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Authors
Balaraman, K.
Ramakrishnan, K.

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Strain Variation and Cross Protection in Citrus Tristeza Virus on Acid Lime

K. Balaraman and K. Ramakrishnan

Cross protection with mild strains of tristeza virus on Galego lime and grapefruit stem-pitting virus has been reported under field conditions (Müller and Costa, 1968, 1972; Fraser *et al.*, 1968; Stubbs, 1964). Evidence is presented in this paper on the occurrence of a multiplicity of natural tristeza virus strains in South India and cross protection with two mild strains on acid lime under field conditions. Earlier results have been published (Balaraman and Ramakrishnan, 1977, 1978). Further results on growth and yield records obtained up to 5 years are reported. Also data on growth and vigor, and yields of lime seedlings preinoculated with mild strains of tristeza and planted in different locations of South India and the persistence of the mild strain under field conditions are presented.

MATERIALS AND METHODS

A survey was conducted and 112 budwood samples thought to be infected were collected from commercial citrus orchards of Andhra Pradesh, Karnataka and Tamil Nadu. These were indexed on acid lime indicator plants, and tristeza virus was successfully transmitted in 70 cases. These were separated from other viruses by transmission to 30-day-old acid lime seedlings with the aphid vector *Toxoptera citricida* Kirk. After 6 months, isolates were classified into six categories (Grades 1 to 6) ranging from very mild to very severe, using the symptoms on acid lime, viz. vein clearing, stem-pitting, stunting, root and shoot loss and girth. For further differentiation, one isolate from each grade was used to inoculate 82 clones belonging to 26 species of *Citrus*, four hybrids, and five other rutaceous hosts.

Cross-protection studies were carried out with two mild strains (S_1 = very mild; S_2 = mild) against a severe (S_5) strain. Initial inoculations were made on 9-month-old lime seedlings, and the seedlings were cut back to a 30-cm single stem for quick expression of symptoms. Challenge inoculation with severe strains was done 8 weeks later. There were six treatments with eight replications. The treatments were: (1) uninoculated control; (2) and (3) inoculated with very mild (S_1) and mild (S_2) strains, respectively; (4) inoculated with the severe strain (S_5); (5) and (6) preinoculated with (S_1) and (S_2), respectively, and later challenge-inoculated with the severe strain. Trees were planted in June 1974 on a 2- x 2-m spacing. Data on vein clearing, stem-pitting, height, circumference, volume and fruit yield were recorded.

EXPERIMENTAL RESULTS

Of 70 isolates from Andhra Pradesh, Karnataka, Tamil Nadu, and Uttar Pradesh, 23 showed mild symptoms, 28 moderate, and 19 severe symptoms (table 1). The symptoms in the field on acid lime trees were duplicated on glasshouse plants. Further, the aphid-transmitted isolates showed similar symptoms.

For differentiating and identifying each strain, the host-virus reaction with 82 clones belonging to different species, hybrids and relatives of citrus against six categorized isolates was studied and infection types were coded from 0 to 5 in order of severity of infection, 0 = complete tolerance and 5 = severe reaction. On the same variety, various degrees of disease severity with different isolates were noted. The types of infection on nine different varieties allowed identification of six isolates as six strains (S_1 to S_5 and HD). These nine hosts were proposed as a set of differential hosts, and their reactions to each strain are presented in table 2.

Reactions to any new isolate of tristeza could be tested on the nine differential hosts and, if the symptoms correspond with those indicated for a particular strain, the identification is complete. If they do not, the isolate may be regarded as a new one. It is likely that many more forms would be recognized if a proper combination of differential hosts were employed. After the type and degree of infection on the differential hosts have been recorded, it is relatively easy to determine the biologic forms or biotypes within a strain.

Cross-protection test. The field experiment planted in June 1974 provided evidence that mild strains (S_1 and S_2) afforded complete protection against the severe strain. Preinoculated and preinoculated + challenge-inoculated (cross-protected) plants grew vigorously for 5 years (table 3). The height and circumference were twice as great in preinoculated and cross-protected plants as in plants inoculated with the severe strain, whereas volumes were seven times as great (fig. 1). Plants inoculated with the severe strain yielded an average of 80 fruit per tree per annum, which were small with little juice. Moreover, these plants did not flower until the fourth year, whereas trees in other treatments fruited in the third year. The preinoculated and cross-protected plants averaged 1355 fruit per tree, 50 per cent more than uninoculated control plants. Apparently, the originally healthy control plants become naturally infected with either severe or mild strains, and those plants with severe strains started to decline, and average growth, circumference, volume, and fruit yield were reduced.

So far, even after 5 years, no breakdown in protection has been noted in the cross-protected plants under field conditions, although they were inoculated with the severe strain. The plants inoculated with the severe strain ceased growth, showed severe vein flecking on the leaves, deep pits on the wood, yellowing of the foliage, and dieback of young twigs.

Persistence of mild strains. The status of the mild strains in inoculated acid lime under field conditions, planted as early as June 1974, was tested by repeated back indexing on lime indicator plants. The S_1, S_2 and S_5 strains expressed their respective degree of symptom intensity, while the cross-protected plants showed only the presence of the mild strain, although they were originally inoculated with mild and severe strains (table 4). Uninoculated control plants were 100 per cent infected with either mild or severe strains. Indexing of individual branches revealed trees infected with mild and severe strains in two different branches of the same tree. In one case,

TABLE 1
RESULTS OF INDEXING OF BUDWOOD COLLECTED AT VARIOUS LOCATIONS IN SOUTH INDIA

Locality	Number of collections	Reaction on acid lime*		
		Mild	Moderate	Severe
Andhra Pradesh	24	8	11	5
Karnataka	35	11	13	11
Tamil Nadu	9	4	2	3
Uttar Pradesh	2	—	2	—
Total:	70	23	28	19

* Reaction based on 10 indicator plants.

TABLE 2

REACTION PATTERN OF PROPOSED SET OF DIFFERENTIAL HOSTS TO IDENTIFY TRISTEZA VIRUS STRAINS

Strains	Variety proposed and its reaction*								
	Cleo mandarin	Shang-yuan	Shun-kokan	Acid lime	L. Rubi	Kikudai-dai	L. Barao	Marsh grapefruit	Pummelo
S_1: Very mild	0	5	0	1	2	3	0	4	5
S_2: Mild	0	0	5	2	2	4	1	5	5
S_3: Moderate	0	1	0	3	3	1	2	0	5
S_4: Moderately severe	0	1	0	4	3	4	1	0	5
S_5: Severe	0	1	0	5	3	2	0	5	5
HD: Severe with corky vein	0	1	3	5	3	5	2	5	5

* 0 & 1, highly tolerant; 2 & 3, moderately tolerant; 4 & 5, highly intolerant. Grades of severity based on lime reaction.

two branches had a severe strain and one branch had a mild strain, and the reverse occurred in another tree.

Preinoculation performance trials. Acid lime seedlings preinoculated with one of the best mild strains (S_1) of tristeza have been tested in six locations in South India. Seedlings inoculated with mild strains made satisfactory growth and were comparable to healthy seedlings. In locations one and two (Tamil Nadu and Andhra Pradesh) (table 5), the mild strain was even milder because of high temperatures ($35 \pm 5°C$) and showed very few vein flecks, and occasional wood pits. The growth and vigor measurements confirm their performance in different agro-climatic regions of South India.

DISCUSSION AND CONCLUSIONS

The survey of citrus orchards revealed the widespread occurrence of tristeza virus strains and its aphid vector *T. citricida.* Indexing on lime indicator plants showed cupping, yellowing, vein clearing, stem-pitting, stunting, and death of roots and rootlets. Earlier, the term "stem pitting" was used to designate a specific disease of grapefruit called "stunt bush" (Oberholzer *et al.*, 1949), but later it was pointed out that the tristeza and stem-pitting disease of grapefruit in Africa are related (Costa *et al.*, 1950). McClean (1950) proved the disease was infectious and transmissible by grafting and by *T. citricida*, which also transmits the tristeza virus.

Fraser (1952) found that young seedlings of Eureka lemon, sour orange, and grapefruit produced seedling yellows symptoms when grafted with tissue from sweet orange and mandarin trees. The same varieties grafted with tissue from field trees of grapefruit, Eureka lemon, and sour orange had occasional vein flecks in the spring flush. At first, Fraser considered seedling yellows to be distinct from tristeza. McClean and van der Plank (1955) suggested that the tristeza virus complex had a stem-pitting and seedling yellows component. However, Knorr and Price (1956) hypothesized that tristeza, stem-pitting, seedling yellows, and the Gold Coast's lime dieback were caused by a single virus which existed as numerous strains. Later, Fraser (1959) considered seedling yellows a reaction to tristeza virus, and stem-pitting as a distinct virus, but was unable to separate them. McClean (1974) considered tristeza a complex of strains and components.

Current observations suggest that the symptoms described above are all due to tristeza virus. These observations

TABLE 3
GROWTH AND YIELD OF ACID LIMES INOCULATED WITH VARIOUS TRISTEZA STRAINS IN A CROSS-PROTECTION EXPERIMENT

Strains inoculated*	Tree height (m)	Trunk circumference (cm)	Canopy volume (m^3)	Vein clearings/ leaf	No. of pits/ 10 cm stem	Fruit yield no./ tree	Fruit yield kg/ tree
Uninoculated control	3.0†	32.0	12.2	4.1	2.7	937	31
Very mild (S_1)	3.8	34.5	17.0	3.0	2.6	1430	51
Mild (S_2)	3.5	32.5	16.1	4.6	3.5	1310	49
Severe (S_5)	1.6	19.0	2.5	13.0	9.9	80	2
Protected$_1$ ($S_1 + S_5$)	3.7	34.0	16.7	3.0	2.5	1420	51
Protected$_2$ ($S_2 + S_5$)	3.4	32.4	15.9	4.8	3.0	1260	47

* Planted June 1974; data recorded to April 1979.

† Mean of 8 replications.

plus evidence presented by Capoor (1965) and Knorr and Price (1956) suggest that tristeza is not a disease complex produced by unrelated viruses, but is caused by a single virus that exists in nature as numerous strains. Much of the earlier confusion arose because of experimentation under uncontrolled conditions. In the present study, possible errors have been minimized by separating all isolates by aphid transmission, raising indicator plants from a single source, and standardization of inoculation procedure.

Few attempts have been made to differentiate tristeza virus strains by differential host reactions. Probably, the reason for not establishing differential hosts for identification is because many plant viruses exist as a large family of strains. Bawden and Kassanis (1947) indicated that many strains of PVY differed in virulence, but remained stable for 3 years when they were transmitted to different plants. In the present study, isolates from each group behaved as stable entities on repeated inoculation to acid lime seedlings, as well as to other citrus types. It therefore appeared worthwhile to explore the possibility of developing a set of differentials for the identification of tristeza virus strains. Attempts have been made to differentiate virus strains on differential hosts. Seven strains (pathotypes) of common bean mosaic virus have been identified with a standard set of differentials (Drijfhout *et al.*, 1978). Similarly, four strains of tungro virus have been identified with six rice varieties (Anjaneyulu and John, 1972), and pepper virus strains have been identified with differential pepper cultivars (Makkouk and Gumpf, 1974; Zitter, 1972).

Based on the infection types on 82 clones of citrus, six isolates of tristeza have been identified as six strains (S_1 to S_5 and HD) with a set of nine differential hosts (table 2). To promote international standardization in tristeza research, recommendations are given for test conditions and procedures, criteria for strain differentiation and maintenance of virus strains.

Symptoms and growth, volume and yield records support the conclusion that the mild srains (S_1 and S_2) afford a high degree of protection against the challenge-inoculated severe strain. Protective effects of mild tristeza strains against stem pitting under field conditions have been tested on Galego lime in Brazil (Müller and Costa, 1968, 1972) and on grapefruit in Australia (Fraser *et al.*, 1968).

Seedlings carrying mild strains and cross-protected seedlings (challenge-inoculated with the severe strain) did not show significant variation in leaf symptoms and stem pitting, indicating complete protection by the mild strain. Planting virus-free trees does not ensure freedom from infection on acid lime in regions where tristeza and its aphid vectors are prevalent. In the present study, all uninoculated plants became naturally infected from adjacent sources. When severe-strain infection occurs at an early age, the plants become stunted and completely unproductive, and they linger on for several years. Under average orchard conditions, the mild strain protected for a number of years.

Further, the preinoculation principle demonstrated in the present study has been extended to different locations of South India, and growth measurements confirm the performance of the mild strain under different agro-climatic regions of South India. The number of trials has since been extended to another 24 locations where lime is extensively grown. Large-scale distribution of preinoculated acid limes has been undertaken, and in 5 years, the entire lime-growing area will be covered. Similar trials of stem-pitting virus on grapefruit in different localities in Australia (Fraser *et al.*, 1968) corroborate the above findings.

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TABLE 4
PERSISTENCE OF MILD STRAINS OF TRISTEZA ON ACID LIME. GROWTH AND SYMPTOM SEVERITY OF ACID LIME INDICATORS USED TO INDEX FIELD TREES INOCULATED WITH DIFFERENT STRAINS

	Mean values of different characters						
Treatments	Height (cm)	Circumference (mm)	Vein flecks/ leaf	Stem pits/ 10 cm stem	Root wt. (g)	Shoot wt. (g)	Reaction
Uninoculated control	68.7*	34.2	7.0	6.1	72	133	Mild, Severe, or both
Very mild strain (S_1)	97.4	51.7	3.6	3.6	120	220	Very mild
Mild strain (S_2)	95.7	49.2	5.3	4.5	116	210	Mild
Severe strain (S_5)	51.6	32.5	13.5	9.9	24	61	Severe
Very mild + Severe strain ($S_1 + S_5$)	96.5	50.1	3.7	3.5	121	217	Very mild
Mild + Severe strain ($S_2 + S_5$)	92.8	49.1	5.2	5.0	113	218	Mild

* Mean of eight plants.

TABLE 5
GROWTH AND VIGOR OF THE PREINOCULATION PERFORMANCE TRIALS ON ACID LIME IN DIFFERENT LOCATIONS OF SOUTH INDIA

Location of the trial	Date of planting	No. of plants		Preinoculated seedlings			Healthy seedlings		
		Preinoculated	Healthy	Height (m)	Circumference (cm)	Volume (m³)	Height (m)	Circumference (cm)	Volume (m³)
Location 1 (Tamil Nadu)	Sept. 1975	40	10	3.3*	28	14.7	3.1	27	13.5
Location 2 (Andhra Pradesh)	Sept. 1975	40	10	2.8	25	11.0	2.9	25	10.6
Location 3 (Karnataka)	Sept. 1976	48	12	2.5	23	8.2	2.5	21	7.7
Location 4 (Karnataka)	Sept. 1977	108	36	1.6	14	3.6	1.6	15	3.5
Location 5 (Karnataka)	Sept. 1977	130	20	1.6	15	3.2	1.6	15	3.3
Location 6 (Karnataka)	Sept. 1977	130	20	1.8	17	3.8	1.7	17	3.8

* Data recorded to April 1979.

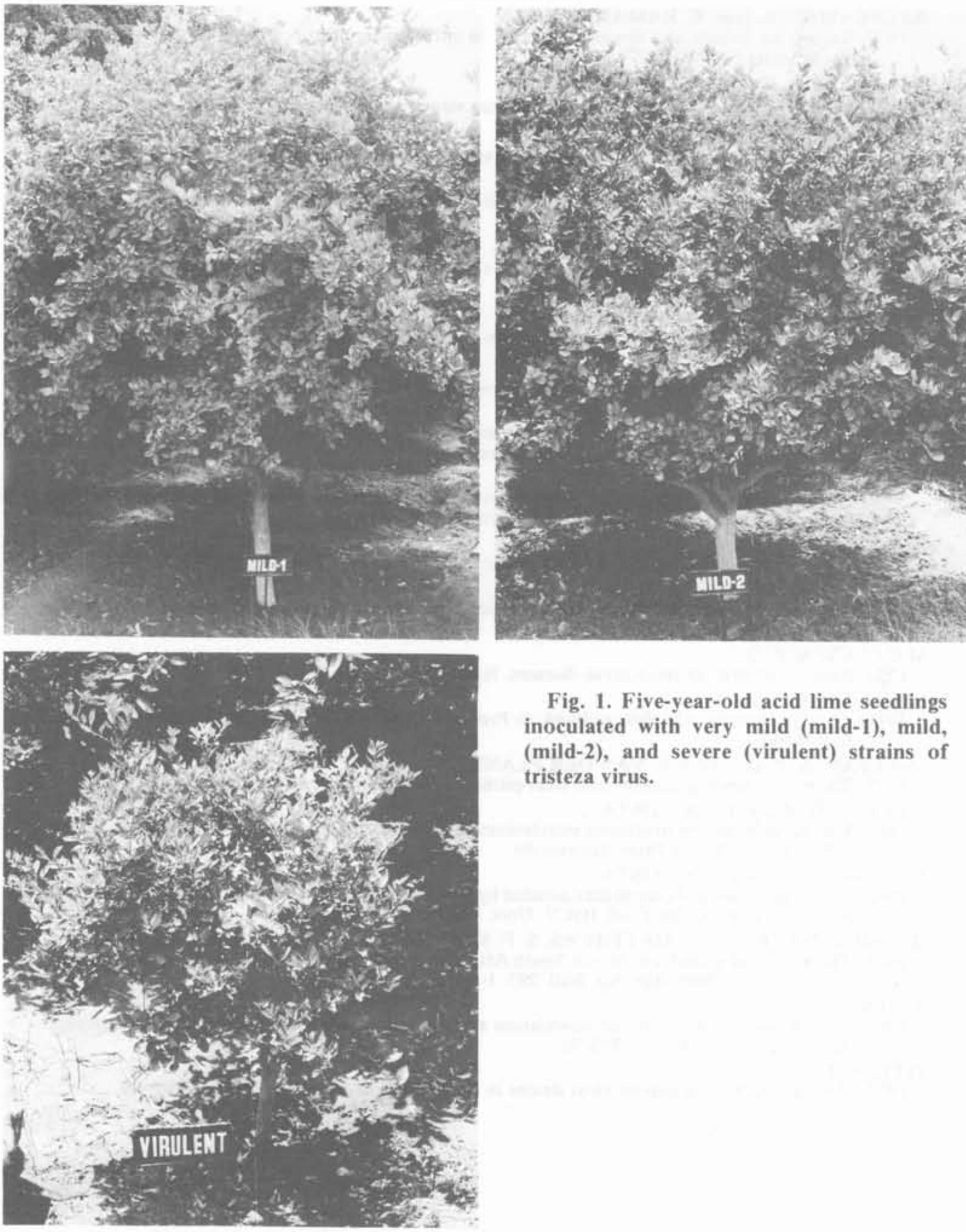

Fig. 1. Five-year-old acid lime seedlings inoculated with very mild (mild-1), mild, (mild-2), and severe (virulent) strains of tristeza virus.

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