Cross Protection Against Citrus Tristeza Seedling Yellows and Stem Pitting Viruses by Protective Isolates Developed in Greenhouse Plants

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ABSTRACT. Protective isolates against citrus tristeza seedlings yellow (CTV-SY) and stem pitting (CTV-SP) were developed in the greenhouse by four methods. Two methods produced a number of highly successful individual protective isolates, nine of which were selected for extensive testing. Six isolates were developed by passage of the virus through symptomless grapefruit seedlings which had previously been inoculated with severe CTV-SY. Three isolates (Code 37, 40, and Z-5) were developed by passage of severe CTV-SY and CTV-SP through *Passiflora* species and back to citrus by *Aphis gossypii*. All nine isolates gave outstanding protection to grapefruit or sweet orange when bud-challenged with the specific source from which they were derived. Two CTV-SY protective isolates showed variable protection when bud-challenged with a number of field isolates of seedling yellows. All six CTV-SY and two CTV-SP attenuated and protective isolates have remained protective over a 4-yr period. However, one isolate (code Z) has shown a tendency to revert back to its severe CTV-SP parent form.

The methods used in these studies provide a relatively rapid means of producing protective isolates against specific CTV-SY or CTV-SP strains, either local or introduced, or for the protection of virus-free, shoot-tip-grafted plants in areas where tristeza is endemic.

Cross protection against the more virulent strains of citrus tristeza virus (CTV) is now practiced in many countries where tristeza is endemic. The search for superior protective strains is an important and continuing worldwide effort. The classical approach to finding protective strains is to search for surviving trees after an epidemic, as in Brazil (4) or India (1). In a recent report (14), another approach was outlined for developing protective strains from existing severe strains which might be tailored to protect against a specific tristeza complex such as small fruit size, stem pitting, excessive stunting or dieback, tree collapse, stock-scion incompatibility, etc. This study was initiated when a very severe citrus tristeza seedling yellows virus (CTV-SY) isolate was discovered spreading in field number 12B, in experimental plots at the University of California, Riverside (UCR) (3, 6). When indexed, budwood from declining trees induced a severe seedling yellows (SY) reaction in seedlings of grapefruit, sour orange, lemon and sweet orange plus severe stem pitting (SP) symptoms in grapefruit and sweet orange, with vein corking in leaves of Mexican lime and sweet orange. This field 12B isolate was found to be highly transmissible by *A. gossypii* (7, 11), and it induced stem pitting and decline in commercial sweet orange, grapefruit and tangelo trees.

Studies were initiated in 1980 to find specific protective isolates which might protect against a challenge inoculation of this severe 12B isolate as well as other CTV-SY and citrus tristeza virus stem pitting (CTV-SP) isolates. In a previous report, four methods were described for obtaining protective isolates (14). These were: 1) by maintaining and testing symptomless and apparently recovered grapefruit, lemon, or sour orange seedlings previously bud-inoculated with specific severe isolates of CTV-SY or CTV-SP; 2) by *A. gossypii* transmission of CTV-SY or CTV-SP in infected grapefruit, lemon or sweet orange seedlings to seedlings of grapefruit, lemon or Mexican lime, for production of attenuated isolates (10); 3) by vector transmission of CTV-SY or CTV-SP from infected sweet orange to Mexican lime using low populations of *A. gossypii* (11) and 4) by *A. gossypii* transmission of CTV-SY and CTV-SP from in-

fected sweet orange to *Passiflora* species and then from *Passiflora* back to Mexican lime (13). All isolates derived by these methods were observed first in Mexican lime and then graft-inoculated from Mexican lime back to grapefruit, lemon, sour orange, and in certain cases sweet orange, to test for the yellows or stem pitting components. A total of 116 mild or attenuated isolates were obtained by these four methods and all were tested for their protective ability against a challenge with the origi-

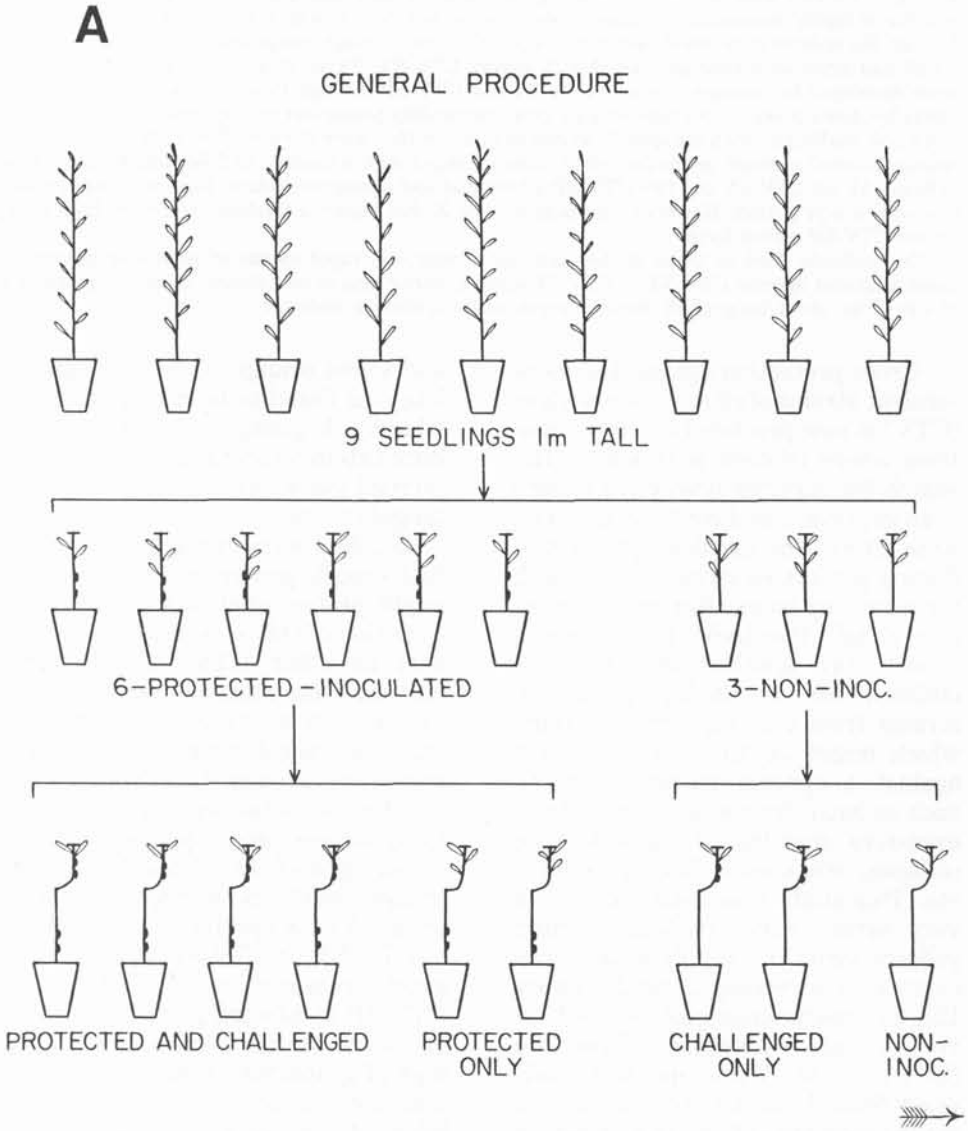

Fig. 1. A) An outline of the general procedure used to evaluate cross protection. Nine seedlings of grapefruit or sweet orange were grown as single shoots to about 1 m. These were cut back and six were protected-inoculated and three left non-inoculated. New growth was trained as a single shoot and after 4 to 6 months, plants were cut back again and challenge-inoculated as shown. Again, a new shoot was trained, and after 4 to 6 months plants were evaluated for seedling yellows and stem pitting. B) The results of a successful inoculation test. Plants are shown in the same sequence as in Fig. 1A. Plants are 8 to 12 months from the initial protection-inoculation. Note the protection in the four plants on the left as compared to the two challenged only plants.

nal isolates from which they were derived. Fifty-two isolates showed some protective potential, and those derived by methods 1 and 4 were found superior (14). This paper reports the results of experiments with some of the most promising isolates in their ability to protect grapefruit and sweet orange seedlings against some virulent forms of CTV-SY and CTV-SP.

MATERIALS AND METHODS

Experiment 1A. Protective ability of six isolates against the CTV-SY isolate from which they were derived. All six protective isolates were derived by method 1, i.e. from grapefruit seedlings which were previously bud-inoculated with known CTV-SY sources, but which showed no SY symptoms in the grapefruit. However, when grapefruit tissue from these symptomless plants was inoculated into Mexican lime, the limes showed positive symptoms for tristeza. The source for the four selected isolates: II, MM, NN, and QQ was a field tree of *Citrus macroptera* code SY 560, CRC 432 from the UCR variety collection. This tree was grown from seed imported in 1914 from the Philippine Islands. The field tree was in decline in 1978, and when budwood from this tree was indexed to indicator seedlings, a severe yellows reaction was observed in grapefruit, sour orange and lemon but no SP was observed in sweet orange or grapefruit (6, 9). The source tree for the two isolates KK and TT was a Parson's Special mandarin, code SY-565, CRC-300, imported from Australia via Florida in 1914. The field tree was not in decline, but when indexed, budwood from this source tree induced strong CTV-SY symptoms in indicator seedlings and no SP in sweet orange or grapefruit. Budwood from the *C. macroptera* and Parson's Special mandarin source trees was graft-inoculated into Madam Vinous sweet orange plants which were held in a screenhouse. Budwood of both selections consistently induced strong seedling yellows reactions in indicator seedlings over a 7-yr period (12).

All potentially protective isolates were transferred by bud inoculation from Mexican lime to sweet orange as

holding or reservoir plants. Two cross-protection experiments were done. The first, (Exp. 1A) was started in October 1983 and the second (Exp. 1B) in October 1985. The inoculation procedures for both experiments are illustrated in fig. 1A. Initially six out of a set of nine 1-yr-old grapefruit seedlings were graft-inoculated with two buds each of the protective isolate and all nine plants were cut back approximately 25 cm above the soil surface. Single shoots were grown and trained to a stake, and when they reached approximately 1 m, about 4 to 6 months after cut back, four protected seedlings and two unprotected seedlings were challenge-inoculated by bud grafts. The set of nine plants was again cut back (fig. 1A). Thus each set of nine seedlings contained four which were protected and challenged, two protected only, two challenged only and one left as a non-inoculated control. The challenge inoculum used for experiment 1A was the CTV-SY source from which the protective isolates were derived. Inoculum buds were cut out (excised) 5 weeks after inoculation. New growth was again trained as a single shoot and when it reached about 1 m in height (in approximately 4-6 months), the shoot was measured and observed for yellows and stunting.

Experiment 1B. Protective ability of two isolates against diverse CTV-SY isolates. The objective of this experiment was to test the ability of a protective isolate derived from one source to protect against a challenge with other diverse CTV-SY sources. This experiment was similar in design to Experiment 1A. Two protective isolates were chosen: Code QQ from *C. macroptera* and Code KK from Parson's Special mandarin. Six sources of challenge inoculum from the UCR variety collection were used, and selected because of their strong yellows reaction in indicator seedlings (12). Grapefruit seedlings were bud-inoculated with the protective inoculum in October 1984, challenged after 6 months, the challenge inoculum removed after 5 weeks, and all plants harvested 5 months after challenge.

Experiment 2. Protective ability of two isolates against eight diverse severe stem pitting isolates of CTV-SP. The two isolates chosen for their protective ability against stem pitting were Codes 37 and 40. Both were derived by aphid transmitting the virus into *Passiflora caerulea* by *A. gossypii* and then transmitting by aphids from *P. caerulea* into Mexican lime (13). The origin of both isolates was a Brazil navel SY-563B, CRC-597 from the UCR variety collection which contained CTV-SY and CTV-SP (8, 9). One positive Mexican lime 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 40A was another source derived by transmitting the virus through *P. caerulea* by *A. gossypii*. It was transmitted to Mexican lime in November 1982; then sub-inoculated to a sweet orange holding plant in March 1983.

The eight challenge isolates were from the UCR variety collection and all induced severe stem pitting in grapefruit. These eight challenge isolates combined with the three protective isolates of codes 37, 37A and 40 gave a total of 24 treatments.

Ten 1-yr-old grapefruit seedlings were used for each treatment: five were inoculated with a protective isolate and five left uninoculated. Seedlings were then cut back and new growth trained as single shoots. After 6 months, all plants were cut back and bud-inoculated with the respective challenge isolates. The challenge inoculum buds were removed 5 weeks after inoculation. New growth was trained to a single shoot and harvested 3 to 4 months after inoculation. A 30-cm length of stem, directly above the harvest cut, was peeled, measured and the pits counted. A calculation was made of the total surface

area and the number of pits per 100 cm^2 of surface area was used as a basis for comparison between treatments.

Experiment 3. Protection of sweet orange seedlings against a challenge with the severe 12B isolate. In January 1982, Code SY-568, commonly known as the 12B isolate, was vector transmitted to *P. gracilis* by *A. gossypii* and again vector transmitted back to Mexican lime (13). This original CTV-infected Mexican lime was coded as Z and its protective ability demonstrated (14). Bud inoculations from Code Z Mexican lime were made to five sweet orange seedlings as holding plants and these were coded as Z-1 through Z-5. Periodic subtransfers were made from code Z-5 to sweet orange. The protective abilities of these subcultures were so similar that the results were combined and labeled as Z-5.

Sixty 14-month-old Madam Vinous sweet orange seedlings trained as single shoots were used. Thirty seedlings were inoculated with the protective isolate code Z-5 and 30 seedlings were left uninoculated. All 60 plants were then cut back as in previous experiments and the new growth again trained as single shoots. After 6 months, 20 of the protected and 20 of the non-protected plants were challenge bud-inoculated with the severe 12B isolate. Ten of the protected and 10 of the nonprotected plants were not challenged and left as controls. Thus, there were five treatments of 10 plants per treatment as follows: A) protected with Z-5 and challenged with the 12B source inoculum with the inoculum removed after 33 days; B) similarly protected and challenged as in A but the challenge inoculum not removed; C) protected as in A but not challenged; D) not previously protected but challenge-bud-inoculated later with the severe 12B isolate to serve as positive controls; inoculum buds were removed after 33 days; E) the same as D but the challenge inoculum not removed. F) not protected or challenged (these were the non-inoculated controls). The plants were again cut back and new growth trained to single shoots. After 3 months, plants were harvested, observed for seedling yellows, measured, calipered and the pits counted as in Experiment 2.

All plants were grown in a protected glasshouse and fertilized using the UC system for plant growth as modified for citrus (5). Greenhouse temperatures were maintained at 26-29/19-20C (maximum day/minimum night).

RESULTS

Experiment 1. Table 1 gives the results for grapefruit seedlings protected with various isolates against a challenge with severe CTV-SY. Tests A and B done 2-yr apart were very similar and results are averaged. All six isolates were highly effective in protecting grapefruit seedlings against a challenge with the severe SY isolate from which they were derived. All 10 nonprotected and challenged controls showed very severe seedling yellows whereas all 48 protected grapefruit plants were negative for yellows. All 24 protected and nonchallenged grapefruit plants were also negative for yellows. The attenuated CTV of the six protective isolates in their respective source plants have shown no tendency to revert back to the original severe SY form, and all appear to be stable after 4 yr. There appeared to be little or no reduction of growth in the protected only or protected and challenged seedlings, whereas the challenged nonprotected controls showed over 50% reduction in growth in addition to showing very small yellow leaves (fig. 1A). After the plants in experiment 1 were cut back for harvest, new growth was trained again as a single shoot, and 3 months later was evaluated for symptoms. The response was similar, indicating that the protective inoculum continued to protect new growth.

TABLE 1
PROTECTION OF GRAPEFRUIT SEEDLINGS BY SELECTED ISOLATES OF CITRUS TRISTEZA VIRUS-SEEDLING YELLOWS (CTV-SY) AGAINST A CHALLENGE BY THE CTV-SY SOURCE FROM WHICH THEY WERE DERIVED. (EXPERIMENT 1[Z])

			CTV-SY Protective Sources[Y]											
			Source II		Source MM		Source NN		Source QQ		Source KK		Source TT	
Plant	Protected	Challenged	Ht. (ca)	SY[X]	Ht. (ca)	SY	Ht. (ca)	SY	Ht. (ca)	SY	Ht. (ca)	SY	Ht. (ca)	SY
1	+	+	90	0	89	0	88	0	78	0	110	0	82	0
2	+	+	66	0	106	0	66	0	76	0	93	0	99	0
3	+	+	80	0	70	0	60	0	113	0	104	0	90	0
4	+	+	99	0	67	0	68	0	80	0	88	0	87	0
5	+	−	90	0	90	0	81	0	85	0	91	0	75	0
6	+	−	90	0	106	0	87	0	75	0	85	0	70	0
7	−	+	34	4+	40	5+	42	5	40	5+	31	5+	35	5+
8	−	+	35	5+	38	5+	48	5	50	5+	36	5+	32	5+
9	−	−	85	0	93	0	118	0	77	0	90	0	79	0

[Z]These results represent the combined readings of two experiments done 2 yr apart.
[Y]Protective sources II, MM, NN, and QQ were derived from *Citrus macroptera* and sources KK and QQ from Parson's Special mandarin. The challenge inoculum was from the original source plants from which the protective inoculum was derived. Inoculum buds were removed after 5 weeks.
[X]Seedling yellows rating scale: 0 = none to 5 = very severe.

Experiment 1A. Table 2 shows the protective ability of isolates QQ and KK when challenged with six isolate of severe CTV-SY from the field collection at UCR. Excellent protection was provided by both isolates against the original sources from which they were derived. In addition, isolate QQ gave excellent protection against five of the six field SY sources, whereas isolate KK also gave excellent but varied protection against five of the six SY-field isolates. The two protective isolates differed somewhat in their protective ability when challenged with the same field isolate. For example, isolate QQ protected against a challenge from SY 568 but failed to protect against SY-566 whereas isolate KK showed 75% protection against SY-566 but failed to protect against SY 568. The data suggest that a protective isolate derived from one source may or may not

TABLE 2
SEEDLING YELLOWS RATING[Z] SHOWING THE PROTECTION OF GRAPEFRUIT SEEDLINGS BY PROTECTIVE SOURCES QQ AND KK AGAINST A CHALLENGE BY SIX SEVERE CITRUS TRISTEZA VIRUS-SEEDLING YELLOWS (CTV-SY) ISOLATES. (EXPERIMENT 1B)

			Protective Source QQ						Protective Source KK					
			CTV-SY Challenge Sources						CTV-SY Challenge Sources					
Plant	Protected	Challenged[Y]	556	560	563	565	566	568	556	560	563	565	566	568
1	+	+	0	0	0	0	5	0	0	0	0	0	0	2+
2	+	+	0	0	0	0	4	2	0	2+	3+	0	0	3
3	+	+	0	0	0	0	4	0	2	2	0	0	0	5+
4	+	+	0	0	0	0	3+	0	0	0	3	0	5	5+
5	+	−	0	0	0	0	0	0	0	0	0	0	0	0
6	+	−	0	0	0	2+	0	2+	0	0	0	0	0	0
7	−	+	5	5	5	5	5	4	5+	3+	5+	5+	3	5+
8	−	+	5	5	3	5	5	4	5	5	5+	5+	5	5+
9	−	−	0	0	0	0	0	0	0	0	0	0	0	0

[Z]Seedling yellows rating scale: 0 = none, 5 = very severe.
[Y]Challenge inoculum buds were removed 5 weeks after graft inoculation.

protect against the CTV-SY component from other sources.

Experiment 2. Previously (14), the code 37 protective isolate was shown to be effective against a number of stem pitting isolates. This experiment was repeated 2 yr later to see if this isolate, as maintained in its original host of Mexican lime (Code 37) or in its holding host of sweet orange (Code 37A), would still be protective. Also tested was a new isolate (Code 40) similarly derived via *P. caerulea* (13). Results are given in table 3 and indicate excellent protection by codes 37, 37A and 40 against a challenge by the original SY-563 isolate from which all three protective isolates were derived. Excellent protection was also achieved when these were challenged by six additional field isolates. The protective isolate code 37 appears to be stable. It has shown no tendency to revert to the stem-pitting form, and the original Mexican lime plant remains vigorous and shows only a very mild tristeza reaction after 4 yr. Code 37 inoculum has given continued protection against stem pitting during this 4-yr period.

Experiment 3. Table 4 gives the results of protection of code Z-5 inoculum against a challenge with the severe 12B isolate. Protection against yellows and stem pitting was complete, whether challenge inoculum buds were removed or allowed to remain. After final harvest some of the remaining protected plants were again challenge-inoculated by bud grafts with the severe 12B inoculum. The new growth was observed 3 1/2 months later and it was totally protected. A third rechallenge gave similar results.

Stability of source Z inoculum. Whereas the protective isolates codes 37, 37A and 40 in their respective holding plants have remained consistent and stable for 4 yr, the plants containing code Z inoculum have shown a tendency to revert and develop stem-pitting symptoms without accompanying yellows (fig. 2). However, by selecting away from the stem pitting, a number of isolates have been maintained in which pits have not yet formed. As seen in Fig. 2, Isolates Z-5B, Z-5D and Z-5E have remained in their hosts without showing extensive pitting for 2 1/2 yr whereas isolates Z-5A and Z-5C are just beginning to break down and are showing pitting. The original isolate Z-1A in sweet orange developed severe pitting after 33 months. The six subcultures of inoculated sweet orange from the original Z-1 isolate ultimately developed severe pitting 9 to 22 months after inoculation in its sweet orange holding plant. There are currently 19 sources of protective Z

TABLE 3
THE NUMBER OF PITS PER 100 cm² OF SURFACE AREA FOR FIVE GRAPEFRUIT SEEDLINGS, NONPROTECTED AND PROTECTED BY SOURCES 37, 37A, AND 40 AND ALL CHALLENGED WITH EIGHT CITRUS TRISTEZA VIRUS-STEM PITTING (CTV-SP) FIELD SOURCES (EXPERIMENT 2)

Challenged isolates[z]	CTV-SP Protective Isolates			Non protected
	Code 37	Code 37A	Code 40	
None	0	0	0	0
26	0	0	0	154
58	8	3	0	94
545	18	0	0	106
583	0	4	0	98
1225	0	0	0	286
7868	41	3	0	198
11118	0	0	0	66
563B[y]	0	0	0	99

[z]Challenge inoculum buds were removed 5 weeks after graft inoculation.
[y]563B is the same source from which codes 37, 37A, and 40 were derived.

TABLE 4
PROTECTION OF MADAM VINOUS SWEET ORANGE SEEDLINGS BY CITRUS TRISTEZA VIRUS (CTV) SOURCE Z5 AGAINST A BUD-GRAFT CHALLENGE WITH THE SEVERE 12B CTV-SEEDLING YELLOWS (SY), CTV-STEM-PITTING (SP) SOURCE (EXPERIMENT 3)

Treatment	Protected with Z5	Challenged with 12B	Challenge inoculum removed[z]	SY[y]	Height (cm)	Stem diam. (mm)	No. of pits per 100 cm² surface area
A	+	+	+	0	88	4.2	3
B	+	+	−	0	100	4.4	1
C	+	−		0	100	4.9	1
D	−	+	+	4.2	69	4.0	928
E	−	+	−	4.1	60	3.7	1,016
F	−	−		0	110	5.3	0

[z]Challenge inoculum buds were excised 33 days after inoculation.
[y]Seedling yellows rating scale of 0 = none to 5 = very severe.

inoculum in sweet orange holding plants under observation; These are 3 yr old or older and are showing no symptoms in sweet orange. They will be held and observed to determine their stability.

DISCUSSION

Two methods for developing protective isolates in greenhouse plants against CTV-SY or CTV-SP appear promising:

1) Testing of symptomless grapefruit, lemon, or sour orange plants previously bud-inoculated with specific severe CTV-SY or CTV-SP isolates, but which show no SY or SP reaction in indicator seedlings. Many

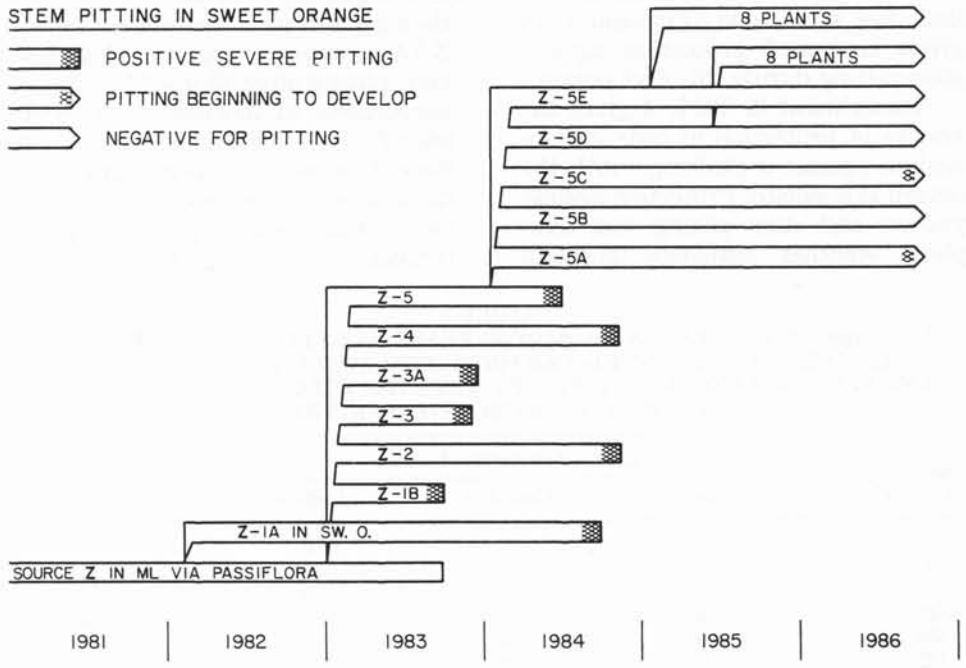

Fig. 2. Development of stem pitting in Z source holding plants. Plant Z was the original Mexican lime derived via vector transmission from *Passiflora gracilis*. Subcultures made to sweet orange (SwO) holding plants Z-1 through Z-5 ultimately reverted back and developed stem pitting (cross-hatch) 9 to 33 months after inoculation. However, five subcultures from Z-5 have remained symptomless for 33 months and 16 subcultures in sweet orange as shown are still symptomless 20 to 22 months after inoculation.

isolates obtained by this method have remained stable and protective over a 4-yr period. This method is similar to that suggested by Wallace and Drake (15), where recovered shoots from CTV-SY-infected plants were shown to be protective. We have observed that budwood taken from CTV-SY positive control plants of grapefruit, sour orange, or lemon will induce consistent SY symptoms in indicator seedlings for up to 1 yr, but after 1 yr there is a tendency for inoculated seedlings to show no yellows symtoms. However, when indexed to Mexican lime or by the enzyme-linked immunosorbent assay (ELISA) (2), CTV was found present in almost all of the symptomless SY indicator plants. For example, in 1,000 individual index tests from seven CTV-SY positive source plants to grapefruit, 42 plants were found negative for SY. However, when these were indexed to Mexican lime almost all were found positive for CTV. Similarly, 8 of 131 sour orange and 11 of 154 lemon plants inoculated with CTV-SY were found negative for SY in their respective index hosts (12). Many of these were found to be positive for CTV when indexed in Mexican lime or by the ELISA. These seedlings have become good sources for protective isolates. They could be classified as the SYT types as described by Wallace and Drake (15).

2) Passage of CTV-SY and CTV-SP through *Passiflora* species by aphid vector provided the second promising method of developing protective isolates. All six isolates developed by this means showed a loss of one or more components of the CTV complex (13, table 3) and three of the six protective isolates (codes 37, 40 and Z-5) have shown exceptional ability to protect against SP in grapefruit and sweet orange, as shown in these studies.

The finding of protective isolates by the four methods previously described (14) present an alternative to the classical approach of selecting surviving trees after a tristeza epidemic occurs. Also, should a new strain become established where tristeza is endemic, or protective strains are needed to inoculate virus-free shoot-tip-grafted selections, these methods provide a means of rapidly developing protective isolates against existing severe local isolates. These studies indicate that protective isolates can be developed from existing local field isolates. Although a protective isolate may be effective against a variety of different severe isolates, these studies suggest that it will be most effective against the severe isolate from which it was derived. Some protective isolates remained effective even when the challenge buds were left intact for the duration of the experiment; and in some tests they were effective when they were left intact for over a 1-yr period with three cutbacks. In one test, plants were repeatedly challenged by bud-graft inoculation at each of the three times plants were cut back and the plants remained protected.

Although these experiments show promise for the rapid development of protective isolates against severe forms of CTV, field tests could not be performed in California. Of the ten protective isolates described in these studies all but one have remained stable in their holding plant hosts. The one exception is the protective code Z isolate which has shown a tendency to revert back to the very severe CTV-SP form. A developed protective isolate should therefore be maintained for a period of time with periodic testing and observation to assure that it will not revert; field testing would be the next step in judging final performance.

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