Movement and Titer of *Citrus tristeza virus* (Pre-immunizing Isolate PB61) Within Seedlings and Field Trees

C. Y. Zhou, P. Broadbent, D. L. Hailstones, J. Bowyer, and R. Connor

ABSTRACT. In Symons sweet orange (SSwO) seedlings grown at 26ºC/22ºC, *Citrus tristeza virus* (CTV) pre-immunizing isolate PB61 was detected by reverse transcription and polymerase chain reaction (RT-PCR) in new roots and shoots as early as 8 days post inoculation (dpi): much earlier than the 44 dpi for detection by ELISA previously reported. First detection of the virus was usually 3 to 6 days earlier by RT-PCR than by direct tissue blot immunoassay, and in young tissue rather than old tissue. The virus was never detected in the oldest leaves. Monitoring CTV movement in 180 SSwO seedlings of two sizes, held in a glasshouse at about 26ºC, first detected the virus at 20 dpi in small plants, 20-40 days earlier than in large ones. The frequency of detection of CTV was higher in new roots (16.1%) than in young shoots (8.9%), suggesting that the virus initially followed the direction of photo-assimilate transport.

The titer of isolate PB61 was monitored in mature red and white grapefruit trees in the field at Dareton (NSW, Australia) from spring to autumn. In Marsh and Thompson grapefruits, PB61 was consistently detected throughout not only the previous season's flush but also the latest flush (hardened, the leaves fully expanded). This suggests that generally PB61 is evenly distributed in these cultivars. The situation for the red grapefruits (Star Ruby and Rio Red) was more variable. In late spring, both the previous season's flush and the latest, hardened flush were consistently positive for the virus. However, by early autumn, the immature summer flush was strongly positive for CTV, while detection in the spring flush was sporadic. Budwood of red grapefruits supplied at this season may thus be negative for CTV. One month later, the summer flush had matured and all flushes were once again strongly positive. These findings have resulted in changes in the distribution dates within the Australian Citrus Budwood Scheme to ensure that all budwood is effectively pre-immunized with PB61.

Index words. CTV preimmunizing isolate, virus movement, virus titer.

Citrus tristeza virus (CTV) has been endemic in Australia since the late 1800s (7). In the 1950's, grapefruit stem pitting almost wiped out the grapefruit industry. Subsequent research (6, 8) led to the use of mild strain cross protection (MSCP), which has effectively maintained commercial grapefruit production in Australia for the past 35 yr.

Uniform distribution within the tree of a pre-immunizing CTV isolate is important for the success of MSCP. The virus must also be able to quickly invade new growth flushes. Any part of a citrus tree that is virus-free, even temporarily, provides a site for an aphid to introduce a severe CTV isolate. Establishment of challenge isolates in islands of virus-free tissue could ultimately result in the breakdown of MSCP (14). Few studies have investigated the movement of CTV isolates within plants during the early post-infection period. In general, these have studied only a small number of plants, and conclusions were based on symptom expression or ELISA. Price (17), Tolba et al. (19), and Chakraborty and Chenulu (4) showed that 8 days post-inoculation (dpi) was the minimum time required for CTV to move from lime inoculum to virus-free lime plants. Gafny et al. (9) monitored the movement of two severe CTV isolates within 8 mo-old sour orange seedlings by ELISA, and first detected CTV infection in the basal parts of the plants and in the root system at 44 dpi, and in the upper parts at 51 to 58 dpi. Analysis by ELISA, however, requires large amounts of tissue, which reduces the number of times each plant can be tested and therefore limits the amount of data that can be collected. Lee et al. (15) used ELISA to study the distribution of CTV within the young shoots

of grapefruit and sweet orange trees in Florida and South Africa. In general, CTV was more evenly distributed in sweet orange than in grapefruit trees in Florida, but occasionally very young flushes were found to be CTV-free. In South Africa, CTV was evenly distributed throughout both grapefruit and sweet orange trees.

In Australia, difficulties have been experienced in pre-immunizing red grapefruits with the mild isolate PB61 (3). The percentage success of pre-immunization was much lower for red grapefruit than for the white Marsh grapefruit (MGF) and pink Thompson grapefruit. CTV could not be detected in the stems of some plants infected with particular sub-isolates PB244, PB245 and PB246 (derived from the severe grapefruit stem-pitting isolate PB219), but was easily detected in the feeder roots of those same plants (20). Occasionally, buds from Marsh grapefruit trees pre-immunized with mild isolate PB61 also tested negative for the virus. Together, these findings indicate either an uneven distribution of CTV within plants or failure of the virus to replicate. This has significant implications for distribution of pre-immunized budwood.

This study investigated the movement and distribution of CTV in citrus plants and whether uneven distribution of the pre-immunizing CTV isolate may contribute to the breakdown of MSCP in grapefruit.

MATERIALS AND METHODS

Movement of mild isolate PB61 within Symons sweet orange (SSwO) seedlings. Eight 8 mo old SSwO seedlings grown in 15 cm pots of UC mix (50 peat:50 sand) with added fertilizer (1), were grown in a growth cabinet at 26°C/22°C (16 h light/8 h dark). Two seedlings were inoculated with two pieces of SSwO bark (about 1 cm in length), infected with mild CTV isolate PB61.

Virus-free brown citrus aphids (*Toxoptera citricida*) were raised on young shoots of virus-free SSwO seedlings in a cage at about 26°C. Virus-free apterous aphids were fed on young shoots of SSwO seedling infected with pre-immunizing CTV isolate PB61 for 24 h to acquire the virus, and 50 were then transferred to the young leaves of each of five SSwO seedlings for 24 h to transmit the virus. A control SSwO seedling was treated with 50 virus-free apterous aphids.

About 10 mg each of old leaf, young leaf, old bark and feeder root was sampled every 3 days. Virus was assayed both by direct tissue blot immunoassay (DTBIA) using a slight modification of the method of Garnsey et al. (10) and by reverse-transcription polymerase-chain-reaction (RT-PCR) using nucleic acid prepared by a rapid micro-extraction method (see below).

For DTBIA, alkaline phosphatase-conjugated IgG against coat protein (CP) of CTV (Sanofi Phyto-Diagnostics, Libourne, France) was used at 1:500 dilution. Presence of the antigen resulted in development of a brown color: a weak positive reaction (a few brown dots in the phloem) was scored as "+"; reaction of about half the phloem with brown dots was scored as "++"; a strong positive (phloem fully brown) was scored as "+++".

The rapid micro-method to extract total nucleic acid (TNA) from CTV-infected leaf, bark, and feeder root tissue, was modified from Mathews et al. (16), and is fully reported elsewhere (12, 20). RT-PCR was conducted in a Hybaid Touchdown™ thermal cycler with primers to the coat protein gene (11). Reverse transcription was conducted according to the manufacturer's recommendations (Promega Corporation). PCR was based on Gillings et al. (11), except we used *Taq* DNA polymerase from Gibco BRL Life Technologies™ and included a touchdown phase in the amplification.

Monitoring the time frame of CTV infection in young feeder roots and young shoots of SSwO seedlings. Mild (PB33, PB61 and PB65) and severe (PB155, PB219 and PB235) CTV isolates/subisolates were graft-inoculated to SSwO seedlings (20-30 plants for each experiment). Seedlings were of either 1 to 1.5 mm stem diameter as measured 10 cm above the soil (referred to as "small") or 2.5 to 3.5 mm stem diameter (referred to as "large"). All conditions were essentially the same within each experiment. Plants were grown in the glasshouse at about 26°C, and the CTV status of young shoots and feeder roots was monitored periodically (20, 30, 40, 50, 60, 90, 180 dpi) using DTBIA.

Distribution of pre-immunizing isolate PB61 in grapefruit trees in the field. Two cultivars of red grapefruit (Star Ruby, 15 trees, and Rio Red, 14 trees), one white grapefruit (Marsh, 11 trees) and one pink grapefruit (Thompson, seven trees) pre-immunized with mild isolate PB61 and planted in Budwood Blocks 2 and 3 at Dareton, NSW, were sampled seasonally to monitor the distribution of CTV. Three to five shoots of the previous season's flush (mature), and of the latest flush (either mature or immature) for each tree, were sampled randomly each time, on four occasions throughout the growing season. The CTV status of each tree was assessed using DTBIA.

RESULTS

Movement of PB61 within SSwO. In SSwO seedling M-1, inoculated by grafting, CTV was detected in both young leaf tissue and young feeder roots (but not in other tissues tested) at both 8 and 11 dpi by RT-PCR assay (Fig. 1), although the DTBIA results were negative. By 14 dpi, both RT-PCR and DTBIA detected the virus in young leaf tissue, young feeder roots and old bark (Fig. 1). Monitoring for the presence of CTV continued up to 80 dpi. CTV was not detected in the oldest leaves by either method during this period.

In contrast to seedling M-1, the other graft-inoculated seedling (M-2) remained CTV-negative by both detection methods through 80 dpi (during which period the plant was monitored every 3 days) and remained negative at the final assay at 106 dpi. Both pieces of inoculating bark were confirmed to be CTV-positive at the time of inoculation, but only one was found positive (though weakly so) at 80 dpi. Bark and a young shoot adjacent to the inocula were negative. Furthermore, Mexican lime seedlings inoculated with bark chips from M-2 at 80 dpi also displayed no symptoms and CTV could not be detected within these indicator plants. Lack of infection was not due to poor union between the inoculum and the plant, since no abnormal callus union was observed when the site

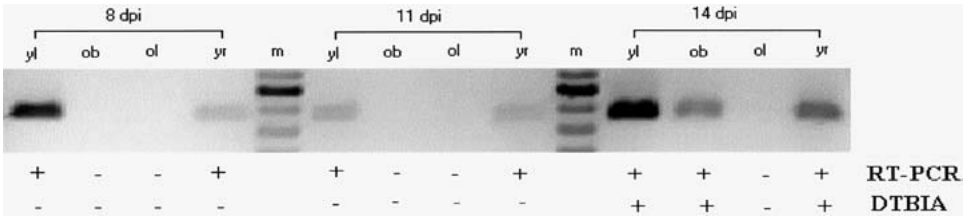

Fig. 1. Monitoring the movement/replication of graft-transmitted mild CTV isolate PB61 within SSwO seedling M-1 by RT-PCR of cDNA specific to the CP gene, and DTBIA. dpi = days post-inoculation; yl = young leaf; ob = old bark; ol = old leaf; yr = young feeder root; m = 100 bp ladder DNA marker (the band with higher density is 800 bp); - = undetectable; + = detectable.

was sectioned and stained with 5% of phloroglucinol-HCl (5) and observed by light microscopy.

CTV was first detected by RT-PCR in aphid-inoculated seedling M-3 in young tissue at 48 dpi, in old bark at 51 dpi and old feeder roots at 54 dpi (Fig. 2). Using DTBIA, CTV was first detected in young feeder roots at 48 dpi and in young leaf at 54 dpi (6 days later than by RT-PCR).

CTV was not detected in the other aphid-inoculated seedlings or the negative control by either method for 80 dpi (assayed every 3 days). The plants were still CTV negative at 106 dpi.

Monitoring CTV infection in young feeder roots and young shoots of SSwO seedlings. Irrespective of the CTV isolate (three mild and three severe), the virus was detected earlier in a greater proportion of small seedlings than larger ones. Generally the virus was detected at the first test (20 dpi) in small seedlings, but 30 to 60 dpi were required before most larger plants tested positive. Infection was detected within 90 dpi in 174 of 180 plants; the remaining six tested negative at 90 dpi and remained negative thereafter. Table 1 summarizes the data for all 180 plants with respect to the tissues in which CTV was initially detected, irrespective of the time of detection. In most cases (71.7%), the virus was detected in both young shoots and feeder roots simultaneously, although the chance of detecting the virus first in feeder roots (16.1%) was greater than that in young shoots (8.9%).

Distribution of PB61 in grapefruit trees in the field. Results are presented in Table 2. The results for Star Ruby and Rio Red grapefruit are also shown in Figs. 3 and 4, respectively.

Table 2 shows that PB61 was consistently detected throughout both the autumn flush (1999) and the maturing spring flush (1999) in the white Marsh grapefruit and pink Thompson grapefruit, in late spring (end of October). In early autumn (early March) 2000, the virus was sometimes not detectable, or only marginally detectable ("-"or "+"), but in most instances it was readily detectable ("++" or "+++") in the spring flush (1999) and the mature summer flush (2000) of both cultivars. By very late autumn (end of May) 2000, the virus was easily detectable in both summer and autumn flushes (2000) of both cultivars. This suggests that generally the virus was evenly distributed in the previous season's flush and the mature summer flush in these two cultivars.

PB61 was consistently detected throughout both the autumn flush (1999) and the maturing spring flush (1999) in the red grapefruits Star Ruby and Rio Red, sampled in late spring (end of October). The situation became more complex when

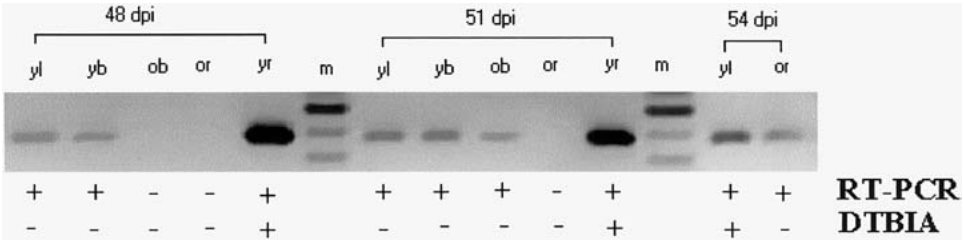

Fig. 2. Monitoring the movement of aphid-transmitted mild CTV isolate PB61 within SSwO seedling M-3 by RT-PCR of cDNA specific to the CP gene, and DTBIA. dpi = days post-inoculation; yl = young leaf; yb = young bark; ob = old bark; or = old feedroots; yr = young feeder roots; m = 100 bp ladder DNA marker (the band with higher density is 800 bp); - = undetectable; + = detectable.

TABLE 1
THE PERCENTAGE OF TISSUES WHERE *CITRUS TRISTEZA VIRUS* WAS FIRST DETECTED IN 180 SYMONS SWEET ORANGE SEEDLINGS

Experiment Code*	CTV PB no.	Number of plants/total number of plants				Plant size**
		Young shoot	Young feeder root	Both	Neither	
1	PB61 (mild)	9/30	8/30	13/30	0/30	Large
2	PB155 (severe)	7/30	9/30	13/30	1/30	Large
Subtotal percentage		26.7%	28.3%	43.3%	1.7%	
3	PB61 (mild)	0/20	5/20	12/20	3/20	Small
4	PB155 (severe)	0/20	0/20	20/20	0/20	Small
5	PB219 (severe)	0/20	2/20	18/20	0/20	Small
6	PB33 (mild)	0/20	1/20	19/20	0/20	Small
7	PB65 (mild)	0/20	2/20	16/20	2/20	Small
Subtotal percentage		0%	10%	85%	5%	
8	PB235 (severe)	0/20	2/20	18/20	0/20	Large
Total percentage		8.9%	16.1%	71.7%	3.3%	

*All experiments were run at the same time except Experiment 8, the time points for CTV detection were 20 days post-inoculation (dpi), 30 dpi, 40 dpi, 50 dpi, 60 dpi, 90 dpi and 180 dpi using direct tissue blot immuno-assay.
**Large seedlings were 2.5-3.5 mm in diameter 10 cm above the soil line, small seedlings were 1-1.5 mm in diameter 10 cm above the soil line.

trees were sampled in early autumn (early March) 2000, as the spring flush (1999) showed a distinct change in detectability (Table 2, Figs. 3 and 4). Many spring-flush shoots (1999) were negative or barely positive, and very few were strongly positive. However, most of the immature summer flush (2000) was strongly positive. One month later (early April), the immature summer flush (2000) had matured, and both the spring flush (1999) and the mature summer flush (2000) were in general strongly positive for the virus. By very late autumn (end of May) 2000, both the summer flush (2000) and the mature autumn flush (2000) were consistently strongly positive. Thus the titer of CTV in the spring flush changed between spring and early autumn: i.e. CTV was present in late spring but at much lower titer when summer flushes were immature, and present again when the summer flush had matured in early April.

Notably, three Star Ruby trees were negative in all flushes tested and on four sampling occasions (231 budsticks tested by DTBIA, and a few by RT-PCR, data not shown). This suggests that budwood used to propagate these trees had not been effectively pre-immunized, i.e., did not contain the virus, even though the mother trees were positive.

DISCUSSION

Movement of CTV within the host. SSwO seedling M-1 provided evidence that PB61 can sometimes be detected at sites distant from the point of inoculation as early as 8 dpi. Seedlings M-1 and M-3 both provided evidence that CTV was transported to and replicated in young tissues, consistent with the suggestion by Sasaki et al. (18) that CTV multiplies mostly in actively-dividing cells, and that the virus follows the vascular movement of carbohydrates through the sieve tubes to the sinks (13), before replicating to a detectable level.

We detected the virus at sites distant from the point of inoculation

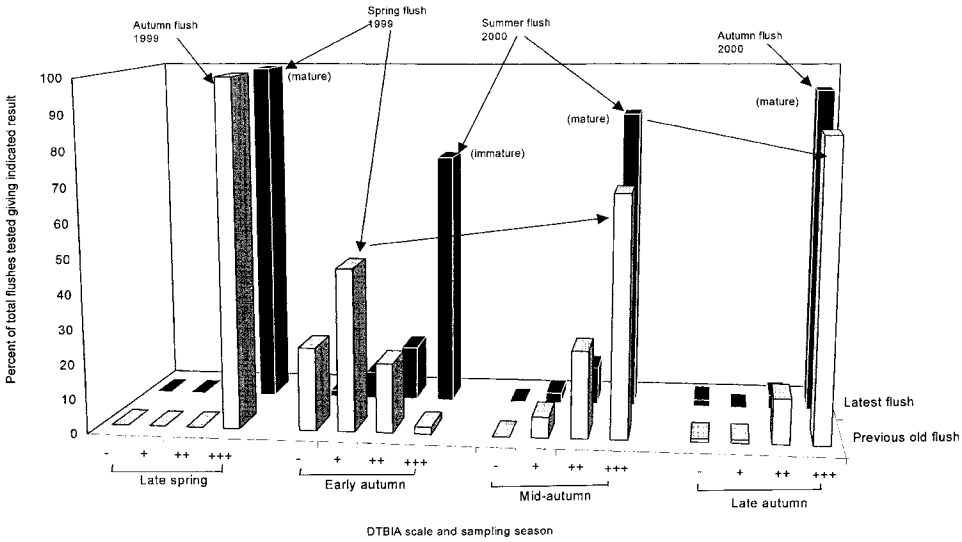

Fig. 3. Distribution as reflected by titer over time of the preimmunizing isolate PB61 in flushes of Star Ruby grapefruit in Budwood Block 3/D at Dareton, NSW, assessed by DTBIA. "-" = negative; "+" = one or a few brown dots of CTV within the phloem, weakly positive; "++" = about half of the phloem shows brown dots of CTV; "+++" = phloem fully brown, strong positive. Actual sampling dates: 23-10-1999 (late spring), 8-3-2000 (early autumn), 3-4-2000 (mid-autumn), 23-5-2000 (late autumn).

much earlier than the 44 dpi in roots, and 51-58 dpi in shoots reported by Bar-Joseph and Nitzan (2) and Gafny et al. (9), who used ELISA to monitor CTV movement. Since 8 dpi was the first time seedling M-1 was moni-

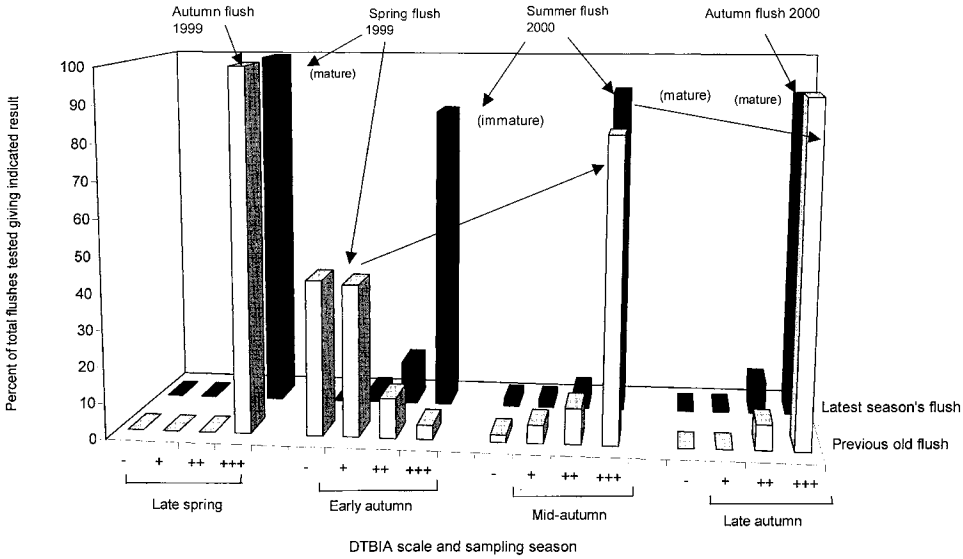

Fig. 4. Distribution as reflected by titer over time of the preimmunizing isolate PB61 in flushes of Star Ruby grapefruit in Budwood Block 3/D at Dareton, NSW, assessed by DTBIA. "-" = negative; "+" = one or a few brown dots of CTV within the phloem, weakly positive; "++" = about half of the phloem shows brown dots of CTV; "+++" = phloem fully brown, strong positive. Actual sampling dates: 23-10-1999 (late spring), 8-3-2000 (early autumn), 3-4-2000 (mid-autumn), 23-5-2000 (late autumn).

TABLE 2
DISTRIBUTION OF PRE-IMMUNIZING *CITRUS TRISTEZA VIRUS* ISOLATE PB61 IN DIFFERENT TISSUES OF FOUR GRAPEFRUIT CULTIVARS IN BUD-WOOD BLOCKS AT DARETON, NSW, IN DIFFERENT SEASONS

Grapefruit cultivar	Type of flush	Number of flushes with indicated DTBIA scales*, in different seasons**															
		Late spring				Early-autumn				Mid-autumn				Late autumn			
		-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Star Ruby	Previous season's flush				38	11	21	9	4		2	9	25	1		2	49
	Latest flush : mature				158		Not available				1	4	32	1	1	11	74
	: immature		Not available			2	7	14	67								
Rio Red	Previous season's flush				52	23	23	6	2	1	2	4	35			4	55
	Latest flush : mature				129		Not available			1	1	2	40	1	1	9	83
	: immature		Not available				3	11	77		Not available				Few available		4
Marsh	Previous season's flush				39	2	8	4	22		Not done					5	41
	Latest flush : mature				106	1	1	2	40		Not done					5	45
	: immature		Not available				Not available				Not done				Few available		3
Thompson	Previous season's flush				21	3	4	7	8		Not done			1		4	24
	Latest flush : mature				68	1	5	4	19		Not done			1	1	7	33
	: immature		Not available				Not available				Not done				Few available		4

*DTBIA: direct tissue blot immuno-assay; DTBIA scales: "-" = negative; "+" = one or a few brown dots of CTV within the phloem, weakly positive; "++" = about half of the phloem shows brown dots of CTV; "+++" = phloem fully brown, strongly positive.
**Actual sampling dates in southern hemisphere: 23-10-1999 (late spring), 08-03-2000 (early autumn), 03-04-2000 (mid-autumn), 23-05-2000 (late autumn).

tored in this experiment, the virus could have been detectable earlier. Why detection of virus in seedling M-1 (tissue inoculation) was much earlier than that in seedling M-3 (aphid transmission) is uncertain. PB61 was transmitted by aphids to only one of the five SSwO seedlings in this experiment (seedling M-3). This low aphid-transmission efficiency was later confirmed in two other experiments (unpublished data), in which transmission rates (50 aphids per plant) were 15% (3/20) and 20% (2/10). These transmission efficiencies are lower than expected, and do not fulfil the requirement of high vector transmissibility for an ideal pre-immunizing isolate suggested by Lee et al. (14).

The earlier detection of CTV by RT-PCR (generally 3 to 6 days) compared with DTBIA presumably reflects the greater sensitivity of RT-PCR, detecting the viral RNA, than DTBIA, which detects the CP.

Monitoring CTV infection in young feeder roots and young shoots. CTV was sometimes first detected in newly developed feeder roots or shoots before it was detectable in other tissue, although frequently it was detected in both young tissues simultaneously. This most likely reflects the limitations of the sampling technique i.e. the initial time period before testing (20 days) and the interval between sampling (10-30 days).

The large number of seedlings (180) tested improved the validity of the results. The probability of first detecting CTV was higher in feeder roots than in young shoots, but the initial direction of movement was not always downward to the roots then up to the shoots as described by Bar-Joseph and Nitzan (2), and Gafny et al. (9).

Distribution of PB61 in grapefruit trees in the field. In all cases in the field, PB61 was readily detected in almost all tissue sections of all flushes tested, in all trees of four grapefruit cultivars, in late spring and late autumn. This suggests that the virus is evenly distributed in these tissues, at these times.

Significantly, however, when the red grapefruits were sampled early in autumn, their latest summer flushes were immature, and their previous spring flushes frequently tested negative or very weakly positive, although the immature flushes tested strongly positive. By late autumn (April), when the latest flushes had matured, the virus was again easily detectable in all tissues of the red grapefruits. The change of virus titer at these times would appear to be due to the effect of nutrient flow. Marsh and Thompson grapefruits flushed earlier than the red ones at Dareton in the summer of 2000 (P. Florissen, pers. comm.), so that the latest flushes on these trees, at the time of sampling, were fully mature.

As both the previous season's flushes and the latest flushes were included in this work, the results should represent a more accurate picture than that obtained by testing only the latest flushes (14). PB61 was evenly distributed in immature summer flushes, but unevenly distributed in the previous season's flushes, and it is this material which is normally distributed to the Australian citrus industry in late summer and early autumn. This suggests a reason for the failure of MSCP in red grapefruits sometimes seen in the field, rather than super-infection of pre-immunized trees by severe CTV isolates borne by aphids. Distribution to the industry of pre-immunized mature budwood in late spring and in late autumn is therefore recommended.

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