Further Evidence on Protective Interference in Citrus Tristeza

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THE PROBLEM of reducing losses induced by tristeza disease was solved for most commercial citrus types by the use of tolerant rootstocks. This, however, did not prevent the damage that resulted from the disease in cases of certain scions that possess somewhat intolerant tissues, such as the Pera sweet orange [*Citrus sinensis* (L.) Osb.], West Indian lime types [*C. aurantifolia* (Christm.) Swing.], and grapefruit varieties (*C. paradisi* Macf.). For these citrus types, yield losses may result from infection with certain stem-pitting strains or strain complexes of the tristeza virus.

One possible control method for the losses induced by tristeza on the above-mentioned citrus types is the pre-immunization of virus-free clones with mild strains or complexes that would protect the plants against further infection with the severe stem-pitting complexes. Protection interference between components of the tristeza virus complex has been reported from several sources (1, 2, 3, 4, 5, 6).

The investigations reported here were undertaken with the aim of finding adequate mild strains or complexes that would not injure the citrus types more susceptible to stem pitting and at the same time would protect them against the field stem-pitting virus complexes.

Material and Methods

SOURCES OF VIRUS ISOLATES.—Mild virus isolates were collected from various citrus-growing areas of São Paulo and especially from orchards where injury due to the severe stem-pitting complexes was more noticeable. Outstanding trees in orchards of Pera sweet orange, Galego lime, and grapefruit varieties that showed mild or less noticeable symptoms were selected as mild virus sources.* In some instances, paired collections were made from the same orchards, one collection from trees with mild or no symptoms and the other from severely affected plants.

Over 70 mild isolates were collected and from these 45 were included in field exposure tests. From 50 mild isolates tested in the greenhouse, only 13 (10 from Galego lime and 3 from Pera sweet orange) induced symptoms that were mild enough to warrant their use in protective interference trials. These were further tested by super-inoculation with severe stem-pitting isolates.

All virus source plants were established by budding in nursery rows at the Main Agricultural Experiment Station of the Instituto Agronômico, Campinas, Brazil.

FIELD EXPOSURE TESTS.—Three nursery plots—Caipira sweet orange, Cleopatra mandarin (*C. reticulata* Blanco), and Rangpur lime (*C. reticulata* var. *austera* hyb.)—were prepared as rootstocks for the field exposure tests. A total of 45 mild and 5 stem-pitting isolates were inoculated on the rootstocks, each isolate on 5 plants of each rootstock. One month after the inoculation, 5 new virus-free clonal lines each of Pera sweet orange, Galego lime, and Ruby Red grapefruit were budded on the inoculated rootstocks so that each isolate was on each of the 5 clones of the 3 citrus types. Growth of the virus-carrying buds was permitted to reach about 10 in. before being trimmed. The scion growth of the new clones was topped and permitted to develop 3 or 4 branches. When the

*The possibility that some of these outstanding plants could represent mutants with tissues of high tolerance to the disease was recognized and investigated.

nursery plants attained the desirable size, they were transplanted to the field and set 13 feet apart in the row with 17 feet between the rows.

In addition to being exposed to field infection, each stock-scion-isolate combination in the orchard was handled as follows: (a) the first 3 plants in the row were kept without further treatment, exposed only to natural infection; (b) the fourth plant of each combination was super-inoculated with 3 buds carrying a challenging stem-pitting isolate (challenger No. 1); and (c) the fifth plant in the row was super-inoculated with the challenging stem-pitting isolate by means of a minimum of 50 aphids (*Toxoptera citricida* Kirk.) bred on the infected virus source (challenger No. 2).

GREENHOUSE TESTS.—The protective effect of mild isolates was evaluated in greenhouse tests by inoculation of small seedlings with the first virus, followed by super-inoculations with the challenging isolate 2 months later. The protective inoculation was made with non-viruliferous aphids from squash that were transferred onto caged young shoots of the virus source plants and permitted to breed. They were then used for inoculation of the very young seedlings. The challenge inoculation was carried out likewise. Healthy controls, as well as healthy controls that were inoculated with the challenging virus only, were prepared.

In preparing blends of mild strains, the components of each blend were inoculated simultaneously by means of 2 leaf pieces each on seedlings of all citrus types to be tested. Six months after the simultaneous inoculation, leaf pieces from the plants that had received the 3 isolates and probably had them in a stable blend were used to pre-immunize seedlings of Pera sweet orange, Galego lime, and Ruby Red grapefruit that were subsequently challenge-inoculated with the stem-pitting isolates.

Experimental Results

FIELD EXPOSURE TESTS.—Three years after the protective inoculation and one and one-half years after planting in the orchard, the effect of 45 mild isolates on the Pera sweet orange and Ruby Red grapefruit scions was practically indistinguishable from the non-inoculated controls. Trees of Pera sweet orange and Ruby Red grapefruit that had been inoculated with only the stem-pitting isolates at the same time when the others were pre-immunized showed only a slightly slower growth and some stem-pitting symptoms. The differences are, however, too small to be reported at present. On the other hand, the reaction of the different isolates on the Galego lime scions was very clear-cut. Out of 45 mild isolates that were used for the protective inoculations, 37 induced medium to severe tristeza symptoms and could not be considered as adequate for protection of this citrus type. Eight of the mild isolates permitted the Galego scions to develop satisfactorily, their type of growth being almost equivalent to that of non-infected control plants. The scoring for growth, tristeza symptoms, and stem pitting of the Galego lime plants pre-immunized with the 8 isolates and further inoculated with the challenging isolates, as well as the respective controls, is shown in Table 1.

GREENHOUSE PROTECTION TESTS OF SINGLE MILD ISOLATES.—A number of mild isolates collected from Pera sweet orange and Galego lime

TABLE 1. FIELD REACTION OF PRE-IMMUNIZED GALEGO LIME SCIONS AFTER SUPER-INOCULATION WITH SEVERE STEM-PITTING ISOLATES

Isolate number	Decline symptoms (s), stem-pitting (p), and growth (g) scoring of Galego lime scions treated as indicated								
	Pre-immunized[c]			Pre-immunized plus challenger No. 1[d]			Pre-immunized plus challenger No. 2[d]		
	s	p	g	s	p	g	s	p	g
Mild 40[a]	1.0	1.0	4.8	1.0	0.6	4.5	1.0	0	4.7
42	1.0	0.7	4.7	1.0	0.3	4.9	1.0	0	4.2
50	1.0	0.7	4.7	1.0	0	4.4	1.0	0.3	4.7
72	1.0	1.0	4.1	1.0	0.5	4.0	1.0	0.5	4.2
128	1.2	1.3	4.3	1.0	1.3	4.4	1.0	1.0	1.0
130	1.0	1.1	3.9	1.0	1.0	4.2	1.0	1.0	4.5
141	1.0	1.0	4.1	1.0	1.0	4.0	1.0	1.0	4.0
142	1.0	1.0	4.2	1.0	1.0	4.7	1.0	1.0	4.2
AVERAGE	1.0	1.0	4.3	1.0	0.7	4.4	1.0	0.6	4.3
Stem pitting 33[b]	4.0	4.4	2.0						
83	3.7	4.8	2.2						
94	4.0	3.2	1.9						
135	4.3	4.8	1.7						
AVERAGE	4.0	4.3	1.9						
Checks, natural infection	2.9	3.5	3.2						
Checks, inoc. challenger No. 1				2.5	2.5	2.6			
Checks, inoc. challenger No. 2							2.5	2.7	3.2
Checks, not infected	0	0	5						

a. Mild isolates used for pre-immunization. Isolate numbers in accession book in the Virus Department.

b. Stem-pitting isolates inoculated on controls at the time other plants were pre-immunized.

c. and d. Figures represent averages based on 9 and 3 plants, respectively.

(s) and (p) Tristeza and stem-pitting symptoms rating, 0 to 5. 0 = no symptoms; 5 = most severe symptoms. Intermediate values for intermediate types of symptoms.

(g) Growth rating, 1 to 5; 1 = poorest growth; 5 = best.

orchards tested in the field were also compared as to their protective value in greenhouse tests. Thirteen of the best mild isolates have been tested so far on Galego lime, Pera sweet orange, and Ruby Red grapefruit seedlings. The results obtained are summarized in Tables 2, 3, and

TABLE 2. GREENHOUSE COMPARISON OF THE PROTECTIVE EFFECT OF SELECTED MILD TRISTEZA VIRUS INOCULATION ON GALEGO LIME SEEDLINGS SUBSEQUENTLY SUPER-INOCULATED WITH SEVERE STEM-PITTING SOURCES

Isolate number and origin		Tristeza symptoms (s), stem pitting (p), and growth (g) scoring for Galego lime seedlings inoculated in the indicated manner								
		Pre-immunized[a]			Pre-immunized and challenger[b]			Challenger only[b]		
		s	p	g	s	p	g	s	p	g
Mild Pera	19	5.0	5.0	1.0						
sweet orange	28	5.0	5.0	1.0						
AVERAGE		5.0	5.0	1.0				5.0[c]	5.0[d]	1.0[e]
Mild	34	2.0	3.0	2.5	2.0	5.0	2.5			
Galego	36	1.0	1.0	4.5	1.0	1.0	4.0			
lime	38	1.0	1.0	4.7	1.6	2.0	3.3			
	40	1.0	1.0	4.5	2.0	1.0	3.7			
	42	1.0	1.0	4.2	1.0	1.0	3.7			
	50	2.3		2.3	3.5		2.0			
	100	1.5	1.0	3.0	3.5		2.0			
	128	2.0	1.0	2.7	4.0	3.0	1.4			
	141	1.2	1.0	3.5	2.0	3.0	3.0			
	142	1.0	1.0	4.5	1.0	3.0	3.7			
AVERAGE		1.4	1.2	3.6	2.2	2.3	2.9	5.0[c]	5.0[d]	1.0[e]
Stem pitting	83	5.0	5.0	1.0						
	94	5.0	5.0	1.0						
AVERAGE		5.0	5.0	1.0						
Controls (not inoculated)		0	0	5.0						

a. and b. Figures represent averages based on 3 and 5 plants, respectively.
c., d., and e. Scoring for control plants inoculated with the challenger at the same time as the pre-immunized plants.

4. They show that the pre-immunization of Galego lime seedlings with the mild isolates from Pera sweet orange induced symptoms already severe, indicating that they were not adequate to pre-immunize this species. Mild isolates from Galego lime plantings gave somewhat contradictory results, especially when the data from the greenhouse tests were compared with those obtained from the field tests. Although the pre-immunized Galego seedlings, subsequently challenge-inoculated, showed

weaker symptoms than those that received only the challenger, there was an increase in symptoms following the super-inoculation that could be assessed by comparison with those seedlings that were only pre-immunized. Satisfactory protection was noted only in the case of isolates 36, 40, and 42 (Fig. 1,F).

TABLE 3. GREENHOUSE COMPARISON OF THE PROTECTIVE EFFECT OF SELECTED MILD TRISTEZA VIRUS INOCULATION ON PERA SWEET ORANGE SEEDLINGS SUBSEQUENTLY SUPER-INOCULATED WITH SEVERE STEM-PITTING SOURCES

Isolate number and origin		Tristeza symptoms (s), stem-pitting (p), and growth (g) scoring for Pera sweet orange seedlings inoculated in the indicated manner								
		Pre-immunized[a]			Pre-immunized and challenger[b]			Challenger only[b]		
		s	p	g	s	p	g	s	p	g
Mild	19	1.5	1.0	3.5	2.0	1.0	3.0			
Pera sweet	28	1.0	1.0	4.3	1.0	1.0	4.0			
orange	66	1.0	2.0	3.5	1.5	5.0	3.0			
AVERAGE		1.2	1.3	3.7	1.5	2.3	3.3	4.0[c]	5.0[d]	2.0[e]
Mild	34	1.1	0	3.5	1.5	0	3.0			
Galego	36	2.0	0	3.0	2.0	2.0	3.0			
lime	38	1.5	0	3.0	1.5	1.0	3.0			
	40	1.2	0	3.8	1.5	0	3.2			
	42	1.2	0	4.0	1.5	1.0	4.0			
	50	1.0	0	4.3	1.0	0	3.7			
	100	1.7	0	2.1	1.7	0	1.7			
	128	1.0	0	4.0	1.0	0	4.0			
	141	1.0	0	4.0	1.5	5.0	3.0			
	142	1.0	0	4.5	1.0	1.0	4.0			
AVERAGE		1.3	0	3.6	1.4	1.0	3.3	4.0[c]	5.0[d]	2.0[e]
Stem pitting	83	3.0	4.0	2.0						
	94	5.0	5.0	1.0						
AVERAGE		4.0	4.5	1.5						
Controls (not inoculated)		0	0	5.0						

a. and b. Figures represent averages based on 3 and 5 plants, respectively.
c., d., and e. Scoring for control plants inoculated with the challenger at the same time as the pre-immunized plants.

Pera sweet orange seedlings protected with mild isolates from Pera and later super-inoculated behaved identically to those that had been protected with mild isolates collected from Galego lime orchards. When compared with the plants that received only the challenger, there was a good indication of protection (Fig. 1,E).

Ruby Red grapefruit seedlings protected with mild isolates from Pera sweet orange showed stronger symptoms than those that received mild isolates from Galego lime. Both groups of isolates, however, gave definite protection against super-inoculation with the severe stem-pitting isolates (Fig. 1,G).

MILD ISOLATE BLENDS VERSUS INDIVIDUAL COMPONENTS.—What have been called mild tristeza isolates in the present paper represent individual field strains or strain complexes of the virus collected from outstanding trees in orchards that were severely affected by stem pitting when the collection was made.

TABLE 4. GREENHOUSE COMPARISON OF THE PROTECTIVE EFFECT OF SELECTED MILD TRISTEZA VIRUS INOCULATION ON RUBY RED GRAPEFRUIT SEEDLINGS SUBSEQUENTLY SUPER-INOCULATED WITH SEVERE STEM-PITTING SOURCES

Isolate number and origin		Tristeza symptoms (s), stem-pitting (p), and growth (g) scoring for Ruby Red grapefruit seedlings inoculated in the indicated manner								
		Pre-immunized[a]			Pre-immunized and challenger[b]			Challenger only[b]		
		s	p	g	s	p	g	s	p	g
Mild Pera	19	2.5	3.6	2.5	2.5	4.0	2.5			
sweet orange	28	2.0	3.0	2.9	2.0	3.0	2.2			
AVERAGE		2.2	3.3	2.7	2.2	3.5	2.3	5.0[c]	5.0[d]	1.0[e]
Mild	34	1.0	0	3.5	1.0	0	3.0			
Galego	36	1.0	0	4.0	1.5	0	3.5			
lime	38	1.0	0	4.5	1.9	0	3.5			
	40	1.0	0	4.0	1.0	0	4.0			
	42	1.0	0	4.2	1.0	0	3.8			
	50	1.0	0	3.5	1.0	0	3.5			
	100	1.6	0	3.5	1.3	0	3.6			
	128	1.8	0	2.6	3.4	0	1.5			
	141	1.0	0	4.3	1.0	4.0	4.0			
	142	1.5	0	3.0	2.0	3.0	3.0			
AVERAGE		1.2	0	3.7	1.5	0.1	3.3	5.0[c]	5.0[d]	1.0[e]
Stem pitting	83	5.0	5.0	1.0						
	94	5.0	5.0	1.0						
AVERAGE		5.0	5.0	1.0						
Controls (not inoculated)		0	0	5.0						

a. and b. Figures represent averages based on 3 and 5 plants, respectively.
c., d., and e. Scoring for control plants inoculated with the challenger at the same time as the pre-immunized plants.

In the course of the present work the possibility was examined that the protective effect of mild tristeza virus isolates could be enhanced by adequately blending them. It was also expected that by mixing mild isolates from different citrus areas, a combination could be obtained that would have a wider protection range. The use of blends for protection

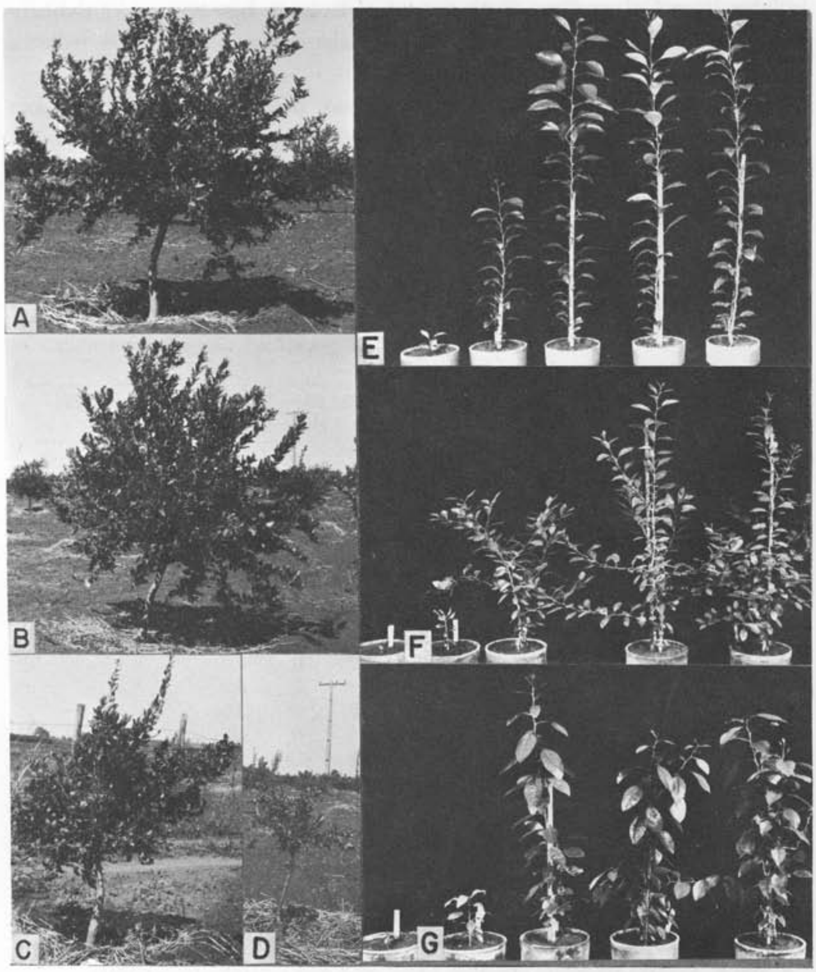

FIGURE 1. *Field interference test. A. Pre-immunized Galego lime plant. B. Pre-immunized and super-inoculated with stem-pitting isolate. C. Non-pre-immunized and naturally infected. D. Control plant inoculated with stem-pitting isolate at the time of pre-immunization. Greenhouse interference test. E., F., and G. Five seedlings each of Pera sweet orange, Galego lime, and Ruby Red grapefruit inoculated (left to right), as follows: (1) with stem-pitting isolates at pre-immunization; (2) with stem-pitting isolates at super-inoculation; (3) pre-immunized with mild isolate and super-inoculated with stem-pitting isolate; (4) with pre-immunizing isolate only; and (5) virus-free controls.*

was conditioned, however, to the fact that the resulting blend must still be mild for the plants to be protected and relatively stable. Also, the combination of certain isolates of different invasion power or the ability to invade different tissues might increase the protection value of the blend when compared with that of the individual components.

An experiment was carried out in which 5 selected mild isolates from different areas, combined in groups of 3, were inoculated comparatively on 3 seedlings each of Pera sweet orange, Galego lime, and Ruby Red grapefruit. Isolates used for the blends were also inoculated individually. Two months after the pre-immunization with blends and individual mild isolates, 1 seedling of each group of 3 was super-inoculated with a severe stem-pitting source by leaf piece grafts, another by means of the vector, and the third was kept only pre-immunized. The results of the test, 4 months after the super-inoculation, are in Table 5. They indicate that the symptoms induced by the blends, as well as by individual components, were practically comparable. No difference in the protective effect of the blends when compared with the individual components was noted.

SPECIFIC EFFECT OF MILD TRISTEZA VIRUS ISOLATES.—Results from comparative inoculations with mild isolates collected from Pera sweet orange, Galego lime, and grapefruit varieties indicated that the effect of the mild isolates was not necessarily parallel when inoculated on the different types of citrus. Thus, many isolates collected as mild from Pera sweet orange and grapefruit varieties induced severe reactions on the Galego lime test plants. Some isolates that were mild for Ruby Red grapefruit scions induced strong stem-pitting symptoms on the Pera sweet orange.

Isolates from Galego lime plants showed a tendency to be milder than those from the other citrus types and behaved generally as mild on Pera sweet orange and Ruby Red grapefruit. Collections made from different orchards showed that those from Galego lime plantings tended to give a much higher frequency of mild isolates than those from Pera sweet orange and grapefruit plantings.

Discussion and Conclusions

The results from protective interference tests carried out in the field and in the greenhouse, described in this paper, supply evidence that in a great number of cases, mild tristeza virus isolates confer protection to pre-immunized Galego lime scions or seedlings against further injury from super-inoculation with severe stem-pitting isolates. This confirms

TABLE 5. Comparison of the protective effect given by mild isolate blends and individual components

Isolates	Tristeza (s) and growth scoring (g) for 3 seedling types treated as follows																	
	Pera sweet orange						Galego lime						Ruby Red grapefruit					
	blend or isolate only		ditto plus				blend or isolate only		ditto plus				blend or isolate only		ditto plus			
			challenge[a]		challenge[b]				challenge[a]		challenge[b]				challenge[a]		challenge[b]	
	s	g	s	g	s	g	s	g	s	g	s	g	s	g	s	g	s	g
40, 50, 130	0	4.5	0	5.0	0	4.5	1.0	4.0	1.0	5.0	2.0	3.5	0	4.0	0	4.0	1.0	5.0
40, 50, 141	0	4.0	0	4.5	0	5.0	1.0	4.5	1.0	4.5	1.0	4.0	1.5	4.5	1.0	4.5	1.0	4.5
40, 50, 19	0	3.0	0	5.0	0	3.5	1.0	4.0	1.0	4.5	1.0	4.5	3.0	3.5	2.0	4.0	2.0	3.5
40, 130, 141	0	5.0	0	5.0	0	2.5	1.5	3.5			1.5	4.0	1.5	3.5	0	4.5	0	3.5
40, 130, 19	0	5.0	0	2.5	0	5.0	2.0	3.0	2.5	4.0	2.5	4.0	1.0	4.5	1.0	4.5	1.0	4.5
40, 141, 19	0	3.0	0	5.0	0	3.0	1.0	4.0	1.0	5.0	1.5	4.0	2.0	4.5	0	4.0	1.0	4.5
50, 130, 141	0	4.0	0	5.0	0	4.5	1.0	4.0	2.0	4.0	2.5	3.5	0	4.0	1.0	4.0	1.5	4.0
50, 130, 19	0	4.5	0	4.5	0	4.5	1.5	4.0	1.0	4.0	1.5	4.0	1.5	4.0	1.5	4.5	1.0	4.0
50, 141, 19	0	4.0	0	5.0	0	5.0	1.0	2.5	2.0	2.5	1.0	4.0	1.5	5.0	1.5	5.0	1.0	4.0
130, 141, 19	0	2.5	0	5.0	0	2.5	2.0	3.0	1.0	4.0	2.0	4.0	1.5	3.5	2.0	4.0	1.5	4.0
Average	0	3.9	0	4.6	0	4.0	1.3	3.6	1.4	4.2	1.6	3.9	1.3	4.1	1.0	4.3	1.1	4.1
19	0	3.5	0	5.0	0	3.5	2.0	3.0	2.0	4.5			1.0	4.0	1.0	5.0	1.5	4.5
40	0	4.0	0	4.5	0	4.0	1.0	4.5	1.0	4.5	1.0	4.0	2.0	4.0	1.0	4.5	2.0	4.5
50	0	5.0	0	5.0	0	3.5	1.5	4.0	1.0	4.0	1.0	4.5	1.0	4.5	1.0	4.5	1.5	5.0
130	0	4.5	0	5.0	0	2.5	2.0	4.0	2.0	4.5	2.0	4.5	2.0	4.5	1.0	4.5	1.5	4.5
141	0	3.5	0	4.0	0	4.5	1.0	4.5	1.0	4.5	1.0	4.5	1.5	5.0	1.0	5.0	1.0	5.0
Average	0	4.1	0	4.7	0	3.6	1.5	4.0	1.4	4.4	1.2	4.4	1.5	4.4	1.0	4.7	1.5	4.7
Barão	0	4.0	0	4.0			4.0	2.0	3.0	2.0			1.0	4.5	2.0	4.0		
120	0	3.5					3.0	3.2			3.0	2.5	1.0	4.0				

a. The challenge inoculations were made by aphids. Virus source Barão B.
b. The challenge inoculations were made by leaf pieces from stem-pitting source No. 120.

previous findings by other investigators (1-6). Whether the protective effect on the Galego plant is permanent or breaks down as time elapses remains to be seen, since the protection effect noted has been recorded for only three years after field exposure and one and one-half years after super-inoculation with the challenging stem-pitting isolates. Protection was more evident under field conditions than in the greenhouse tests.

No difference was noted in the field between decline symptoms or growth rate of Pera sweet orange and Ruby Red grapefruit scions pre-immunized and super-inoculated, or controls exposed only to natural infection. Even the scions that had been inoculated with stem-pitting isolates at the time the others were pre-immunized showed a type of growth only slightly slower than the others, although stem pitting was present in them. On the other hand, Pera sweet orange and Ruby Red grapefruit seedlings in the greenhouse showed considerable differences when pre-immunized and challenge-inoculated plants were compared with those that received only the challenging inoculation, thus indicating that protection was operative. This is considered an indication that greenhouse protection tests are more effective for these citrus types and that results from field tests require a longer period for evaluation.

Mild isolates from one citrus type are not necessarily mild to others. Thus, mild isolates from Pera sweet orange were generally rather severe on Galego lime scions. It was noted, however, that mild isolates from Galego lime tended to be mild for other citrus types. Although the effect of the tristeza virus isolate seems to be rather specific, it might be suggested that the Galego lime tissues are highly sensitive to the effects of the tristeza virus and better suited to indicate virus sources that are really mild in their effect on citrus tissues.

Blends of mild isolates induced slight tristeza symptoms not any stronger than those resulting from infection with the individual isolates. Also, there was no gain in protection by the use of blends in comparison with the individual isolates. It is expected, however, that in the long run protective interference would be better achieved by the inoculation of adequate blends collected from different areas or plant sources. These blends would afford protection to a wider range of severe tristeza complexes because they would combine isolates of different invasive power and relationships.

Galego lime orchards were a better source for mild isolates than were Pera sweet orange and grapefruit groves. This is believed to result from the high sensitivity of the Galego plants to the tristeza virus, and consequently, when budwood is collected for multiplication there is a tendency for selection on the part of the nursery man that will lead to the choice of trees carrying mild tristeza complexes.

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