ITALUS HORTUS
Vol. 31 (2024), Pages 43-55
doi: 10.26353/j.itahort/2024.1.4355
Original Research Paper

"Extreme hybrids" from the Australian citrus rootstock breeding program

Malcolm W. Smith[1*], Debra L. Gultzow[1], Michael Reid[1], Jessica E. Huie[1] and Toni K. Newman[1,2]

[1] Bundaberg Research Station, Department of Agriculture and Fisheries, 49 Ashfield Road, Bundaberg 4670, Queensland, Australia
[2] retired 2020
[*] Corresponding author: malcolm.smith@daf.qld.gov.au

Received: 26 February 2024; Accepted: 25 April 2024; Published: 31 May 2024

Abstract: Citrus is one of the most genetically diverse fruit trees used by humans, and yet few rootstock breeders have ventured beyond a narrow range of parents. For the last 25 years we have explored wider graft and sexual compatibility within the citrus subfamily (Aurantioideae) than any previous attempts, and have identified new genera and species for rootstock breeding. Whilst the vast majority of this work has met with failure, it has identified factors such as sexual incompatibility, late-acting lethality and poor field adaptation as the reasons why many citrus relatives are not currently amenable to rootstock breeding. A major breakthrough early in the program was the discovery that Oceania citrus species are extremely sensitive to Citrus tristeza virus (CTV) when used as rootstocks, and the realisation that resistance must be introgressed if we are to ever discover useful traits masked by this disease sensitivity. Bridging hybrids were required to transfer this virus resistance and a newly discovered species (*Citrus wakonai*) was employed to speed-up the process. After three generations of crossing, our "extreme hybrids" with Oceania parentage now show commercial performance equivalent to industry standards. By providing citrus growers with a range of high-performance rootstocks from extremely complex genetic backgrounds we can increase the biological diversity of orchards without compromising production. Our hope is that these "extreme hybrids" may help in the battle against Huanglongbing (HLB) disease.

Keywords: crop relatives; huanglongbing; citrus tristeza virus; introgression; domestication; late-acting lethality.

1. Introduction

Rootstocks have shaped the history of world citrus production, providing solutions to devastating problems like phytophthora and CTV and allowing production to expand into regions limited by factors such as low temperatures, salinity and drought. The first deliberate attempt to breed citrus rootstocks is credited to Walter Swingle in the late 1800s although this was a fortuitous spin-off from scion breeding for cold tolerance. Nonetheless it demonstrated the potential of interspecific hybridisation and the parents Swingle used have remained largely unchanged since that time. There are few tree crops where rootstocks have demonstrated impacts on as many traits as is the case with citrus. Consequently, it is not surprising that rootstock breeding programs now exist in many citrus producing countries and have provided significant economic benefits to industry. These programs aim to address a wide range of issues including tree size control, graft compatibility, disease resistance, edaphic constraints, and fruit quality.

Regardless of the ongoing debate around citrus taxonomy, the extent of genetic diversity of sexually compatible species is enormous and has been long recognised by plant breeders. It is therefore perplexing why more of this genetic diversity has not been exploited and why most current rootstock breeding programs rely on a very narrow range of parents. Distant relatives have been evaluated as rootstocks (Bitters et al., 1964, 1969, 1977; Caruso et al. 2020; Yoshida 1996) but seldom when hybridised with other species (Reforgiato et al. 2009). Even fewer programs have developed and tested hybrids beyond the F1 (Bowman et al. 2021; Grosser et al. 2015).

We sort to address these questions by developing a multi-generational breeding program, using highly diverse parents. Our objective was to obtain “extreme hybrids” whose performance as rootstocks was comparable to existing industry standards.

2. Materials and Methods

Seed was obtained from a diverse range of citrus species and relatives and sown at Bundaberg Research Station (BRS) where they were maintained in the nursery until of sufficient size for grafting (approximately 18 months). An additional 17 Rutaceae species, distantly related to *Citrus*, could not be obtained as seed and were instead purchased as small seedlings from native plant wholesalers and were potted-up and grown-on in the BRS nursery. This rootstock material, representing 22 different genera and 60 species from the Rutaceae family, was used to assess graft compatibility with ‘Eureka’ lemon and ‘Imperial’ mandarin. Mango, *Mangifera indica* seedlings (cv. ‘Kensington Pride’) were included as a non-Rutaceae control. All rootstock plants showed uniform growth and morphology consistent with their species designation even though the majority of the accessions are assumed to be non-apomictic. Pathogen-tested budwood of ‘Eureka’ lemon and ‘Imperial’ mandarin was sourced exclusively from the Australian budwood scheme (Auscitrus). This budwood contains strains of CTV that are now endemic in southern Australia, but is free of CTV strains causing seedling-yellows, grapefruit and orange stem-pitting and also free of other citrus pathogens. The presence of endemic CTV strains in the budwood ensured that all rootstocks were uniformly exposed to this virus at the time of grafting. All grafting was undertaken in the BRS nursery during favourable growing conditions, using vigorous rootstocks and by the same grafter (MWS). Replication of the 82 different rootstock-scion combinations ranged from four to 16 individual plants, with most replicated eight times.

Observations during the nursery propagation stage provided information on initial graft compatibility and pointed to those combinations that were sufficiently robust for testing under field conditions. Field trials using these robust combinations and a range of other rootstocks were established in commercial orchards near Bundaberg in 2002 (‘Eureka’ lemon scion, 28 rootstocks) and 2004 (‘Imperial’ mandarin scion, 31 rootstocks). Treatment replication ranged from six to 15 trees, with most replicated eight times. Field performance was assessed at regular intervals for the first six years in the ‘Eureka’ lemon trial, and for the first 12 years in the ‘Imperial’ mandarin trial, and the information progressively used to guide new parent selection.

Simultaneously with the rootstock trials, hybridisation experiments were conducted between diverse parents in an attempt to generate unique genetic combinations. For the more distant relatives, the newly described *C. wakonai* (Forster and Smith, 2010) was used as the maternal parent on account of its extremely high fertility and short juvenile period. CTV resistance was assessed for all hybrids by bud inoculation and serological testing (Smith et al., 2016; Yoshida 1993) using Rough lemon (*C. jambhiri*) carrying a diverse range of CTV strains. New growth on the hybrids was monitored serologically every six months for a minimum of two years with the infected Rough lemon bud remaining in place. Hybrids that permitted replication of the virus were discarded, except when a CTV-resistant parent was not available for intergeneric crossing.

Annual results from these initial rootstock and hybridisation experiments guided the choice of breeding parents and the traits for which they were screened. Surviving hybrids were propagated via cutting (Albrecht et al. 2017; Sykes, 2010) and used to establish new field rootstock experiments.

3. Results

Graft compatibility results for ‘Eureka’ lemon and ‘Imperial’ mandarin on species of varying taxonomic proximity are shown in Table 1. *Citrus* species were considered “Very close”; those within the

Table 1. Graft compatibility testing results for 'Imperial' mandarin and 'Eureka' lemon grafted on a wide range of species of differing taxonomic proximity to *Citrus*. Oceania species are shown in bold.

Genus	Species	Scion	Buds sprout[1]	Nursery shoot length[2]	Suitable for field planting	Initial field growth
			Taxonomic proximity: VERY CLOSE			
Citrus	*aurantium* (hybrid)[3]	'Imperial'	Y	85	Y	good
Citrus	***australasica***	'Imperial'	Y	60	Y	very poor
Citrus	***australis***	'Eureka'	Y	109	Y	modest
Citrus	*erythrosa*	'Imperial'	Y	102	Y	good
Citrus	***garrawayi***	'Eureka'	Y	1	N	
Citrus	***garrawayi***	'Imperial'	Y	45	Y	poor
Citrus	*ichangensis*	'Imperial'	Y	75	Y	very poor
Citrus	***inodora***	'Eureka'	Y	72	Y	very poor
Citrus	***inodora***	'Imperial'	Y	50	Y	very poor
Citrus	*junos*	'Imperial'	Y	92	Y	good
Citrus	*reticulata*	'Imperial'	Y	100	Y	good
Citrus	***wakonai***	'Eureka'	Y	29 & 45	Y	modest
Citrus	***wakonai***	'Imperial'	Y	47	Y	very poor
Citrus	***warburgiana***	'Eureka'	Y	72	Y	poor
Citrus	***warburgiana***	'Imperial'	Y	85	Y	very poor
Citrus	***wintersii***	'Eureka'	Y	25	Y	very poor
			Taxonomic proximity: CLOSE			
Atalantia	*ceylanica*	'Eureka'	Y	100	Y	modest[4]
Atalantia	*ceylanica*	'Imperial'	Y	65	Y	modest
Atalantia	*paniculata*	'Eureka'	Y	10	N	
Atalantia	*paniculata*	'Imperial'	Y	2	N	
Citropsis	*daweana*	'Imperial'	Y	28	Y	poor
Citropsis	*gabunensis*	'Eureka'	Y	80	Y	modest[4]
Citropsis	*gabunensis*	'Imperial'	Y	40	Y	poor
Citropsis	*gillentiana*	'Eureka'	Y	118	Y	modest
Citropsis	*schweinfurthi*	'Eureka'	Y	112	Y	modest
Clymenia	***polyandra***	'Eureka'	Y	22	Y	modest
Clymenia	***polyandra***	'Imperial'	Y	40	Y	poor
Fortunella	*hindsii*	'Eureka'	Y	38 & 48	Y	poor-good
Fortunella	*japonica*	'Eureka'	Y	23	Y	poor
Fortunella	*oblata*	'Eureka'	Y	134	Y	modest
Fortunella	*polyandra*	'Eureka'	Y	87	Y	poor
Severinia	*disticha*	'Eureka'	Y	90	Y	modest[4]
			Taxonomic proximity: MODERATELY DISTANT			
Aegle	*marmelos*	'Eureka'	Y	9	Y	poor[4]
Aegle	*marmelos*	'Imperial'	Y	10	N	
Aeglopsis	*chevalieri*	'Eureka'	N			
Aeglopsis	*chevalieri*	'Imperial'	Y	3	N	

continue next page

Table 1 (continued)

Genus	species	Scion	Buds sprout[1]	Nursery shoot length[2]	Suitable for field planting	Initial field growth
			Taxonomic proximity: DISTANT			
Bergera	***crenulata***	'Eureka'	Y	45	N	
Bergera	***crenulata***	'Imperial'	Y	12	N	
Bergera	*koenigii*	'Eureka'	Y	50	Y	poor[4]
Bergera	*koenigii*	'Imperial'	Y	20	N	
Clausena	*anisata*	'Eureka'	Y	32	Y	poor[4]
Clausena	*anisata*	'Imperial'	Y	5	N	
Clausena	***brevistyla***	'Eureka'	Y	1	N	
Clausena	***brevistyla***	'Imperial'	Y	10	N	
Clausena	*excavata*	'Eureka'	N			
Clausena	*excavata*	'Imperial'	N			
Clausena	*harmandiana*	'Eureka'	Y	1	N	
Clausena	*harmandiana*	'Imperial'	N			
Clausena	*lansium*	'Eureka'	Y	41	Y	very poor
Clausena	*lansium*	'Imperial'	Y	40	Y	very poor
Clausena	**sp.NT**	'Eureka'	N			
Glycosmis	*pentaphylla*	'Eureka'	Y	2	N	
Glycosmis	*pentaphylla*	'Imperial'	N			
Glycosmis	***trifoliata***	'Eureka'	Y	7	N	
Micromelum	***minutum***	'Eureka'	Y	74	Y	very poor
Micromelum	***minutum***	'Imperial'	Y	60	N	
Murraya	***ovatifoliolata***	'Eureka'	Y	20	N	
Murraya	***paniculata***	'Eureka'	Y	40	Y	very poor[4]
Murraya	***paniculata***	'Imperial'	N			
			Taxonomic proximity: VERY DISTANT			
Acronychia	***acidula***	'Eureka'	Y	1	N	
Acronychia	***oblongifolia***	'Eureka'	Y	1	N	
Acronychia	***pubescens***	'Eureka'	N			
Acronychia	***suberosa***	'Eureka'	N			
Acronychia	***wilcoxiana***	'Eureka'	N			
Casimiroa	***edulis***	'Eureka'	Y	2	N	
Casimiroa	***edulis***	'Imperial'	Y	5	N	
Dinosperma	**sp.**	'Eureka'	Y	1	N	
Melicope	***xanthoxyloides***	'Eureka'	Y	2	N	
Melicope	***rubra***	'Eureka'	Y	5	N	
Flindersia	***australis***	'Eureka'	Y	1	N	
Flindersia	***bennettiana***	'Eureka'	Y	1	N	
Flindersia	***bourjotiana***	'Eureka'	Y	2	N	
Flindersia	***brayleyana***	'Eureka'	N			
Flindersia	***collina***	'Eureka'	N			
Flindersia	***xanthoxyla***	'Eureka'	Y	1	N	
Geijera	***salicifolia* var. *latifolia***	'Eureka'	N			
Lunasia	***amara***	'Eureka'	N			
Melicope	***elleryana***	'Eureka'	N			
Melicope	***micrococca***	'Eureka'	N			
Melicope	***vitiflora***	'Eureka'	Y	2	N	
Sarcomelicope	***simplicifolia* subsp. *simplicifolia***	'Eureka'	N			
			Taxonomic proximity: UNRELATED (different Family)			
Mangifera	*indica*	'Eureka'	Y	1	N	

Table 1 (footnote)

1. Indicates whether scion buds started to 'sprout' after propagation.
2. Length of scion growth (cm) at about 10 months after propagation.
3. 'Guotou chen' is likely a *C. aurantium* hybrid and has been shown to tolerate Australian strains of CTV causing quick decline, in contrast to true *C. aurantium* which fails when used as a rootstock in Australia.
4. Some combinations were not included in the replicated field trials (see below) but were field planted at BRS for observation.

Subtribe Citrinae "Close"; species within the Tribe Citreae "Moderately distant"; those within the Aurantioideae "Distant"; and Rutaceous species outside Aurantioideae were considered "Very distant". Listed taxon often have multiple synonyms and are frequently being rearranged, particularly at the genus level. We have generally followed the current botanical circumscription including the removal of genera such as *Eremocitrus*, *Microcitrus* and *Poncirus*, and the recent reinstatement of the *Bergera* genus.

Rootstocks that induced good growth in the 'Eureka' lemon scion during the nursery phase (Table 1) were chosen for inclusion in a replicated field experiment. This field experiment also included three Oceania species as interstocks between 'Troyer' rootstock and the 'Eureka' lemon scion. Nine conventional citrus accessions were included for comparison with the Oceania *Citrus* species as well as other unusual rootstocks that showed promise during nursery testing. The experiment was planted adjacent to a commercial orchard of 'Eureka' lemon on 'Volkamer' rootstock in December 2002. Tree growth for the first 71 months of these combinations is shown in Table 2, together with their initial shoot length during the nursery phase.

Rootstocks that induced good growth in the 'Imperial' mandarin scion during the nursery phase (Table 1) were included in a field trial that was planted at a commercial citrus orchard in November 2004. This experiment included a range of Asian *Citrus* species (including *C. erythrosa*, *C. ichangensis* and *C. reticulata* accessions) to assess performance relative to the Oceania species. A further nine commercial rootstocks were included as controls. The canopy development of Imperial mandarin on these 31 different rootstocks is shown in Table 3, with eight of the combinations declining soon after planting (including all of the Oceania species).

Rootstock hybrids were generated ever year from controlled crosses, and new field trials planted every 2-3 years. To date, more than 5,000 hybrids have been screened for various traits and 885 of these tested in replicated field trials. CTV quickly emerged as a major constraint in using wild citrus relatives and a program of deliberate inoculation and serological screening was required. More than 3,000 individual hybrids have been generated and screened for CTV replication. A range of donor parents for CTV resistance were used, most of which were assumed to have inherited this trait from their Trifoliata parent (*Citrus trifoliata*, syn. *Poncirus trifoliata*). However, we also included potential new sources of CTV resistance discovered during regular serological testing of the germplasm collection at Bundaberg Research Station (Volk et al. 2023). Some of these donor parents are listed in Table 4 below, along with their frequency of CTV resistant progeny.

Field trials now contain multiple rootstocks with shared parentage, making it possible to compare performance for different species. One example is shown below, were the 17 rootstocks containing *C. glauca* parentage, in a trial with 'Premier' mandarin field planted in November 2020, are listed in Table 5. This table demonstrates changes in tree health at four dates after planting and the tree replication that existed at planting and then 38 months later.

Replants in the above trial with 'Premier' mandarin have include the new rootstock 'F438' which was not available when the experiment was initially established. This is a complex hybrid containing four different Citrus species (C. australis, C. glauca, C. trifoliata and C. wakonai) and tree establishment has been excellent (Figure 1A). Another field experiment contains 138 different rootstocks under 'Imperial' mandarin scion and was planted in November 2016. It contains multiple hybrids with Oceania species, including 13 hybrids that have C. australasica parentage, of which the most promising continues to be 'ICA12' and is pictured four years after planting in Figure 1B. Wide differences in field performance between rootstocks with identical parentage demonstrates the importance of field-screening large numbers of individual hybrids, replicated via cuttings.

Table 2. Tree size of Eureka lemon on 28 rootstocks at various ages from planting to almost six years in the field, along with the initial nursery shoot length. Oceania species are shown in bold.

Treatment		Nursery shoot length[1]	CSA 17[2]	CSA 28	CSA 40	CSA 71
		Oceania *Citrus* species				
Citrus	***australis***	109	6.1	8.3	11.1	11.3[3]
Citrus	***inodora***	72	1.5	1.7	1.9	
Citrus	***wakonai*** (Acc. 1)	45	5.2	6.8	8.0	5.8
Citrus	***wakonai*** (Acc. 2)	29	4.5	6.3	10.6	16.4
Citrus	***warburgiana***	72	4.4	4.7	5.2	0
Citrus	***wintersii***	25	1.6	2.2	2.6	0
Troyer + inter.[4]	***glauca***	43	11.1	19.5	21.0	35.6
Troyer + inter.	***wintersii***	49	9.9	16.7	18.4	22.1
		Non-*Citrus* relatives				
Citropsis	*gilletiana*	118	4.7	7.1	12.5	15.2
Citropsis	*schweinfurthii*	112	4.7	7.4	13.1	10.1
Clymenia	***polyandra***	22	7.1	10.9	16.8	12.3
Fortunella	*hindsii* (Acc. 1)	38	9.0	14.3	19.9	27.6
Fortunella	*hindsii* (Acc. 2)	48	4.3	7.0	10.9	0
Fortunella	*japonica*	23	1.8	2.1	2.4	0
Fortunella	*obovata*	134	8.4	12.5	17.5	7.2
Fortunella	*polyandra*	87	8.8	11.6	19.3	8.0
Clausena	*lansium*	41	0.9	1.0	0.9	0
Micromelum	***minutum***	74	0			
Troyer + inter.	***Clymenia***	65	11.9	12.9	14.7	12.4
		Non-conventional rootstocks				
Tangerine	'Ponkan'	103	13.9	20.5	34.1	49.4
Tangerine	'Tankan'	106	8.5	8.8	14.9	7.0
Citrumelo	'US119'	123	12.1	16.9	29.1	33.3
		Conventional rootstocks				
Lemon	'Volkamer'	100	11.4	15.9	28.5	41.6
Tangerine	'Cleopatra'	79	10.8	11.1	17.5	11.4
Citrange	'Benton'	65	16.1	21.8	35.6	43.1
Citrandarin	'Cox'	68	14.5	22.4	38.0	42.1
Citradias	'Fraser'	37	14.1	22.5	36.7	49.6
Citrange	'Troyer'	87	13.1	14.4	18.2	27.1

1. Scion length (cm) at the end of the nursery phase.
2. Canopy surface area (m^2) at 17, 28, 40 and 71 months after field planting.
3. Trees on *C. australis* collapsed shortly after 71 months with severe stem pitting.
4. 30 cm interstock on Troyer rootstock.

Table 3. Tree size of Imperial mandarin on 31 rootstocks at three ages, from planting to 144 months (12 years) in the field, along with the initial nursery shoot length. Oceania species are shown in bold.

Treatment		Nursery shoot length[1]	CSA 35[2]	CSA 41	CSA 144
		Oceania *Citrus* species			
Citrus	***australasica***	51	0.7	1.6	0.0
Citrus	***garrawayi***	30	0.0	0.0	0.0
Citrus	***wakonai***	60	1.3	1.1	0.0
Citrus	***warburgiana***	68	0.3	0.0	0.0
Citrus	***wintersii***	57	0.0	0.0	0.0
		Non-*Citrus* relatives			
Atalantia	*ceylanica*	64	3.1	3.4	0.0
Clymenia	***polyandra***	40	0.0	0.0	0.0
		Non-conventional rootstocks			
Aurantium[3]	'GoutouchengD2'	88	12.3	13.9	25.2
Aurantium[3]	'GoutouchengD3'	83	12.6	14.5	27.0
Erythrosa	'Anjiang hongju'	106	11.8	13.2	31.2
Erythrosa	'Caoshi xiangju'	101	15.5	18.5	34.6
Erythrosa	'Zhuhongju'	92	11.8	13.8	31.3
Ichangensis	'No. 4'	72	4.7	5.4	14.0
Ichangensis	'2-3'	78	3.6	3.9	13.4
Junos	'Xiecheng'	91	10.9	13.6	40.1
Reticulata	'Gulin jinqianju'	105	14.1	18.6	42.4
Reticulata	'Hongju'	91	13.5	14.6	36.5
Reticulata	'Hongpi suanju'	109	12.3	15.3	34.7
Reticulata	'Jiangjing suanju'	93	14.1	14.9	27.7
Reticulata	'Jinju'	105	12.6	14.4	37.9
Reticulata	'Nianju'	102	13.9	15.4	37.2
Reticulata	'Shantou suanju'	100	8.8	11.4	30.3
		Conventional rootstocks			
Junos	'Barkley'[4]	93	11.1	13.4	34.1
Reticulata	'Cleopatra'	96	10.8	13.1	37.6
Citrandarin	'Cox'	52	11.7	15.1	39.1
Citradias	'Fraser'	68	10.3	12.1	36.3
Citrange	'Savage'	91	2.8	3.0	0.0
Citramelo	'Swingle'	84	14.3	16.6	35.6
Citrange	'Troyer'	66	12.1	14.2	42.8
Citrange	'Troyer 341'	56	14.0	17.1	41.9
Citrandarin	'US812'	84	14.6	18.0	36.2

1. Scion length (cm) at the end of the nursery phase.
2. Canopy surface area (m^2) at 35 and 41 months after field planting.
3. 'Guotou chen' is likely a *C. aurantium* hybrid and has been shown to tolerate Australian strains of CTV causing quick decline, in contrast to true *C. aurantium* which fails when used as a rootstock in Australia.
4. 'Barkley' started out in this experiment as a non-conventional rootstock but did so well that it is now used commercial and is considered a conventional rootstock.

Table 4. Eight of the donor parents used in attempts to introgress CTV resistance in new hybrids, and the frequency of resistant progeny obtained.

CTV Donor parent	Hybrids screened	% CTV resistant hybrids
Citrus trifoliata	880	45
US119 [(*paradisi* × *trifoliata*) × *sinensis*]	87	23
US812 (*reticulata* × *trifoliata*)	132	141
3831 (*reticulata* × *trifoliata*)	98	61
14Q055 (*reticulata* × *trifoliata*)	231	51
GLA77 [(*wakonai* × *glauca*) × *trifoliata*]	14	64
Fortunella japonica (syn. *C. japonica*)	40	23
Citrus glauca	60	0

These hybrids from 'US812' tested CTV-free at the end of the 2-year screening process but later became CTV-positive, indicating that they were disease-escapes.

Table 5. Seventeen rootstocks with *C. glauca* parentage established in a field experiment using 'Premier' mandarin scion, planted in November 2020. Tree health was assessed on four different dates after planting and the loss of replicates recorded. Troyer data is included for comparison.

Code	Parentage (species)	Tree health[1] (months after planting)				Replication	
		12	15	22	38	Initial	38
'GLA56'	(wakonai × glauca) × trifoliata	0				1	0
'F164'	(wakonai × glauca) × (reticulata × trifoliata)	1	4	4.7	4	3	1
'F410'	[(wakonai × glauca) × trifoliata] × (wakonai × australis)	1.5	0			3	0
'F31'	(wakonai × glauca) × Citropsis schweinfurthii	2.5	0			2	0
'F210'	(wakonai × glauca) × Fortunella japonica	3	0			7	0
'F188'	(wakonai × glauca) × (reticulata × trifoliata)	3.5	2	1.3	0	2	0
'F148'	(wakonai × glauca) × (reticulata × trifoliata)	4	2	2.5	5	1	1
'F220'	(wakonai × glauca) × (reticulata × trifoliata)	4	4.2	4.6	8.3	3	3
'F154'	(wakonai × glauca) × (reticulata × trifoliata)	5	4	5	10	2	1
'F184'	(wakonai × glauca) × (reticulata × trifoliata)	6	5	5	10	1	1
'F244'	(wakonai × glauca) × (reticulata × trifoliata)	6	5	4.7	0	1	0
'F208'	(wakonai × glauca) × (reticulata × trifoliata)	6	6.5	7.7	3.5	2	2
'F134'	(wakonai × glauca) × (reticulata × trifoliata)	6	4.8	5.3	9.2	4	4
'F96'	(wakonai × glauca) × (reticulata × trifoliata)	7	6.5	6.8	10	2	2
'F155'	(wakonai × glauca) × (reticulata × trifoliata)	7	4.5	6.4	8.3	2	2
'F231'	(wakonai × glauca) × (reticulata × trifoliata)	7	4.8	6	9.2	9	9
'F193'	(wakonai × glauca) × (reticulata × trifoliata)	8	5.8	6	7.1	4	4
'Troyer'	sinensis × trifoliata	7.5	6.1	8.3	9.8	4	4

Tree health rated visually by three assessors on a scale from 0=dead to 10=extremely health

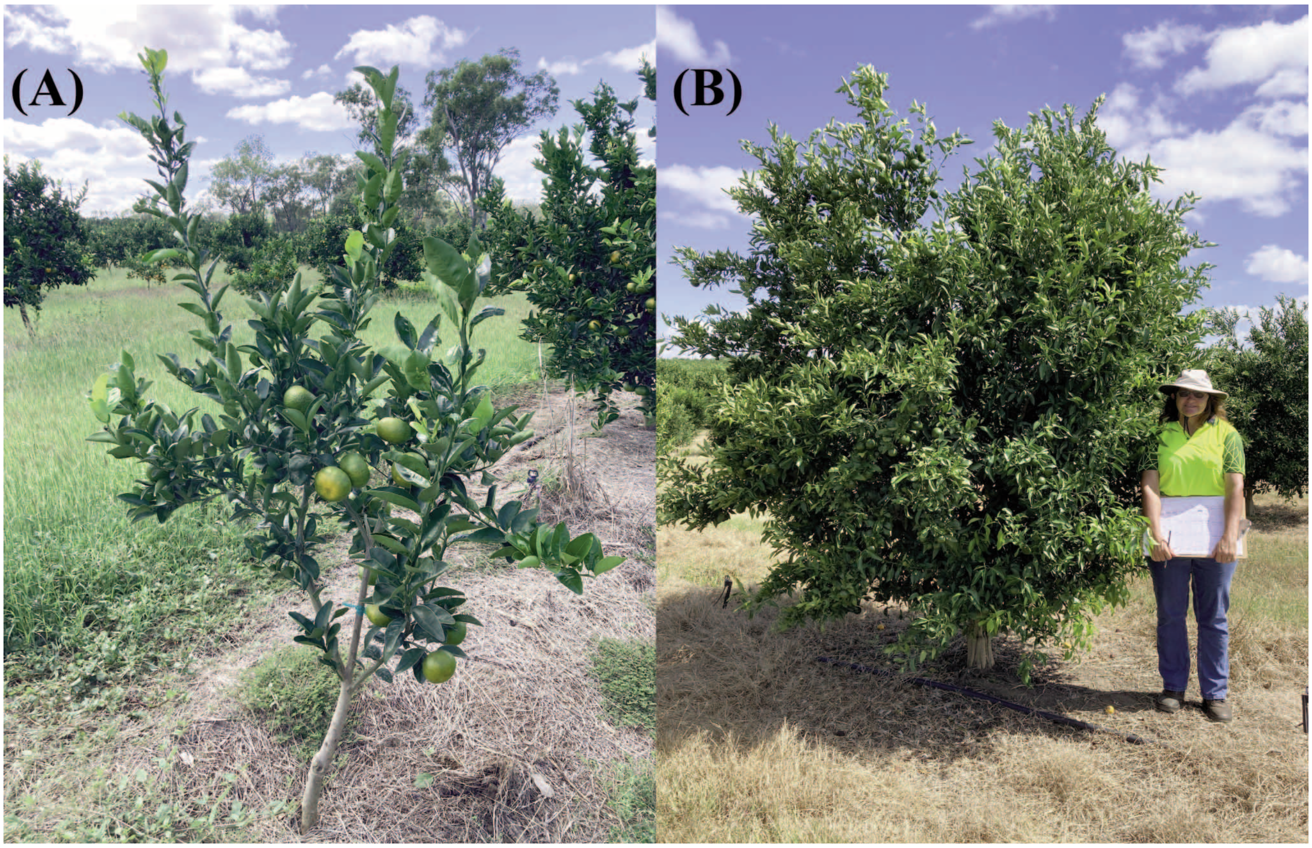

Figure 1. Successful "extreme hybrids" as rootstocks: **(A)** 18-month old 'Premier' mandarin on 'F438' [[(wakonai × glauca) × trifoliata] × (wakonai × australis)]; **(B)** four year old 'Imperial' mandarin on 'ICA12' [(wakonai × australasica) × trifoliata].

4. Discussion

Graft compatibility testing showed that of the 81 combinations tested (Table 1) about 22% resulted in buds that never "sprouted" (even though many of the graft sticks remained green on the rootstock for up to nine months) and a further 20% resulted in very minimal (1-2 cm) bud growth, followed by eventual scion death. The lowest compatibility tended to be with the "Very distant" species and none of the 21 non-Aurantioideae species showed any promise. A surprising number of combinations involving the tribe Clauseneae showed some level of compatibility and some even progressed to field planting. Of most interest within this tribe were *Micromelum minutum* and *Bergera koenigii* (syn. *Murraya koenigii*) and both warrant further investigation. The six species of *Clausena* showed limited promise and the often repeated statement that "…*Clausena lansium* can be used as a rootstock for Citrus…" (Swingle and Reece, 1967 p. 211) is not supported by our data. Our poor results with another species in this genus, *Clausena excavata*, also contrasts strongly with results of Bitters et. al (1964) who found it to be extremely promising. Curiously, Eureka lemon grafted on *Mangifera indica* (included in the experiment as a 'control') produced some shoot development and remained viable for more than eight months. Such a result demonstrates the need for caution in assuming that initial bud development indicates compatibility. Within the "close" relatives, *Citropsis* and *Clymenia* showed great promise with the former being one of the easiest to bud with very fast scion growth-rates. *Citropsis* is an outstanding rootstock in the nursery, but it quickly loses its vigour when field-planted. In a smaller experiment with Murcott tangor, trees on *Citropsis schweinfurthii* survived for six years, but were extremely stunted and never fruited. Most of the Oceania *Citrus* species showed high graft compatibility during the nursery phase, with growth rates comparable to conventional rootstocks. However, once these trees were planted in the field they quickly declined and most died.

Wild citrus relatives present many challenges as rootstock breeding parents, which may explain why previous attempts to use them have been short-lived (e.g. Breedt et al. 1988) and why most current programs have persisted with a narrow range of proven parents. Nonetheless, we have identified CTV sensitivity as one of the major challenges and demonstrated that it can be overcome using conventional breeding with the virus resistance source found in *C. trifoliata*. However, many wild relatives will not readily hybridise with *C. trifoliata*, or the resulting hybrids from wide crosses show high levels of late-acting lethality (Smith et al. 2013). This problem can be solved by selecting F1 trifoliata parents that have high levels of virus resistance (Smith et al. 2016) or by creating new bridging hybrids using *C. wakonai*, which have the added advantage of a shorter juvenile period. Although CTV resistance is generally considered to be controlled by a single dominant gene (Gmitter et al. 1996) for which *C. trifoliata* is heterozygous, segregation data from our deliberate inoculations show that hybrids resistant to replication generally represent less than half of the population. This is consistent with our previous study showing the non-donor parent impacts virus replication (Smith et al. 2016) and the work of Hearn et al. (1993) and Yoshida (1983) who obtained only 1/3 resistant hybrids when using 'US119' and *C. trifoliata* respectively as the donor parents. Our early use of 'US812' as a donor parent for CTV was a mistake brought about by field phenotyping which suggested it was resistant to virus replication. All hybrids with 'US812' have now been removed from the program because even the low percentage of "resistant" hybrids (~14%, see Table 4) later proved to be disease-escapes. Virus replication studies show that US812 supports a high virus titre and that infection can be detected in 82% of seedlings within six months of inoculation (Smith et al. 2016). All of these findings are supported by our recent molecular testing showing that 'US812' lacks the CTV resistance gene, and illustrates the value of combining molecular markers and serological testing when screening rootstock hybrids for CTV resistance. More importantly, it is essential that prospective parents are properly tested (molecular and conventional) for CTV resistance before using them in a rootstock breeding program.

In many cases it has not been possible to introgress CTV resistance into distant relatives because our F1 hybrids between distant taxa are entirely sterile (Smith et al. 2013) and neither parent carried the CTV resistance gene. These F1s have been vegetatively propagated as rootstocks and sometimes produce good nursery trees but they quickly fail once planted in the field (Table 5). We have not been able to find any hybrids with *Atalantia*, *Buxifolia*, *Citropsis*, *Fortunella* or *Hesperathusa* (syn. *Naringi*) that perform adequately as rootstocks under field conditions, despite generating more than 600 hybrids between these five genera (crossed with *C. wakonai*) and testing the 40 strongest of them as multiple trees in field experiments. The remarkable sexual compatibility between these five genera and *Citrus* (Caruso et al. 2020, Fig. 7.3 pg. 136) is a result of the high fertility of *C. wakonai*. Whether such high fertility has been retained in any of our new CTV-resistant *C. wakonai* hybrids has not been tested but could potentially offer a way to generate intergeneric hybrids with the necessary CTV resistance.

Field trials have shown wide phenotypic variation in rootstock effects (e.g. tree health, vigour, fruit production) even between hybrids derived from identical parents. This creates rich opportunities to select for the desired trait expression. Whilst this is partly due to the diverse parents being used in the program, it may also be a consequence of the more advanced generations at which crossing is occurring. Citrus rootstock breeding has often returned to the original parents even though breeders have long known that maximum segregation occurs in the F2. By using diverse parents and multiple generations of breeding we have potentially increased phenotypic variation. Consequently, it is important to use large families (lots of individuals from the same cross) to ensure this phenotypic variation is adequately exposed and sampled.

Concomitant with wide phenotypic variation has been the opportunity to "fix" important traits within the breeding population. A strong focus on CTV resistance (absence of virus titre) has created a large pool of parents carrying the necessary gene, and in some cases both parents are resistant. Similarly, we have tried to "fix" apomixis in our populations (Smith et al. 2019). Molecular markers have proven extremely useful for both of these traits and all of our hybrid populations are now screened using SNPs linked to these two traits (Montalt et al. 2023; Ohta et al. 2015).

The frequent occurrence of individual hybrids that are CTV resistant and grow well in the nursery, but then quickly decline once field planted, has stimulated an interest in finding genomic regions associated with rootstock "domestication". Having large numbers of promising hybrids that quickly decline after field planting (and for no obvious reasons like CTV or phytophthora) suggests an opportunity to improve the efficiency of rootstock breeding when using distant relatives.

During the course of this breeding program, exciting new knowledge emerged suggesting that the Oceania citrus species may possess tolerance/immunity to HLB (e.g. Alves et al. 2022; Ramadugu et al. 2016; Weber et al. 2022). Whilst these observations are based on scion performance not rootstocks, the potential implications are nonetheless worth exploring given that we now have advanced germplasm based on these Oceania species and have already resolved the CTV constraint. Alves et al. (2022) have suggested that full resistance to the casual agent of HLB exists within some Oceania citrus species with no detectable bacterial titre. This raises the possibility of having rootstocks that contain no bacteria in their root system even if the scion is infected, the disease implication of which are unknown. However, we would advise extreme caution to those who think these Oceania species can be used as rootstocks without first addressing CTV.

5. Conclusions

Unique citrus rootstocks have been created whose performance is equivalent to existing commercial choices. These new rootstocks capture wild species and genera that have never been successfully deployed in world citriculture. Large scale testing with different scions and under a range of climatic and edaphic conditions is now required, using the rootstock hybrids that are currently inducing the best tree health and productivity. Recognition of the importance of CTV resistance and development of a breeding methodology to introgress it has converted previously disastrous germplasm into hybrids that may ultimately play a part in solving the HLB outbreak that is currently destroying citrus production in many parts of the world.

Funding: This project has been funded by Hort Innovation, using the Citrus Industry research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture. Initial funding support was provided by the Queensland Citrus Improvement Scheme and the research work has been support throughout by the Queensland Government via the Department of Agriculture and Fisheries.

Acknowledgments: We are indebted to the many citrus growers/managers who have generously hosted rootstock experiments on their properties, including Russell Baker, Anne and Warren Bayntun, Troy Emmerton, Nick Furey, Brian Gorman, Tom Jameson, Mick Matthews, Craig Pressler, Ian Shepherd, Craig Spencer, Will Thompson, Mark Trott, and Nick Ulcoq. Patricia Barkley, Mark Hoult, Andrew Miles, John Owen-Turner and Wayne Parr have provided valuable technical advice throughout the project. Robert Krueger and Paul Forster have provided botanical advice and germplasm. Member of the Queensland Citrus Improvement Scheme Committee (John Owen-Turner, Allen Jenkin, Troy Emmerton, Nick Ulcoq, Peter Young, Wayne Parr) were instrumental in providing funding support to start this breeding program.

Conflicts of Interest: The authors declare no conflict of interest.

References

Albrecht, U., M. Bordas, B. and Lamb, B. Meyering and Bowman, K.D. (2017) 'Influence of propagation method on root architecture and other traits of young citrus rootstock plants', *HortScience,* 52(11), pp. 1569-1576. doi 10.21273/HORTSCI12320-17

Alves, M.N., Raiol-Junior, L.L., Girardi, E.A., Miranda, M., Wulff, N.A., Carvalho, E.V., Lopes, S.A., Ferro, J.A., Ollitrault, P. and Peña, L. (2022) 'Insight into resistance to 'Candidatus Liberibacter asiaticus,' associated with Huanglongbing, in Oceanian citrus genotypes', *Frontiers in Plant Science,* 13, 1009350-1009373. doi 10.3389/fpls.2022.1009350

Bitters, W.P., Brusca, J.A. and Cole, D.A. (1964) 'The search for new citrus rootstocks', *The California Citrograph*, 49(11), pp. 443-448.

Bitters, W.P., Cole, D.A. and Brusca, J.A. (1969) 'The citrus relatives as citrus rootstocks', *Proceedings First International Citrus Symposium*. Riverside California, USA, 16-26 March 1968. California: Riverside Color Press, 1, pp. 411-415.

Bitters, W.P., Cole, D.A. and McCarthy, C.D. (1977) 'Citrus relatives are not irrelevant as dwarfing stocks or interstocks for citrus', *Proceedings of the International Society of Citriculture*. Orlando Florida, USA 1-8 May 1977. DeLeon Springs: E. O. Painter Printing Co., 2, pp. 561-567.

Bowman, K.D., McCollum, G. and Albrecht, U. (2021) 'SuperSour: a new strategy for breeding superior citrus rootstocks', *Frontiers in Plant Science*, 12, 741009. doi 10.3389/fpls.2021.741009

Breedt, H.J., Lange, J.H.d. and Vincent, A.P. (1988) 'The value as rootstocks of species related to the genus Citrus: Onderstamwaarde van spesies verwant aan die genus Citrus', *Information Bulletin, Citrus and Subtropical Fruit Research Institute, South Africa*, 187, pp. 17-20.

Caruso, M., Continella, A., Modica, G., Pannitteri, C., Russo, R., Salonia, F., Arlotta, C., Gentile, A. and Russo, G. (2020) 'Rootstocks influence yield precocity, productivity, and pre-harvest fruit drop of Mandared pigmented mandarin', *Agronomy*, 10(9), pp. 1305-1317. doi 10.3390/agronomy10091305

Caruso, M., Smith, M.W., Froelicher, Y., Russo, G. and Gmitter, F. G.Jr (2020) 'Traditional breeding', in Talon, M., Caruso, M. and Gmitter, F.G.Jr (eds.). *The Genus Citrus*. United Kingdom: Elsevier, pp. 129-148. ISBN 978 0 12 812163 4

Forster, P.I. and Smith, M.W. (2010) 'Citrus wakonai P.I.Forst. & M.W.Sm. (Rutaceae), a new species from Goodenough Island, Papua New Guinea', *Austrobaileya,* 8(2), pp. 133-138.

Gmitter, F.G., Xiao, S.Y., Huang, S., Hu, X.L., Garnsey, S.M. and Deng, Z. (1996) 'A localized linkage map of the citrus tristeza virus resistance gene region', *Theoretical and Applied Genetics*, 92(6), pp. 688-695. doi 10.1007/BF00226090

Grosser, J.W., Barthe, G.A., Castle, B., Gmitter, F.G. and Lee, O. (2015) 'The development of improved tetraploid citrus rootstocks to facilitate advanced production systems and sustainable citriculture in Florida', *Acta Horticulturae*, 1065, pp. 319-327. doi 10.17660/ActaHortic.2015.1065.38

Hearn, C.J., Garnsey, S.M. and Barrett, H. (1993) 'Transmission of Citrus Tristeza Virus resistance from citrus breeding line US119', Abstract, *HortScience*, 28(5), pp. 483.

Montalt, R., Cuenca, J., Vives, M.C., Mournet, P., Navarro, L., Ollitrault, P. and Aleza, P. (2023) 'Genotyping by sequencing for SNP-based linkage analysis and the development of KASPar markers for male sterility and polyembryony in Citrus', *Plants*, 12(7), pp. 1567. doi 10.3390/plants12071567

Ohta, S., Endo, T., Shimada, T., Fujii, H., Shimizu, T., Kita, M., Kuniga, T., Yoshioka, T., Nesumi, H., Yoshida, T. and Omura, M. (2015) 'Construction of genetic linkage map and graphical genotyping of pseudo-backcrossed F 2 (BC′ 2) progeny to introduce a CTV resistance from Poncirus trifoliata (L.) Raf. into Citrus by introgression breeding', *Tree Genetics and Genomes,* 11(1), pp. 797. doi 10.1007/s11295-014-0797-y

Ramadugu, C., Keremane, M.L., Halbert, S.E., Duan, Y.P., Roose, M.L., Stover, E. and Lee, R.F. (2016) 'Long-term field evaluation reveals huanglongbing resistance in Citrus relatives', *Plant Disease,* 100(9), pp. 1858-1869. doi 10.1094/PDIS-03-16-0271-RE

Reforgiato Recupero, G., Russo, G., Recupero, S., Zurru, R., Deidda, B. and Mulas, M. (2009) 'Horticultural evaluation of new Citrus latipes hybrids as rootstocks for citrus', *HortScience,* 44(3), pp. 595-598. doi 10.21273/HORTSCI.44.3.595

Smith, M.W., Gultzow, D.L. and Newman, T.K. (2013) 'First fruiting intergeneric hybrids between Citrus and Citropsis', *Journal of the American Society for Horticultural Science*, 138(1), pp. 57-63. doi 10.21273/JASHS.138.1.57

Smith, M.W., Newman, T.K., Gultzow, D.L., Parfitt, S.C. and Barkley, P.B. (2016) 'Citrus tristeza virus replication and movement in seedlings of 71 rootstock genotypes', *Citrus Research and Technology*, 37(2), pp. 156-164. doi 10.4322/crt.ICC041

Smith, M.W., Webb, M., Gultzow, D.L., Newman, T.K., Innes, D., Dillon, N., Owen-Turner, J. and Xu, Q. (2019) 'Application of a MITE Citrus apomixis marker in the Australian rootstock breeding program', *Acta Horticulturae,* 1230, pp. 1-6. doi 10.17660/ActaHortic.2019.1230.1

Swingle, W.T. and Reece, P.C. (1967) 'The botany of Citrus and it's wild relatives', in Reuther, W., Webber, H. J. and Batchelor, L. D. (eds) *The Citrus Industry: History, world distribution, botany and varieties.* Berkeley, University of California. 1: 190-430.

Sykes, S.R. (2010) 'The effect of nucellar seedling and micropropagated clonal rootstocks on the performance of Imperial and Sunset mandarins', *Proceedings of the International Society for Citriculture*. Wuhan, China, 26-30 October 2008. 1, pp. 44-49.

Volk, G.M., Gmitter, F.G. and Krueger, R.R. (2023) 'Conserving Citrus diversity: From Vavilov's early explorations to genebanks around the world', *Plants*, 12(4), pp. 814-827. doi 10.3390/plants12040814

Weber, K.C., Mahmoud, L.M., Stanton, D., Welker, S., Qiu, W., Grosser, J.W., Levy, A. and Dutt, M. (2022) 'Insights into the mechanism of Huanglongbing tolerance in the Australian finger lime (Citrus australasica)', *Frontiers in Plant Science*, 13, pp. 1019295-1019318. doi 10.3389/fpls.2022.1019295

Yoshida, T., Shichijo, T., Ueno, I., Kihara, T., Yamada, Y., Hirai, M., Yamada, S., Ieki, H. and Kuramoto, T. (1983) 'Survey of citrus cultivars and hybrid seedlings for resistance to citrus tristeza virus (CTV)', *Bulletin of the Fruit Tree Research Station*, 10, pp. 51-68.

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, pp. 33-43.

Yoshida, T. (1996) 'Graft compatibility of Citrus with plants in the Aurantioideae and their susceptibility to citrus tristeza virus', *Plant Disease*, 80(4), pp. 414-417.