon.

4. The occurrence of varying proportional mixtures of different strains of tristeza virus has been demonstrated by subcultures from plants with experimentally induced mixtures and from plants in the heat-treatment tests.

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