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Artigo/Article

Citrus tristeza virus replication and movement in seedling trees of 71 rootstock genotypes

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SUMMARY

Citrus tristeza virus (CTV) replication and movement were studied in 1-year-old seedlings of 71 rootstock genotypes[i], by inoculation with buds of an Imperial mandarin carrying multiple endemic strains of CTV including those causing seedling-yellows and quick-decline, but free of orange-stem-pitting strains. A virus-free Rough lemon bud was inserted 30-40 mm above the infected bud on each of the 965 nursery trees to study virus movement. A further subset of 226 trees of the same rootstock genotypes were budded with virus-free Imperial mandarin to serve as a control. Virus replication was detected (using direct tissue blot immunoassay) in most seedlings within six months of budding, with levels of infection indicating significant differences between nucellar selections, hybrid families, and within hybrid families. Genotypes lacking *Poncirus* in their pedigree were rapidly colonised by the virus, while those with *Poncirus* parentage were often either resistant or slow to replicate CTV. Large differences in the percentage of infected seedlings from *Citrus* x *Poncirus* hybrid families indicate that transmission of resistance is complex and not independent of the seed parent. CTV moved rapidly even in resistant genotypes with 100% of virus-free Rough lemon buds acquiring the virus within three months of budding. Tolerance to the diseases caused by CTV is an essential requirement of rootstocks used in Australia and this work has helped to describe initial virus replication in existing and potentially new commercial rootstocks.

Index terms: resistance breeding, germplasm, segregation, *Poncirus*, virus.

Replicação e movimento do vírus da tristeza dos citros em 71 genótipos de porta-enxertos

RESUMO

A replicação e o movimento do vírus da tristeza dos citros (CTV) foram estudados em mudas de 1 ano de idade de 71 genótipos de porta-enxertos, por inoculação com brotos de tangerina Imperial com múltiplas estirpes endêmicas de CTV, incluindo aquelas que causam amarelecimento e declínio rápido, mas livres de estirpes stem-pitting. Uma borbulha de limão rugoso sem vírus foi inserida

[i] The term 'genotype' is used through this paper to describe material derived from an individual seed-lot. In some cases these individuals may be genetically identical nucellar seedlings, whilst in other cases the individuals are genetically distinct hybrids derived from the same parents (full-sibs).

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30-40 mm acima da borbulha infectada em cada uma das 965 mudas para estudar o movimento do vírus. Um outro subconjunto de 226 plantas dos mesmos genótipos de porta-enxertos foi enxertado com tangerina Imperial sem vírus para servir de controle. A replicação do vírus foi detectada (usando imunoensaio direto de transferência de tecido) na maioria das mudas, no prazo de seis meses da enxertia, com níveis de infecção indicando diferenças significativas entre seleções nucelares e famílias híbridas, e dentro de famílias híbridas. Os genótipos que não possuíam *Poncirus* em sua composição genética foram rapidamente colonizados pelo vírus, enquanto aqueles com parentesco de *Poncirus* foram em sua maioria resistentes ou lentos para replicar CTV. Grandes diferenças na porcentagem de mudas infectadas dos híbridos *Citrus x Poncirus* indicam que a transmissão de resistência é complexa e não independente da semente parental. CTV moveu-se rapidamente, mesmo em genótipos resistentes com 100% de borbulhas de limão rugoso sem vírus, adquirindo o vírus dentro de três meses da enxertia. A tolerância às doenças causadas por CTV é um requisito essencial dos porta-enxertos usados na Austrália e esse trabalho ajudou a descrever a replicação inicial do vírus em porta-enxertos comerciais e potencialmente novos.

Termos de indexação: melhoramento para resistência, germoplasma, segregação, *Poncirus*, vírus.

INTRODUCTION

CTV tolerance is an essential requirement of rootstocks used in Australian citriculture (Broadbent, 1988). Debilitating strains of the virus have been present in Australia since the 1800s and the virus is spread rapidly by *Toxoptera citricida* under field conditions. Consequently, any new rootstock must be able to perform in the presence of the virus. While the biology of this virus has been extensively studied (Karasev & Hilf, 2010) and the genetics of resistance defined (Garnsey et al., 1981; Yoshida, 1993; Gmitter et al., 1996; Mestre et al., 1997), horticultural aspects of the host/pathogen/environment interaction are still to be fully explored. This inadequate understanding of horticultural aspects of CTV is illustrated by the recent discovery that genotypes long-considered to be resistant to the virus apparently permit replication within the root system (Harper et al., 2014).

Although it is obviously desirable to categorise genotypes as either resistant or susceptible, in practice this has been difficult, and such a simplistic approach overlooks important horticultural and commercial implications. For example, genotypes may be considered resistant in some parts of the world and yet permit virus replication in others, because of the presence of different virus strains. Similarly, tolerant genotypes may support high virus titre and yet still perform well as rootstocks without showing any disease symptoms associated with CTV. This complex interaction between host/pathogen/environment creates challenges for citrus breeders trying to establish an acceptable level of CTV tolerance in addition to the many other disease and horticultural attributes demanded in commercial rootstocks.

World citriculture relies exclusively on one species, *Poncirus trifoliata*, and its hybrids for CTV resistance. The mechanism of this resistance has been well studied and is generally accepted to be via a single gene *Ctv* (Mirkov et al., 2010) such that hybrids with *P. trifoliata* segregate 1:1 for resistance. However, Mestre et al. (1997) suggest the existence of a second gene *Ctm* involved in the movement of virus within the host and interacting with the *Ctv* gene. Unfortunately these genetic studies seldom involve more than a single strain of CTV, which is tested on a very limited range of germplasm. The presence of additional CTV strains can quickly alter segregation ratios and cause a rootstock that is considered resistant in some countries to be reclassified as susceptible in others. As an example, Troyer and Swingle are generally considered resistant to CTV replication in most parts of the world (e.g. Garnsey et al., 1987) and yet they often test positive when grown in Australia. Disease response can also differ between regions as was dramatically demonstrated with Savage rootstock. In New Zealand it is considered an outstanding rootstock for mandarins (Currie et al., 2000) but in Australia, Imperial mandarin trees on Savage developed severe CTV stem pitting and died within three years of planting. Perhaps the late Herb Barrett, long-time citrus breeder with the USDA in Florida, better understood the genetic complexity of CTV resistance breeding as demonstrated by his strategy of progeny-testing resistant hybrids by hybridising them with both 'non-CTV-infectible' clones as well as 'CTV-infectible' clones (Barrett, 1990).

Understanding the way in which CTV initially replicates and moves within different seedlings may help to identify genotypes and techniques for more efficient

disease screening. The purpose of this experiment was to test CTV replication in a wide range of rootstocks. Such information may help in the choice of new commercial rootstocks, demonstrate whether past breeding programs have developed replication-resistant varieties, and point to better techniques to screen for CTV resistance in future rootstock breeding programs.

MATERIALS AND METHODS

Rootstock genotypes

Two main types of germplasm were tested - nucellar seedlings from breeding programs plus existing commercial varieties, and hybrid seedlings from controlled pollinations using *P. trifoliata*.

Nucellar seedlings

Genotypes were from breeding programs conducted in California, New South Wales (NSW) and Queensland (Qld), as well as existing commercial rootstock varieties. Twelve seed lots were supplied by the University of California in February 2008 and the resulting seedlings were then used in this experiment. Budwood of 21 selections from the NSW breeding program was obtained and the resulting nursery trees were field planted between October 2005 and June 2007 at Bundaberg Research Station (BRS). Twelve of these selections fruited for the first time in 2008 and seeds were collected for use in this experiment. Seeds from five selections of the Qld breeding program were collected in 2008 along with ten commercial varieties from source trees at BRS. Seedlings were visually culled for off-types to reduce the possibility of any zygotic or tetraploid plants making it into the experiment.

Hybrid seedlings

P. trifoliata pollinations were conducted on 27 seed parents at BRS in August 2007, along with Benton pollinations onto Ellendale, and the resulting seed sown the following April. For polyembryonic seed parents, only seedlings with trifoliate leaves were propagated and used in the experiment.

Nursery production

Seedlings were grown within an aphid-proof screenhouse from the time of sowing until the completion of the experiment. After initial germination and growth in bulk-containers, the required numbers of seedlings were transferred into individual 5L polybags at 3-months of age. The potting media was a 3:1 (v:v) mix of composted pinebark and 6mm blue-metal, with the addition (per 500L) of 1kg dolomite, 200g milled superphosphate, 300g Osmoform (30.5% urea formaldehyde, 7.5% ureic nitrogen), 130g trace element mix, 950g slow release 15:9:11 (N:P:K 8-9 month), and 400g granular wetter. Plants were drip-irrigated three times per week, fertilised fortnightly, and kept free of pests and diseases. Seedlings were checked for the presence of CTV by blotting onto nitrocellulose paper using the direct tissue blot immunoassay (DTBIA) methodology of Garnsey et al. (1993), prior to the commencement of budding.

Virus inoculation

Approximately 16 trees of each genotype were chip-budded, at a height of 200-300mm above the potting mix, between 27th and 29th January 2010. Premium budwood (high health status material indexed for major graft transmissible diseases) of Imperial mandarin was supplied by AusCitrus, Dareton (Order No. 00008841) from the same field-grown source tree (B2R5) used to supply Australian commercial nurseries. At the same time a chip-bud of virus-free Rough lemon (from a seedling tree grown within the screenhouse) was inserted 30-40mm above the Imperial bud on each of the nursery trees. Bud sticks of both the Imperial and Rough lemon budwood were blotted onto nitrocellulose paper to later confirm their CTV status. A total of 965 nursery trees were budded using the virus-infected Imperial budwood. In an adjacent bay of the screenhouse a further 226 nursery trees from the same genotypes were double-budded as above, but using virus-free Imperial budwood supplied by AusCitrus, Sydney. These budwood sticks were also blotted to confirm their virus-free status.

Buds were unwrapped 16 days after budding, and trees immediately cut-back to 30 mm above the Rough lemon bud. Secateurs and budding knives were soaked in 10% bleach between trees to reduce unintentional virus transfer.

On the 21st April 2010 (3 months after budding) trees were tested for CTV using DTBIA. This testing method is well established in CTV research, and has the added

advantage of confirming the signal is confined to the phloem, thus avoiding false-positives. Three shoots were sampled and blotted from each tree: a shoot from the Imperial bud, a shoot from the Rough lemon bud, and a shoot from the rootstock emerging above the Rough lemon bud (Figure 1).

Each shoot was immediately double blotted onto nitrocellulose paper. The resulting sheets were developed within 48h using anti-CTV detection antibody (Agdia Inc. USA) and signal development with NBT-BCIP alkaline phosphatase substrate (Sigma-Aldrich Inc. USA). Developed sheets were visually rated under a dissecting microscope, using a 5-point score where 0 = negative, 1 = negative?, 2 = positive?, 3 = weak positive, 4 = positive, 5 = extremely positive. Ratings were completed within 2h of development once the sheet was sufficiently dry to handle. The above process was repeated on shoots collected on the 23rd July 2010 (6 months after budding) and on the 27th July 2010 (to confirm CTV-negative trees). New growth was managed on each tree to ensure material could be tested from all three shoot types.

After completion of the third round of CTV testing (27th July 2010) the Rough lemon bud/shoot was cut off and the Imperial bud allowed to develop into a tree suitable for commercial planting. Measurements of tree height and trunk circumference were made of all trees on the 7th March 2011 (13 months after budding). In early August 2011 the trees were removed from the aphid-proof screenhouse and planted on a commercial orchard in Emerald, Queensland. Each tree had a unique code throughout the nursery experiment and field planting so that future field data can be linked back to individual tree performance during the CTV testing phase.

RESULTS AND DISCUSSION

DTBIA results from the first round of testing indicated that 100% of the virus-free Rough lemon buds had become infected within three months of budding. Given that the Rough lemon budwood was virus-free (confirmed by DTBIA), that the rootstock seedlings were also virus-free at the time of budding (confirmed by DTBIA), and that aphids could not access the plants, then the most likely explanation is that the virus moved from the infected Imperial bud through the rootstock and into the initially virus-free Rough lemon bud 30-40 mm above it. This conclusion is further supported by the Rough lemon buds remaining virus free on rootstocks that were budded with virus-free Imperial budwood. Thus none of the genetic material tested was capable of preventing, or even slowing, the movement of CTV through the rootstock seedling.

Table 1 shows the CTV status of 39 nucellar rootstock genotypes at six months after bud inoculation. Seedling numbers per genotype were generally 15 or 16, but varied from 3 to 31. Consequently, the right hand column indicates the percentage of seedlings that could confidently be classified as CTV positive, derived by adding scores 3, 4, and 5, and dividing by the total number.

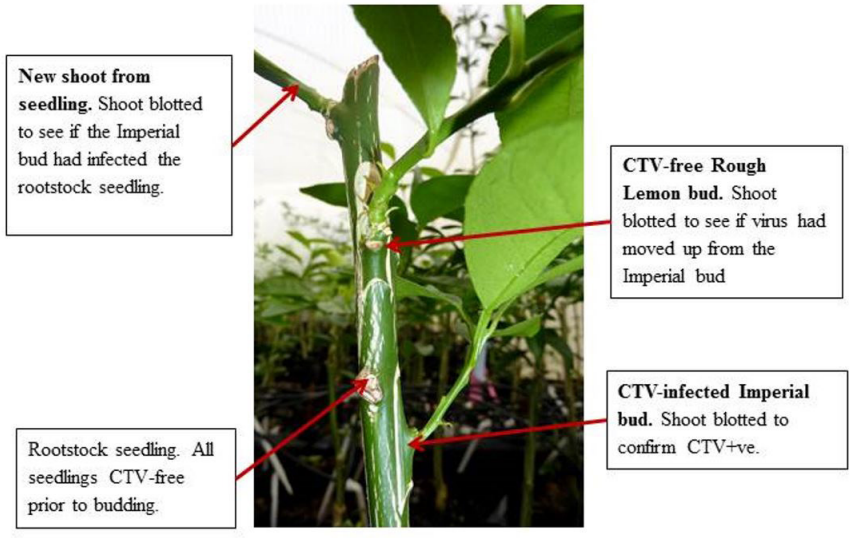

Figure 1. Method of testing CTV replication and movement in individual nursey seedlingls.

Table 1. CTV ratings for seedlings of 39 nucellar genotypes, six months after bud inoculation

Nucellar rootstock	Breeding program	*Poncirus* parent	CTV severity category[a], 6 months post inoculation (No. of seedlings)							CTV-positive[b] (%)
			0	1	2	3	4	5	Total	
3784	NSW	yes					15		15	100
3796	NSW	yes				8	8		16	100
3802	NSW	yes	4			5	6		15	73
3806	NSW	yes	5	2	4		2		13	15
3812	NSW	yes		2	2	5	6		15	73
3816	NSW	yes				1	2		3	100
3817	NSW	yes	3	2	3	2	6		16	50
3822	NSW	yes					15		15	100
3831	NSW	yes	8	3	3		2		16	13
3834	NSW	yes	1			11	3		15	93
3835	NSW	yes	2	2	1	3	7		15	67
4033	NSW	yes	6	1	2	1	6		16	44
02C017	BRS	no				2	11		13	100
02C018	BRS	no				4	12		16	100
05C009	BRS	no					14	1	15	100
Bakers	BRS	no				3	13		16	100
14Q055	BRS	yes	11	2	1		1		15	7
C22 (Bitters)	California	yes	3		5	2	6		16	50
C32	California	yes	5	3	3	3	1		15	27
C35	California	yes					15		15	100
C54(Carpenter)	California	yes				12	2		14	100
C57 (Furr)	California	yes					12		12	100
C146	California	yes				2	11		13	100
58-220-2	California	yes				8	4		12	100
59-24-8	California	yes		1		2	9		12	92
63-199-31	California	yes	1	2	3	9			15	60
63-199-49	California	yes	15	9	1		6		31	19
62-109-1	California	yes					6		6	100
62-137-2	California	yes		1	1	18	9		29	93
Benton	Commercial	yes	6	2	1	5			14	36
H639	Commercial	yes	5	4	1		4		14	29
Swingle	Commercial	yes					15		15	100
*P.trifoliata*Tri22	Commercial	yes	9	1		5	1		16	38
Troyer	Commercial	yes	5	1	2	5	2		15	47
US812	Commercial	yes	1		1	6	3		11	82
Cleopatra	Commercial	no				5	12		17	100
Rangpur	Commercial	no					13		13	100
Rough Lemon	Commercial	no					15	1	16	100
C. volkameriana	Commercial	no					11		11	100
Total			**90**	**38**	**34**	**127**	**286**	**2**	**577**	**72**

[a]Intensity of DTBIA signal (purple colouration induced by NBT-BCIP alkaline phosphatase substrate) rated for each individual seedling, where 0 = no colouration to 5 = extremely purple colouration, assessed at 30X magnification: [b]Combined severity categories 3 to 5 divided by Total.

There are clear differences between genotypes, with 19 of the 39 genotypes being 100% infected within six months of bud inoculation. All eight genotypes lacking *Poncirus* in their pedigree were 100% infected. Amongst these non-*Poncirus* hybrids there are five rootstocks from the Bundaberg breeding program. These are polyembyronic mandarin and orange hybrids that were included mainly for their potential impact on fruit granulation, and clearly they have no capacity to restrict virus replication. Indeed one of these hybrids, Bakers, was the only genotype in the whole experiment to show a seedling-yellows reaction when exposed to the infected Imperial budwood.

Some *Poncirus* hybrids, which we know from our extensive field testing at BRS to be capable of CTV replication, showed only intermediate levels of infection after six months, suggesting that *Poncirus* parentage may help to restrict virus replication even when it is not prevented. Two such examples are Troyer and Benton, field trees of which always test positive, but only 50% of their seedlings were infected after six months. This may suggest that the early testing of multiple seedlings could be a way of identifying better parents, even in genotypes that will eventually become positive.

Information is already available on the replication of Australian endemic CTV strains in some of the genotypes listed in Table 1. This includes DTBIA field testing of seed-source trees at BRS as well as ELISA testing of leaves and bark by Broadbent & Gollnow (1993) in NSW. Results are consistent, with the exception that Troyer was resistant in the work of Broadbent & Gollnow (1993) but is moderately susceptible in Table 1, and always tests CTV-positive in field trees at BRS.

The presence of some CTV positive seedlings of *P. trifoliata* was unexpected and indicates that even resistant genotypes can intermittently host the virus under ideal conditions (such as close proximity to an infected bud). Garnsey et al. (1987) observed a similar phenomenon in Swingle citrumelo which was considered resistant in their environment but produced some trees that tested positive. They found that CTV replicated to some extent in tender tissue but did not persist in mature leaves. This illustrates the importance of testing multiple seedlings on multiple occasions. Accidental inclusion of zygotic seedlings is not considered an adequate explanation for the occurrence of positive seedlings amongst a batch of otherwise negative seedlings, because their frequency is too high. Many of the commercial rootstocks included in this experiment have low rates of zygotic seedling production, significantly less than the frequency of positive seedlings found in our work. The results show that it is not always possible to classify genotypes as either replicating or not-replicating CTV. Instead they may be classified into a larger number of categories ranging from 'strongly-resistant-to-CTV-replication' to 'highly-supportive-of-CTV-replication' based on the number of seedlings that become infected, how quickly they become infected, and how strong a DTBIA signal they give (indicative of titre). Variability in CTV titre levels between rootstock genotypes is consistent with previous reports (e.g. Garnsey et al., 1981, 1987; Broadbent & Gollnow, 1993).

Based on a low number of seedling infections, slow rate of virus detection and weak DTBIA signal, only six of the nucellar genotypes show strong resistance to CTV replication, and the best of these [14Q055, 3831, 63-199-49, Tri22 (control)] are being progeny tested for their ability to transmit resistance. Whether any of these are more resistant than *Poncirus* (i.e. transgressive segregation) is also worth investigating by more extensive bud inoculation with different CTV strains. Conventional thinking would explain the high resistance of these genotypes simply via the inheritance of the *Ctv* (and possible *Ctm*) gene from their *Poncirus* parent, but as breeders we need to be open to the possibility that high selection intensity may enable us to exceed the resistance level of the donor parent. Although *Poncirus* is still widely regarded as the best source of CTV resistance, we know that resistance-breaking (RB) CTV strains exist (Harper et al., 2010) and that *P. trifoliata* itself is only heterozygous for the known gene(s) conferring resistance. It would be interesting to test whether RB-CTV strains such as those present in New Zealand, were inhibited in any of the strongly resistant genotypes shown in Table 1.

Table 2 shows CTV replication in 30 segregating hybrid families generated using *P. trifoliata* pollen, along with two distant but graft compatible monoembryonic genera. Each plant within a family is a genetically distinct individual even though they share the same parentage (siblings) and hence we may expect a wider spread of results than for the nucellar seedlings shown in Table 1. The number of genotypes with 100% infection after six months is clearly lower than with the nucellar rootstocks, largely on account of the involvement of *Poncirus* in generating most of these families. And whilst there were few families where infection approached 100%, there were also few families with very low levels of infection (scores 0, 1, and 2). Despite this absence of genotypes with low percentage infection scores,

Table 2. CTV ratings for seedlings of 32 hybrid seedling families, six months after bud inoculation

Hybrid family	Type of seed parent	CTV severity category[a], 6 months post inoculation (No. of seedlings)							CTV-positive[b] (%)
		0	1	2	3	4	5	Total	
01C011×Pt[c]	mandarin	6		1	3	6		16	56
05C009×Pt	mandarin	4	1		1	6		12	58
05C023×Pt	mandarin	1		1		2		4	50
Arrufatina×Pt	mandarin	1				2		3	67
AustClem×Pt	mandarin	3	3	1	2	7		16	56
Daisy×Pt	mandarin	3	1	2	1	5	1	13	54
Ellendale×Benton	mandarin	2		1	2	5		10	70
Ellendale×Pt	mandarin		1	1	2	11		15	87
Encore×Pt	mandarin	2			7	5		14	86
Fallglo×Pt	mandarin	4	1	1	1	9		16	63
Fina×Pt	mandarin	3		1	2	9		15	73
Fremont×Pt	mandarin	2	1	1	1	11		16	75
IM111×Pt	mandarin	2	2	1		7	1	13	62
Imperial×Pt	mandarin	6		1	2	6		15	53
Marisol×Pt	mandarin	4			1	9		14	71
Monarch×Pt	mandarin	3			3	7		13	77
Nules×Pt	mandarin	5			1	9		15	67
Oroval×Pt	mandarin	3		2		8	1	14	64
Temple×Pt	mandarin	1	2	1	1		11	16	75
Umatilla×Pt	mandarin	1			2	11		14	93
Wilking×Pt	mandarin	2	1	1	2	7		13	69
Hamlin×Pt	sweet orange		1			1		2	50
Chinotto×Pt	sour orange		1			1		2	50
Seville×Pt	sour orange	3			1	12		16	81
Limonera×Pt	lemon		2	2		8	1	13	69
Rangpur×Pt	lemon	2		1		10		13	77
RoughLemon×Pt	lemon	2				5		7	71
Carters×Pt	pomelo	3	4	2	1	5		15	40
K15×Pt	pomelo	3		3		5	4	15	60
StarRuby×Pt	pomelo					2		2	100
Citropsis	out-group					8	2	10	100
Micromelum	out-group					8	8	16	100
Total		**71**	**21**	**24**	**36**	**207**	**29**	**388**	**70**

[a]Intensity of DTBIA signal (purple colouration induced by NBT-BCIP alkaline phosphatase substrate) rated for each individual seedling, where 0 = no colouration to 5 = extremely purple colouration, assessed at 30X magnification: [b]Combined severity categories 3 to 5 divided by Total: [c]Pt = *Poncirus trifoliata*.

there were CTV-negative (score 0) individuals in all but five of the 30 hybrid families. These individuals warrant further inoculation and assessment over a longer period of time to confirm they are not disease escapes. None-the-less, the presence of these putatively-resistant hybrids indicates a potential to select individuals with low CTV replication even from families with high overall scores.

It seems clear from the results that the seed parent influences the distribution of CTV sensitivity. In families like Imperial×*P. trifoliata* (Pt) and 01C011×Pt almost half of the seedlings were still free of CTV after six months, while other families like Ellendale×Pt, Temple×Pt and Fremont×Pt had few if any CTV-free seedlings. Obvious vein-clearing symptoms were seen in seedlings of Temple×Pt,

Fremont×Pt and Monarch×Pt, and these are some of the same families that produced few replication-resistant hybrids. Thus, assessment of early CTV replication in hybrid families may help to identify superior parents to cross with *Poncirus*, by measuring the initial frequency of resistant individuals. Our data suggests that both parents play a role in the inheritance of CTV resistance even when the non-donor parent is highly susceptible to infection.

Resistance was less common than susceptibility within all but one (Carters×Pt) of the 30 hybrid families. Even after just six months, at least 50% of hybrids were replicating the virus, and we might expect that numbers would increase if the plants had been tested again after 12 months. In some families such as Umatilla×Pt, Ellendale×Pt and Encore×Pt, more than 80% of the seedlings were infected within six months. There were also differences in the intensity of the DTBIA signal observed from different families, Temple×Pt being particularly unusual in severity with eleven of the sixteen hybrids in the highest category (score 5).

The results above help to demonstrate the horticultural complexity of CTV and the diseases it causes. All ten commercial rootstocks shown in Table 1 are considered tristeza tolerant and yet most of them rapidly became infected with CTV. This has important implications for breeding strategy and is why most rootstock breeding programs have used 'disease reaction' rather than virus 'presence/absence' as the selection criteria. Consequently, many new rootstocks are free of disease symptoms even though they allow virus replication, as shown in Table 1. Whilst selecting such genotypes ensures a greater proportion of the breeding population is retained, it is far more complex and time consuming than simply retaining those few hybrids that prevent virus replication. For example Bordignon et al. (2004) measured field symptoms for five years before deciding which of their hybrids were tolerant. By contrast, our results show that it is possible to identify resistant hybrids during the nursery phase within 12 months of bud inoculation. While the number of retained hybrids is relatively low, resistant individuals are found in all crosses with *Poncirus* (when family size is approximately 10 or greater). Furthermore, Mestre et al. (1997) recommends complete suppression of CTV as a better strategy for long term management of disease, in preference to developing tolerant hybrids.

Based on these results, the Queensland breeding strategy is to retain only those hybrids that prevent CTV replication. While useful commercial germplasm is undoubtedly discarded, this is outweighed by the CTV issue being resolved quickly, enabling the program to then focus on the fruit quality impacts of new rootstocks. Development of a large and diverse collection of resistant germplasm (our F_1 hybrids) will also enable more effective incorporation of CTV resistance in the next generation of hybrids, including the possibility of homozygous parents transmitting resistance to all of their progeny. The finding that the non-donor parent influences CTV replication will also enable us to capture additional genes that may be involved in the disease response.

The number of different parents used over the last 100 years of international citrus rootstock breeding is remarkably small. Not only do most hybrids share one parent (*P. trifoliata*) and at the same generation level (F_1), but diversity in the second parent is also limited (e.g. often being Sweet orange, Sunki or Cleopatra mandarins). Aside from some preliminary work at Indio (Furr et al., 1963), most breeding programs have settled on this same narrow range of parents without any evidence to suggest they are better (or worse) than other potential parents. Bill Bitters, who spent decades evaluating rootstocks in California and is largely responsible for the commercial emergence of Troyer, was well aware of the poor foundation on which parental choices were made by rootstock breeders. Indeed, he states in relation to the breeding of C32 and C35 that the Ruby blood has never been used as a rootstock and has nothing to recommend it. One wonders how much better Troyer, Carrizo, C32, and C35 citranges might have been if the sweet orange female parent would have been a more desirable and proven rootstock type" (Bitters 1986, p. 107).

Results from our experiment suggest that selection of the non-donor parent can significantly impact outcomes, even within such a well-studied trait as CTV resistance. The conventional wisdom that the CTV performance of progeny is derived solely from the *Poncirus* parent may be wrong and our data suggest that a far wider range of germplasm should be included in the initial phase of rootstock breeding programs.

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REFERENCES

Barrett HC (1990) US119, an intergeneric hybrid citrus scion breeding line. HortScience 25(12): 1670-1671.

Bitters WP (1986) Citrus Rootstocks: their characters and reactions. 236 p. (Nemeth M, Kahn T, Krueger RR & Siebert T, eds. digital version). Available from: <http://www.citrusvariety.ucr.edu/links/documents/Bitters.pdf>. Accessed: 17 Oct. 2016.

Bordignon R, Medina-Filho HP, Siqueira WJ & Teofilo Sobrinho J (2004) The genetics of tolerance to tristeza disease in citrus rootstocks. Genetics and Molecular Biology 27(2): 199-206.

Broadbent P (1988) Disease and pest resistance of citrus rootstocks: potentials for improvement. In: Walker RR (Ed). Citrus breeding workshop. Camberra: Commonwealth Scientific and Industrial Research Organisation, p 74-86.

Broadbent P & Gollnow BI (1993) Selecting disease-resistant citrus rootstocks. Australian Journal of Experimental Agriculture 33(6): 775-780.

Currie AJ, Sutton PG, Machin T & Anderson P (2000) Miyagawa satsuma mandarin rootstock trial in New Zealand. Proceedings of the International Society of Citriculture, Orlando, p. 485-486.

Furr JR, Carpenter JB & Hewitt AA (1963) Breeding new varieties of citrus fruits and rootstocks for the southwest. Journal of the Rio Grande Valley Horticultural Society 17: 90-107.

Garnsey SM, Barrett HC & Hutchison DJ (1981) Resistance to citrus tristeza virus in citrus hybrids as determined by enzyme-linked immunosorbent assay. Phytopathology 71: 875.

Garnsey SM, Barrett HC & Hutchison DJ (1987) Identification of citrus tristeza virus resistance in citrus relatives and its potential applications. Phytophylactica 19: 187-191.

Garnsey SM, Permar TA, Cambra M & Henderson CT (1993) Direct tissue blot immunoassay (DTBIA) for detection of citrus tristeza virus (CTV). Proceedings of the 12th International Organisation of Citrus Virologists, Riverside, CA, p. 39-50.

Gmitter FG, Xiao SY, Huang S, Hu XL, Garnsey SM & Deng Z (1996) A localized linkage map of the citrus tristeza virus resistance gene region. Theoretical and Applied Genetics 92(6): 688-695.

Harper SJ, Cowell SJ, Robertson CJ & Dawson WO (2014) Differential tropism in roots and shoots infected by Citrus tristeza virus. Virology 460-461: 91-99.

Harper SJ, Dawson TE & Pearson MN (2010) Isolates of Citrus tristeza virus that overcome *Poncirus trifoliata* resistance comprise a novel strain. Archives of Virology 155(4): 471-480.

Karasev AV & Hilf ME (2010) Citrus tristeza virus complex and tristeza diseases. Saint Paul: American Phytopathological Society Press. 260 p.

Mestre PF, Asins MJ, Carbonell EA & Navarro L (1997) New gene(s) involved in the resistance of *Poncirus trifoliata* (L.) Raf. to citrus tristeza virus. Theoretical and Applied Genetics 95(4): 691-695.

Mirkov E, Yang ZN, Rai M, Molina JJ, Roose ML & Ye XR (2010) Toward positional cloning of the Citrus tristeza virus resistance gene. In: Karasev AV & Hilf ME (Eds). Citrus tristeza virus complex and tristeza diseases. Saint Paul, MN: American Phytopathological Society Press, p. 187-201.

Yoshida T (1993) Inheritance of immunity to citrus tristeza virus of trifoliate orange in some citrus intergeneric hybrids. Bulletin of the Fruit Tree Research Station 25: 33-43.

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