Hort Innovation

Final Report

QLD Citrus Improvement Scheme: Finding Better Rootstocks for Australia

Project leader:

Malcolm Smith

Delivery partner:

The Department of Agriculture and Fisheries, QLD

Project code:

CT13004

Project:

QLD Citrus Improvement Scheme: Finding Better Rootstocks for Australia – CT13004

Disclaimer:

Funding statement:

This project has been funded by Hort Innovation, using the citrus research and development levy, co-investment from Queensland Department of Agriculture and Fisheries, Queensland Citrus Improvement Scheme and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

ISBN 978 0 7341 4484 3

Published and distributed by: Hort Innovation

Level 8
1 Chifley Square
Sydney NSW 2000

Telephone: (02) 8295 2300

www.horticulture.com.au

Content

Summary

The aim of this project was to breed and develop superior rootstocks that would help support the future commercial viability of Queensland and Australian citrus growing. One new rootstock was released and is now entering commercial production. It represents germplasm distinctly different from currently available rootstocks thus expanding the genetic diversity of the Australian citrus industry. This new rootstock was named 'Barkley' in recognition of Mrs Patricia Barkley (Broadbent), one of Australia's most distinguished citrus researchers. A second new rootstock is poised for commercial development as final testing has been expanded. The influence of rootstocks on fruit quality is no more strongly felt than is the case with citrus – and the choice growers make at the time of planting influences economic viability for the life of the orchard. Through a series of integrated breeding and evaluation experiments, this project has delivered new information and unique germplasm to give growers better choices for modern orchards, and deliver improved fruit quality to consumers.

The project was built around five key experiments that captured research and development activities across the full spectrum of "risk verses return". Indeed, some of the activities that seemed "left-field" at the start of the project and with a high risk of failure now show promise of a high return on investment as new germplasm with commercial application. For example, some native Australian citrus species have been used in the breeding program and after multiple generations of crossing are now showing promise in trials on commercial citrus orchards. In their primitive state, these species are highly susceptible to viral infection and they die within a few years of planting. Conventional breeding has solved this problem and created unique germplasm that is now beginning to reveal useful traits such as resistance to phytophthora diseases. This represents an important step forward for international breeding efforts.

Large replicated trials have been established on commercial orchards with almost 500 different genotypes currently being field tested at five diverse locations. Many thousands more hybrids were screened for a range of important traits before choosing these genotypes for field trials. Techniques to efficiently screen for Citrus tristeza virus resistance, phytophthora tolerance, salt tolerance and apomixis have been developed and refined during the project. Large families of hybrids can now be quickly screened for most of the traits that are needed by the Australian citrus industry. Close industry collaboration, monitoring and input have ensured this complex project stayed on track, achieved all of the promised outputs and delivered outcomes that are useful to commercial citrus businesses.

Keywords

citrus, breeding, rootstocks, disease resistance, salt tolerance, graft compatibility, fruit quality, citrus relatives, germplasm, citrus tristeza virus, phytophthora, apomixis

Introduction

Better rootstocks are a zero-cost solution to many problems affecting commercial citrus production. Of particular interest is the opportunity to improve fruit quality. For example, Australia's most important mandarin variety 'Imperial' is plagued by granulation problems which threaten to undermine consumer confidence in the category as a whole. This makes 'Imperial' the obvious scion of choice for rootstock research, and success in previous research demonstrates how quickly growers will adopt new rootstocks that show promising results. It is conservatively estimated that around 100,000 new trees are planted commercially in Queensland each year, and nationally the figure is in excess of 300,000. There is a significant opportunity to improve orchard performance by ensuring that these new planting utilise improved rootstock germplasm.

International citrus rootstock research dates back more than a century and continues to be a driving force in modern citriculture development. There has been a steady turn-over of rootstock varieties with the increasing pressures brought about by disease, changing horticultural practices and market demands. Queensland research in the 1960s identified disease resistant rootstocks that were able to handle occasional salt problems during drought years as well as producing better quality fruit for the consumer. Additional Queensland research during the 1990s identified another rootstock that further improved fruit quality, and extended orchard longevity.

Rootstock research continued at Bundaberg Research Station during the early part of the century, all be it without funding support and consequent restrictions on activity. However this situation changed dramatically in 2013 when CT13004 "Qld Citrus Improvement Scheme: finding better rootstock for Australia" provided the necessary resources to up-scale the research. This project brought a commercial focus to the research activity and captured an opportunity to get some quick industry outcomes by tapping-into the genetic progress and germplasm enhancement that has occurred over the previous 10 years. New rootstock germplasm (both national and international) had already been obtained and established in the trials that were then evaluated as a component of this project providing a system for benching marking with the best work internationally. Of greater significance was the rootstock breeding work captured by this new project. There is no other rootstock breeding work in Australia, although major programs are underway in a small number of places including Brazil, Florida and Spain. Not surprisingly, their breeding objectives and commercial focus are different from Australian industry needs. While the size and funding levels of these overseas programs are vastly superior to anything that could be justified in Australia, they lacked the broad genetic diversity and unique germplasm that characterise breeding populations at Bundaberg Research Station.

The work was not funded from national citrus industry levys. Instead, the project team had to rely on the generosity of the not-for-profit industry organisation 'Queensland Citrus Improvement Scheme Inc.' and the willingness of the Queensland Department of Agriculture and Fisheries to invest Royalty Funds. These contributions were enthusiastically matched by HortInnovation who quickly recognised the value of the project and agreed to a Voluntary Contribution project. Committee members of the Queensland Citrus Improvement Scheme Inc (QCIS) have life-long involvement in commercial citrus production and are committed to ensuring their industry remains economically viable and internationally competitive. They capture a diverse range of expertise, are recognised as leaders in their professions, operate internationally, and they share the view that new ideas and technology are essential to industry viability. As successful business people they brought to the project a focus on efficiency, performance and the paramount importance of producing commercially useful outputs. Their pragmatic approach helped to ensure the project stayed on track, and that the funds they invested were used to produce long-term industry outcomes.

The research itself was conducted by the citrus team at BRS. Their work is internationally recognised and they are well connected with key programs around the world. They are the only program to have discovered and named a new species (*Citrus wakonai* P.I.Forst. & M.W.Sm.) the Type Specimen of which is housed at BRS. Further cementing their position as a leader in germplasm utilization is their world-1st success in

hybridising the sexually incompatible genera Citrus and Citropsis. They have presented papers at world conferences, published in leading peer-reviewed journals, and serve on international Scientific Advisor Panels. Their rootstock research experience included not only citrus but also mango and macadamia, giving a unique insight into how genetic improvements to the root system could best generate useful industry outcomes. The BRS citrus team have extensive practical experience, have developed very efficient hybrid generation and screening procedures, have a thorough understand of the obstacles and opportunities associated with rootstock research, and are committed to generating outputs that improve grower returns. The project served to strengthen these skills and develop new ones such as phytophthora and salt resistance screening. It also provided an opportunity to link with DAF-funded molecular work on apomixis and generate publications that have helped to strengthen collaborations with other international citrus research groups.

The project was built around five key experiments that captured research and development activities across the full spectrum of “risk verses return”. Indeed, some of the activities that seemed “left-field” at the start of the project and with a high risk of failure now show promise of a high return on investment as new germplasm with commercial application. The project delivered the promised fast turn-around in industry adoption with one new rootstock now being supplied commercially and a second new rootstock about to enter large-scale commercial testing. Innovative research, a commitment to international collaboration, and a solid theoretical foundation built on more than 100 years of citrus research literature, have produced a project which has found better rootstocks for Australia and develop a genetic basis for continued improvement well into the future.

Methodology

A significant portfolio of experiments was evaluated and further expanded during the project period. The table below (Table 1) summarises these experiments and the data that was collected. More detail is provided for most of these experiments in the subsequent discussion within this section of the report. Some of these experiments are ongoing and data will be collated and prepared for publication (where appropriate) once the individual field trials are complete. For experiments where the work is complete, a copy of the associated data summaries and publications is attached.

Table 1: Summary of experiments assessed during the project period.

Experiment	Field planted	Location	Treatments	Datum trees	Annual data collection		
					Tree growth[a]	Productivity	Fruit quality[b]
1	2004	Gayndah	34	420	2007-16	2014-16	2007-16
2	2010[c]	BRS	71	1,191	CTV replication and movement		
3	2011	Emerald	151	891	2014-18	2014-18	2014-18
Extra D[d]	2012	Mareeba	9	54	2015	2015	2015
4	2013	Gayndah	245	490	2015-18	2015-18	2015-18
4	2014[c]	BRS	582	582	CTV screening		
Extra A[d]	2016[c]	BRS	1,407	1,407	Phytophthora screening		
5	2017[c]	BRS	2,272	2,271	CTV & phytophthora screening		
Extra B[d]	2017[c]	BRS	182	194	Salt screening		
Extra C[d]	2017[c]	BRS	79	79	Apomixis screening		
Extra E[d]	2017	Gayndah	17	90	2018	n.a.	n.a
5	2018	Wallaville	138	605	2017-18	2018	n.a

a: includes measures of tree height, width and canopy volume, as well as trunk circumference 100mm above and below the graft union.
b: includes measurements of granulation, fruit size, rind thickness, Brix, acid, digital photo.
c: nursery experiment commencement date.
d: not part of the original project document but added as a complementary activity.

Experiment One: Gayndah 'Imperial' Rootstock Experiment

Background:

An 'Imperial' rootstock trial was propagated in 2003 and planted on a commercial orchard at Gayndah in November 2004. It contained 34 different rootstocks including Chinese germplasm, selections from local and overseas rootstock breeding programs, and some species native to Australia and PNG. External funding for this work dried-up in January 2006 just as trees were starting to carry fruit. However, because of the substantial effort that had gone into negotiating access to, and obtaining the germplasm, it was decided to maintain basic fruit quality assessments annually. Consequently data exists for the period 2007 to 2012, but had not been entered or processed prior to CT13004.

This project compiled and analysed existing information on tree survival and growth, and anticipated longevity (based on graft union benching and cracking) along with fruit quality data to determine the impact of rootstocks on fruit size, acidity, Brix and granulation. Given the high seasonal variation in 'Imperial' granulation, and the critical importance of this quality trait to the marketing of Australian mandarins, the project also supported the continued annual collection of basic fruit quality data so that the spatial and temporal nature of granulation could be examined over a 10 year period. This information was useful not only in terms of rootstocks but also in helping to understand the recalcitrant problem of granulation. Information was conveyed to nurserymen and growers in relation to the performance of current rootstock varieties as well as the new ones that showed promise. Indeed, the Project Management Committee were so impress with one of the new rootstocks when they visited the trial site on 25th October 2016 that they insisted all growers be made aware of the results. A field day was organised and coincided with the launch of the new rootstock 'Barkley' which is now being produced by the national citrus seed scheme (AusCitrus).

This field trial was the oldest experiment in the project (planted 2004) and all necessary information had been collected and the trial finalised by June 2016. This reflects the desire of the project team to move onto new experiments, which incorporate knowledge gained from previous experiments, rather than continue to collect the same information for multiple decades. Previous work by the project team had demonstrated that there was only limited value in extending rootstock experiment beyond about 10 years (Smith et al. 2004) and this is consistent with the views of other international researchers (e.g. Castle, 2010). During the life of this experiment many new techniques had been developed to increase the efficiency of field data collection, and this knowledge was also of benefit to new experiments established by the project. Methods to complete quality assessment in the field (Figure 1), rather than carting large numbers of fruit samples back to the lab, represent an important technique to improving rootstock research efficiency.

Figure 1: The project team members assessing the Bakers Gayndah rootstock experiment in March 2014. Most fruit quality assessments were completed in the field, and in subsequent years further improvements in efficiency were achieved through the use of a portable shade canopy and the digital image capture of granulation samples.

Summary performance data.

To coincide with the Field Day at the trial site (7th March 2017), the project team summarised all available information and provided a handout to those in attendance. Rootstocks of interested were colour-coded on the handout and matched to colour coded labels that were placed on the different trees in the trial. This information is presented below:

Draft Performance Data: For use at the joint field day, Gayndah March 7th 2017.

This information is intended for the use of field day participants only, to aid in their on-site assessment of rootstock performance. Explanation of the data will be provided during the field day.

Canopy Surface Area (m2)

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Barkley	11	14	17	22	26	27	28	31	32	34
Anjiang hongju	12	13	17	24	30	24	25	28	28	31
Caoshi xiangju	15	19	24	27	33	32	33	34	36	35
Hongpi suanju	12	15	20	23	26	31	33	31	35	35
Xiecheng	11	13	18	22	23	28	30	30	33	40
Zhuhongju	12	14	19	21	27	27	28	28	32	31
Cleopatra	11	13	18	24	28	28	29	32	38	38
Nianju	14	15	20	26	28	31	32	33	37	37
US812	15	18	22	28	31	31	34	34	36	36
Cox	12	15	19	20	27	30	32	34	37	39
Hongju	13	15	19	23	28	30	31	33	36	37
Jinju	13	14	17	23	28	30	32	33	37	38
Goutou D2	12	14	17	21	27	29	31	32	28	25
Goutou D3	13	14	17	21	26	26	24	28	29	27
Swingle	14	17	20	24	31	31	34	35	38	36
Shantou suanju	9	11	15	20	27	26	27	29	29	30
Gulin jinqianju	14	19	22	28	32	34	36	38	42	42
Jiangjing suanju	14	15	17	26	25	26	26	25	27	28
Ichang No.4	5	5	6	8	12	11	11	12	11	14
Ichang 2-3	4	4	5	6	7	10	9	11	11	13
Troyer	12	14	19	28	29	34	33	31	39	43
Troyer341	14	17	22	28	33	34	36	36	37	42
Fraser	10	12	14	18	25	26	26	29	33	36

Crop Load (canopy fruit density 0=no fruit, 10=extremely dense)

	2014	2015	2016
Barkley	4.65	5.64	6.75
Anjiang hongju	3.65	5.11	5.80
Caoshi xiangju	4.95	5.03	5.16
Hongpi suanju	3.93	4.82	4.79
Xiecheng	4.34	3.64	6.16
Zhuhongju	4.80	4.48	5.24
Cleopatra	3.40	4.29	5.29
Nianju	3.15	4.82	4.69
US812	4.15	4.63	5.47
Cox	3.30	4.06	6.19
Hongju	3.30	4.52	5.46
Jinju	2.68	5.00	4.59
Goutoucheng D2	4.05	5.29	6.98
Goutoucheng D3		6.76	4.69
Swingle	4.78	5.25	7.81
Shantou suanju	3.93	4.07	7.29
Gulin jinqianju	4.08	5.01	5.65
Jiangjing suanju	2.80	4.96	6.72
Ichang No.4	6.34	5.36	4.57
Ichang 2-3	3.78	3.14	4.86
Troyer	5.65	4.44	7.06
Troyer341	4.60	3.20	7.93
Fraser	3.70	2.96	7.55

Average Fruit Weight (g)

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Barkley	163	122	112	95	96	95	106	102	120	87
Anjiang hongju	142	119	105	96	90	93	101	87	108	86
Caoshi xiangju	143	98	106	90	94	96	101	84	109	90
Hongpi suanju	135	103	102	93	89	90	108	83	107	80
Xiecheng	146	120	109	93	93	95	105	84	111	83
Zhuhongju	140	118	104	89	98	95	105	79	111	87
Cleopatra	152	112	98	94	84	91	93	82	102	83
Nianju	148	108	101	94	84	91	100	84	105	84
US812	157	109	106	94	95	102	100	98	111	88
Cox	147	110	105	93	99	103	108	96	115	85
Hongju	138	113	106	91	97	95	100	77	102	78
Jinju	148	114	113	97	94	96	101	87	110	86
Goutou D2	133	108	103	97	89	89	105	105	106	71
Goutou D3	135	100	101	94	91	93	107	92	99	86
Swingle	146	114	112	98	95	96	105	89	120	77
Shantou suanju	152	115	95	98	94	91	98	94	122	73
Gulin jinqianju	143	109	105	96	90	97	104	86	109	85
Jiangjing suanju	163	108	109	93	93	95	103	96	112	86
Ichang No.4	192	122	119	108	98	109	106	90	98	98
Ichang 2-3		115	131	119	100	127	105	101	107	74
Troyer	152	113	108	92	93	93	99	90	129	85
Troyer341	156	117	107	98	101	95	106	105	125	82
Fraser	143	115	111	95	93	99	105	101	124	78

Granulation (0=none, 5=extremely granulated)

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Barkley	1.65	2.02	1.42	0.75	1.83	1.30	1.09	0.45	1.00	1.29
Anjiang hongju	1.92	1.58	1.06	1.08	2.08	1.77	1.35	1.06	0.99	1.74
Caoshi xiangju	1.63	1.57	0.86	0.92	2.03	1.58	1.41	0.48	0.83	1.84
Hongpi suanju	1.49	1.42	0.76	1.13	1.43	1.21	1.20	0.73	0.70	1.31
Xiecheng	1.44	1.88	1.01	0.71	1.33	1.19	0.95	0.82	0.88	1.16
Zhuhongju	1.78	1.47	0.77	0.95	2.15	1.39	1.60	0.43	0.88	1.53
Cleopatra	2.03	1.42	0.58	0.82	1.42	0.78	0.72	0.80	0.83	1.28
Nianju	1.73	1.37	0.92	0.68	1.40	1.20	1.20	1.23	0.67	1.35
US812	1.53	1.58	0.94	1.44	1.98	1.73	1.43	0.96	1.13	1.44
Cox	1.50	1.30	1.20	1.78	2.21	1.44	1.40	1.08	0.96	1.18
Hongju	1.53	1.35	0.86	0.83	1.17	1.24	1.18	0.80	0.80	1.39
Jinju	1.38	1.78	1.06	1.11	1.73	1.12	1.35	1.62	0.95	1.53
Goutou D2	1.62	1.40	1.01	1.23	2.41	1.68	1.18	0.97	0.92	2.34
Goutou D3	1.85	1.55	1.16	1.11	2.15	2.18	0.98	0.77	1.06	2.08
Swingle	1.79	1.20	0.99	1.07	1.55	1.64	0.92	1.36	0.94	1.26
Shantou suanju	1.48	1.97	1.06	1.83	1.99	1.67	1.15	1.03	1.22	1.33
Gulin jinqianju	1.75	1.20	0.63	0.65	1.65	1.48	1.33	1.13	0.89	1.61
Jiangjing suanju	1.66	1.37	0.79	1.33	1.69	1.54	0.88	1.03	0.83	1.55
Ichang No.4	2.46	2.42	1.60	1.79	2.49	1.63	1.34	0.78	1.23	2.88
Ichang 2-3		2.02	1.67	1.66	3.11	1.92	1.56	1.63	1.48	1.72
Troyer	1.85	1.67	1.02	1.37	1.83	2.17	0.87	1.16	1.24	1.60
Troyer341	1.90	1.50	0.99	1.34	1.93	2.23	1.16	1.50	1.20	1.67
Fraser	1.92	2.08	1.42	1.52	2.28	1.79	0.89	1.05	1.28	1.24

Australian Citrus Standard (based on BrimA)

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Barkley	122	114	114	115	112	105	121	142		110
Anjiang hongju	127	125	118	124	122	115	122	155		117
Caoshi xiangju	126	121	123	119	123	116	118	148		107
Hongpi suanju	124	115	115	118	124	108	111	137		104
Xiecheng	125	118	114	119	116	107	115	143		110
Zhuhongju	125	121	120	119	122	119	117	150		110
Cleopatra	131	120	114	124	122	103	114	135		100
Nianju	126	116	114	115	118	103	104	144		89
US812	123	119	124	121	118	105	123	145		103
Cox	115	112	121	121	115	102	109	141		106
Hongju	130	130	124	118	117	108	114	135		102
Jinju	120	113	112	114	117	98	111	132		100
Goutou D2	129	121	120	121	118	108	116	148		122
Goutou D3	133	122	125	123	122	122	123	162		124
Swingle	123	128	126	126	119	111	121	153		112

Shantou suanju	124	118	119	109	116	105	115	140		115
Gulin jinqianju	129	122	120	118	116	108	111	147		109
Jiangjing suanju	122	125	125	121	116	103	119	144		110
Ichang No.4	121	115	113	114	118	118	119	134		113
Ichang 2-3		119	112	111	122	117	116	144		119
Troyer	125	123	121	122	115	107	127	146		119
Troyer341	125	116	121	124	120	109	124	148		117
Fraser	126	115	116	116	117	109	119	146		109

Brix (degrees)

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Barkley	10.1	9.7	10.2	9.8	10.1	10.2	10.8	12.7		11.7
Anjiang hongju	10.5	10.4	10.6	10.2	10.6	10.8	11.4	13.4		12.4
Caoshi xiangju	10.6	10.5	10.7	9.9	10.5	10.8	11.2	13.2		12.3
Hongpi suanju	10.2	10.0	10.4	10.1	10.9	11.3	11.4	13.4		12.5
Xiecheng	10.3	10.0	10.2	10.0	10.4	10.6	11.3	13.4		12.2
Zhuhongju	10.4	10.2	10.7	10.0	10.5	11.0	11.3	13.2		12.1
Cleopatra	10.4	9.9	10.2	10.4	10.7	10.6	11.6	13.1		12.2
Nianju	10.3	10.2	10.4	10.1	10.7	10.7	11.3	13.9		12.5
US812	10.2	10.1	10.6	10.2	10.4	10.6	11.6	13.0		12.0
Cox	9.8	9.8	10.4	9.8	10.3	10.2	11.0	12.4		11.7
Hongju	11.0	11.1	11.0	10.5	10.6	11.4	11.2	13.6		12.5
Jinju	10.2	9.9	10.2	9.9	10.3	10.2	11.1	13.0		12.2
Goutou D2	10.6	10.1	10.3	9.9	10.1	10.6	10.4	12.8		12.4
Goutou D3	10.6	10.3	10.6	9.7	10.3	11.1	10.8	13.5		12.1
Swingle	10.2	10.8	10.8	10.4	10.5	10.9	11.3	13.3		12.1
Shantou suanju	10.2	10.0	10.5	9.5	10.1	10.4	11.0	12.9		11.9
Gulin jinqianju	10.7	10.3	10.9	10.1	10.5	10.5	11.3	13.6		12.5
Jiangjing suanju	10.1	10.4	11.0	10.0	10.2	9.9	11.1	12.8		12.3
Ichang No.4	9.4	9.5	10.0	9.4	9.7	10.4	11.1	12.0		11.1
Ichang 2-3		10.3	10.1	9.3	9.9	10.4	10.9	12.2		12.1
Troyer	10.3	10.4	10.6	10.0	10.3	10.9	11.4	13.4		11.9
Troyer341	10.5	10.0	10.7	10.1	10.4	10.8	11.2	13.1		11.9
Fraser	10.3	9.7	10.0	9.7	10.0	10.1	10.9	11.9		11.3

Understanding granulation

Granulation in 'Imperial' mandarin remains one of the biggest fruit quality challenges for the Australian citrus industry. This project provided a truly unique data set in which the extent of granulation was measure for a large number (140) of individual trees for 10 consecutive seasons. As a prelude to more detailed modelling by physiologists, the project took a preliminary look at this data in an attempt to better understand the problem. It was already known that the trait of granulation was extremely difficult to accurately phenotype.

Fruit granulation data was collected every season from 2007 to 2016. Ten replicates (centre tree of a 3-tree plot) were available for the 'Troyer' rootstock treatment and so the analysis has been based on this treatment. Table 2 shows the average granulation of 10 individual trees over 10 consecutive seasons.

Table 2: Granulation level of 10 individual 'Imperial'-on-'Troyer' trees over 10 consecutive seasons of cropping, rated on a scale 0=no granulation to 5=complete granulation, Gayndah 2007-2016.

	Tree Number										
	29	**44**	**116**	**143**	**194**	**197**	**287**	**305**	**344**	**383**	**Average**
2007	1.42	1.42	1.75	1.67	1.83	2.42	1.92	2.50	1.83	2.00	1.88
2008	0.58	1.33	0.75	2.42	1.25	1.83	2.00	2.00	2.08	1.58	1.58
2009	0.88	1.29	1.13	0.58	0.38	0.79	0.33	1.71	1.54	1.42	1.00
2010	0.92	0.92	1.17	1.42	1.42	1.46	1.75	1.58	1.50	1.42	1.35
2011	2.08	1.83	1.33	2.33	1.25	2.33	2.00	1.92	1.58	2.17	1.88
2012	1.83	1.75	2.33	2.75	1.79	3.42	1.96	2.29	2.17	1.71	2.20
2013	0.75	0.50	0.58	1.42	1.17	1.83	1.75	0.58	0.42	1.13	1.01
2014	1.25	1.58	0.75	1.00	1.42	1.42	2.17	0.88	1.25	1.58	1.33
2015	0.58	1.17	1.75	1.50	1.79	2.25	1.67	1.46	1.50	1.50	1.52
2016	0.83	1.08	1.25	1.75	2.08	2.58	2.08	1.75	1.17	1.33	1.59
Total	1.11	1.29	1.28	1.68	1.44	2.03	1.76	1.67	1.50	1.58	1.54

It is clear from Table 1 that the 2012 and 2011 seasons were the worst for granulation whereas 2009 and 2013 had fewer problems. In a bad year (2012) granulation for the 10 trees ranged from 1.71 to 3.42, and even in a good year (2009) it ranged from 0.33 to 1.71. Such wide within-season variation between genetically identical trees makes it difficult to describe rootstock genetic effects on granulation. Averaged over 10 seasons, Tree 197 had almost twice the granulation (2.03) of Tree 29 (1.11), and this between-tree variation was almost as large as the seasonal variation over the 10 years (1.00 vs 2.20). To remove some of the seasonal variation, the data was re-examined using a 3-year rolling average. Results are shown in Figure 2.

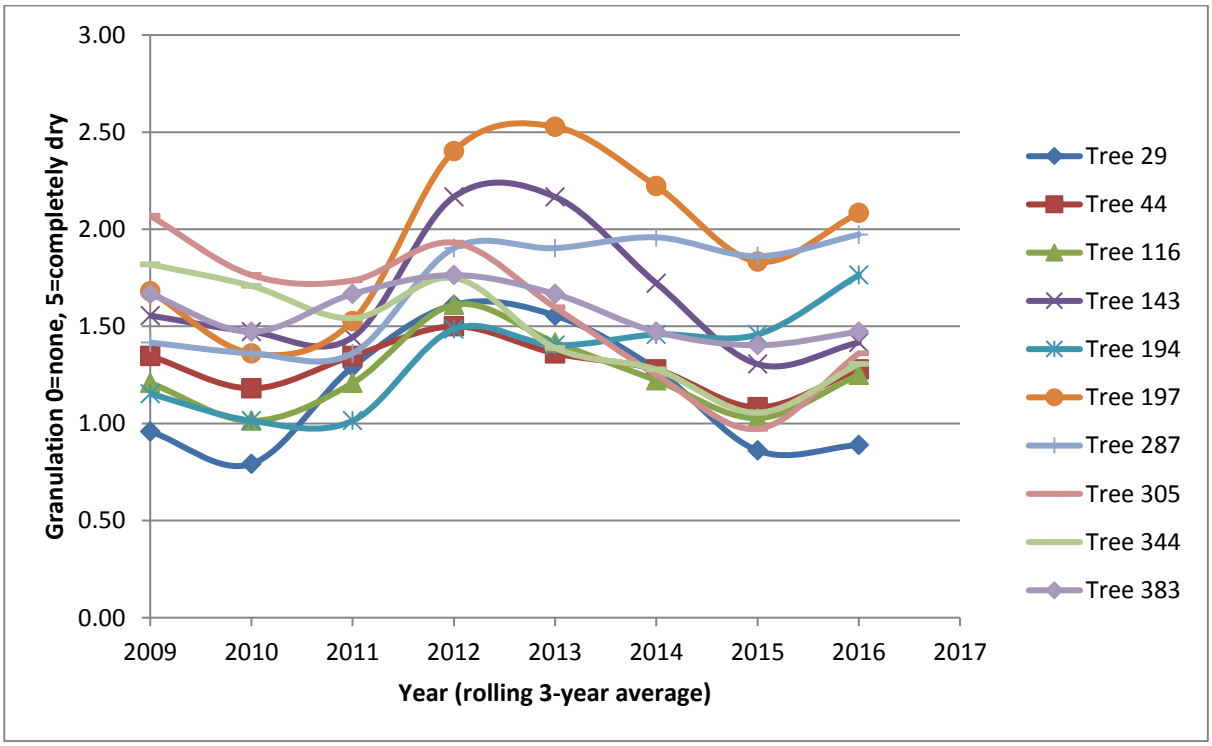


Figure 2: Seasonal variation in granulation for 10 individual trees of 'Imperial'-on-'Troyer', using a 3-year rolling average such that data for 2009 is the mean of 2007-2009, data for 2010 is the mean of 2008-2010 etc.

These results illustrate that individual trees do not all follow the same pattern of seasonal variation. Thus while trees like 197, 143 and 29 show a sharp increase in granulation toward the middle of the experiment and then quickly improve again, other trees like 194 and 287 tend to steadily increase throughout the life of the experiment. Even with a 3-year rolling average some trees range widely over the course of the experiment (e.g. 197, 29) where as others (e.g. 44) remain fairly constant. Variation between individual trees is further tested in Figure 3 where we examine whether granulation performance in the first 5 years of the experiment can be used to predict granulation in the second half of the experiment.

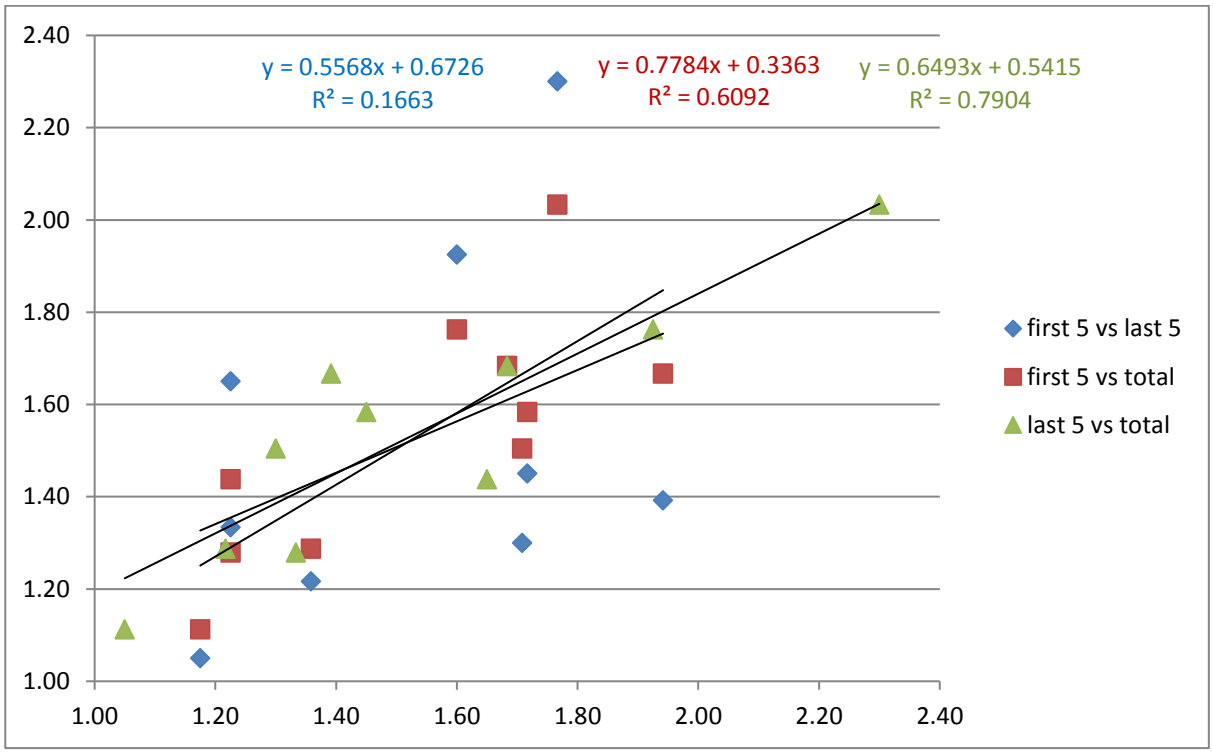


Figure 3: Relationship between average granulation in the first-5-years and last-5-years of the experiment for 10 individual trees of 'Imperial' on 'Troyer'.

There was a weak relationship between granulation in the first 5 years and granulation in the last 5 years (R2=0.1663). This relationship becomes stronger when compared with granulation over the total 10 year period (as would be expected because of co-correlation), and even stronger when granulation in the last 5 years of the experiment was compared with granulation over the whole 10 years. This relationship warrants further assessment on a different data set (different experiment) as it tends to suggest that results from older trees provides a more accurate indication of overall granulation of individual trees.

Results in Figure 2 suggested that individual trees like 29 and 197 show wide seasonal variation in granulation, in contrast with trees like 44 and 116. Consequently, we were interested in seeing whether trees 29 and 197 also had higher levels of variation within the fruit samples collected each season; the granulation rating for each tree is based on a 12-fruit sample (based on previous experience) and variation between individual fruits in this sample can be high. To complete this analysis, the standard deviation of the 12 fruit sample was calculated for each season and then a 3-year rolling average of standard deviation plotted in Figure 4.

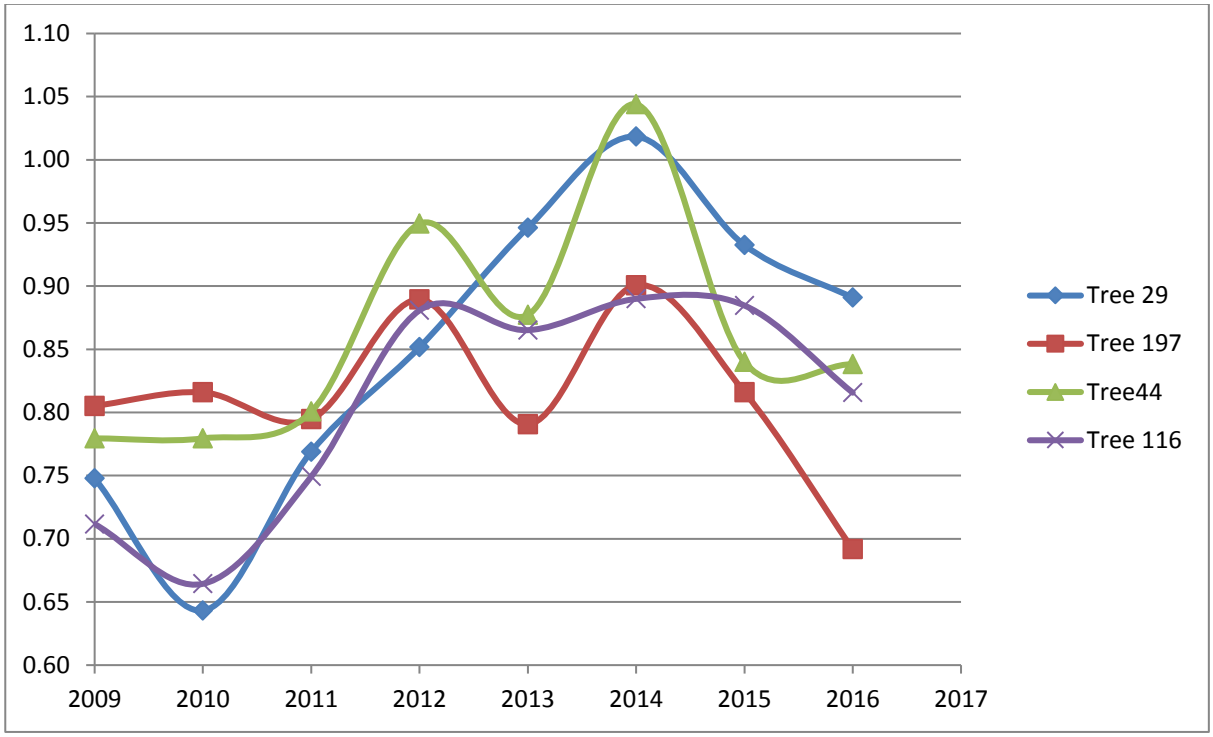


Figure 4: Variation of within-sample-variability (standard deviation of a 12 fruit sample) for 4 individual trees that showed high seasonal granulation variability (29, 197) and low seasonal granulation variability (44, 116) over a 10 year period (3-year rolling average).

The 10-year average standard deviations for the 4 trees were very similar (range 0.79 to 0.84) and it can be seen from Figure 3 that trees with high seasonal variation (29, 197) were no more, or less, variable within the 12-fruit sample. This suggests that the variation within the 12-fruit sample is not an indication of the likelihood of a particular tree having higher seasonal granulation variability. An unexpected consequence of plotting the data in Figure 3 is that trees 44 and 197 show a similar pattern of change over the life of the experiment, and likewise trees 29 and 116 have a similar pattern that is distinctly different from 44 and 197. Understanding the variability within-samples (12-fruit per tree) and between-trees (of genetically identical rootstock/scion combinations) will greatly aid the task of understanding the factors that cause granulation in 'Imperial' mandarin. Data collected during this rootstock project, while primarily aimed at identifying rootstock effects on granulation, will be of use to any future studies of granulation and will be made freely available to anyone involved in this challenging task.

Clear rootstock differences in 'benching' at the graft union were observed, with the new rootstock 'Barkley' showing vastly superior performance to 'Troyer', and and even exceeding the performance of 'Cleopatra' (which is normally considered to have a smooth graft union with 'Imperial') (Figure 5). This trial also showed how a rootstock like 'Swingle' can perform well in the first few years and then be disastrous in terms of long-term graft compatibility (Figure 6).

Figure 5: **Graft union compatibility was a major consideration for growers inspecting the field trial at the launch of 'Barkley' rootstock in March 2017. The LHS shows how 'Troyer' develops incompatibility at around 10-15 years old resulting in sucker production, canopy decline and eventually tree death. Even the preferred choice for compatibility 'Cleopatra' RHS shows a more pronounced graft union than the new rootstock 'Barkley'.**

Figure 6: **Breeding team member Toni Newman with an extreme example of the impact of delayed graft incompatibility. 'Pinching' at the graft union is causing canopy defoliation and death. The fruit is unmarketable and trees soon die after reaching this stage.**

Experiment Two: CTV Replication and Movement in Advanced Rootstocks

Background:

Citrus tristeza virus (CTV) tolerance/resistance is essential for any rootstock used in Australia. The biology and horticultural implications of CTV are very complex, but from a practical viewpoint it is essential to know which rootstocks will enable commercial production and which will decline. In terms of future breeding it is also necessary to differentiate between rootstocks that allow the virus to replicate without affecting tree performance, verses those rootstocks that are truly resistant and will not allow virus replication. To address these needs an extensive range of germplasm was grown under screen house conditions (to prevent natural infection from aphids), then deliberately inoculated using CTV-infected budwood. Each tree was simultaneously budded with virus-free budwood a short distance above the infected bud. New shoots from the rootstock, infected bud, and clean bud were later serologically tested to determine whether the rootstock supported CTV replication, movement or both. The project enabled the processing of this background information on CTV performance to determine the commercial value of these new rootstocks, as well as selecting parents for better informed breeding. Consequently, the new project completed the description of 1,400 individual plants from 70 different genotypes that were assessed for CTV movement and replication. It also supported the serological testing of field-grown trees that were propagated on these different rootstocks using both CTV-infected and CTV-free 'Imperial' budwood. Mandarins are recognised as symptom-less carriers of this virus even though small differences in performance have been previously noted between CTV-infected and CTV-free trees. The project showed that trees were rapidly infected under commercial orchard conditions but that initial virus status had no impact in terms of growth and production. The role of rootstocks with differing CTV reaction was also assessed for their impact on field infection and early tree performance.

An unexpected benefit of this component of the project was that it pointed the breeding team toward genotypes that could transmit two critically important rootstock traits, namely CTV resistance and apomixis. At the start of the project in 2013 it was believed that these two traits could be introgressed from *Poncirus* trifoliata, but results soon showed that *Poncirus* was a poor source of the apomixis trait. Consequently, we needed to find another genotype that could transmit apomixis but that also had the excellent CTV resistance acquired from *Poncirus*. Data from Experiment Two provided exactly this required information. As a consequence, all subsequent pollinations in the rootstock breeding work employed these identified genotypes and Poncirus is no longer used in Australian rootstock breeding. The Project has pioneered this strategy and our results created vigorous debate at the recent International Citrus Biotechnology Conference in Uruguay (April 2018) with other international breeders who persist in the use of *Poncirus*. By combining the use of the gentoypes identified in Experiment Two, with new molecular markers for apomixis, we have been able to reverse the conventional crossing direction (apomictic pollen parent rather than apomictic seed parent) and thus rapidly generate very large segregating populations to which high selection intensity can be applied for multiple traits.

Trees for this experiment where propagated in the insect-proof facility at BRS to prevent contamination by aphid vectors of CTV. The deliberate bud inoculation and serological testing were also carried out within this structure.

Figure 7: (a) Trees with the insect proof facility at BRS having been bud inoculated and then (b) individually blotted onto nitrocellulose paper and then test for the presence of virus using a CTV antibody

Details of the process and findings of this component of the project were presented as a poster at the International Citrus Congress in Brazil in September 2016 (self-funded) and published as a manuscript in *Citrus Research & Technology*:

Citrus R&T (e-ISSN 2236-3122)
Citrus Research & Technology, v. 37, n. 2, 2016
http://dx.doi.org/10.4322/crt.ICC041

Artigo/Article

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

Malcolm Wesley Smith[1], Toni Karen Newman[1], Debra Lorraine Gultzow[1], Sigrid Carola Parfitt[1] & Patricia (Broadbent) Barkley[2]

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).

[1] Bundaberg Research Station, Department of Agriculture & Fisheries, Queensland, Australia
[2] Retired Citrus Pathologist, PO Box 46, Mulgoa 2745, New South Wales, Australia
Corresponding author: Malcolm Wesley. Smith, Bundaberg Research Station, Department of Agriculture & Fisheries, 49 Ashfield Road, Bundaberg 4670, Queensland, Australia. E-mail: malcolm.smith@daf.qld.gov.au

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.

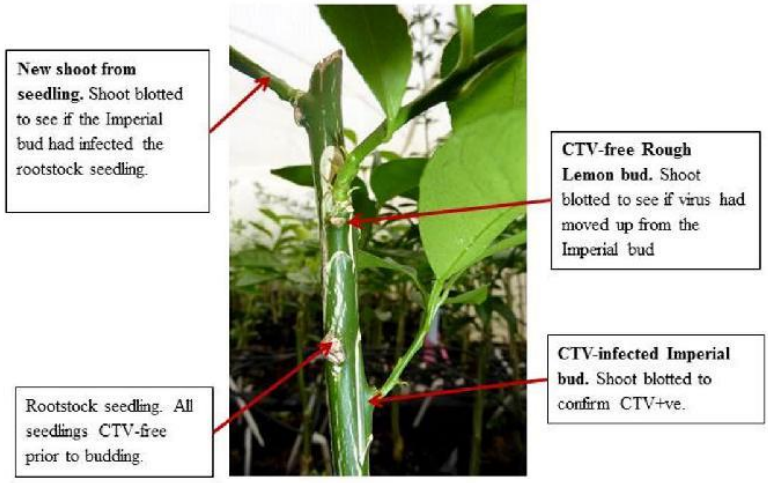


Figure 1. Method of testing CTV replication and movement in individual nursey seedlingls.

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.

ACKNOWLEDGEMENTS

Andrew Miles and Andre Drenth lobbied strongly for the construction of the aphid-proof screenhouse at BRS, which then made it possible to do this work. AusCitrus provided pathogen tested budwood free of charge which was organised and sent by Tim Herrmann and Nerida Donovan. NSW Department of Primary Industries and University of California supplied germplasm for testing and we thank

Graeme Sanderson, Sandra Hardy, Claire Federici and Mikeal Roose for their efforts. The research was funded by the Queensland Department of Agriculture & Fisheries, and this report prepared as part of a collaborative project (CT13004) with the Queensland Citrus Improvement Committee and Horticulture Innovation Australia.

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.

Received: November 15, 2016
Accepted: May 01, 2017

Experiment Three: Emerald 'Imperial' Rootstock Experiment

Background:

More than 50 years of research, in breeding programs in both NSW and California, has gone into developing a series of potential rootstocks that had yet to be commercialised. Recognising an opportunity, this advance germplasm was introduced to BRS in 2005 and 2008 and quickly propagated into a new rootstock experiment which was planted on a commercial property at Emerald in August 2011. Twelve rootstocks from the NSW program and 12 from California were included, with 'Imperial' mandarin again chosen as the scion (because of its commercial significance AND quality problems). A further 42 rootstocks representing hybrids bred at BRS and industry standards were included to bring the number of different genotypes to 66. Of these, 49 were included with both CTV-infected and CTV-free 'Imperial' budwood. This brought the total number of treatments to 151, with 891 trees in the experiment. The project assessed the impact of this extensive range of rootstocks on 'Imperial' fruit quality and tree performance. The site was visited annually just prior to harvest and each plot assessed for fruit size, juice acid and Brix, and granulation. Graft unions were measured along with canopy development both before and after the commencement of annual tree topping by the orchard owner. Trees were rated for health, and a new and innovative technique developed for efficiently assessing crop load.

Included within this trial were 27 full-sib families generated from monoembryonic seed parents. Each of these families was treated as a different rootstock, when in fact every tree **within** each family is genetically different – unlike the normal polyembryonic rootstocks where each tree is genetically identical to the parent tree (due to apomixis). One of the purposes of these 27 full-sib families was to test whether such families actually result in more variability compared to nucellar rootstocks. It is commonly believed that commercial rootstock varieties must be nucellar, so that resulting orchards are uniform. Evidence from rootstock experiments with mango and avocado indicate that this is not the case. If the same can be demonstrated for citrus then it may be possible to incorporate increased genetic diversity into commercial orchards without compromising uniformity. Furthermore, family selection rather than mass selection is more effective at improving traits with low heritability (such as yield) so this trial tested a new methodology to improve traits that are normally difficult to improve. Although not originally recognised at the start of the project, this new methodology also dove-tailed very effectively with the strategy of acquiring the apomixis and CTV-resistant traits from the pollen parent (see discussion in Experiment Two) and adds to the strong theoretical foundation underling the rootstock breeding approach used in the Bundaberg program.

The trial site was intentionally chosen because it was considered of marginal suitability for citrus, and so tested the 66 rootstocks for phytophthora and survival on poorer re-plant ground. High quality "citrus soils" in a virgin state are now quite rare even in Australia, and there seem little point in testing rootstock under such ideal conditions. It is far more sensible to identify rootstocks that enable high quality production on marginal country where infrastructure already exists. The first harvest and assessments were completed in April 2014 and subsequently in March/April for 2015, 2016, 2017 and 2018. Thus five seasons of data were successfully captured during the project period and this information has already pointed us to one particularly promising rootstock. While still too early for wide-scale promotion to industry, a commercial quantity of seed of this promising rootstock has been supplied to the collaborating grower who has agreed to test it on a larger scale. It has excellent resistance to CTV, produces consistent yields on moderate size trees and shows good graft union compatibility. Early results from this experiment were also used to guide the choice of parents in our hybridisation work and these decisions, based on very preliminary observations, have been substantiated by consistent performance in subsequent seasons. Thus this field trial has not only identified a potential new commercial rootstock but also identified parents for future breeding. Furthermore, in what is one of the most

surprising findings of the whole project, this field trial has revealed a new potential source of vigour control in rootstock breeding. It is planned to explore this discovery in a future project.

Despite these successes, we have been unable to identify a rootstock to solve the long-standing problem of 'Imperial' granulation. While it is possible to identify rootstock that consistently give high levels of granulation, the tasks of finding genotypes that consistently reduce the problem is far more challenging. However, some potential parents for reduced granulation were identified based on the first few seasons of data and have been used as parents to breed new genotypes. These new hybrids are now starting to come through the screening program and hopefully can be evaluated in future work.

Figure 8: (a) Trees of 'Imperial' on 66 different rootstocks propagated at BRS and ready for dispatch to the trial site at Emerald. (b) Trees laid out in the required experimental design, with 3-tree plots and each tree carrying a code associated with its individual performance while in the nursery at BRS. (c) trees shortly after the completion of planting, Emerald, 4th August 2011..

Figure 9: Field crew strip-picking one of the 40 Individual calibration trees in April 2017. This process was repeated each season so that the visual estimate of crop load (from 852 datum trees) could be correlated with actual fruit production to produce a predicted yield for each tree. Note also the extensive and non-fruiting vegetative growth that has occurred after topping to a height of 3m eight months earlier.

Figure 10: Major differences in scion vigour have been induced by some of the rootstocks being tested in this experiment. Note the significant size reduction of the centre three trees in the photograph compared with the rootstock trees on either side. Note also the extreme amount of vegetative growth that has occurred on the vigorous tree following topping 8 months earlier.

Figure 11: (a) A photographic record has been made of every plot in this experiment for the 5 seasons of fruit assessment conducted during the project period. Because the trial design involves 3-tree plots, the top 4 fruit are from the northern tree in the plot, the centre 4 fruit from the centre tree of the plot, and the bottom row of 4 fruit from the southern tree in the plot, with a combined juice samples. (b) arrangement used to record images and (c) collecting other data associated with each sample.

Figure 12: Graft union compatibility is an important consideration, particularly for scions such as 'Imperial'. The rootstock on the left has produced a smooth union indicative of a long-lived combination. This rootstock has other promising attributes and seed has been supplied for wider scale commercial testing. The rootstock on the right is 'X639' and is clearly unsuitable for use with 'Imperial'. The trees have been unproductive and fruit have shown an unusual physiological rind breakdown.

A new method for estimating tree yield has been developed by the project. . This involves estimating crop load using a visual rating system in which each tree is given a 'fruit density' ratings (on a 0 to 10 scale) by five people working independently. Four sets of 10 adjacent trees are then chosen at random and the fruit from each of these 40 trees individually harvested and weighted. The 'fruit density' ratings are then calibrated using these 40 actual tree yields and the equation used to estimate yields for each tree in the experiment. A simple linear equation was derived with an R-square value 0.71 in 2015, 0.70 in 2016 and 0.73 in 2017, which is sufficient to differentiate between low and high yielding rootstocks in this experiment (Figure 13).

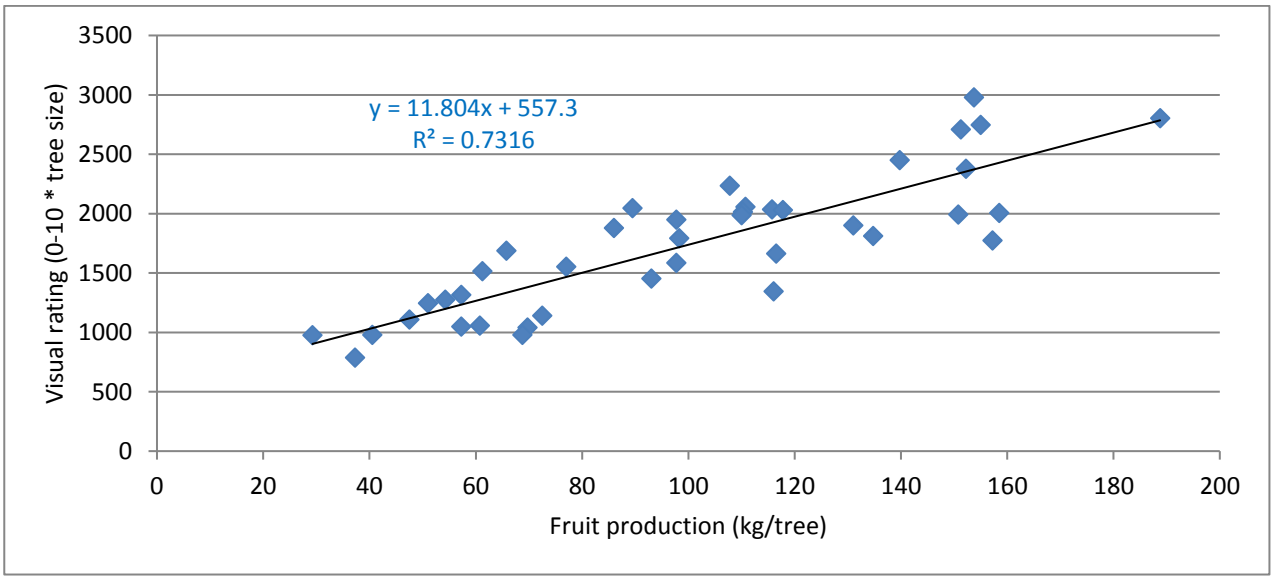


Figure 13: Yield calibration equation generated from 40 individual 'Imperial' trees at the Emerald rootstock trial in April 2017. Data from the visual rating score (Y axis) provides a reasonable estimate of the actual weight of fruit on each tree (X axis). This equation was then used to estimate yield for all 891 trees in the experiment.

Experiment Four: Breeding new *P.trifoliata* hybrids with CTV resistance

Background:

P. trifoliata (known as "tri") is an inedible citrus relative that has made an extraordinary contribution to modern citriculture. While direct use as a rootstock has many limitations, its use as a parent in rootstock breeding has resulted in hybrids that acquire resistance to CTV, phytophthora, and produce good fruit quality. Intellectual property considerations and commercialisation constraints have made it increasingly difficult to acquire new rootstocks for testing and even more complex if any of these show promise and need to be made available to growers. Indeed, the BRF citrus team reasoned that it may be faster and more efficient to breed our own rootstock hybrids, than to negotiate the long path with getting access and use agreements for externally bred material. To test this proposition a series of hybrids with *P. trifoliata* were created in 2007/8 and when they were of sufficient size deliberately inoculated using budwood infected with CTV. Serological testing and confirmation over a two year period identified a range of hybrids truly resistant to this virus. Cuttings were taken from each of these young CTV-resistant plants and used to produce nursery trees for a future rootstock experiment, while the original seedling was field planted at BRF to enable it to pass through the extended juvenile period prior to its first production of fruit. The project CT13004 enabled the continuation of this large-scale rapid development process. Of the more than 700 *P. trifoliata* hybrids produced, the work had already discovered 235 that were resistant to CTV and propagated them as cutting ready for testing as rootstocks. Using 'Imperial' as the scion variety, this project conducted the budding of these rootstocks and their field-planting on a commercial orchard in 2013 (Sheppherds Gayndah Rootstock Experiment). Tree survival and growth were assessed throughout the project, and three seasons of fruit quality data were also generated within the project period. By the end of the project, many of the source trees at BRS had passed through their juvenile phase and were producing fruit/seed. Indeed, a small number of the hybrids, that had completed their transition through the juvenile phase, were incorporated into the 2016 and 2017 pollination plans, based in part on their performance in the field trial at Gayndah. It was originally envisaged that outstanding performers from the rootstock experiment could be propagated using seed from the source trees and supplied to nurserymen for semi-commercial testing. However, the discovery of low levels of apomixes when using *P. trifoliata* as a parent meant that such seed would not come true-to-type. This was an important discovery with implications for more efficient breeding in the future, and when combined with the knowledge gained from Experiment One it pointed the breeding team to a new strategy for rootstock breeding. While *P. trifoliata* is well recognised as an outstanding parent in rootstock breeding, it is remarkable how little attention has been given to the other parent used in the cross. Indeed, there is no information anywhere in the world to say which parents are most likely to give hybrids that result in the best fruit quality – despite the fact that rootstocks are well recognised as one of the best ways to change fruit quality! The 700 BRs hybrids were produced using 27 different seed parents all crossed with *P. trifoliata*, so data from this experiment may eventually help to fill this knowledge gap (once a few more years of growth, productivity and fruit quality data become available).

Figure 14 (a) Trees in Experiment Four, 20 months after planting showing difference in early tree establishment on these single-tree plots. (b) trees in 2018 (5 years after planting) with the breeding team illustrating how some rootstock treatments have induced a highly productive canopy of short stature while other rootstocks have produced vigorous trees with very limited fruit. On the far LHS is another rootstock with a large and productive canopy.

Figure 15: Some of the variability in granulation seen between individual trees at Experiment Four during the 2017 assessment season.

Experiment Five: The Commercial Value of "Extreme hybrids" as Rootstocks

Background:

There is a huge amount of genetic diversity in *Citrus* and its close relatives but, other than *P. trifoliata*, very little of this has ever made any impact on commercial citrus growing. Past breeders, particularly in the USA, have attempted to capture useful traits from this material but failed, and most current breeding programs use a very narrow range of germplasm (just a few orange, mandarin and grapefruit varieties crossed with *P. trifoliata*). A concerted effort by the BRs citrus team has seen the Queensland germplasm collection expand to be one of the most diverse in the world, and pollination experiments have identified the reasons why past breeders failed with such material and how these problems can be overcome. Consequently, there are now available what might be called "extreme hybrids" that capture the genetics of distant relatives and put them in a background that should make them commercially applicable. This project supported the evaluation and continued development of 'extreme hybrids' focusing on their value as rootstocks for commercial citrus production. Material was propagated into rootstock experiments and rapidly assessed for likely commercial suitability. Tested material included the first fruiting intergeneric hybrids ever produced between *Citrus* and *Citropsis*, as well as a series of Australian Desert Lime [*Citrus glauca* (syn. *Eremocitrus glauca*)] hybrids that had been bred with CTV resistance. These 'extreme hybrids' ended up in two large field experiments that were planted during the project period. Information on the potential commercial merit of some of these 'extreme hybrids' was generated as a result of data collected from these field experiments as well as additional nursery experiments. To overcome delays caused by juvenility and sterility, these 'extreme hybrids' were propagated as cutting and then budded with 'Imperial' before field planting. Any new germplasm must be suitable for efficient nursery production, so data on nursery growth rates, survival and ease of budding was collected and many hybrids discarded simply because of poor nursery growth. Some of the parents included in this component of the project, which have never been successfully incorporated into conventional citrus production, include *Citrus glauca* (Australian Desert Lime, Lime Bush), *C. australasica* (Finger Lime), *C. australis* (Australian Round Lime), *C. garrawayi* (Mt White Lime), *C. wintersii* (PNG species), *C. wakonai* (unique to BRF), *Severinia buxifolia* (Box Orange), and four *Citropsis* species (African cherry oranges). One of the key reasons why much of this germplasm has never lived up to expectations, is that it is highly sensitivity to CTV. Consequently, a major activity in the project was to introgress CTV-resistance into this unique germplasm via conventional breeding and then screen it by direct inoculation and serological testing. The BRS citrus team has identified a number of alternative sources of CTV-resistance in addition to *P. trifoliata*, and experiments designed to test the heritability of these exciting new sources of resistance was initiated during the project. Eventually it is hoped to use molecular methods to confirm if any new heritable sources of CTV-resistance are conferred by the same genes involved as those from *P. trifoliata*. Some preliminary work toward this objective was undertaken in a related project (see Additional project related activity C) in which we attempted to verify molecular markers for CTV-resistance that had been developed and published by other international research groups.

To speed-up the development and commercial release of better rootstocks, the project has relied on cutting generated from young hybrid seedlings. This overcomes the long (5-10 year) delay waiting for hybrids to start to produce seed and also overcomes issues created by non-apomicitic hybrids. In the case of 'extreme hybrids' it also solves the problem of infertility which is common in wide crosses (which may never produce seed). Our process for producing cutting is simple, efficient, and well suited to dealing with a large number of different genotypes at the same time. It is shown pictorially below (Figure16):

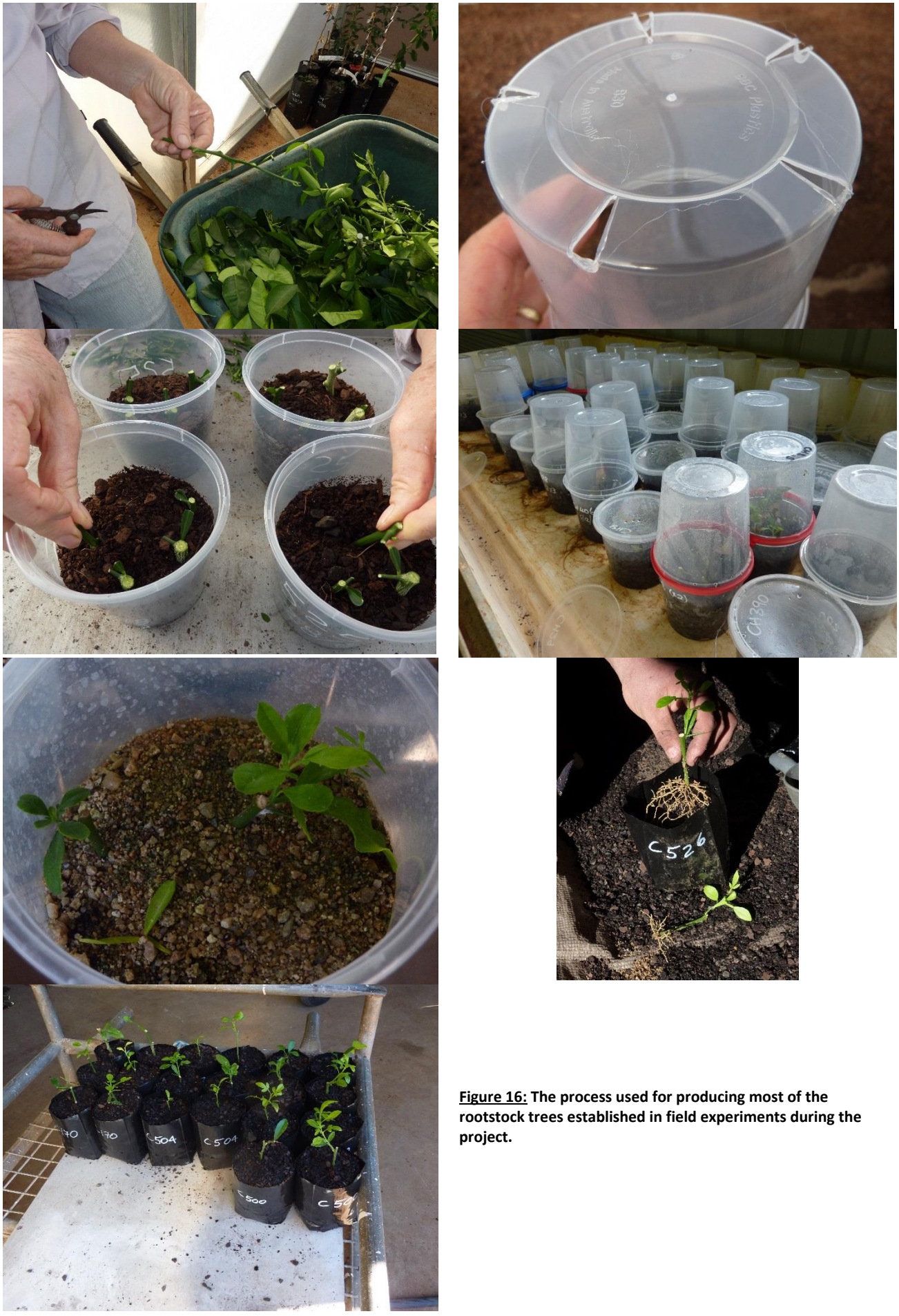


Figure 16: **The process used for producing most of the rootstock trees established in field experiments during the project.**

It has proven difficult to generate hybrids from many of the citrus relatives, although the availability of potted mature trees within the nursery facility at BRS has enable crosses to be made whenever flowers become available (Figure 17).

Figure 17: Early fruit set on a citrus relative being used to generate 'extreme hybrids' for testing as rootstocks. In this case a CTV-resistant hybrid with *C. glauca* parentage (GLA81) has been pollinated with a CTV-resistant hybrids with *C. australasica* parentage (10Q019). The pedigree of the offspring is:
{[(*C.wakonai* x *C. glauca*) x *P.trifoliata*] x [(*C.wakonai* x *C. australasica*) x *P.trifoliata*]}.

Hybrids that survived the process of CTV resistance testing, phytophthora screening, were able to be propagated as cuttings, and that produced adequate growth during the nursery phase were budded with 'Imperial' budwood (Figure 18). Trees that grew adequately were then established at a very challenging site (heavy waterlogging soils, low-lying frost prone) on a commercial property at Wallaville (Figure 19)

Figure 18: **Rootstock trees of 'extreme hybrids' propagated from cutting prior to budding (note the distinct morphology compared with normal citrus rootstocks). (b) Breeding team member Deb Gultzow inspecting rootstock trees shortly after the 'Imperial' buds have been unwrapped and the rootstock tops removed. (b) Same trees one month later showing variability in the rate of bud take.**

Figure 19: **(a,b)Planting 'extreme hybrid' rootstocks on a commercial site at Wallaville, 3rd November 2016. Note the heavy clay soil and low-lying drainage gully. This site was completely inundated during both the 2013 and 2015 Burnett River floods and represents an excellent location to test the resilience of these new and unique genetic combinations. (c) trial site 7 months after planting.**

Measurement of early tree survival and growth has shown a strong rootstock effect amongst these 605 trees. For example, of the 7% of trees that died within 12months of planting, 40% were from just 3% of hybrids (involving *Citropsis schweinfurthii*, *Citrus glauca*, and *Atalantia ceylanica* parentage). More importantly, the experiment has already demonstrated that sibling performance can be vary widely. In the case of rootstocks with *C. australasica* parentage, the 13 hybrids in the trial have shown markedly different initial growth (Figure 20).

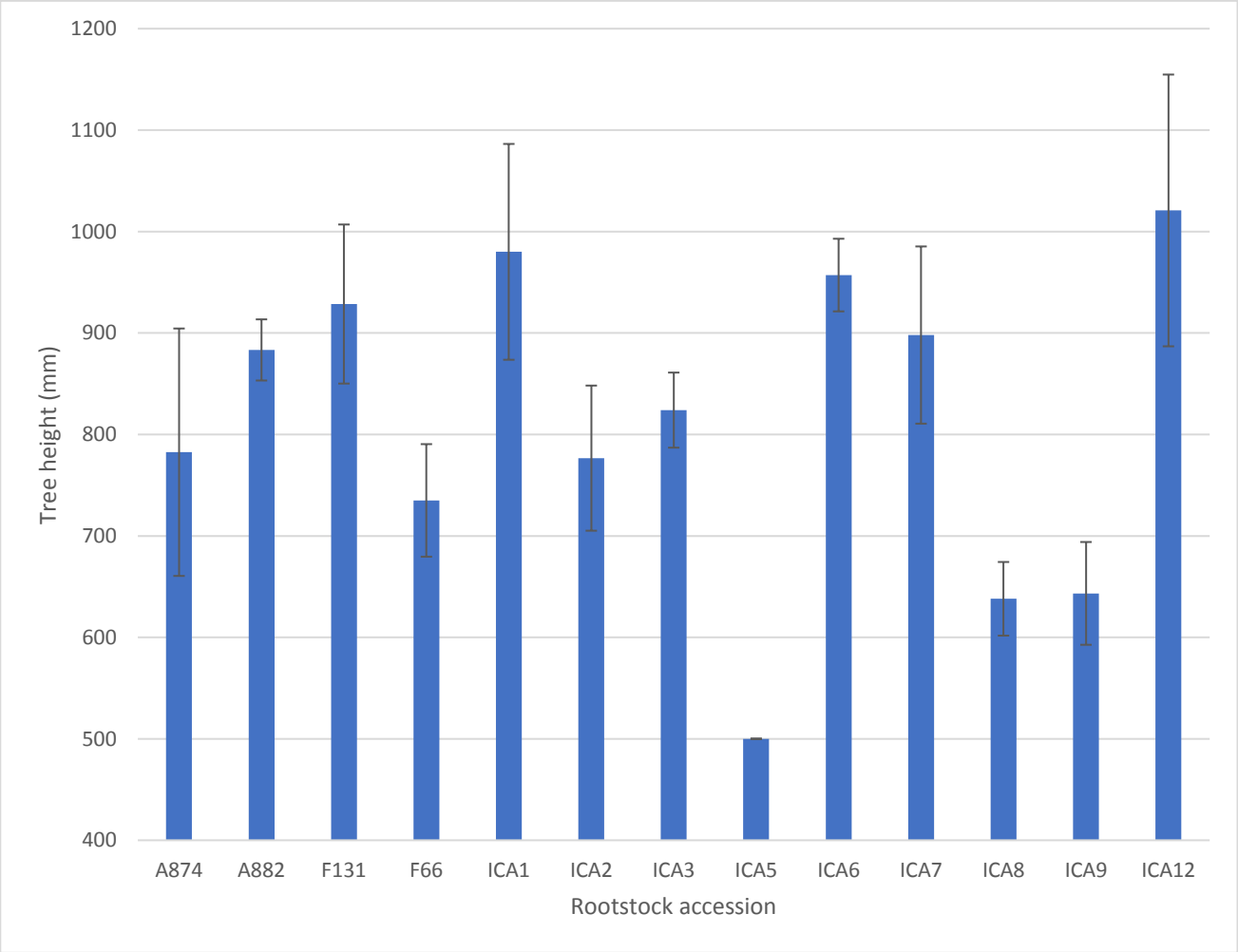


Figure 20: The comparative performance of 13 different rootstocks that contain *Citrus australasica* in their parentage. Tree height (mm) is used as an indicator of how well they have established in the first six months after field planting, June 2017, Wallaville, Queensland.

This is an important finding for a number of reasons. Firstly it demonstrates the value of testing a large number of sibling when using wide crosses, secondly it reveals the existence of wide segregation within families, and thirdly it indicates the need for caution when choosing which sibling(s) to use in future crosses. For example, in the continuing breeding work at BRS the hybrid 'ICA5' has been used extensively as a parent, partly because it flowers freely and sets well. However it can be seen from Figure 20 that it has very poor early tree establishment when used as a rootstock, and we may be better to use hybrids like 'ICA1' and 'ICA12' as future parents. This illustrates how data from the various field trials is being fed back into the breeding program to help guide new breeding work.

Additional project related activity: A. High-throughput phytophthora screening

Phytophthora diseases have been causing problems for citrus growers for almost 150 years, and a great deal of research has been published. There is an additional body of knowledge held by a handful of very competent citrus pathologists with decades of practical field experience. Collectively, this literature and knowledge was used to develop a test method for high-throughput screening of germplasm. Many modifications were made to this method during the course of the project and the discussion below explains how we eventually arrived at a method well suited to the low-resource high-throughput breeding approach used at BRS. We have retained considerable detail in the following discussion so that it may be useful to future investigators, in both citrus and other crops, who have a similar methodology requirement.

Initial test method

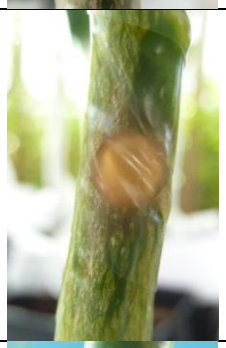
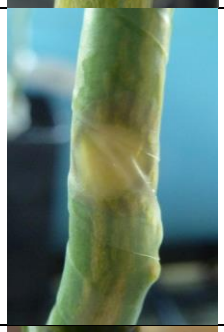
Despite very promising initial results with phytophthora trunk inoculations onto potted nursery trees (25th Feb and 15th May 2015) when tested on a small number of plants (85), this screening procedure largely failed when we moved on to use it on a larger scale (558 plants on 20-21 July 2015). It was clear within a week of inoculations that this large scale screening had failed (for inoculations on the 15th May, symptoms were seen within 3 days). The reason for this contrast in performance was unknown but needs to be resolved before spending another 2 days repeating the exercise. The problem could have been the host, the pathogen, the environment, or any combination of these. Consequently, an experiment was designed to investigate environmental effects in the first instance, and developed a range of post-inoculation treatments that would create different environmental conditions.

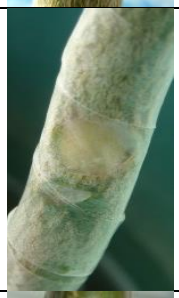
1. Bud, wrap, place in PPH[a] (as per previous inoculations).
2. Bud, wrap, cover with damp cloth, enclose in clingwrap, place in PPH.
3. Bud, wrap, place in humid coolroom (ambient temperature).
4. Bud, wrap, place in humid & heated coolroom (32-35°C).
5. Bud, wrap, place in seed store (6°C).
6. Bud, wrap with wider budding tape, place in PPH.
7. Bud, wrap, enclose in garbage bag, place in PPH.
8. Bud, cover with damp cloth, wrap with wider budding tape, place in PPH.
9. Bud, wrap, place in bucket full of water (up to soil level), place in PPH.
10. Bud, wrap, place in SRA glasshouse (with evaporative coolers running).

[a]Photoperiod glasshouse, passive glasshouse, low humidity

Seedlings of Sweet orange growing in 500mL forestry tubes were used for all the treatments. Three trees were randomly allocated to each of the treatments, giving a total of 30 trees. Weather conditions at the time of the experiment were dry and mild. The humid/heated dark coolroom set at about 32-37°C had a very good "oppressive" tropical feel. The unheated coolroom was not as warm (~23°C) but still had near 100% humidity. Temperatures in the PPH stayed between about 15-27°C, with low humidity. Trees were assessed 9 days after treatment with results consistent between the three replicates (Table 3).

Table 3: **Results obtained from 10 different combinations of treatments designed to elicite a phytophthora disease response on Sweet orange seedlings.**

Treatment	Wood browning	Bark browning	Callus	Moisture under tape	Comment	
1 Wrap PPH	slight	no	no	moderate		
2 Wrap Dampcloth Clingwrap PPH	yes	severe	no	wet	Best treatment	
3 Wrap humidCR ambient	yes	slight	no	wet	2nd best treatment	
4 Wrap humidCR heated 32C	slight	no	yes	moderate		
5 Wrap Fridge 6C	no	no	no	dry		

6 Wide tape PPH	slight	no	no	dry	Hopeless tape	
7 Wrap Seal in bag PPH	slight	1 tree	no	moderate		
8 Dampcloth Wide tape PPH	slight	Possibly	no	dry	Hopeless tape	
9 Wrap BucketH20 PPH	v. slight	no	no	dry		
10 Wrap glasshouse	slight	no	low	moderate		

The best treatment was “2” followed by “3”. The wider tape (and any treatments involving it: 6, 8) was a failure presumably because it did not remain moist under this tape. It is important to note that this ‘wider tape’ also differed from the normal wrapping tape in that it was textured (embossed) and so may not have sealed as well as a smooth tape. Placing trees in a bucket of water didn’t seem to help much, even though pots were flooded to the top of soil level. Trees with the least symptoms were those from the coolroom “5” closely followed by “1” and those that dried out. The heated humidified room “4” did not give good phytophthora symptoms but probably had the best callus; presumably the high temperature (>32°C) had prevented symptoms from developing (cf. mild temperature high humidity “3”). These results suggested that it was important to have ‘free water’ around the infection site, and simply keeping the root system wet was no substitute for this. While normal budding tape is effective in preventing desiccation it does not retain ‘free

water' around the infection site. These results suggest that modification to the post-inoculation 'environment' may help improve this screening procedure. There are many practical considerations in how this is best done. We need to avoid having to shift trees to humidified coolrooms, or having laborious wrapping and wetting procedures. A simple inoculation technique that gives quick and consistent results was the goal. A new experiment was designed to test these hypotheses.

Consequently, the following experiment was set-up on the 12th Oct 2015:

1. Two rootstocks, Sweet orange, Troyer
2. Eight treatments
    a. Bud, wrap, dampcloth, clingwrap, PPH
    b. Bud, wrap, dampcloth, clingwrap, foil, PPH
    c. Bud, dampcloth, clingwrap, PPH
    d. Bud, dampcloth, clingwrap, foil, PPH
    e. Bud (non Pn.), wrap, dampcloth, clingwrap, PPH
    f. Bud, dampcottonbud, clingwrap, PPH
    g. Bud, wrap, dampcloth, clingwrap, dark box, PPH
    h. Bud, dampcottonbud, wide waterproof tape, PPH
    i. (actually 'J') wet trunk (50uL/l Tween 20), bud, wrap, PPH, wet daily

This gave a total of 18 treatments, and with 3 replicates required 54 trees. Conditions at the time of treatment were mild and dry. Trees were assessed at 8 days and again at four weeks after treatment (Figure 21).

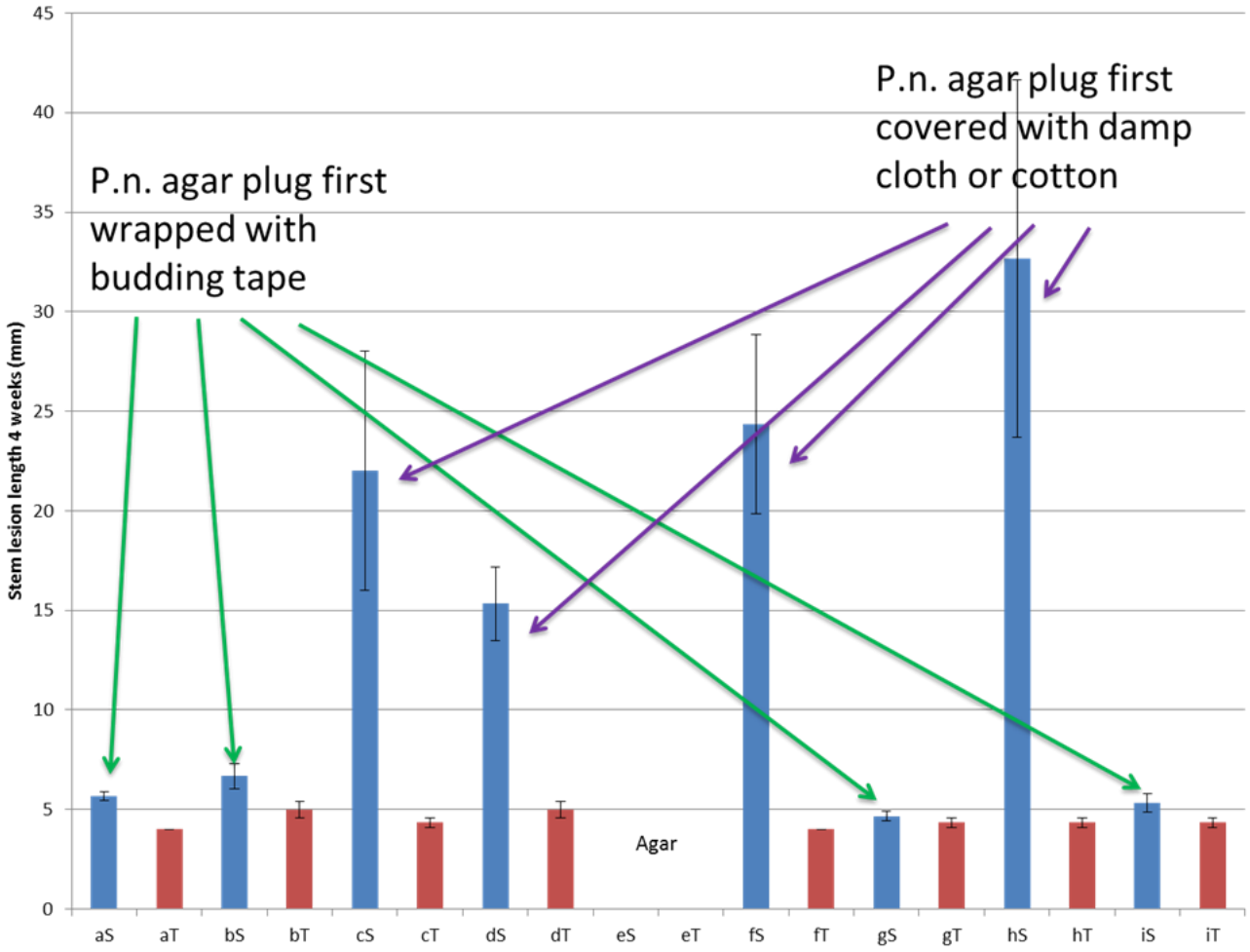


Figure 21: Effect of 'environmental' conditions on the development of phytophthora symptoms following stem punch inoculation. S=Sweet orange, T=Troyer, a-e refer to different post-inoculation treatments, see text above.

The non-phytophthora inoculation (e) was white and clean and demonstrated that the response in other treatments was not simply due to the trunk remaining wet. Mild symptoms (wood browning) on the 'Troyer' in (h) probably suggests that it is not resistant but instead is tolerant, and illustrated the need to measure lesion length when rating stocks for sensitivity to this pathogen. There seemed to be better symptoms where the agar was not wrapped with budding tape (c, d, f, h) rather than where it was wrapped (a, b, g, j) which again hinted at the importance of having free-water around the infection site. It was difficult to say if the damp cloth was any better/worse than the cotton bud. However it was clear that daily misting of wrapped trees does not lead to good symptoms, consistent with our belief that there needs to be free-water around the infection site.

Detached stem technique

A preliminary experiment was set-up on the 22nd February 2016 to test the response of detached citrus stems to punch inoculations with phytophthora. The breeding team had read some papers with other plants (eg Jarrah) where the technique had been moderately useful, and had given rapid results, particularly at elevated temperatures. We chose three citrus types (Rough lemon, CH390, F59) and inoculated ~40mm stem sections (2mm punch) with five different phytophthora cultures. Within two days, clear symptoms were developing on the Rough lemon stem sections treated with *P. citrophthora* (53412), and possibly some other combinations. Second best results were with *P. palmivora* and also *P. nicotianae*).There were definitely no symptoms on *P. cactorum*. Results improved further over the next few days and after five days were very obvious (Figure 22).

Figure 22: Detached stem screening technique use to test the pathogenicity of various Phytophthora species and accessions, with a range of common citrus hosts. The accession 53412 was chosen for future disease screening of new hybrids.

Six days after treatment, the stems were assessed with the following observations: Best results with '535412' giving a severe reaction on Rough lemon and mild on CH390 and F59. Next best was '53986', with good results on all three but particularly CH390 and F59. Third best was '63631' with a mild response except on F59 which did not respond. Second worst was '4093' which gave a mild response on Rough lemon but not on CH390. '51744' gave no response on any of the stem sections.

To confirm these exciting results, a new experiment was set up on the 26th February 2016 using a greater range of citrus types (hosts) and a greater range of phytophthora accessions (pathogens). There were seven different citrus types: Rough lemon, Flying Dragon, Cleopatra, Sweet orange, Swingle, Troyer, and Sour orange. The six different phytophthora accession cultures were *P. citrophthora* (53412, 41805), *P. nicotianea* (4093, Troyer), *P. palmivora* (53986), and *P. cactorum* (51744). Each of the 42 different treatments was replicated on three different stem sections. Some browning of the agar pugs was beginning to happen one day after treatment.. By two days there seemed to be good symptoms on the Rough lemon and Sour orange with '53412', (*P. citrophthora*) but symptoms were unclear for the other treatment combinations. Better symptoms developed over the next few days and by seven days there was no further improvement in symptoms. It was clear from this experiment that '53412' (*P.citrophthora*) give very fast and severe reactions, and is the obvious choice for future germplasm screening. Perhaps the next best choice is '53986' (*P. palmivora*).
The newly developed technique was subsequently applied in the screening of thousands of new hybrids at BRS. This experience lead to a number of recommendations to help ensure a dependable disease response:

1. Only use humidifiers, not heaters, in the coolroom (this will help prevent the stems drying). Combine the steam humidifier with the electronic humidifiers to get a constant cloud of fog.
2. Watch that the temperature does not climb too high (perhaps keep under 25 C). Only have one steam humidifier running at a time to prevent overheating.
3. Use freshly prepared agar plates (prepared no more than 2 weeks prior to use).
4. Keep freshly prepared plates at room temperature NOT in a fridge.
5. Ensure control sticks (Rough lemon, Sweet orange, Troyer) are included in every tray.
6. Preferably use a coolroom where good results have been obtained in the past. Some 'environments' seem to give better results than others, for unknown reasons.
7. Symptoms should appear within a week, and misting with a phytophthora broth does not seem to help.
8. Avoid applying phos acid (or any other fungicide) to the plants that are going to be inoculated (preferably never, but certainly for 2-3 months prior to screening).
9. The most likely causes of the failure of screening batches are using old phytophthora cultures, using coolroom 'environments' that allow the detached stems to dry out, or allowing temperatures to rise too high during symptom development.

In summary, this project has developed a fast, efficient and effective technique to screen large amount of citrus germplasm, and in a way that does not contaminate nursery propagation areas.

Additional project related activity: B. Salt screening

Although not originally included within the project proposal, the breeding team recognised an opportunity to incorporate salt tolerance in the breeding objectives. With the endorsement of the Project Management Committee, this work sort to refine existing salt screening protocols and develop the necessary equipment to apply salt stress and measure its impact on hybrids. A automated drip irrigation system was designed and constructed by the breeding team to allow the regular application of a known amount of saline water to individual pots (Figure 23a-b). Plants were measured on a regular basis and the amount of salt was increased every two weeks eventually resulting in severe symptoms on some hybrids. Inclusion of control cultivars and one of the parents enable confirmation that the treatments had been severe (Figure 23c).

Figure 23: (a-b) Equipment constructed to perform salt response phenotyping on citrus hybrids. A salt solution of know concentration was injected daily into each pot. (c) eventually resulting in visual symptoms of salt burn and some tree death.

At the end of the treatment period, plants were assessed for biomass, and leaf samples were then ground for chlorine determination (see full explanation below). A special purpose chloridometer was acquired for this purpose (Figure 24a-b) and proved useful in conjunction with visual ratings of salt damage to leaves and roots (Figure 24c).

Figure 24: (a,b) Chloridometer used to measure leaf chloride concentration of hybrids after 115 days of salt treatment, eventually reaching 11.g/L sodium chloride with an EC of 21mS. Plants had also been sampled for leaf chloride content prior to the salt treatment and these samples were similarly processed using the chloridometer (c) root health was also checked at the end of the treatment period.

The salt screening procedure was commenced on the 31st March 2017, using the hybrids that remained after two rounds of phytophthora screening and CTV testing. The hybrids, growing in 2.8L bags were cut back to 200mm above soil level on the 8th and 23rd February, and then allowed to re-shoot for 5 to 7 weeks.

Salt treatments started on the remaining 182 hybrids on the 31st March 2017 with daily injection (10am) of 100mL of solution. The initial concentration was 1.45g/L sodium chloride, which had an EC of 2.84mS. During the 115 days of salt treatment, the concentration was increased each ~14 days by an extra 1.45g/L sodium chloride, such that the final concentration applied from the 11th July to 24th was 11.6 g/L sodium chloride with an EC of ~21mS. This daily treatment continued until the 24 July 2017, when plants were then subject to a 16 day drought (24th July to 10th August) with regular weighing of pots to calculate water usage of each hybrid.

Four leaves were taken from the middle of the shoot on the 10th August and dried (60°C) before being ground. The plants were also rated for visual salt damage in terms of marginal necrosis, leaf drop and overall salt sensitivity (Figure JJJ).

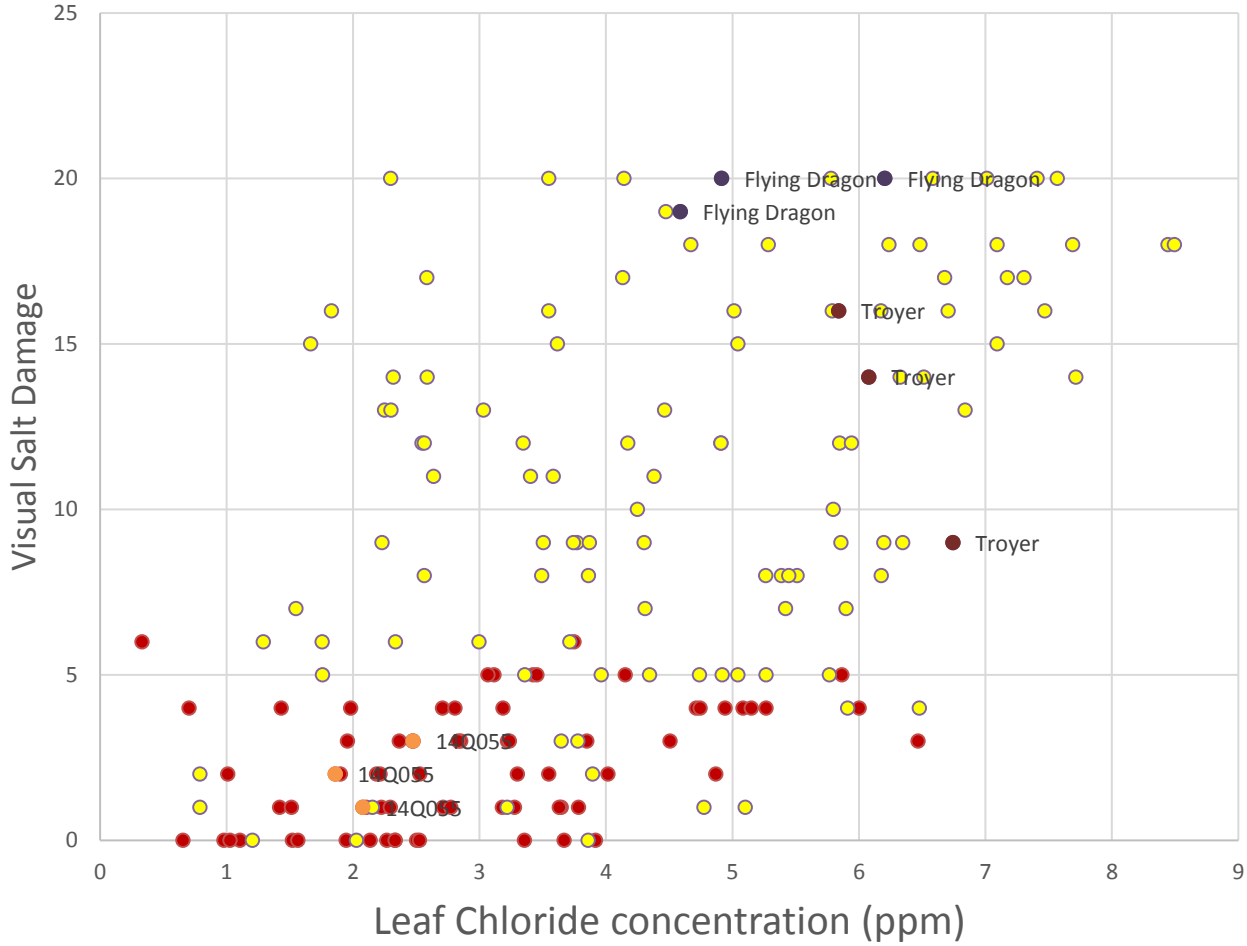


Figure 25: Relationship between visual salt damage rating and leaf chloride concentration for 171 hybrids plus three individual plants of three different control rootstocks. Values in yellow were excluded from the breeding program while those in red were retained for further development.

On the 16th August, plants were cut-back to 1-2 nodes above where the previous shoot had emerged (after being cut back in February 2017) and the bottom section (150mm) used for phytophthora testing. A 50mm stem section of approximately 4mm diameter was collected for wood and bark volume determination. The remainder of the stem was used to produce cuttings, (which did not do very well perhaps as a result of their high salt content). Leaf samples from the best 71 hybrids were sent to Natalie Dillon on the 4th Oct 2017 for DNA extraction ahead of testing for the MITE apomixis marker(see Additional project related activity below).

Processing of leaf samples was completed in the week 4-8th September 2017, and the results used to confirm culling decisions based on visual symptoms. Leaf samples had been collected from each individual hybrid both before the salt treatment started, and after the salt treatment was complete.

A total of 171 hybrids were tested for leaf Cl levels after salt treatment with the results shown below

Cross	No of hybrids	Mean Cl	Minimum Cl	Maximum Cl
Encore*14Q055	34	3.142928931	0.329767442	6.511737589
Encore*63-199-49	15	3.10329915	0.700755814	6.481262626
Encore*Tri	4	3.766999774	2.264861111	5.082212389
K15*14Q055	49	4.14570293	0.977931034	7.71277027
K15*63-199-49	69	4.205891026	1.008211009	8.493229167
Grand Total	171	3.86868995	0.329767442	8.493229167

From the sample of 171 hybrids, a group of 84 were ***chosen*** as likely salt-tolerant based on their leaf symptoms. Results for these hybrids are shown below:

Cross	No. of hybrids	Mean Cl	Minimum Cl	Maximum Cl
Encore*14Q055	21	2.743919068	0.654461538	5.864158879
Encore*63-199-49	4	2.522037042	0.700755814	4.773465347
Encore*Tri	4	3.766999774	2.264861111	5.082212389
K15*14Q055	24	3.281418928	0.977931034	6.47505102
K15*63-199-49	31	3.128990288	1.008211009	6.46722973
Grand Total	84	3.075298578	0.654461538	6.47505102

Results for the 87 hybrids ***excluded*** based on leaf symptoms are as follows:

Cross	No. of hybrids	Mean Cl	Minimum Cl	Maximum Cl
Encore*14Q055	13	3.787483325	0.329767442	6.511737589
Encore*63-199-49	11	3.314667189	1.663979592	6.481262626
K15*14Q055	25	4.940844213	1.289090909	7.71277027
K15*63-199-49	38	5.084415311	1.756238532	8.493229167
Grand Total	87	4.625603557	0.329767442	8.493229167

For comparison purposes, if we had chosen 84 hybrids based solely on their low Cl levels then the results would have looked like the following:

Cross	No. of hybrids	Mean Cl	Minimum Cl	Maximum Cl
Encore*14Q055	21	2.032084314	0.329767442	3.643472222
Encore*63-199-49	12	2.502580949	0.700755814	3.545
Encore*Tri	2	2.74034127	2.264861111	3.215821429
K15*14Q055	23	2.616538198	0.977931034	3.613173077
K15*63-199-49	26	2.371183475	1.008211009	3.629404762
Grand Total	84	2.381149684	0.329767442	3.643472222

A major disadvantage if we had selected based on just leaf Cl levels is that we would have selected less K15 hybrids, and we are hoping that this may be a good source for HLB tolerance. This discrepancy between leaf symptoms and Cl levels in different families may indicate that some families have better salt tolerance than others, even if their leaf Cl levels are higher. In the final wash-up, the leaf Cl level had limited bearing on which hybrids were retained. This decision was based more on visual health of the plants after salt treatment plus re-assessment of phytophthora reaction. We finally arrived at 69 hybrids to retain and the leaf Cl levels for these are shown below:

Cross	No. of hybrids	Mean Cl	Minimum Cl	Maximum Cl
Encore*14Q055	14	2.699055878	0.329767442	5.864158879
Encore*63-199-49	3	1.771560941	0.700755814	3.184491525
Encore*Tri	3	3.950725889	2.264861111	5.082212389
K15*14Q055	21	3.068158265	0.977931034	5.999230769
K15*63-199-49	28	3.041898127	1.008211009	6.46722973
Grand Total	69	2.963087583	0.329767442	6.46722973

Twenty of these 69 hybrids (29%) had a leaf Cl level lower than '14Q055' (2.14%, known to be a good Cl excluder) and 68 hybrids (99%) had a leaf Cl level lower than 'Troyer' (6.22%). Thus, this population should contain individual hybrids that are very good under saline conditions, given that they have been selected on both visual symptoms and leaf Cl levels following exposure to very high levels of salt.

Four 'control' rootstocks were contained within the experiment and leaf chloride levels for the three individual replicates of each are shown below:

Rootstock	Rep1	Rep2	Rep3	Average
14Q055	1.86	2.08	2.47	2.14
FlyingDragon	4.59	4.91	6.20	5.23
Troyer	5.84	6.08	6.74	6.22
Benton	7.17	7.01	7.30	7.16

It is clear from these results that '14Q055' has considerable ability to restrict Cl accumulation in its leaves, and this is consistent with previous screening work that has been done with this rootstock and with its growth performance in our salt-treatment experiment. 'Flying Dragon' and 'Troyer' had similar leaf Cl levels even though 'Flying Dragon' suffered more severely from the salt treatment. It was not until toward the end of the experiment that 'Troyer' started to show symptoms of salt damage. 'Benton' did very poorly but this could be because they were extremely small plants when they were entered into the experiment, and we know that salt screening should only be done once plants are about 12 months old and of a reasonable size (see Sykes 1985 Table 5).

The decision to include '14Q055' as a parent was a good one in terms of salt tolerance. (Both it and 63-199-49 have been shown to accumulate Cl levels similar to Cleopatra, which is commonly used in salt marginal areas). It accumulated on average 2.14% Cl, and we were able to make some selections from both Encore and K15 that had lower levels than this. In the case of Encore*14Q055 we had 9 out of 34 hybrids with leaf Cl% lower than 2.14%, and 4 of these are being retained (4 were culled because of poor phytophthora performance). The 9th one (K659) does not have %phloem data (for unknown reasons) and was not originally going to be selected, however it did okay in phytophthora screening and has the lowest leaf Cl levels so will be retained (the leaf Cl level for this sample really should be checked because it seems unusually low). In fact, all of the retained 69 hybrids should be rechecked for Cl levels using the existing samples, and then comfirmed with a new experiment containing replicates of each hybrid (in contrast with the single seedling that was tested in the current experiment). Of the 48 hybrids of K15*14Q055, only two of them had a leaf Cl level lower than

their 14Q055 parent and both (554, 493) are included for future work. However 40 of these hybrids had lower leaf Cl levels than 'Troyer' and all 22 of the selections made from this family have lower leaf Cl levels than 'Troyer'. Fifteen of these selections had leaf Cl below 3.5%.

The rootstock '14Q055' seems to have a lot going for it, and it was a wise decision to include it in our crossing program during the 2015 pollinating season. It was included because of promising early performance in the rootstock experiment at Emerald, and subsequent results have confirmed this observation. Although we have not sufficiently processed the granulation data, it continues to show good graft union compatibility and has been a healthy productive tree. We also know that it has excellent CTV resistance and is able to transmit this trait (see Smith et al. 2016). It has moderate to good tolerance to phytophthora and this may be a trait in which improvements can be targeted.

Sykes (1985) indicated that there was a relationship between leaf Cl levels before and after salt treatment but provided no data or indication of the strength of such a relationship. We were keen to pursue his suggestion as a possible way of identifying salt tolerant hybrids without the need to subject them to salt treatment. To do this, we sampled each hybrid before the commencement of the salt treatment so that we could then compare the results with those found in the leaves after treatment. It is clear from our results that some kind of relationship does exist, and that even *prior* to salt treatment (using plants grown with low salt water) there are differences between hybrids that relate in some way to the differences seen *after* salt treatment. However this relationship is of no use from a breeding/screening point-of-view because hybrids can have very low initial Cl levels (eg <0.05%) prior to treatment, but then produce very high leaf levels after salt treatment (eg >7%). For example, if we were to select individuals with leaf Cl lower than 0.5% then we would have mistakenly selected many hybrids that accumulate very high Cl levels in the presence of salt. Consequently we should not waste time and resources sampling and testing leaves prior to salt treatment. The relationship between our visual assessment of salt tolerance, and the actual leaf Cl at this same time is shown below:

Additional project related activity: C. Molecular marker verification

An internally funded DAF project was undertaken to investigate the validity of molecular markers that had been published by various international research groups for important traits in citrus breeding. These traits were apomixis, CTV-resistance and seedlessness. The only markers that proved accurate and useful were some very recent (2017) markers for apomixis. Because this work had clear synergy with the CT13004 rootstock project, the internal report on this project as well as the manuscript prepared and accepted for Acta Horticulturae are presented below:

Department of Agriculture and Fisheries

Assessment of molecular markers for improving citrus selections

Agri-Science Queensland Innovation Opportunity

8th June 2017

Matthew Webb and Malcolm W. Smith

Queensland Government

This publication has been compiled by Malcolm W. Smith and Matthew Webb of Horticulture and Forestry Science, Department of Agriculture and Fisheries.

Summary

In this investigation, we sought to identify co-dominant molecular markers that would allow for more effective genotyping of three important traits in the Queensland Department of Agriculture & Fisheries (DAF) citrus breeding program, namely: seedlessness, homozygous citrus tristeza virus (CTV) resistance, and early detection of apomictic/polyembryonic individuals. All three traits warrant the application of molecular markers because they meet the criteria of being:

1. A trait of economic significance
2. Technically difficult to phenotype by conventional methods
3. Time consuming to phenotype by conventional methods
4. Likely controlled by a simple genetic mechanism.

Published literature and personal communication with international researcher were used to identify marker systems and primers worthy of testing on a diverse range of DAF germplasm that had been accurately phenotyped for these three traits. This quickly revealed that published markers for seedlessness had failed to work in independent testing by two laboratories in the USA, and so we decided to postpone our validation until more reliable markers become available. For the other two traits, a range of primers were identified for testing on DAF germplasm that was segregating for CTV resistance and apomixis (ability to produce clonal seedlings).

Available CTV resistance markers did not correlate with virus resistance/susceptibility phenotyping although they accurately reflected the parentage of hybrids. The results confirmed that two accessions in the DAF breeding program mediate CTV resistance via a mechanism independent of genes linked to currently available molecular markers. For broad spectrum resistance to CTV in Australia these additional genetic resources could play an important role, and the development of associated markers may represent a worthwhile future project.

In a stroke of good fortune, a new molecular marker system for apomixis appeared in the literature half way through our project. We were one of the first international groups to validate this system, and confirmed a close association between the marker and apomixis in DAF germplasm. This marker represents an enormous step forward in the breeding of citrus rootstocks that are readily adapted to commercial use. Options are now being explored to deploy this innovative technology in the DAF citrus breeding program.

Table of contents

Background

Citrus is the most important fruit crop in the world, and has attracted a concerted international research effort to develop molecular markers that will aid breeding. Long generation times (5-15 years) and large plant size (~500 trees/ha) obstruct genetic progress but also make citrus an ideal candidate for the deployment of markers. There is the potential to identify useful hybrids during the seedling stage, more than 10 years before they would normally display the trait of interest. Despite this potential and significant research investment by the world's largest citrus growing nations, very few useful molecular markers have emerged and none are routinely employed in breeding programs.

Whilst the DAF citrus research effort has deliberately focussed on conventional breeding techniques, the team keep a keen eye on published literature and have close contacts with most international citrus programs, recognising the value that new technologies could bring to the program. By early 2016 there were published markers for three important traits that warranted evaluation. They each met the criteria of: economically significant; difficult to phenotype by conventional methods; slow to phenotype by conventional methods; and under simple genetic control. These were molecular markers that were linked to the traits of seedlessness, citrus tristeza virus resistance, and apomixis (whether seed give rise to zygotic or nucellar plants). All three traits are important in DAF citrus scion and rootstock breeding projects.

Seedlessness is as desirable as it is difficult to achieve. Every international breeding program employs a range of techniques to produce seedless germplasm and all are being used by DAF. An approach currently finding favour is the introgression of a seedlessness gene from a non-conventional citrus relative. Access to this gene has already been achieved through collaboration with the University of Florida, with F1 and BC1 families in field plantings at Bundaberg awaiting fruit production (to verify seed content). According to the literature, these families should segregate for seedlessness in a pattern consistent with a single dominant gene. RAPD markers have been published for this gene. Citrus trees flower only once per year and so deployment of these molecular markers would enable flowering hybrids to be used as pollen parents as soon as they flower, reducing generation times by 12 months and ensuring only hybrids carrying the gene were field planted (halving the size of progeny blocks).

Citrus tristeza virus (CTV) is the agent of one of the most destructive citrus diseases in the world, with germplasm tolerance/resistance playing a critical role in its effective management. Resistance is generally believed to be controlled by a single dominant gene and a range of markers linked to this gene have been published since the 1990s. The DAF breeding program has used conventional virus inoculation and serological detection techniques to develop a diverse range of hybrids that are resistant to CTV. Consequently, new hybrids can now be produced using parents that both carry CTV resistance. However, our conventional detection technique is not able to distinguish between hybrids that are homozygous or heterozygous for the resistance. A co-dominant molecular marker would enable us to identify the homozygous resistant hybrids, without having to resort to expensive and slow breeding techniques such as progeny-testing.

Some citrus cultivars have the capacity to produce clonal offspring via seed (apomixis). This trait has been extremely valuable in rootstock breeding because it enables new hybrids to be commercially deployed as seed. However, citrus has a very long juvenile period (5-15 years) so breeders must wait until fruit is produced before they can examine the seed and determine which new hybrids will come true-to-type. Attempts to develop molecular markers for this trait have met with mixed success, but it is of such importance to breeding efficiency that this trait warranted inclusion in the project.

Existing DAF breeding projects did not have the financial or technical capacity to evaluate these molecular markers and so an Innovation Opportunity project was developed to link the citrus breeding expertise, knowledge and international contacts of the Bundaberg based breeding program, with the biotechnology understanding and capacity of scientists at ESP.

Project Objectives

The objective of this project was to evaluate published and recently developed DNA markers for the economically important traits of seedlessness, CTV resistance and apomixis, using germplasm from the DAF citrus breeding program.

Immediately prior to the investigation, information became available to suggest that available markers for seedlessness are not consistently associated with this phenotype. Consequently, a decision was made to commit all project resources to the other two traits of CTV resistance and apomixis.

CTV-resistance studies primarily sought to identify a co-dominant molecular marker that can rapidly identify individuals homozygous for a dominant, broad-spectrum resistance allele derived from *Poncirus*. Investigations of apomixis endeavoured to identify co-dominant markers that can facilitate early detection of this trait in a diverse range of *Poncirus* and *Citrus* species/hybrids.

Methodology

Cultivars and DNA extraction

Twenty-one cultivars representing a diverse range of genotypes from the DAF breeding program were selected for analysis with available CTV and apomixis-associated markers. Each cultivar had previously been phenotyped for CTV resistance and apomixis/polyembryony using conventional methods (direct tissue-blot immunoassay (DTBIA) and seed embryo morphology). DNA was extracted from plant leaves using a commercial kit (QIAGEN) for subsequent marker assays. DNA quality and quantity was assessed via spectrophotometry, electrophoresis and PCR amplification with quality control primers (data not shown).

CTV resistance markers

Two CAPS markers (CTV103 and Ks9005) that had previously been associated with the CTV resistance locus of *Poncirus* and which had the potential to differentiate between homozygous and heterozygous individuals were initially assessed. Methods used were essentially as described by Ohta *et al* 2015. Additional primers (CTV102R_topless_F and _R) were also designed to identify SNP's associated with this locus (Ohta et al 2011) but were not used in the current investigation due to time limitations. PCR primer sequences used in this part of the investigation are detailed in Table 3.

Apomixis/Polyembryony markers

Primer sequences for three unpublished SSR markers (cf-ag07, ji-tc04 and cf-ag06) reported to co-segregate with apomixis/polyembryony in a *Citrus maxima* (monoembryonic) x *Poncirus trifoliata* (polyembryonic) population were kindly provided by Mikeal Roose (University of California). PCR primer sequences and a selection of *Poncirus*, *Citrus*, and *Citrus x Poncirus* hybrids were provided to AGRF for subsequent PCR amplification, separation and detection. Primer sequences are provided in Table 3.

During our investigation Wang *et al* (2017) published a paper describing a MITE insertion associated with apomixis in various *Citrus* species. Two of the published primer sets (mite_p1 and mite_p2) were used on all available DNA extracts as codominant markers for the detection of this MITE insertion. An

additional primer set (CitRWP) designed to account for possible sequence variation within this region was similarly tested. Sequences for each of these primers are listed in Table 3.

Prior to publication of the report by Wang *et al* (2017) we planned to examine SNP's occurring within an apomixis-associated region for use as co-dominant markers within *Citrus* species (Nakano *et al* 2012). Due to the efficacy and ease of the assay outlined by Wang *et al* (2017), however, investigations with these markers were discontinued. PCR primer pairs ordered for this project aim (067sgc05, 048F02T, 002F02T, dg17) are detailed in Table 3.

Results

The *Poncirus* CTV resistance region is not always associated with a strong CTV-resistant phenotype.

Two co-dominant CAPS markers (CTV103 and Ks9005) were analysed within twenty-one cultivars from the DAF breeding collection in order to identify individuals containing *Poncirus*-derived CTV resistance alleles (Ohta *et al* 2015). Both primer sets allowed for effective differentiation of distinct allele variants typically associated with *Citrus* and *Poncirus* species ('a' and 'b' respectively - Table 1). In accordance with results presented by Ohta *et al* (2015), the *P. trifoliata* cultivar "Flying Dragon" was found to be heterozygous when the CTV103 marker was used and similar results were obtained with a locally available *P. trifoliata* cultivar ("Queensland trifoliata - QLDTRI"). Further, it was found that all of the *Citrus* x *Poncirus* hybrids tested in our collection were heterozygous at this locus.

It is important to note that the presence of the CTV103 'b' allele did not always correspond with a CTV resistant phenotype. In particular, the *Citrus* x *Poncirus* hybrid "Swingle" was shown to possess an 'ab' genotype identical to that of *P. trifoliata*, however it appears to be highly susceptible to CTV replication. Moreover, the *Citrus* x *Poncirus* hybrids "Benton", "Troyer" and "US812" appear to display inferior resistance compared to *P. trifoliata,* despite possessing the same 'ab' genotype. Our results also confirmed that two citrus species, sour orange hybrid "11Q003" and the pummelo cultivar "K15", appear to mediate CTV resistance via an independent mechanism.

Analysis of results with the Ks9005 marker indicated that *P. trifoliata* cultivars are homozygous for the 'b' allele, as has been previously reported by Ohta *et al* (2015). Similar to results obtained with CTV103, our investigations indicated that the Ks9005 'b' allele was present within all *P. trifoliata* derived hybrids but its presence did not simply correlate with CTV resistance (Table 1).

Agarose electrophoresis results obtained with markers Ks9005 and CTV103 are shown in Figure 1.

MITE insertions upstream of the CitRWP gene are associated with apomixis/polyembryony in a diverse range of *Citrus* species and hybrids but not in *Poncirus trifoliata*.

Wang *et al* (2017) recently determined that insertion of an approximately 200bp MITE sequence upstream of the CitRWP gene is associated with apomixis in an extensive range of *Citrus* species. Consequently, we sought to analyse all available DNA samples for the presence of this MITE sequence using three different primer sets (mite_p1, mite_p2 and CitRWP) that collectively encompass known sequence diversity in this region from *Citrus* species. Our investigations revealed that the presence of a MITE sequence ('b' allele) generally correlated well with a polyembryonic phenotype in a diverse range of *Citrus* species and hybrids (Table 1). However a notable exception was the sour orange hybrid "11Q003" which was found to contain a copy of the 'b' allele but which possesses a monoembryonic phenotype, and has been confirmed as non-apomictic by progeny testing using *P. trifoliata*. It is also important to note that a MITE insertion was not detected in both

polyembryonic *P. trifoliata* parents ("Flying Dragon" and "Queensland trifoliata - QLDTRI") as well as the polyembryonic *Citrus x Poncirus* hybrid "Swingle".

Table 1. – Association of co-dominant marker genotypes with CTV resistance and apomixis/polyembryony phenotypes in a range of *Citrus* and *Poncirus* species and their hybrids.

			Phenotypes		*CTV-resistance PCR Genotypes*		*Polyembryony SSR Genotypes*			*Polyembryony MITE PCR Genotypes*
Extract No	Type	Cultivar	CTV resistance	Embryo Type	Ks9005	CTV103	cf-ag06	cf-ag07	ji-tc04	CitRWP mite_p1 mite_p2
1	*mandarin*	01C011	Susceptible	Mono	aa	aa	cc	cc	cd	aa
2	*wild lime*	09Q005	Susceptible	Mono	aa	aa				aa
3	*kumquat*	14Q021	Susceptible	Mono	aa	aa				aa
4	*mandarin*	DAISY	Susceptible	Mono	aa	aa	cd	cf	cd	aa
5	*mandarin*	ELLENDALE	Susceptible	Mono	aa	aa	cc	cc	dd	aa
6	*mandarin*	IM111	Susceptible	Mono	aa	aa				aa
16	*sour orange hybrid*	11Q003	Solid resistance	Mono	aa	aa	ae	ab	eg	ab
17	*pummelo*	K15	Solid resistance	Mono	aa	aa	ab	ab	df	aa
7	*mandarin*	FREMONT	Susceptible	Poly	aa	aa	cc	cf	cd	ab
8	*mandarin*	KARA	Susceptible	Poly	aa	aa				ab
9	*mandarin*	MURCOTT	Susceptible	Poly	aa	aa	cc	cf	cc	ab
10	*Poncirus hybrid*	3831	Solid Resistance	Poly	ab	ab				ab
11	*Poncirus hybrid*	14Q055	Solid Resistance	Poly	ab	ab	ff	cc	bc	ab
12	*Poncirus hybrid*	63-199-49	Solid Resistance	Poly	ab	ab				ab
15	*Poncirus hybrid*	US119	Solid Resistance	Poly	ab	ab				ab*
18	*Poncirus hybrid*	BENTON	Partial Resistance	Poly	ab	ab				ab
19	*Poncirus hybrid*	SWINGLE	Susceptible	Poly	ab	ab	ee	ee	ac	aa
20	*Poncirus hybrid*	TROYER	Partial resistance	Poly	ab	ab				ab
21	*Poncirus hybrid*	US812	Partial resistance	Poly	ab	ab				ab
13	*Poncirus*	FLYING DRAGON	Solid Resistance	Poly	bb	ab	ef	cd	ab	aa
14	*Poncirus*	QLDTRI	Solid Resistance	Poly	bb	ab				aa

* Data missing for mite_p1

In addition to detection of the MITE insertion, we also sought to examine whether SSRs within three separate marker regions (cf-ag06, cf-ag07 and ji-tc04) could be used to differentiate apomictic/polyembryonic cultivars. Primer pairs targeting these regions detected 6-7 different alleles (a-g) within a selection of samples that included various polyembryonic and monoembryonic *Citrus* varieties, *P. trifoliata* (polyembryonic) and a range of polyembryonic and monoembryonic *Citrus* x *Poncirus* hybrids (Table 1). For each of these three marker regions, we found no consistent correlation between observed genotypes and polyembryony in a range of *Citrus* species. In particular, we failed to reliably detect any alleles that were consistently shared between *P. trifoliata* and two different polyembryonic *Citrus* x *Poncirus* hybrids.

Agarose electrophoresis results obtained with the MITE PCR primers (mite_p1, mite_p2 and CitRWP) are shown in Figure 2. Size data for the SSR primers (cf-ag06, cf-ag07 and ji-tc04) is shown in Table 2.

Conclusions/Significance/Recommendations

In this investigation, we sought to identify co-dominant molecular markers that could allow for more effective genotyping of CTV-resistant citrus cultivars and early detection of apomictic/polyembryonic individuals.

Our investigations confirmed that marker CTV103 allows for effective differentiation of cultivars that are either homozygous or heterozygous at the associated CTV-resistance locus. It is anticipated that it will find utility in our breeding program for identifying homozygous cultivars that can readily transmit the *Poncirus* CTV103 resistance allele to all progeny and for further characterisation of its role in generating a resistant phenotype. Ohta *et al* (2015) indicate that marker Ks9005 does not consistently

co-segregate with CTV103 and a CTV-resistant phenotype, however it may be useful for identifying hybrids that have flanking, undesirable *Poncirus* genes removed during meiosis.

Previous studies indicate that broad-spectrum CTV resistance derived from *P. trifoliata* involves a single dominant gene linked to CTV103 (Ohta *et al* 2015). However, we did not consistently associate CTV resistance with a single copy of the *Poncirus* CTV103 resistance allele. Cultivars such as "Troyer" and "Swingle" are considered resistant to CTV in many parts of the world, although our phenotyping (Smith *et al* 2017) for these, and many other cultivars heterozygous for CTV103, is more consistent with Roose (1996) in showing CTV replication within supposedly resistant cultivars. This suggests the need for a more complex model of CTV resistance, as has been previously proposed by Mestre *et al* (1997). Our results appear to concur with those of Harper *et al* (2014), who similarly observed that the hybrid cultivar "Swingle" had reduced resistance compared to *P. trifoliata* when challenged with a range of CTV strains. Interestingly, our phenotyping studies (Smith *et al* 2017) also indicate that *P. trifoliata* is not completely resistant to the mixture of CTV strains used in our experiment and will host the virus whilst an infected bud of a susceptible genotype is attached. Other researchers have similarly observed only partial resistance of *P. trifoliata* to CTV infection (Harper *et al* 2014) and the existence of resistance breaking strains of CTV have previously been described (Yokomi *et al* 2017).

Our studies indicate that the sour orange hybrid "11Q003" and pummelo variety "K15" represent potential sources of useful CTV-resistance genes that are not detectable with the CTV103 marker. Available evidence from our breeding program suggests that resistance mediated by "K15" is not inherited in a simple, dominant manner whilst "11Q003" has been found to readily transmit CTV resistance to its progeny. Further investigation of the genetic basis of these traits and concomitant development/testing of additional molecular markers for selective breeding purposes represents a potential avenue for future research.

Our investigations support the hypothesis that insertion of a MITE sequence upstream of the CitRWP gene is associated with ovule-specific gene expression and concomitant apomixis/polyembryony in many citrus varieties (Wang *et al* 2017). A notable exception in our study was the sour orange hybrid "11Q003" which was found to contain a MITE insertion, yet is monoembryonic and produces only zygotic seedlings. It would be relatively simple to determine if there are additional disruptive mutations within the CitRWP gene of this cultivar that are responsible for this phenotype.

Whilst the MITE marker appears to be a promising tool for identifying apomictic/polyembryonic *Citrus* species, *Poncirus* species appear to employ a distinct molecular mechanism to achieve this trait. In accordance with this, we were unable to detect a MITE insertion in two *P. trifoliata* cultivars. We were also unable to detect a MITE insertion in one of the polyembryonic *Citrus x Poncirus* hybrids (cv. "Swingle") and it is considered possible that this hybrid inherits this trait from its *Poncirus* parent.

It has previously been proposed that *Poncirus*-derived apomixis/polyembryony is associated with a single dominant allele (Kepiro and Roose 2009). Using three different SSR markers, we were unable to detect any alleles that were consistently shared between *P. trifoliata* and two different polyembryonic *Citrus x Poncirus* hybrids. It may be prudent to test additional hybrids to confirm this observation, in particular with hybrids that have a confirmed monoembryonic *Citrus* parent. In addition, Kepiro and Roose (2009) describe a co-dominant marker (EMB-6P) that may give more reliable results. However, the authors of the MITE marker paper are due to publish details of a new molecular marker for identifying apomictic *Poncirus* hybrids (Qiang Xu – *pers. comm.*) and this may be the preferred option for future investigations.

Key Messages

Scientific literature concerning the use of molecular markers in plant breeding is often misleading, and in the hands of the ill-informed can lead to rash decisions and poor allocation of breeding resources. Experiments are often conducted under such tightly controlled conditions and with such limited genetic diversity that the results can barely be repeated let alone extrapolated. Traits that are thought to be under simple genetic control are often found to be more complex. Sometimes an existing conventional screening technique can be used or developed which will provide the same result more efficiently.

For these reasons it is important to keep abreast of molecular developments in important international crops like citrus, and to critically evaluate potentially useful molecular markers as they become available. By doing so we can quickly identify useful technology, avoid wasting resources on markers that don't work, and develop a better understanding of the complexity of the important commercial traits we are working to improve.

The confirmation of a molecular marker that can distinguish between monoembryonic and polyembryonic hybrids represents an enormous step forward in the efficient breeding of new citrus rootstocks.

Where to next

An efficient methodology needs to be developed so that the MITE apomixis marker can be incorporated into the DAF citrus breeding program. This current project has demonstrated that it is an accurate marker for an economically important trait that is difficult and extremely slow to phenotype by conventional methods. Developing a procedure so that this marker can be used efficiently to screen thousands of new hybrids is the next critical step.

Failure of molecular markers for seedlessness and CTV resistance reinforce the complexity of these traits and the need to improve conventional screening techniques, including a better progeny-testing methodology. Developing test-parent genotypes that flower and fruit at a very young age would enable potential parents to be genotyped faster than the 5-10 years it currently takes. More DAF germplasm should be tested with CTV103 to find new hybrids that are homozygous for the resistance allele. This will help to build robust disease resistance suitable for Australian production conditions.

New molecular markers will continue to emerge, as a result of the enormous research investments being made by the world's major citrus producing countries. For example, it is anticipated that markers linked to Huanglongbing (HLB) tolerance will become available in the next few years and could be useful in our pre-emptive breeding efforts for this devastating disease, which is currently exotic to Australia. Any such new markers need to be tested to ensure they accurately describe the phenotype of DAF citrus breeding germplasm.

Acknowledgements

The following DAF staff are acknowledge for their contribution to the success of this project; Natalie Dillon, Deb Gultzow, Louise Hucks, David Innes, and Toni Newman. We also acknowledge the following international colleagues for their guidance and provision of unpublished data: Fred Gmitter

(University of Florida), Mike Rouse (University of California) and Qiang Xu (Huazhong Agricultural University, Wuhan). We are indebted to Patricia Barkley for promptly alerting us to the publication of Wang (2017).

Budget Summary

Description	**Amount ($)**
PCR primers	208.56
Lab equipment	17.45
Lab reagents/consumables	561.55
AGRF genotyping service	609.95
Total:	1397.51

References

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

Kepiro JL and Roose ML. (2009). AFLP markers closely linked to a major gene essential for nucellar embryony (apomixis) in *Citrus maxima* x *Poncirus trifoliata*. Tree Genetics and Genomes 6:1-11.

Mestre PF, Asíns MJ, Carbonell EA and Navarro L. (1997). New gene(s) involved in the resistance of *Poncirus trifoliata*(L.) Raf. to *Citrus tristeza virus*. Theor Appl Genet 95:691-695.

Nakano M, Shimada T, Endo T, Fujii H, Nesumi H, Kita M, Ebina M, Shimizu T, Omura M. (2012). Characterization of genomic sequence showing strong association with polyembryony among diverse *Citrus* species and cultivars, and its synteny with *Vitis* and *Populus*. Plant Sci 183: 131–142.

Ohta S, Endo T, Shimada T, Fujii H, Shimizu T, Kita M, Kuniga T, Yoshioka T, Nesumi H, Yoshida T, 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 Genet Genomes 11: 797.

Ohta S, Endo T, Shimada T, Fujii H, Shimizu T, Kuniga T, Yoshioka T, Nesumi H, Yoshida T, Omura M. (2011). PCR primers for marker assisted backcrossing to introduce a CTV resistance gene from Poncirus trifoliata (L.) Raf. into Citrus. J Japan Soc Hort Sci 80: 295–307.

Roose, M. L. (1996). Rootstock breeding at the University of California, Riverside. Proceedings of the International Society for Citriculture 2: 1254.

Smith MW, Newman TK, Gultzow DL, Parfitt SC, Barkley (Broadbent) P. (2017) Citrus tristeza virus replication and movement in seedling trees of 71 rootstock genotypes. Citrus Research and Technology. accepted.

Wang X, Xu Y, Zhang S, Cao L, Huang Y, Cheng J, Wu G, Tian S, Chen C, Liu Y, Yu H, Yang X, Lan H, Wang N, Wang L, Xu J, Jiang X, Xie Z, Tan M, Larkin RM, Chen LL, Ma BG, Ruan Y, Deng X, Xu Q. (2017). Genomic analyses of primitive, wild and cultivated citrus provide insights into asexual reproduction. Nat Genet 49(5):765-772.

Yokomi RK, Selvaraj V, Maheshwari Y, Saponari M, Giampetruzzi A, Chiumenti M, Hajeri S. (2017). Identification and Characterization of *Citrus tristeza virus* Isolates Breaking Resistance in Trifoliate Orange in California. Phytopathology 107(7):901-908.

Appendix

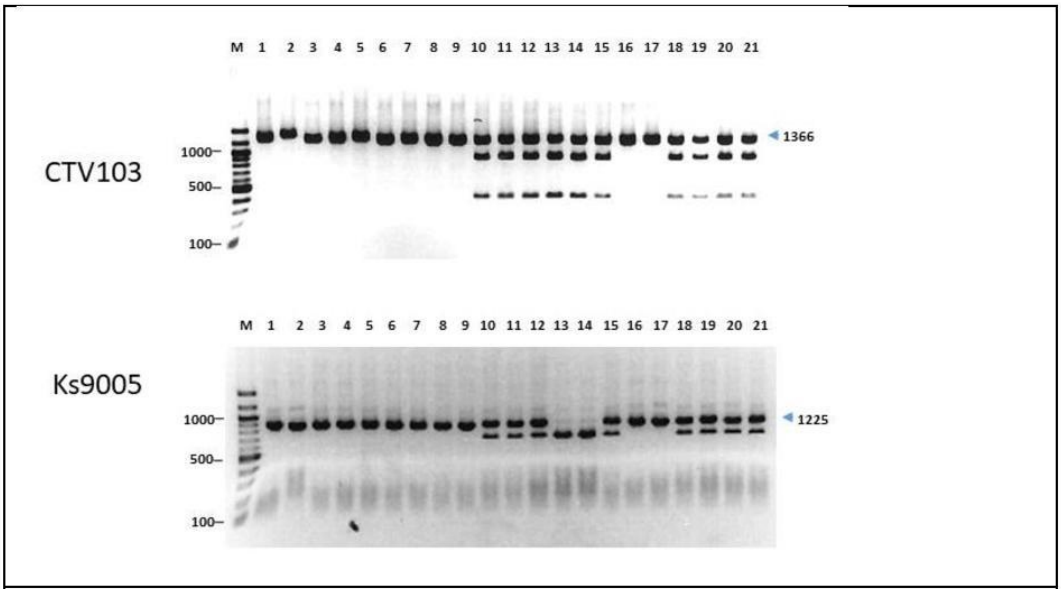


Figure 1. – Co-dominant CAPS markers associated with CTV resistance. Sample numbers correspond to the extract numbers detailed in Table 1. Marker names and the sizes (in base pairs) of major bands in the DNA marker (M) are shown on the left. Expected sizes of the undigested PCR products are marked with a blue arrow.

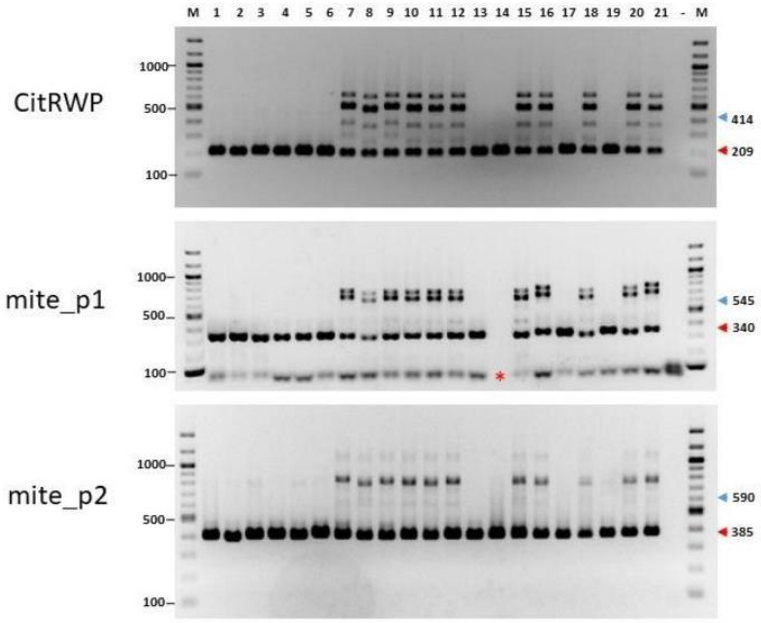


Figure 2. – Detection of MITE insertion events associated with apomixis/polyembryony. Sample numbers correspond to the extract numbers detailed in Table 1. PCR primer pair names and the sizes (in base pairs) of major bands in the DNA marker (M) are shown on the left. Expected sizes of PCR products that do not contain a MITE insertion are marked with a red arrow. Expected sizes of PCR products that contain a MITE insertion are marked with a blue arrow. PCR products containing a MITE insertion typically migrate slower than expected, perhaps due to DNA secondary structure (Qiang Xu – *pers. comm.*). Missing data is marked with a red asterisk.

Table 2. – Apomixis/polyembryony SSR genotypes of *Citrus*, *Poncirus* and *Citrus* x *Poncirus* hybrids.

Sample Name	Marker	Allele 1	Allele 2	Size 1	Size 2
CIT001	cf-ag06	190	190	190.86	190.86
CIT004	cf-ag06	190	192	190.7	192.6
CIT005	cf-ag06	190	190	190.76	190.76
CIT007	cf-ag06	190	190	190.83	190.83
CIT009	cf-ag06	190	190	190.76	190.76
CIT011	cf-ag06	200	200	200	200
CIT013	cf-ag06	198	200	198.09	200
CIT016	cf-ag06	184	198	184.76	198.09
CIT017	cf-ag06	184	186	184.84	186.76
CIT019	cf-ag06	198	198	198.11	198.11
CIT001	cf-ag07	264	264	264.35	264.35
CIT004	cf-ag07	264	278	264.17	277.62
CIT005	cf-ag07	264	264	264.35	264.35
CIT007	cf-ag07	264	278	264.36	277.76
CIT009	cf-ag07	264	278	264.31	277.73
CIT011	cf-ag07	264	264	264.35	264.35
CIT013	cf-ag07	264	270	264.06	269.89
CIT016	cf-ag07	254	256	254.48	256.37
CIT017	cf-ag07	254	256	254.46	256.5
CIT019	cf-ag07	272	272	271.9	271.9
CIT001	ji-tc04	226	232	226.39	232.32
CIT004	ji-tc04	226	232	226.37	232.3
CIT005	ji-tc04	232	232	232.33	232.33
CIT007	ji-tc04	226	232	226.35	232.34
CIT009	ji-tc04	226	226	226.31	226.31
CIT011	ji-tc04	206	226	206.78	226.34
CIT013	ji-tc04	202	206	202.67	206.61
CIT016	ji-tc04	236	242	236.22	242.2
CIT017	ji-tc04	232	240	232.33	240.34
CIT019	ji-tc04	202	226	202.82	226.3

Assessment of molecular markers for improving citrus selections, Department of Agriculture and Fisheries, 2017

Sample Names CIT001 – CIT019 correspond to extract numbers 1-19 in Table 1. Adjusted allele sizes and raw data sizes are in base pairs (bp).

Table 3. – Primer details.

Description	Primer Name	Primer Sequence	Product size (bp)
Ohta *et al* 2015.CTV resistance primers	Ks9005_RsaI_f	GAGGCTGGGGAGAGATT	1225
	Ks9005_RsaI_r	GAAATTATTTTTGGCACCCT	1225
	CTV103_RsaI_f	CCCAAGAATTCATTTTTAGAG	1366
	CTV103_RsaI_r	GCCCTGAGAGGAACTTC	1366
Ohta *et al* 2011.CTV resistance primers	CTV102R_topless_F	GCAGACGGGTTGATACGACAACG	370
	CTV102R_topless_R	GCAAATTCCAACTGGGAGGACAC	370
Wang *et al* 2017.*Citrus* Apomixis / polyembryony primers	CitRWP_R_65_7	CGGTCTCTTAACTGCCTCTCGCGCCT	209/414
	CitRWP_F_66_6	TGGCCCTAGGATCYGCCATGCAWAACTGA	209/414
	mite_p1_R	GTACCGAATTACCMCCCATAA	340/545
	mite_p1_F	GTAGGATTTGGGTTATTGATG	340/545
	mite_p2_R	TCTGGTTCATTGAGAATCCGCG	385/590
	mite_p2_F	ATCATGTGGGTCATGGTAC	385/590
Roose (unpublished). *Poncirus* apomixis / polyembryony SSR primers	cf-ag07_F	GAGGAACTTGAATGGGCTGA	258
	cf-ag07_R	AAATACGGAAGCGAATGGTG	258
	ji-tc04_F2	GCTGACACGCACACWTTCAT	228
	ji-tc04_R	ATGATCGAAAATCTGACGGC	228
	cf-ag06_F	TGTTTTGCTTTGTGCATGGT	182
	cf-ag06_R	ACCATGCAAGGAGTTTCCAC	182
Nakano *et al* 2012. *Citrus* apomixis / Polyembryony SNP primers.	067sgc05_F	GCATTTGAATTCTTGCAACG	574
	067sgc05_R	GAAGAGATTAGAATGGGAGCC	574
	048F02T_F	CAACAAGACCCAAGTAATAAGCAC	470
	048F02T_R	CTTCGTTTAGTAGAGCATCACC	470
	002F02T_F	AAGGCTGTTAATAGTGGCGA	546
	002F02T_R	CTGAATCAAATCCATAGGTGTC	546
	dg17_F	TCTTTCACCATCTTTCTCCT	810
	dg17_R	GGGTCATTTAGTCTCAACTCTC	810

Application of a MITE *Citrus* apomixis marker in the Australian rootstock breeding program

M.W. Smith[1a], M. Webb[1], D.L. Gultzow[1], T.K. Newman[1], D. Innes[1], N. Dillon[1], J. Owen-Turner[2], Q. Xu[3]

[1]Department of Agriculture and Fisheries, Queensland, Australia; [2]Queensland Citrus Improvement Scheme Inc., Australia; [3]Huazhong Agricultural University, China.

Abstract

Australia's citrus breeding efforts are small by international standards, and unashamedly focused on conventional approaches. Molecular markers had not yet been used in the program because they failed to meet our four essential criteria of being: linked to a trait of economic significance; technically difficult to phenotype by conventional methods; temporally difficult to phenotype by conventional methods and; likely controlled by a simple genetic mechanism. This situation changed dramatically with the 2017 publication of a miniature inverted-repeat transposable element (MITE) marker that co-segregated with the *Citrus* apomixis trait. This met all of the above four criteria and was quickly verified on local germplasm. Application of this MITE marker is now a standard screening procedure in our rootstock breeding research. An extensive network of field rootstock trials is used to identify parents for rootstock breeding, and the resulting segregating populations are nursery-screened within 18 months of sowing for tolerance to phytophthora, resistance to CTV, and salt exclusion using conventional screening techniques. Hybrids that survive this screening are then assessed for apomixis using the MITE marker. Monoembryonic and polyembryonic hybrids are both useful for future breeding, but those with apomixis have more immediate commercial application. Consequently, putative apomictic hybrids are propagated in greater numbers (via cuttings) to maximise replication and data precision in rootstock field trials. Use of the MITE marker has enabled maximum replication of putative apomictic hybrids, dramatically reducing the size and cost of field trials and hastened the establishment of seed-source trees. We consider it to be the first useful molecular marker in citrus breeding.

Keywords: clonal rootstocks, segregation, selection intensity, nucellar,

INTRODUCTION

Citrus is the most important fruit crop in the world, and has attracted a concerted international research effort to develop molecular markers that will aid breeding. Long generation times (5-15 years) and large plant size (~500 trees/ha) obstruct genetic progress but also make citrus an ideal candidate for the deployment of markers. There is the potential to identify useful hybrids during the seedling stage, more than 10 years before they would normally display the trait of interest. Despite this potential and significant research investment by the world's largest citrus growing nations, very few useful molecular markers have emerged and been routinely employed in breeding programs.

Some citrus genotypes have the capacity to produce clonal offspring via seed (apomixis). This trait has been extremely valuable in rootstock breeding because it enables new hybrids to be commercially deployed as seed. However, citrus has a long juvenile period (5-15 years) so breeders must wait until fruit is produced before they can examine the seed and determine which new hybrids will come true-to-type. For some breeding programs this means that there is a ~7 year hiatus between the production of hybrids and the next step of establishing field trials, in order to ensure that resources are only spent assessing the rootstock performance of hybrids that are apomictic. Other breeding programs proceed directly to field trials (via cuttings or tissue culture) but with the risk that outstanding rootstocks may turn out to be non-apomictic and less

attractive to commercial nurseries. While citrus can be easily propagated from cuttings or tissue-culture, and some minor rootstocks are already propagated in this way, industry experience shows a strong commercial preference for apomictic genotypes (Roose, 1996) from which genetically uniform high-health-status rootstocks can be propagated at low-cost and with low-technology. Attempts to develop molecular markers for this trait have met with mixed success (Kepiro and Roose, 2010), perhaps compounded by the more recent understanding that the apomixis trait inherited from *Poncirus trifoliata* is less commercially useful than when the apomixis trait is acquired from the *Citrus* genus. This realisation prompted us to cease using *P. trifoliata* as a paternal breeding parent, and instead search for alternative genotypes that possess the useful traits of *P. trifoliata* [such as *citrus tristeza virus* (CTV) resistance] but also contain the *Citrus* source of apomixis. While this overcomes the need to use apomictic seed parents, and the difficulties of distinguishing hybrid from nucellar seedlings (Kepiro and Roose, 2007), it does not solve the problem of identifying which hybrids possess the apomixis trait.

A small research project was conducted in 2016-17 to identify and validate published molecular markers (for seedlessness, CTV resistance and apomixis) that may have application within Australian citrus breeding activities (Webb and Smith, 2017). During this project a new marker system linked to *Citrus* apomixis was published (Wang et al., 2017) and it was quickly incorporated into our testing. Here we report on the validation of this marker system and how it is being applied in our rootstock breeding program.

MATERIALS AND METHODS

Cultivars, hybrids and DNA extraction

An initial test population of 21 cultivars representing a diverse range of genotypes used in the Department of Agriculture and Fisheries, Queensland breeding program were selected for analysis. Each cultivar had previously been phenotyped for apomixis/polyembryony using conventional methods including seed embryo morphology, seedling number production, and progeny-testing. DNA was extracted from plant leaves using a commercial kit (QIAGEN) for subsequent marker assays. DNA quality and quantity was assessed via spectrophotometry, electrophoresis and PCR amplification with quality control primers (data not shown).

Following successful validation, a second population consisting of advanced hybrids from the rootstock breeding program were genotyped for apomixis using the MITE marker. These advanced hybrids were too young to have been phenotyped for apomixis but their pedigree data was used to hypothesise the existence and segregation of the *Citrus* apomixis gene within these families.

Apomixis/Polyembryony markers

The two published primer sets of Wang et al. (2017), namely 'mite_p1' and 'mite_p2', were used on all available DNA extracts as codominant markers for the detection of the MITE insertion. An additional primer set 'CitRWP', designed to account for possible sequence variation within this region, was similarly tested. Sequences for each of these primers are listed in Table 1. PCR conditions for 'mite_p1' and 'mite_p2' primer pairs were as described by Wang et al. (2017). PCR conditions for the 'CitRWP' primer pair consisted of 95˚C for 1 min, followed by 35 cycles of 15 s at 95˚C, 20 s at 63˚C, 10 s at 52˚C and 80 s at 72˚C, followed by a final extension of 3 min at 72˚C. Products were subsequently visualised via agarose gel electrophoresis with Midori Green stain.

Table 1. Primer details for three primer sets used to identify a MITE insert in citrus germplasm (based on Wang et al. 2017). Expected product sizes are given for the absence/presence of the MITE insertion.

Primer name	Primer sequence	Product size (bp)
CitRWP_R_65_7	CGGTCTCTTAACTGCCTCTCGCGCCT	209/414
CitRWP_F_66_6	TGGCCCTAGGATCYGCCATGCAWAACTGA	209/414
mite_p1_R	GTACCGAATTACCMCCCATAA	340/545
mite_p1_F	GTAGGATTTGGGTTATTGATG	340/545
mite_p2_R	TCTGGTTCATTGAGAATCCGCG	385/590

mite_p2_F	ATCATGTGGGTCATGGTAC	385/590

RESULTS

Test population

Our investigations revealed that the presence of a MITE sequence ('A' allele) generally correlated well with a polyembryonic phenotype in a diverse range of citrus species and hybrids. Table 2 indicates the origins of each of these 21 cultivars, their known apomictic/non-apomictic phenotype, and their MITE genotype. The MITE marker was particularly accurate in discriminating within *Citrus*-based cultivars (Extracts 1-9), with the notable exception of the sour orange hybrid '11Q003'. This hybrid was found to contain a copy of the 'A' allele but possesses a monoembryonic phenotype, and has been confirmed as non-apomictic by progeny testing using both *P. trifoliata* and *C. reticulata*. The marker was more problematic in predicting an apomictic phenotype in *Poncirus*-based cultivars, reinforcing the view that this trait has a different genetic mechanism in *Citrus* and *Poncirus*. The results confirm that *P. trifoliata* (Extracts 13-14) does not possess the MITE insert, and that most *Poncirus*-hybrids used as commercial rootstocks (Extracts 10-21) have acquired the apomixis trait from their *Citrus* parent (and possibly also from their *Poncirus* parent). A notable exception is 'Swingle' citrumelo (Extract 19) which does not possess the MITE insert but is known to be apomictic. Other researchers have noted this discrepancy with 'Swingle' citrumelo in their validation of these markers (P. Aleza, pers. commun., 2017).

Table 2. Test population of 21 cultivars used to relate embryony phenotype to MITE insert genotype

Extract No.[1]	Type	Cultivar	Phenotype	MITE genotype
1	mandarin	01C011	non-apomictic	aa
2	wild lime	09Q005	non-apomictic	aa
3	kumquat	14Q021	non-apomictic	aa
4	mandarin	Daisy	non-apomictic	aa
5	mandarin	Ellendale	non-apomictic	aa
6	mandarin	IM111	non-apomictic	aa
16	sour orange hybrid	11Q003	non-apomictic	Aa
17	pummelo	K15	non-apomictic	aa
7	mandarin	Fremont	apomictic	Aa
8	mandarin	Kara	apomictic	Aa
9	mandarin	Murcott	apomictic	Aa
10	Poncirus hybrid	3831	apomictic	Aa
11	Poncirus hybrid	14Q055	apomictic	Aa
12	Poncirus hybrid	63-199-49	apomictic	Aa
15	Poncirus hybrid	US119	apomictic	Aa
18	Poncirus hybrid	Benton	apomictic	Aa
19	Poncirus hybrid	Swingle	apomictic	aa
20	Poncirus hybrid	Troyer	apomictic	Aa
21	Poncirus hybrid	US812	apomictic	Aa
13	Poncirus	FlyingDragon	apomictic	aa
14	Poncirus	QldTri	apomictic	aa

1. Extract No. corresponds with gel lanes in Figure 1

Figure 1 shows agarose electrophoresis results for this test population for each of the three primer sets. Similar results were obtained with all three primer sets and 'CitRWP' was chosen for future analysis when analysing large numbers of samples.

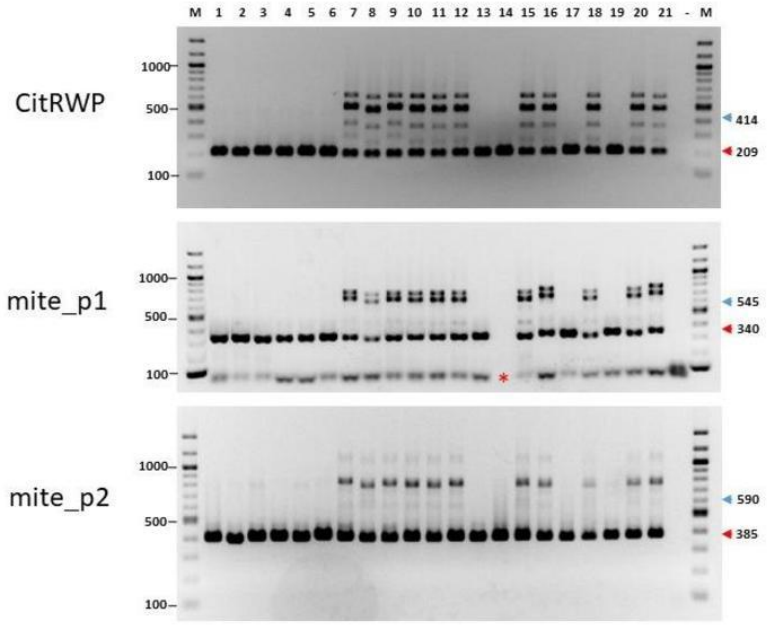


Figure 1. Detection of MITE insertion events associated with apomixis/polyembryony. Sample numbers correspond to the extract numbers detailed in Table 2. PCR primer pair names and the sizes (in base pairs) of major bands in the DNA marker (M) are shown on the left. Expected sizes of PCR products that do not contain a MITE insertion are marked with an arrow at 209, 340 or 385. Expected sizes of PCR products that contain a MITE insertion are marked with an arrow at 414, 545 or 590. PCR products containing a MITE insertion typically migrate slower than expected, perhaps due to DNA secondary structure. Missing data is marked with an asterisk.

Hybrid population

Table 3 provides subsequent genotyping results for 79 hybrids from four families in our rootstock breeding program. The two pollen parents ('63-199-49' and '14Q055') are *Poncirus*-hybrids that carry the *Citrus* MITE insert and are known to be polyembryonic. The three seed parents are monoembryonic and do not contain the *Citrus* MITE insert. 'K15' is a pummelo (*C. maxima*), 'Encore' is a mandarin (*C. reticulata*) and 'ICA5' is a complex hybrid of *C. wakonai*, *C. australasica* and *P. trifoliata*. The results indicate that inheritance of the MITE marker did not deviate from an expected 1:1 ratio at a statistically significant level ($p<0.05$) when assessed with a Chi-square (χ^2) test. However, it is noted that the observed frequency of heterozygous progeny did appear to be low when '63-199-49' was used as a pollen parent.

Table 3. Segregation for a MITE apomixis marker in four families from the Australian rootstock breeding program.

Seed parent (aa)	Pollen parent (Aa)	No. hybrids	MITE segregation		χ^2 test (p value)
			aa	Aa	
K15	63-199-49	27	18	9	0.08
K15	14Q055	21	8	13	0.28
Encore	14Q055	15	9	6	0.44
ICA5	14Q055	16	8	8	1.00
Total		79	43	36	0.43

DISCUSSION

In this investigation, we sought to identify a co-dominant molecular marker that could allow for early detection of apomictic/polyembryonic individuals, and to examine how such a marker may be segregating in our existing rootstock breeding program. Moreover, we wanted to develop a system to incorporate this technology in a way that may improve breeding efficiency and maximise genetic gain.

Our investigations support the hypothesis that insertion of a MITE sequence upstream of the CitRWP gene is associated with ovule-specific gene expression and concomitant apomixis/polyembryony in many citrus varieties (Wang *et al* 2017). Markers linked to this MITE sequence appear to have application across much of the germplasm currently employed in the Australian rootstock breeding program. The genotyping for this marker support the notion that apomixis in *Citrus* and *Poncirus* are under separate genetic control, and endorse our previous decision to move away from *P. trifoliata* as a pollen parent in rootstock breeding. This strategy has only been made possible by the identification of *Poncirus*-hybrids with a level of CTV resistance equivalent to that of their *P. trifoliata* donor parent (Smith et al., 2017). The current study has shown that the MITE marker is segregating in our advanced populations, with hybrids acquiring the MITE insert from their paternal parent. It is thus possible to transmit both CTV resistance and apomixis from the paternal parent and use monoembryonic seed parents so that large segregating populations can be readily generated.

The capacity to efficiently generate large segregating populations that contain genes for both CTV resistance and apomixis, creates the opportunity to apply high selection intensity for a number of other traits that are considered necessary in commercial citrus rootstocks. This high selection intensity for multiple traits is likely to significantly improve the rate of genetic gain. As an example, the first two families in Table 3 ('K15' × '63-199-49' and 'K15' × '14Q055') are the last remaining 48 hybrids from an original population of 556 seedlings germinated in 2015. These original seedlings were screened for CTV resistance and phytophthora tolerance by which time the surviving 118 individuals were old enough [>12 months, (Sykes, 1985)] to be validly assessed for salt tolerance. Forty-eight hybrids were identified as having good salt tolerance and of these 22 are putative apomicts (Table 3). Sykes (2010) showed that apomictic genotypes propagated either as cuttings or seedlings gave identical results in terms of rootstock performance. Consequently, our 22 putative apomicts have been vegetatively propagated for field trials, while source-trees have been established for future seed supply (and phenotype confirmation). Thus it has taken little more than three years to reduce the breeding population by 96% and establish field trials with genotypes likely to have good tolerance to CTV, phytophthora and salt, and that can be propagated by seed.

CONCLUSION

The new MITE marker for apomixis (Wang et al. 2017) is the first citrus molecular marker to meet all four of our essential criteria:

- ☑ A trait of economic significance
- ☑ Technically difficult to phenotype by conventional methods
- ☑ Time consuming to phenotype by conventional methods
- ☑ Likely controlled by a simple genetic mechanism.

This marker has shown a close association with the apomixis trait in a range of breeding material, and is segregating in advanced rootstock breeding populations. Marker testing has been readily incorporated into our breeding system and already reduced the size and expense of field trials. Use of the marker enables breeders to select parents prior to phenotyping and thus shortens the breeding cycle. It also resolves the dilemma posed by Kepiro and Roose, (2007) in which breeders never attempt certain crosses because of a lack of nucellar embryony, and circumvents the need to breed rootstocks that must be vegetatively propagated. Our breeding strategy of having paternal parents with high levels of CTV resistance and *Citrus* apomixis genes makes it possible to efficiently generate large segregating families from diverse germplasm, apply high selection intensity for a range of commercially important traits, and then use the MITE marker to identify apomictic hybrids for vegetative propagation and inclusion in field trials.

ACKNOWLEDGEMENTS

We acknowledge the assistance of Louise Hucks and Patricia Barkley. Marker validation was made possible through funds from an Agri-Science Queensland Innovation Opportunity grant. Rootstock breeding and evaluation has been funded by Hort Innovation through a Voluntary Contribution Project with co-investment from Horticulture & Forestry Science Royalty Funds and the Queensland Citrus Improvement Scheme Inc. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Literature cited

Kepiro, J.L., and Roose, M.L. (2007). Nucellar embryony. In Citrus Genetics, Breeding and Biotechnology, I. Khan ed (Wallingford, UK: CAB International), p. 141-149.

Kepiro, J.L., and Roose, M.L. (2010). AFLP markers closely linked to a major gene essential for nucellar embryony (apomixis) in Citrus maxima x Poncirus trifoliata. Tree Genet Genomes *6*, 1-11.

Roose, M.L. (1996). Rootstock breeding at the University of California, Riverside. Proc. of the Int. Soc. for Citriculture *2*, 1254.

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 Res. and Technol. *37*, 156-164.

Sykes, S.R. (1985). Effects of seedling age and size on chloride accumulation by juvenile citrus seedlings treated with sodium chloride under glasshouse conditions. Australian J. of Exp. Agric. *25*, 943-953.

Sykes, S.R. (2010). The effect of nucellar seedling and micropropagated clonal rootstocks on the performance of Imperial and Sunset mandarins. Proc. of the Int. Soc. for Citriculture *1*, 44-49.

Wang, X., Xu, Y., Zhang, S., Cao, L., Huang, Y., Cheng, J., Wu, G., Tian, S., Chen, C., Liu, Y., *et al.* (2017). Genomic analyses of primitive, wild and cultivated citrus provide insights into asexual reproduction. Nat. Genet. *49*, 765-772.

Webb, M., and Smith, M.W. (2017). Assessment of molecular markers for improving citrus selections. In Agri-Science Queensland Innovation Opportunity (Brisbane, Australia: Queensland Department of Agriculture & Fisheries), pp. 14.

Additional project related activity: D. K15 pomelo rootstock trial

This activity was not include in the original project document but was later incorporated without additional budget demands. A rootstock trial had been established in North Queensland in January 2012. This trial was designed by the breeding team and the rootstocks grown and budded at BRS. It was planted by the then DAF horticulturist Matt Weinert, who was eager to develop better rootstocks for pomelo in northern Australia. The current NQ horticulturist Yan Diczbalis was similarly eager to assess the trees and gleam any useful information that the established trees might yield.

The project leader travelled to Mareeba (north Queensland) 11th-14th May 2015 to assess a rootstock experiment in conjunction with NQ colleagues. We had propagated the trees for this trial at BRS in 2010 (Figure 26) and they were subsequently planted near Mareeba in January 2012.

Figure 26: Trees of K15 pomelo grafted onto nine different rootstocks at BRS and ready for dispatch to the north Queensland commercial test site, December 2011..

This experiment contains nine rootstocks carefully selected for their potential to be commercially appropriate for pomelo production in the tropics. The rootstocks were Benton, Troyer, Swingle, Volkameriana, US812, C22, C54, C57 and C146. There had never been any rootstock work done with pomelo in Australia, and very little internationally, so any information gleamed from this trial was likely to be of commercial interest.
The trial site is a commercial lime and pomelo enterprise with a long-term commitment to citrus production in tropical Australia. Soil conditions were severe, with a high clay content seldom considered suitable for citrus. None-the-less, two of the rootstocks have performed well while the other seven have done extremely poorly (Figure 27).

Figure 27: Part of the K15 pomelo rootstock experiment , 12th May 2015.

It is unusual to see such a dramatic effect of rootstock, particularly when many of the poor performers are important commercial rootstocks in other parts of Australia and overseas. The rootstock 'Swingle' has proven outstanding at this site and the collaborating grower is now using this rootstock for all their new plantings. It is a clear recommendation for pomelo grown under similar conditions.

Additional project related activity: E. Rootstocks for new scion varieties

Most of the field experiments established by the project used 'Imperial' mandarin as the scion variety because of its many fruit quality problems and as a 'canary' to identify rootstock that could improve scion fruit quality. Toward the end of the project the breeding team and Project Management Committee became interested in testing some of the emerging rootstocks on a wider range of scions. This resulted in a number of rootstocks being included in the commercial test sites used for the final evaluation of hybrids from the Mandarin Hybridisation Project (Figure 28).

Figure 28: Newly planted rootstock trials designed to test the performance of soon-to-be-released scions varieties on some of the recently developed rootstock germplasm. Gayndah 22nd August 2017.

A total of 17 rootstocks are being evaluated with four different scions (12C006, 12C009, 13C006, 15C001) (see Table 1). The first results from these trials will become available in 2019.

Outputs

The original project document stated that "Each of the five experiments in this project will generate clear outputs that are easily measured and have a specified delivery date. In many cases, preliminary information of potential commercial value will become available before the output is complete, and any such information will be communicated as soon as it is discovered." The project was successful in delivering on all of these 14 promised outputs and evidence to support this is provided against each of them below:

Experiment One

1. Best rootstock choice for 'Imperial' amongst 34 different varieties based on their fruit quality (including granulation) during the first 6 years of fruiting. Described rootstocks to include the Chinese ACIAR material, endemic species, US812 and existing commercial rootstocks.

Delivered: December 2013

Compilation of an extensive amount of data collected for a long and continuous run of seasons provided a clear "winner" worthy of commercial release. The project team provided growers with a summary of the performance of each rootstock and then allowed them to wonder freely through the trial site prior to harvest in 2017 (where all rootstock treatments had been labelled). This combined activity of providing performance data alongside first-hand inspection of the trial site provided a very convincing case to support the relative merits of each rootstock. As a result, the new rootstock 'Barkley' has been entered into the national citrus seed supply scheme (AusCitrus). Not only does it offer an additional choice for citrus growers, but it greatly expands the genetic diversity of commercial rootstocks available in Australia, being from a distinctly different genetic origin.

2. Tree health, growth, union compatibility and expected longevity of 'Imperial' on 34 different rootstocks after 10 years in the field.

Delivered: February 2015

Annual data collection continued during the project period so that a full 10 seasons of fruit production, quality and tree growth/development was captured. Australian horticulture increasingly sufferes from research data sets (and associated conclusions!) based on just a couple of seasons results collected from young trees. Such information can be very misleading because it does not reflect commercial reality. As an example, the rootstock 'Swingle' performed very well in the early years but was a disaster and mostly dead by the end of the experiment due to delayed graft incompatibility. This long run of data has already been useful in guiding rootstock breeding activities.

3. Interpretation of seasonal variation, tree location, past history, and rootstock effects on granulation during the first 10 years of fruiting.

Delivered: June 2016

The large data set provided an opportunity to examine some aspects of granulation, being unique in terms of its spatial and temporal coverage. Preliminary examination further reinforce the enormous complexity of the granulation issue and the need for a solid scientific effort to help resolved this, and other related, quality issues. This data set is likely to remain the most complete information on spatial, temporal and rootstock effects on granulation and is likely to useful in

future research on this topic, including the testing of various hypotheses.

Experiment Two

4. CTV movement and replication in 70 different rootstock varieties. Identify which of these are truly resistant.

Delivered: June 2014

The project was able to locate a few genotypes (just 4) that are truly resistant to virus replication, and at a level at least equivalent to *Poncirus trifoliata*. These new virus-resistant genotypes have already been incorporated into the breeding program and the heritability of their resistance confirmed. The work has emphasised the value of a project that combines phenotyping, commercial evaluation, and breeding because it has been confirmed that replication-resistant genotypes can transfer this trait to their progeny whereas replication-susceptible genotypes do not give rise to any resistant offspring. Many international rootstock breeding programs continue to use parents that our phenotyping research has shown are not truly resistant to this important virus, and our breeding research has shown that these cannot produce resistant progeny. These results have been published and have attracted international interest.

5. Determine any impact of initial freedom from CTV on 'Imperial' grown on 49 different rootstocks.

Delivered: ongoing, final May 2018

This experiment confirmed that strains of CTV currently present in Australia are only an issue for 'Imperial' growers in terms of rootstock choice. Having 'Imperial' budwood that is initially free of CTV offers no advantage, partly because trees become rapidly infected by aphids once they are planted in the field, but mostly because 'Imperial' mandarin is a symptomless carrier of the virus.

Experiment Three

6. Fruit quality and tree performance of 'Imperial' grown on 24 non-commercialised rootstocks bred in NSW and California in the 1950-60s. Make commercial recommendations based on the first 5 years of fruiting.

Delivered: 2015 onwards, final May 2018

Four seasons of fruit quality data were generated from this particular experiment during the project period (the first light crop of fruit in 2014 was removed by the grower to allow proper tree development) along with extensive tree performance information. This data revealed one very promising rootstock which has already been supplied (from the seed source tree at BRS) to the collaborating grower who has agreed to test it on a wider commercial scale. This rootstock is also now being tested more widely in other BRS-based citrus research to test if it warrants wider commercial release. Furthermore, the experiment has confirmed the adequate performance of a rootstock that has recently attracted commercial interest ('C22') and the disastrous performance of 'X639' which has been promoted by some individuals but is clearly unsuitable for 'Imperial'. All of this new knowledge has important commercial value and has been generated within the life of the project. It provides a basis on which to make preliminary commercial recommendations and is knowledge that the Australian industry is now utilising via publications such as the Mandarin Production Manual (Hardy et al 2018) and presentations at Technical Forum.

7. Fruit quality and tree survival of 'Imperial' grown on 27 full-sib families during the first 6 years of growth. Comparison of variability between full-sib families and nucellar rootstocks. Recover shoots of the best individual trees from the best performing families and establish as seed-source trees.

Delivered: ongoing, final May 2018

A complete set of performance data has been collected annual (2013-2018 inclusive) during the project period for the 27 full-sib families. This field performance data follows on from the nursery performance data collected during the nursery phase for these same genotypes (a published outcome of Experiment One). Although the experiment is designed with 3-tree plots, the data collection process ensured that information was collected for every individual tree, recognising that they were genetically distinct siblings rather than clonal nucellar seedlings. A few more seasons of data are required before this robust data set (generated from individual measurements on 892 trees) can be used to compare variance between nucellar and full-sib rootstocks. Although no individuals have as yet been identified within the full-sib families that warrant establishment as seed-source trees (via shoot recovery), the project team have discovered potential new sources of vigour control. Crosses of these exciting new full-sib families have been repeated in recent annual spring pollinations and hope to be explored further in a future project. This illustrates the value of exploring a broad range of parents in rootstock breeding and the high heritability of important traits within particular families.

8. Recommend new rootstock varieties for inclusion as seed source trees, in anticipation of future commercial demand.

Delivered: May 2015

Preliminary data from this experiment points to one particularly promising new rootstock. Three mature source trees of this genotype already exist at BRS and were sufficient to supply a few thousand seeds for wider scale testing by the collaborating grower. Data generated over the next few seasons will determine whether further seed source trees should be established. This follows the system established with the launch of the new 'Barkley' rootstock, which had now been propagated for commercial seed production by AusCitrus.

Experiment Four

9. Identify new CTV resistant P. trifoliata hybrids that give significantly improved 'Imperial' fruit quality, in the first 3 seasons of cropping. Seed supplied to nurserymen for semi-commercial testing.

Delivered: 2017 onwards, final May 2018

Fruit quality testing for this experiment has not yet identified any individual hybrids that completely solve the major problem of granulation. Although seed is now available from many of these hybrids, we now know that these genotypes have low levels of apomixes because of their *P. trifoliata* donor parent. Although there has been no

10. Identify the relative merit of 27 different parents, combined with P. trifoliata, in terms of improved fruit quality when used as rootstocks. Recommend best parental combinations for future rootstock breeding.

Delivered: 2016 onwards, final May 2018

Three parent have been identified that appear to have superior merit in terms of improved fruit quality when their progeny are used as rootstocks. Rootstock effects on fruit quality can be difficult to phenotype (large seasonal and special variation) so a few more seasons of data are required before we can be confident with this finding. None-the-less this information was incorporated into the 2016 and 2017 pollination program so that large families could be generated for future testing. In addition, the project has identified parents with differing effects on scion vigour and this information was also incorporated into recent crossing program. Thus this experiment has generated useful new knowledge useful in identifying the best parental combinations for future rootstock breeding. This moves rootstock breeding from "what we want to achieve" to "how we are going to achieve it" and creates opportunities for more purposeful and efficient breeding activity.

Experiment Five

11. Nursery performance data of 'extreme hybrids' and their early survival under field conditions.

Delivered: 2014 onwards, final May 2018

Many citrus relatives are "reluctant-participants" in breeding, defying the conventional wisdom that citrus relatives are readily cross-compatible. They flower inconsistently, produce limited pollen/seed, and set poorly. Subsequently any progeny that are obtained often suffer a range of malodies from failure-to-germinate to late-acting-lethality. A common problem has been 'extreme hybrids' that grow well in the nursery but then die-back to ground level before re-shooting. All of this nursery performance data has been recorded for the various wide crosses that have been attempted. Our pragmatic approach has been to discard any genotypes that are like to create problems during the nursery phase. Despite this heavy culling for nursery performance, and the similarly heavy culling based on CTV resistance phenotyping, a large number of 'extreme hybrids' have made it through this process ready for field testing. These hybrids have shown a wide range of performance in terms of early survival under field conditions. High rates of tree death in the field experiment (planted >>>) are clearly associated with particular genotypes. Conversely, some of the 'extreme hybrids' have established very well, at least on par with conventional rootstocks, and these crosses are now being repeated. There is wide segregation within families with some genotypes performing poorly while their siblings are outstanding. This demonstrates the value of a conventional approach to rootstock breeding where distinctly different parents are used to generate genetic diversity.

12. Fruit quality of 'Imperial' mandarin grown on a diverse range of 'extreme hybrids' as rootstocks. Recommend any that should be included as seed source trees in anticipation of future commercial demand.

Delivery: 2012 onwards, final May 2018

Having now identified 'extreme hybrids' with field establishment equivalent and better than conventional commercial rootstocks, the next challenge is to find some that significantly improve fruit quality. Depending on project continuance, this may occur in the 2019 fruiting season as trees in the Wallaville Imperial Experiment produce their first commercial crop. Early indications from the limited number of 'extreme hybrids' in the Gayndah Imperial Experiment (and the small number of trees that have already fruited in the recently planted Wallaville Imperial Experiment) are that granulation will remain a challenge. Although it is premature to recommend any for future commercial testing, it should be noted that five of the promising early hybrids have been included in new rootstock trials planted under four scions at a commercial site near Gayndah.

13. Develop CTV resistant hybrids incorporating diverse germplasm that has never previously been used in commercial citrus production.

Delivered: 2014 onwards, final May 2018

Introgression of CTV resistance is a corner-stone of the BRS rootstock breeding program. Not surprisingly, these efforts have continued by attempting to develop diverse germplasm that carries (and can transmit) this trait. The project has been successful in generating and phenotyping CTV-resistant hybrids with citrus relatives that have never been used in commercial citrus production. This has created new opportunities for increasing the genetic diversity employed in Australian citrus orchards. The development of *C. australasica* hybrids with CTV resistance warrants particular mention because some of these are showing excellent early tree establishment at an edaphically challenging commercial test site.

14. Test the heritability of CTV-resistance from sources other than P. trifoliata.

Delivered: June 2017

The project has confirmed that sources other than *P.trifoliata* are capable of transmitting CTV-resistance to their progeny. This knowledge is of major importance to the program because it has enable us to use better parents, rather than having to continually return to *P.trifoliata*. Indeed, the new breeding strategy is to never again use *P. trifoliata* in rootstock breeding. By using new sources of CTV-resistance it is now possible to capture additional traits, and to develop breeding populations in which genes of interest become fixed within the population. Having such advanced breeding populations puts the program in a strong position to tackle new challenges as they come along, and is why issues such as vigour control can now be addressed. Another important finding from the project is that rootstock genotypes that are tolerant of CTV diseases but not resistant to CTV replication, are not able to transmit CTV-resistance to their progeny. This has made it critically important for the BRS program to accurately phenotype all potential parents prior to use (see Experiment One) because some rootstock that people claim are resistant to CTV (e.g. Swingle,Troyer, US812) allow higher titres of the virus to accumulate. Our attempts to validate published molecular markers for CTV-resistance (Additional project related activity C) failed to find any convincing correlation between any of these markers and our phenotyping data (via bud inoculation and serological confirmation). Indeed biotechnological approaches to solving CTV have been a resounding failure despite major research invested by multiple international research groups over many decades. One such group, suggested this was because "…more than one gene in the locus may be involved in resistance to CTV or that the role of other loci was overlooked." And in attempting to explain why clonal propagations of the same transgenic line display highly variable response to CTV concluded that "…factors other than the genetic background of the transgenic plant may affect the resistance phenotype displayed by transgenic plant propagations." (Moreno et al. 2008). Perhaps not the first time that molecular biologists have failed to deliver on their promises of a quick fix. By contrast, future investment in conventional breeding approaches to introgessing new sources of CTV-resistance stand a good chance of generating useful industry outcomes at minimal expense.

Outcomes

The extensive (and potentially confusing) 14 project outputs listed above were all focused on generating one simple outcome: better rootstock choices that improve the commercial viability of Australian citrus growing.

Citrus growers are continually making new plantings, as markets dictate changing varieties or as orchards grow old and become too difficult to manage and become unproductive. By providing these growers with rootstocks that give better fruit quality, longer lived trees and improved disease resistance the project has already had a positive outcome on industry without any need to change grower behaviour (they are already going to be making new plantings).

Consequently, the outcomes of this project can be evaluated in terms of the changing demand for particular rootstock varieties. In the short term, the success of the project may be predicted by the relative improvement in performance of the new rootstocks compared to the current range of choices available to nurserymen and growers. Given the extensive range of advanced material already established in field trials, the scale of new hybrid generation and screening, and the extreme genetic diversity of the breeding work, it should come as no surprise that this project has already released one new rootstock 'Barkley' to Australian growers, has a second rootstock entering commercial production and has a range of diverse genotypes poised to deliver new genetics for the Australian citrus industry. As predicted in the original project document, this project has identify better rootstocks that have been quickly adopted by industry.

Monitoring and evaluation

Industry Adoption

The aim of this project was to give industry access to better rootstocks. Therefore the adoption target was nurseries and growers seeking this new genetic material. The extent to which newly identified rootstocks are used in the annual production/planting of nursery trees (estimated at ~100,000 in Queensland plus ~300,000 nationally) will be the best measure of industry adoption. Historically, there has often been a very long delay between when better rootstocks are identified and when they become important commercial rootstocks. This delay is often because of inadequate initial availability of planting material and poor communication links between research and commercial nurseries/orchards. BOTH of these problems were addressed in this project by having the organisation that supplies seed controlling the research agenda and information. The benefits of this linkage a clear, with one new rootstock already available to industry, a second rootstock being tested commercially, and a research program firmly focused on generating industry outcomes. 'Benton' as a rootstock for 'Imperial' was an example of rapid adoption of research results by growers. In this example it was about four years between when the research results were first communicated and when seed orders from nurseries started to increase. It was therefore anticipated that we might expect to see industry adoption toward the end of the project, and increasing thereafter. This has indeed been the case with demand for 'Barkley' seed exceeding supply, even though the rootstock was only launched in March 2017.

The close collaboration between DAF and QCIS was a success factor in the project, capturing complimentary expertise in areas such as orchard and nursery management, breeding, and business management. Locating major rootstock experiments on commercial orchards was also a success factor because of the economic reality it brings to the work. There was some risk of trial sites being terminated ahead of plan, but this never happened, and in any case there was sufficient flexibility within the project to adjust resources into other experiments and hence retain prospects for industry adoption. Knowing that new rootstocks are tested under local conditions and managed by commercial orchardists undoubtedly aids adoption, in addition to being able to view the trees themselves. Gaining access to germplasm remains one of the major impediments to breeding worldwide and can delay projects for many years. This obstacle had already been overcome in this project because the hard work of negotiation access has already occurred and much of the germplasm was already established in field trials.

Communication and publicity

The project has maintained an active communication and publicity profile (for examples the Publciations section of this report). In addition, there are three activities that warrant specific mention within the context of monitoring and evaluation, those being the Project Management Committee process, the 'Barkley' rootstock launch field day, and the International Society of Citrus Nurserymens Conference visit to inspect the project.

Project Management Committee

A major contributor to the success of this project has been the Project Management Committee (Figure 29), made up mostly of Queensland Citrus Improvement Scheme members. They have provided review and guidance on an annual basis and directed the breeding team toward issues that are of most relevance to the Australian citrus industry.

Figure 29: Members of the Project Management Committee meeting at BRS on the 8th October 2014 [Left to Right: Malcolm Smith (project leader), Wayne Parr, Alan Jenkins (president), John Owen-Turner (secretary), Nick Ulcoq, Peter Young, Troy Emmerton].

The Project Management Committee have met in each year of the project (23Jul2013, 8Oct2014, 13Oct2015, 25Oct2016, 19Oct2017) and been provided with a detailed update of project activities. Invariable this has been met with protracted and useful discussion that helped to shape future work. An example the PowerPoint presentation prepare for the final Project Management Committee meeting (19th October 2017) is shown below:

Department of Agriculture and Fisheries

Queensland Citrus Improvement Scheme: finding better rootstocks for Australia. CT13004

Update on project activities: 36th Annual General Meeting of the QCIS Inc.,
19th October 2017, Bundaberg Research Station

Hort Innovation

QLD CITRUS IMPROVEMENT SCHEME INC

Queensland Government

Today's presentation

- Agreed CT13004 activities and progress toward objectives
- Additional activities linked to and made possible by CT13004
- How it all fits together

Department of Agriculture and Fisheries

Project completion date: 31st May 2018

Five key experiments make up CT13004:

1. Gayndah 'Imperial' rootstock experiment
2. CTV replication and movement in advanced rootstocks
3. Emerald 'Imperial' rootstock experiment
4. Breeding new *P.trifoliata* hybrids with CTV resistance
5. The commercial value of "Extreme hybrids" as rootstocks

Department of Agriculture and Fisheries

1. Gayndah 'Imperial' rootstock experiment

Project Committee visit 12 months ago, wanted action.

Carefully managed politics and ego-stroking enabled a successful field-day.

Unanimous agreement to market the new rootstock as 'Barkley'.

Strong interest from local and interstate growers.

Source trees propagated by AusCitrus.

Department of Agriculture and Fisheries

1. Gayndah 'Imperial' rootstock experiment

CT13004 has identified a new rootstock (Barkley) that warrants wider commercial testing (regions/scions).

It is genetically unique from ALL other rootstocks currently used in Australia.

It is highly graft compatible, producing healthy long-lived trees that crop consistently with good quality fruit. It does not solve granulation.

2. CTV replication and movement in advanced rootstocks

Nursery-based experiment to test virus resistance of new rootstocks.
Gayndah experiment (1.) showed that CTV tolerance was critical for Australia.
71 rootstocks were tested by graft inoculation followed by serological testing.

Troyer, Swingle, C35, US812 are not resistant to CTV, contrary to popular belief.

They are okay for commercial use but not for breeding.

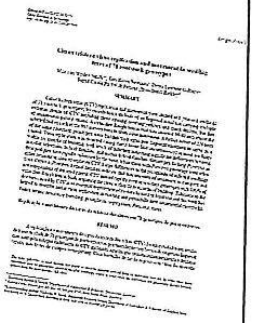
We found 3 rootstocks that are resistant. These are useful in our breeding of new rootstocks.

2. CTV replication and movement in advanced rootstocks

Thoroughly CTV tested rootstocks went into the field trial at Emerald (3.)

Found new parents for breeding CTV resistance...no long need to rely on *P.trifoliata*...time to "move on"!

Publication in the Brazilian journal 'Citrus Research & Technology', with due acknowledgement of CT13004 partners.



3. Emerald 'Imperial' rootstock experiment

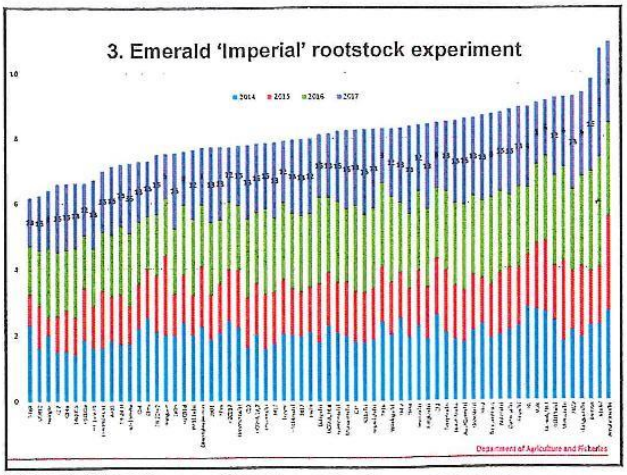
Feeling forgotten and ignored? .....so were many rootstocks.
Neglected Californian and NSW germplasm were given a chance to perform. Along with Bundaberg hybrids.

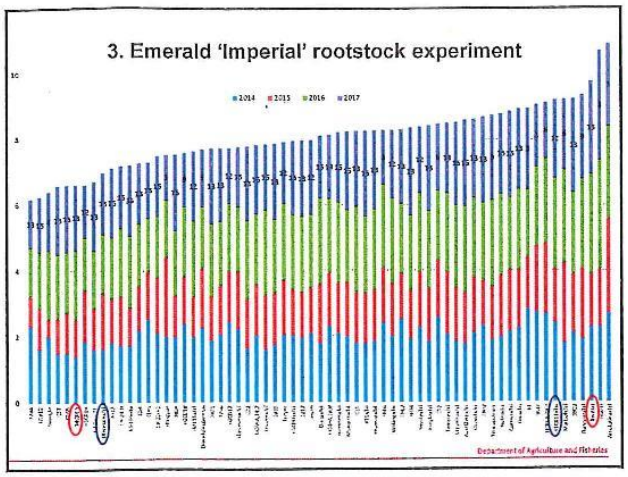
Total of 66 different rootstocks, 891 trees.

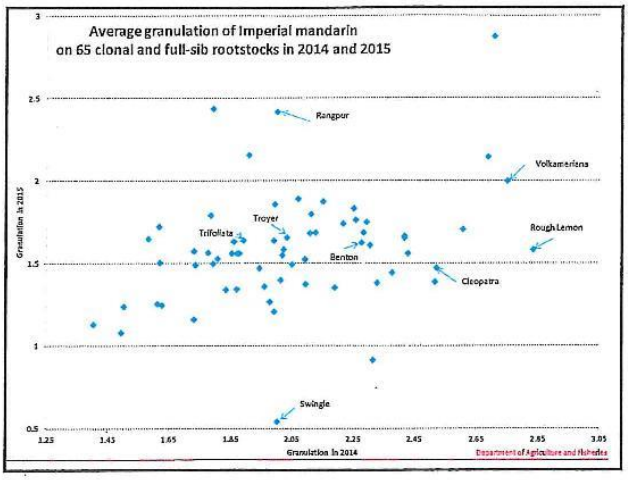
Planted 2011, fruit production and quality data collected in 2014, 2015, 2016 and 2017.

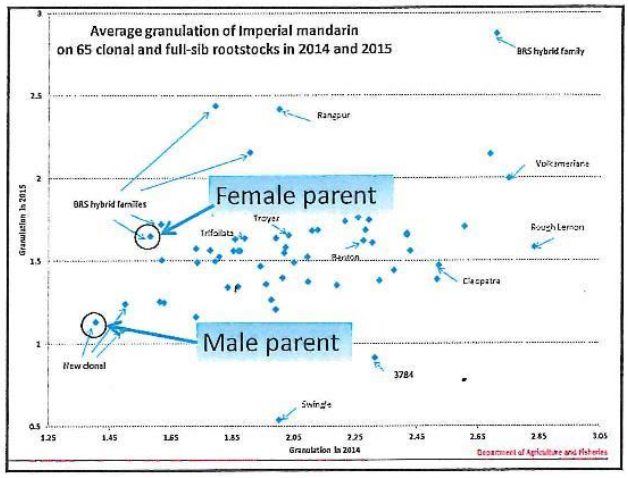
Find new commercial rootstocks AND identify the best parents for rootstock breeding.

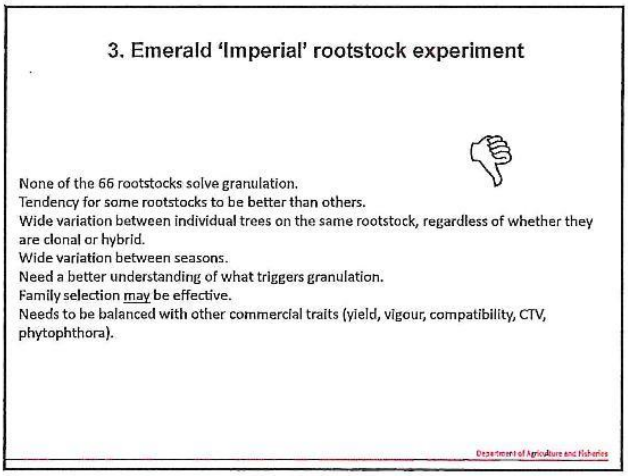
Hopes of discovering a rootstock that would solve granulation.

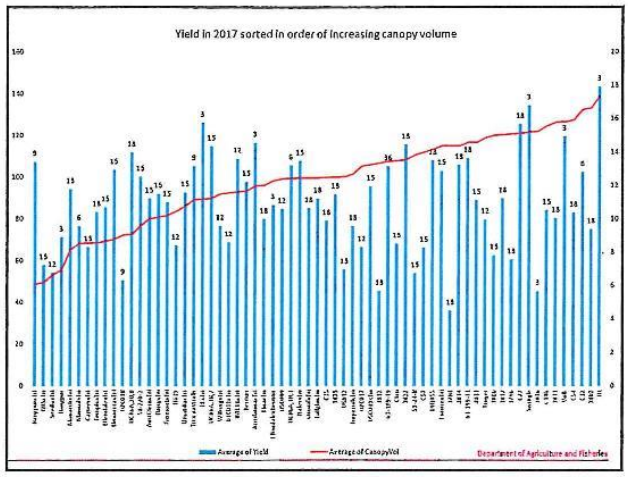

3. Emerald 'Imperial' rootstock experiment
2014
2015
2016
2017
Department of Agriculture and Fisheries

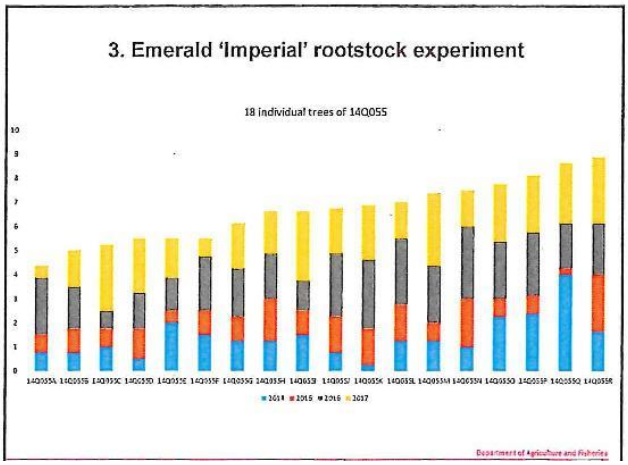

3. Emerald 'Imperial' rootstock experiment
2014
2015
2016
2017
Department of Agriculture and Fisheries

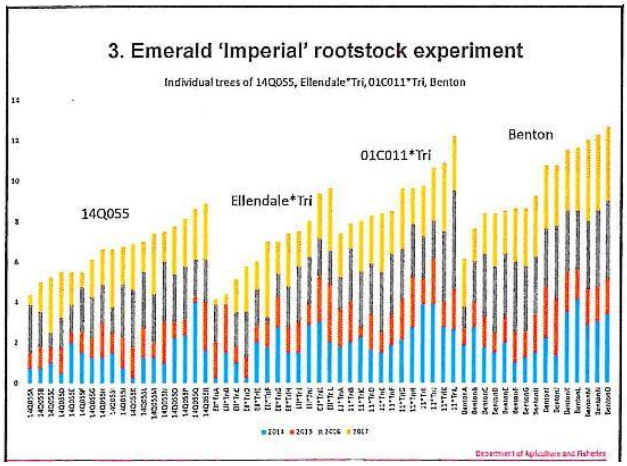

Average granulation of Imperial mandarin
on 65 clonal and full-sib rootstocks in 2014 and 2015
Rangpur
Volkameriana
Troyer
Trifoliata
Rough Lemon
Benton
Cleopatra
Swingle
Granulation in 2015
Granulation in 2014
Department of Agriculture and Fisheries


Average granulation of Imperial mandarin
on 65 clonal and full-sib rootstocks in 2014 and 2015
BRS hybrid family
Rangpur
Volkameriana
BRS hybrid families
Female parent
Troyer
Trifoliata
Rough Lemon
Benton
Cleopatra
Male parent
New clonal
3784
Swingle
Granulation in 2015
Granulation in 2014
Department of Agriculture and Fisheries


3. Emerald 'Imperial' rootstock experiment
None of the 66 rootstocks solve granulation.
Tendency for some rootstocks to be better than others.
Wide variation between individual trees on the same rootstock, regardless of whether they are clonal or hybrid.
Wide variation between seasons.
Need a better understanding of what triggers granulation.
Family selection may be effective.
Needs to be balanced with other commercial traits (yield, vigour, compatibility, CTV, phytophthora).
Department of Agriculture and Fisheries

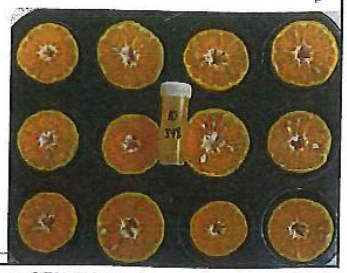

Yield in 2017 sorted in order of increasing canopy volume
Average of Yield
Average of CanopyVol
Department of Agriculture and Fisheries


3. Emerald 'Imperial' rootstock experiment
18 individual trees of 14Q055
2014
2015
2016
2017
Department of Agriculture and Fisheries


3. Emerald 'Imperial' rootstock experiment
Individual trees of 14Q055, Ellendale*Tri, 01C011*Tri, Benton
14Q055
Ellendale*Tri
01C011*Tri
Benton
2014
2015
2016
2017
Department of Agriculture and Fisheries

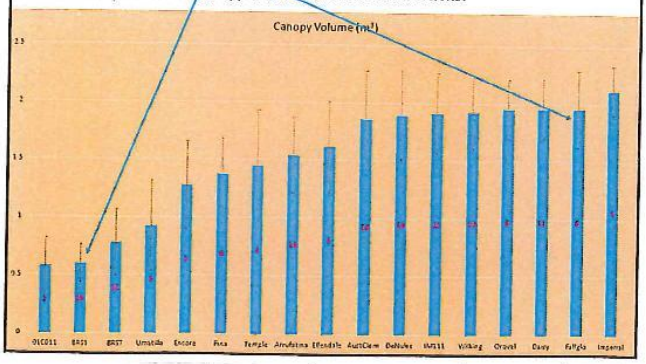
4. Breeding new P.trifoliata hybrids with CTV resistance

Field trial near Gayndah airport, planted April 2013.
245 different rootstocks (227 BRS *P.trifoliata* hybrids), 490 trees.
Fruit assessments done in 2016 and 2017.
Good fruit quality, very little granulation so far.
Big differences in tree size and productivity.



4. Breeding new P.trifoliata hybrids with CTV resistance

Can we breed vigour-controlling rootstocks?
e.g. hybrids of Fallglo*P.trifoliata tend to be vigour-inducing rootstocks.
e.g. hybrids of BRS1*P.trifoliata tend to be dwarfing rootstocks.
Would the same thing happen with Murcott or Eureka scions?



4. Breeding new P.trifoliata hybrids with CTV resistance

Objective: Identify "good" parents so that rootstock breeding can target the desired trait(s).

Successful Method: Lots of different hybrids from lots of different parents.

"Vigour control" is the first trait where we have demonstrated a strong parental influence.

In the coming years it is expected that "good" parents for other traits will emerge from this trial site.

"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" (Bill Bitters 1986).

Rootstock breeding should be *deliberate* ....not *random*

Department of Agriculture and Fisheries

5. The commercial value of "extreme hybrids" as rootstocks

Useful traits can be "hidden" in seemingly useless germplasm.

Australia has many wild citrus relatives but they have never proven useful to citrus growers, (despite many attempts around the world over the last 100 years).

They were a disaster when used as rootstocks under Imperial at Gayndah in 2004.

Why?.....CTV.

We have bred these wild citrus to capture CTV resistance from *P.trifoliata*.

CT13004 enabled field testing of this unique germplasm.

5. The commercial value of "extreme hybrids" as rootstocks

Planted November 2016, Wallaville.
138 different rootstocks, 605 trees.
Screened for CTV and phytophthora.
Huge genetic diversity, including "extreme hybrids" of inter-generic and inter-specific crosses.

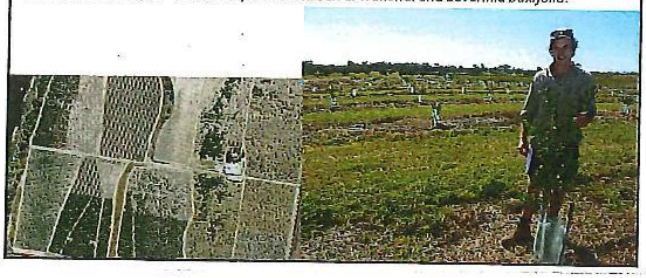
5. The commercial value of "extreme hybrids" as rootstocks

Good tree establishment on a challenging site (heavy soil, low-lying, frost, salt).

Clear differences between rootstocks even after just 6 months.

One of the best early performers is 'ICA12', an "extreme hybrid" with *C. australasica* (Fingerlime) parentage.

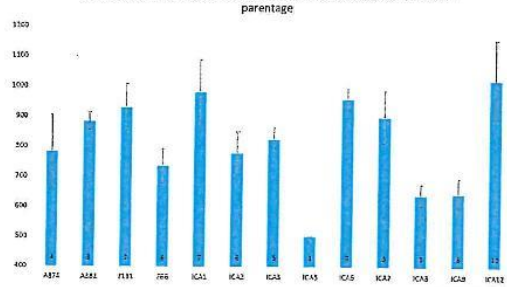
Another is 'Bux13', an "extreme hybrid" between *C. wakonai* and *Severinia buxifolia*.



5. The commercial value of "extreme hybrids" as rootstocks

Siblings (hybrids with the same parentage) do not all perform the same.
We need to find "good" parents and assess multiple siblings.

Tree Height of Imperial trees on 13 different rootstocks with *C.australasica* parentage



Department of Agriculture and Fisheries

Additional activities linked to and made possible by CT13004: Phytophthora screening

Not originally part of CT13004, but necessary to incorporate phytophthora screening into breeding program.

Simple screening technique developed.

"Harsh" technique, most hybrids fail.

Additional activities linked to and made possible by CT13004: Apomixis

Some citrus varieties produce zygotic seed (monoembryonic) and every seedling is **genetically different.**
e.g. Imperial, Daisy.

Other citrus varieties produce nucellar seed (polyembryonic) and every seedling is **identical** to the mother.
e.g. Murcott, Sweet orange, Troyer.

New rootstock varieties **should** be polyembryonic.
Convenient and commercially attractive.

Department of Agriculture and Fisheries

Additional activities linked to and made possible by CT13004: Apomixis

This trait is called Apomixis, and is controlled by just a few genes.
At least one parent must be apomictic.

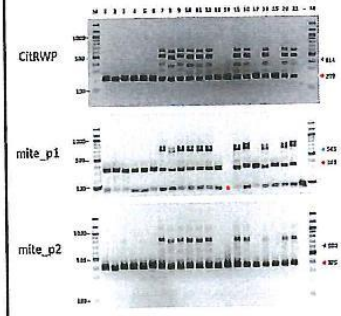
Some hybrids will be monoembryonic and some will be polyembryonic, have to wait ~7 years before seed is produced to check.

Breeders have been trying (without success) to find a molecular marker, so that polyembryonic hybrids could be identified at a much earlier stage.

DAFQ awarded an "Agri-Science Queensland Innovation Opportunity" 12-month project to test existing markers for **apomixis**, CTV and seedlessness
1. A trait of economic significance
2. Technically difficult to phenotype by conventional methods
3. Time consuming to phenotype by conventional methods
4. Likely controlled by a simple genetic mechanism.

Department of Agriculture and Fisheries

Additional activities linked to and made possible by CT13004: Apomixis



Accurate molecular marker for Apomixis !!

Additional activities linked to and made possible by CT13004: Salinity

Salinity problems in southern Australia largely solved?

Periodic salt issues in the Burnett River catchment remain.

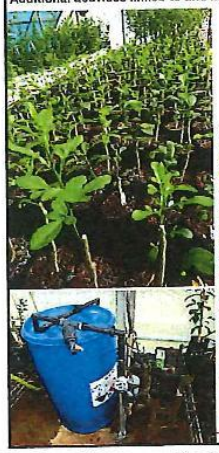
Some level of genetic tolerance essential.

Large body of existing knowledge.

Not part of CT13004 but an important commercial issue.

Department of Agriculture and Fisheries

Additional activities linked to and made possible by CT13004: Salinity



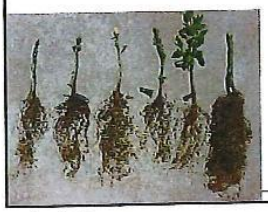
171 hybrids treated daily with salty water, for 17 weeks.

1.45g/L increasing to 11.6gl/L sodium chloride (seawater is ~35g/L)

Flying Dragon, Troyer and 14Q055 controls.
Water 21mS Leachate 27mS

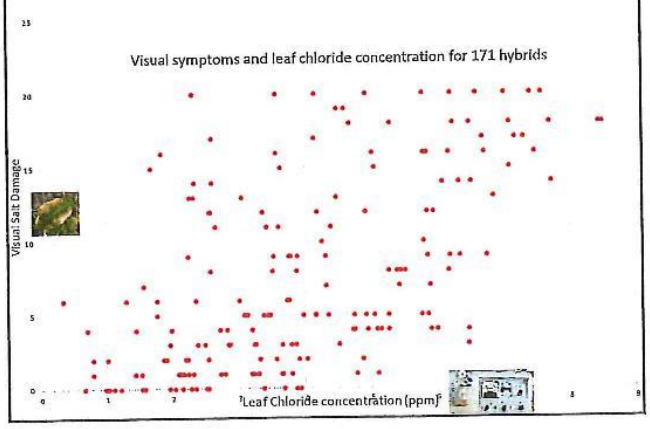
Additional activities linked to and made possible by CT13004: Salinity

1. Visual symptoms of salt damage
2. Leaf chloride concentration

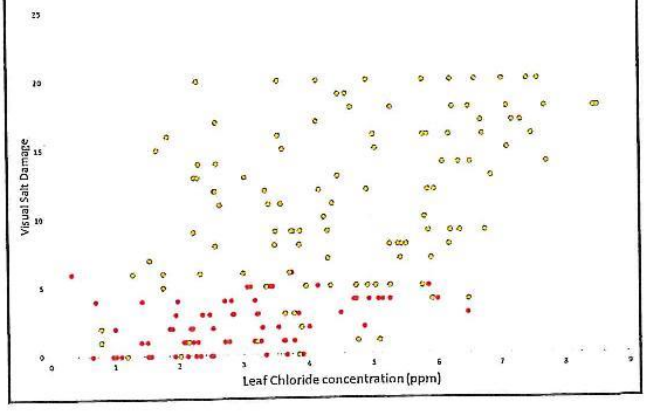


Additional activities linked to and made possible by CT13004: Salinity

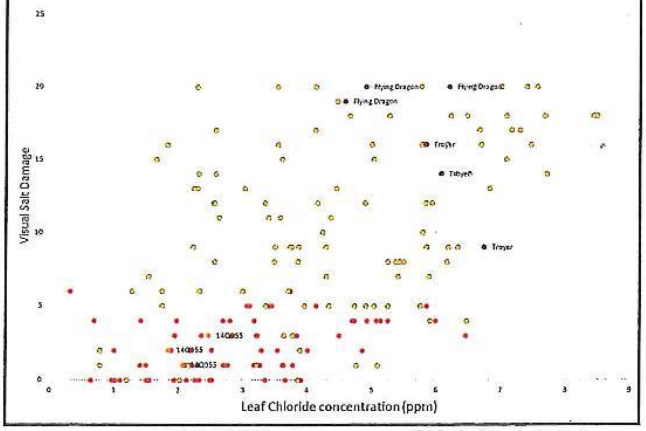
Visual symptoms and leaf chloride concentration for 171 hybrids



Additional activities linked to and made possible by CT13004: Salinity



Additional activities linked to and made possible by CT13004: **Salinity**



Additional activities linked to and made possible by CT13004: **non-Imperial scions**

Recent plantings from the Mandarin Hybridisation Project (CT15017), have incorporated a range of new and experimental rootstocks.

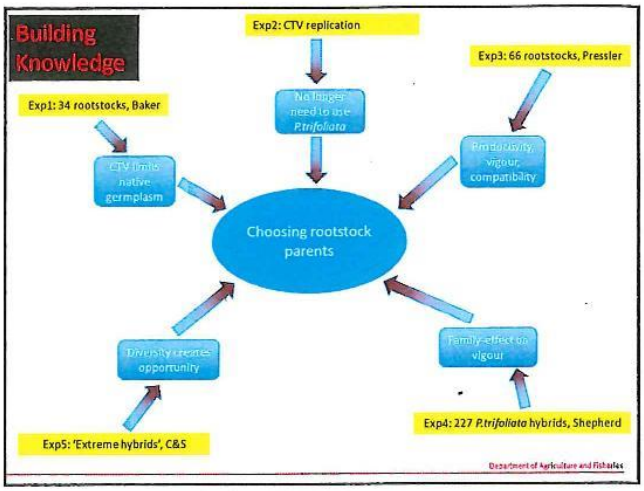
These have occurred at two sites in Mundubbera and one site near Gayndah.

Some of the rootstocks include: 3784, 3831, 3834, 3835, C35, C54, C146 14Q055, ICAS, E397, Benton, Fraser, Cox, CH414, GLA55 US812, UCR6A38/3 (62/103/1), and UCR6A38/8 (62/137/2).

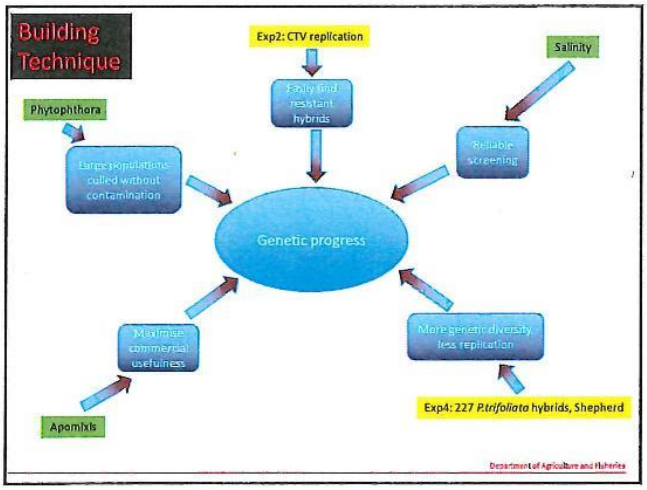
Additional activities linked to and made possible by CT13004: **publicity**

Barkley Field-day March 2017
Mildura Technical Forum presentation March 2017
Australian Citrus News March 2017
Australian Tree Crops Magazine April 2017
Good Fruit & Veg Magazine May 2017
National Variety Group update June 2017
International Society of Citrus Nurserymen July 2017
CQU lecture September 2017
TropAg Conference November 2017

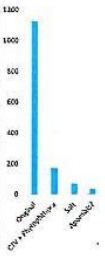
Building Knowledge



Building Technique



Family	Original hybrids	After phytophthora and CTV screening	After salt screening	After apomixis
Encore*14Q055	175	34	15	7?
Encore*63-199-49	30	15	3	2?
Encore*Tri	16	4	3	0?
K15*14Q055	556	49	21	11?
K15*63-199-49	346	69	28	14?
Total	**1,123**	**171**	**70**	34?



Rapid and heavy culling of a large population.
97% removed within 2 years of sowing.
Ability to tackle new issues using well-adapted populations.

CT13004 Project document states:

Outputs will include

- fruit quality data from large-scale field trials established under commercial conditions,
- local performance results for rootstock germplasm from national and international breeding programs,
- new virus-resistant *P. trifoliata* hybrids
- unique genotypes incorporating genes from distant genera,
- new hybrids that combine Australian endemic *Citrus* species with the necessary traits for commercial success, and
- genetic information on which parents give the best rootstock hybrids in terms of fruit quality.

CT13004 Project document states:

Outputs will include

- fruit quality data from large-scale field trials established under commercial conditions,
- local performance results for rootstock germplasm from national and international breeding programs,
- new virus-resistant *P. trifoliata* hybrids
- unique genotypes incorporating genes from distant genera,
- new hybrids that combine Australian endemic *Citrus* species with the necessary traits for commercial success, and
- genetic information on which parents give the best rootstock hybrids in terms of fruit quality.

THANKYOU

'Barkley' rootstock field day

At the instigation of the Project Management Committee, a field day was held to launch the new rootstock, named in honour of Patricia Barkley, one of Australias most distinguished citrus researchers (Figure 30). Details on the technical aspects of the field day are contained under Esperiment One above. The field day was a great success and generated significant positive publicity for the work.

Figure 30: (a) Mrs Patricia Barkley (Broadbent) addressing growers at the launch of the new rootstock 'Barkley' named in her honor, Gayndah 7th March 2017. She had introduced this germplasm from China in the 1980s in a collaborative project between NSWDPI and ACIAR. (b) During her visit to Queensland she reviewed many of the other project activities and trial sites and provided useful feedback.

International Society of Citrus Nurserymen

The International Society of Citrus Nurserymen held their conference in Australia during the project period, providing an excellent opportunity to showcase project work. The pre-conference tour was organised by Wayne Parr (Golden Grove Nursery) and the breeding team provide him with every assistance to ensure international delegates were exposed to extensive technical information. They visited two rootstock trial sites at Wallaville on the 20th of July 2017 and then spent the remainder of the day at BRS inspecting the breeding program. A hand-out was given to all delegates to explain the Wallaville experiments and this is attached below:

Handout for Delegates participating in the

International Society of Citrus Nurserymen pre-Conference Tour

20th July 2017, Rootstock Breeding and Evaluation

New rootstock are being bred by the **Queensland Department of Agriculture and Fisheries** and tested at nine commercial sites. These field trials contain more than 3,500 trees and almost 500 unique rootstock genotypes. The objective is to create rootstocks that improve scion fruit quality, but with preliminary breeding and screening to first ensure that new rootstocks are resistant to CTV, tolerant to phytophthora, graft compatible and with good field adaptation. The work is currently sponsored by local industry (**Queensland Citrus Improvement Scheme Inc.**) and IP royalties (**Queensland Department of Agriculture and Fisheries**) through a **Hort Innovation** project CT13004 *'Qld Citrus Improvement Scheme: finding better rootstocks for Australia'*. Two rootstock trials are planted on Spencer Ranch, Wallaville:

Imperial mandarin on 138 rootstocks

Planted November 2016, single-tree plots, clay to clay-loam, potential for frost and flooding.

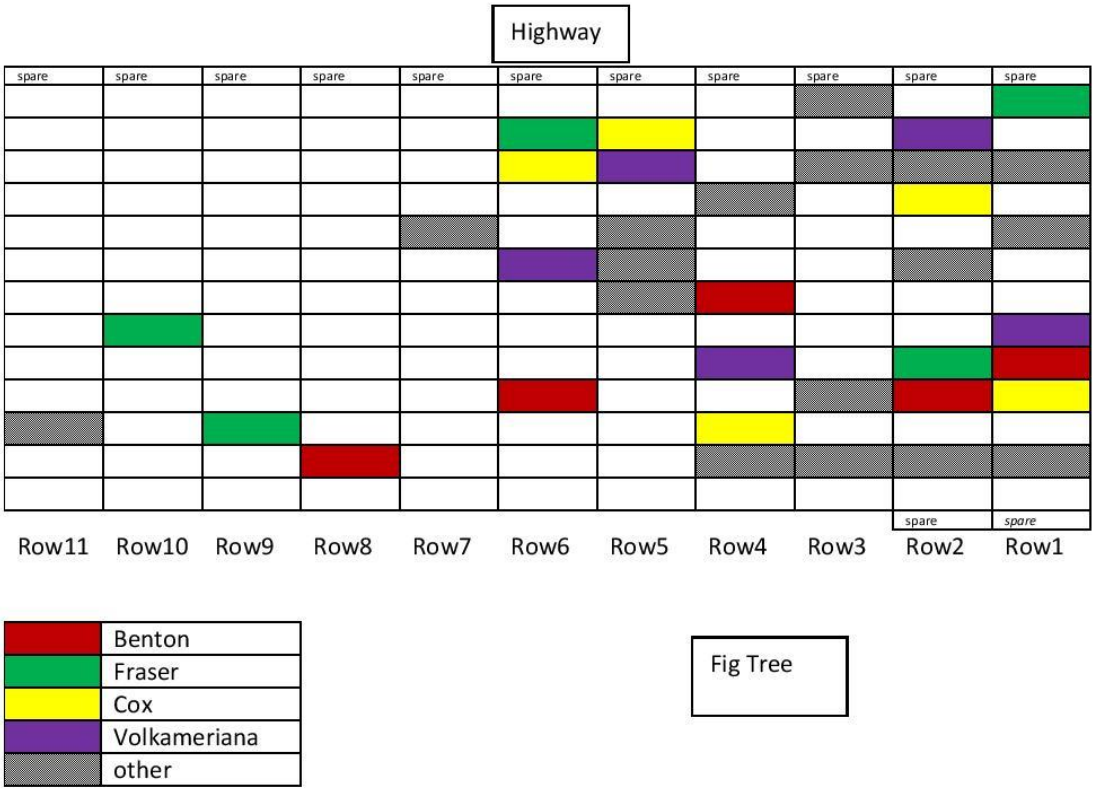
This trial contains an extremely high level of genetic diversity. Hybrids have been produced using the genera *Atalantia, Citropsis, Fortunella* and *Severinia*, as well as the usual *Citrus* and *Poncirus*, and cuttings then used to produce the rootstocks for this experiment. For example, there are 7 different hybrids of *Citrus wakonai* x *Severinia buxifolia* as well as 6 hybrids of *C. wakonai* x *Fortunella japonica* and 3 of *C. wakonai* x *Atalantia ceylanica*. *Clymenia polyandra*, *Hesperathusa crenulata* and *Swinglea glutinosa* are included as seedlings along with the control standards of Benton, Barkley, US812 and Rough lemon. The Australian citrus relatives (formerly *Microcitrus* and *Eremocitrus*) feature heavily in our breeding program as we attempt to address their disastrous performance as rootstocks in our original field trials planted >10 years ago. Using bridging species and capturing CTV resistance from *Poncirus* has enabled us to produce a range of new genetic material with Australian relatives in their parentage that will hopefully show much improved field adaptation. There are 32 different hybrids that have *Citrus glauca* (formerly *Eremocitrus glauca*) as a grandparent and 28 hybrids with *Citrus australasica* (formerly *Microcitrus australasica*) as a grandparent. These derive from much larger hybrid families that were heavily culled for CTV resistance and phytophthora tolerance.

The oral presentation we gave at ISCN Chongqing, China in 2008 showed how citrus relatives were performing disastrously in our field trials. Today, in less than 10 years, we can show that conventional breeding has reversed this situation. Hybrids with *Citrus australasica* and *Severinia buxifolia* are already showing excellent field establishment, and along with other citrus relatives may finally be poised to make a useful contribution to commercial citriculture.

Eureka lemon on 28 rootstocks

Planted December 2002, single-tree plots, sandy loam, well drained and frost free.

This experiment contains rootstocks used or recently available for Eureka lemon in Australia, namely Benton, Cox, Fraser and Volkameriana. As 15-year-old trees, the site provides some indication of their relative merit. Other rootstocks of interest included Troyer (normally considered incompatible), Cleopatra (which suddenly collapsed at about 2 years of age) and US119 (an experimental scion variety that has excellent CTV resistance and has performed surprisingly well). The other 21 rootstocks were mostly citrus relatives (*Fortunella, Micromelum, Clausena, Citropsis*) many of which died soon after planting, and have no commercial potential. The long term survival (15 years) of Eureka on *Citropsis schweinfurthii* and *Citropsis gilletiana* has been surprising, although these trees have remained small and unthrift.



Queensland Government

QLD CITRUS IMPROVEMENT SCHEME INC

Hort Innovation

Representatives were present from all citrus growing regions of the world, and most of them own nursery operations far larger than anything that could ever be imagined in Australia (Figure 31). None-the-less they were impressed by the project work and eager to learn more about it. There has been much follow-up seeking access to results and germplasm once it becomes commercialised. Contacts made during the visit were utilised by the project leader when he subsequently undertook a self-funded study tour to Tucuman Argentina and was able to visit the massive nursery operations of some of the delegates who had come to the ISCN Australian conference.

Figure 31: Wayne Parr (far LHS) ISCN Conference Tour Organiser, with some of the international delegates on the ISCN pre-conference tour to Queensland, inspecting a rootstock trial near Bundaberg, 20th July 2017.

Recommendations

It is recommended that the new rootstock 'Barkley' be trialed on a wider commercial scale and under a range of edaphic and management conditions. Additional new rootstock releases are anticipated in the next few years. Innovative opportunities now exist to explore new traits in the Australian rootstock breeding program such as reduced scion vigour and outstanding phytophthora resistance. There are exciting prospects for continued improvements to the Australian citrus industry through the breeding and development of better rootstocks.

Refereed scientific publications

Journal article

Smith, M. W., Newman, T. K., Gultzow, D. L., Parfitt, S. C., & Barkley, P. B. (2016). Citrus tristeza virus replication and movement in seedlings of 71 rootstock genotypes. *Citrus Research and Technology, 37*(2), 156-164.

Smith, M. W., M. Webb, D. Gultzow, T. Newman, D. Innes, N. Dillon, J. Owen-Turner and Q. Xu (2018). Application of a MITE Citrus apomixis marker in the Australian rootstock breeding program. *Acta Horticulturae* (accepted).

Whole book

Hardy, S., Barkley, P., Treeby, M., Smith, M., & Sanderson, G. (2017). *Australian mandarin production manual* (ISBN 978 1 76058 056 8): State of New South Wales 318pg.

Chapter in a book or Paper in conference proceedings

Smith, M. W. (2015). Mandarin breeding and commercialization in Australia. International Symposium on Strategy for Promoting Citrus Cultivar Improvement and Commercialization, Jeju, South Korea, The Korean Society for Citrus and Subtropical Climate Fruits. 129.

Smith, Malcolm Wesley, Matthew Webb, Debra Gultzow, Toni Newman, David Innes, Natalie Dillon, John Owen-Turner, and Qiang Xu. (2018) Application of a Mite Citrus Apomixis Marker in the Australian Rootstock Breeding Program.. *4th International Symposium on Citrus Biotechnology: Book of Abstracts* 244.

Smith, M.W. (2017) Improved rootstock for 'Imperial' mandarin. In: Damiani, J., et al. Citrus Technical 2017 Forum + Field Day. Citrus Australia Pty Ltd. Mildura.

Smith, M.W. et al. Traditional Breeding (Chapter 7) In: Talon, M., Caruso, M. and Gmitter, F. *The Genus Citrus* (ISBN 978 0 12812 163 4) Elsevier (due for publication October 2018) 720pg.

Oral presentations

Smith, M.W. (2013) Mandarin breeding & variety commercialisation. Citrus Australia Ltd Pre-season Workshop, Gayndah 12th March 2013. 20 slides.

Smith, M.W., Gultzow, D.L., Newman, T.K. (2013) Rootstock project development status. Mundubbera, 23rd July 2013. 6 slides.

Smith, M.W. (2014) Rootstock breeding. Citrus Australia Ltd Pre-season Workshop, Gayndah 21st March 2014. 15 slides.

Smith, M.W., Gultzow, D.L. and Newman, T.K. (2014) CT13004: Update on project activities. 33rd AGM of the Queensland Citrus Improvement Scheme Inc. Bundaberg, 8th October 2014. 19 slides.

Smith, M.W. (2015) Scion & rootstock breeding: Update on commercialisation of varieties. Citrus Australia Ltd Pre-season Workshop, Gayndah 24th February 2015. 17 slides.

Smith, M.W. (2015) Mandarin and rootstock breeding. National Citrus Technical Forum. Mildura, 17th March 2015. 27 slides.

Smith, M. W. (2015). HIA Ltd visit to Bundaberg Research Station. Bundaberg, 1-2 June 2015. 62 slides.

Smith, M.W., Gultzow, D.L. and Newman, T.K. (2015) CT13004: Update on project activities. 34th AGM of the Queensland Citrus Improvement Scheme Inc. Bundaberg, 13th October 2015. 18 slides.

Smith, M.W. (2015) Mandarin breeding & commercialisation in Australia. 1st International Citrus Breeders Workshop. Jeju, South Korea, 11th November 2015. (self-funded). 22 slides.

Smith, M.W. (2015) Identification and screening for disease resistance. Phytophthora mini-Conference. Brisbane, 11th December 2015. 19 slides.

Smith, M.W. (2016) Growing citrus in Queensland. Citrus Field Day Qld Regional Advisory Committee. Bundaberg, 16th June 2016. 20 slides.

Smith, M.W. (2016) Citrus breeding in Australia. 2nd International Citrus Breeders Workshop, Salto, Uruguay. 29th September 2016. (self-funded) 27 slides.

Smith, M.W., Gultzow, D.L. and Newman, T.K. (2016) CT13004: Update on project activities. 35th AGM of the Queensland Citrus Improvement Scheme Inc. Gayndah, 25th October 2016. 19 slides.

Smith, M.W. (2017) Improved rootstocks for 'Imperial' mandarin. National Citrus Technical Forum. Mildura, 2nd March 2017. 26 slides.

Smith, M.W. (2017) Citrus breeding in Australia. International Society of Citrus Nurserymen Pre-conference Tour. Bundaberg, 20th July 2017. 26 slides.

Smith, M.W. (2017) Research in agriculture. Central Queensland University Guest Lecture. Bundaberg, 25th September 2017. 72 slides.

Smith, M.W., Gultzow, D.L. and Newman, T.K. (2017) CT13004: Update on project activities. 36th AGM of the Queensland Citrus Improvement Scheme Inc. Bundaberg, 19th October 2017. 40 slides.

Smith M.W., Gultzow D.L., Newman T.K. Tran N.T. and Miles A.K. (2017) Impacts of plant breeding on the Australian mandarin industry. Session: Ensuring the health and growth of horticulture. Convenor Prof A. Drenth. TropAg Conference, Brisbane, 20th November 2017. 18 slides.

Smith, M.W. (2017) Citrus breeding and commercialization in Australia. 3rd International Citrus Breeders Workshop. Acireale, Italy. December 2017. (self-funded) 45 slides.

Smith, M.W., Webb, M., Gultzow, D.L., Newman, T.K., Innes, D., Dillon, N., Owen-Turner, J. and Xu, Q. (2018). Application of a MITE Citrus apomixis marker in the Australian rootstock breeding program.Las Brujas, Uruguay, 16th April 2018. (self-funded) 21 slides.

Smith, M.W. (2018) Citrus breeding in Australia. Estacion Experimental Agroindustrial "Obispo Colombres". Tucuman, Argentina, 25th April 2018. (self-funded). 33 slides.

References

CASTLE, W. S. 2010. A career perspective on citrus rootstocks, their development, and commercialization. *HortScience,* 45**,** 11-15.

PEDRO, M., SILVIA, A., R., A.-M. M., JOSÉ, G. & LEANDRO, P. 2008. Citrus tristeza virus: a pathogen that changed the course of the citrus industry. *Molecular Plant Pathology,* 9**,** 251-268.

SMITH, M. W., NEWMAN, T. K., GULTZOW, D. L., PARFITT, S. C. & BARKLEY, P. B. 2016. Citrus tristeza virus replication and movement in seedlings of 71 rootstock genotypes. *Citrus Research and Technology,* 37**,** 156-164.

SMITH, M. W., SHAW, R. G., CHAPMAN, J. C., OWEN-TURNER, J., LEE, L. S., MCRAE, K. B., JORGENSEN, K. R. & MUNGOMERY, W. V. 2004. Long-term performance of 'Ellendale' mandarin on seven commercial rootstocks in sub-tropical Australia. *Scientia Horticulturae,* 102**,** 75-89.

SYKES, S. 1985. A glasshouse screening procedure for identifying citrus hybrids which restrict chloride accumulation in shoot tissues. *Australian Journal of Agricultural Research,* 36**,** 779-789.

Intellectual property, commercialisation and confidentiality

This project generated information and germplasm of potential commercial value. It utilised existing IP much of it owned by DAFF Qld or licensed to DAFF Qld through Material Testing Agreements with third parties. Some of the material that has been evaluated is public domain and therefore has no commercial restrictions and if proved beneficial can be made freely available to the industry. Other material was included under testing agreements, so negotiations are required with the owners prior to commercial supply to industry. This was the case with the launch of the 'Barkley' rootstock which had been introduced to Australia through a joint project between NSWDPI and ACIAR in the 1980s. These negotiations were somewhat protacted but eventually resulted in an agreement whereby AusCitrus could propagate source trees for future seed sales to Australian growers. Details of all germplasm and any crosses made and their ownership was outlined at the start of the project and contained within Milestone 102. Background IP (including all DAFFQ owned IP) equity was also established in this report. Equity is determined by each party's project inputs and background IP contributions based on the information reported on in Milestone 102. More recently, NSWDPI and DAFQ have re-negotiated a germplasm exchange agreement original set in place in 2005, so that any promising genotypes can be incorporated into the breeding program. The original agreement prevented breeding, which undermined the value of any phenotyping activities conducted with this germplasm. The new agreement allows breeding to occur.

All material trialed on farm sites have been covered by Material Transfer (non-propagation) Agreements to ensure protection of the IP prior to commercialisation. These agreements have been established by DAFQ in collaboration with the participating industry partners. It is DAFQ policy that field trials cannot be established on non-DAFQ premises without a finalised MTA in place. All project activities have met this requirement.

Acknowledgements

The outstanding technical and intellectual inputs of **Debra Gultzow** and **Toni Newman** throughout the life of this project have made it possible for the project leader to meet, and exceed, the optimistic targets established at the outset. This small breeding team (3 people) have an industry-focused, hands-on approach that is fast disappearing from regional Australia. **John Owen-Turner** was a champion for the project long before it developed, having lobbied for many years within the Queensland Citrus Improvement Scheme Inc for their reserve funds to be allocated to rootstock breeding and research. The board members of QCIS, including **Alan Jenkins**, **Nick Ulcoq**, **Wayne Parr**, **Peter Young**, **Troy Emmerton** and **Dan Papacek** supported and guided the project, and at their own expense, willingly traveled long distances to participate in day-long meetings that were so critical to keeping the project on track. DAF staff including **Jodi Campbell**, **Rod Edmonds** and **Roger Broadley** are thanked for their enthusiastic support in development of the project proposal, and with the continuous and dependable involvement of Rod Edmonds, subsequent staff including **David Innes**, and **Jodi Huffer** have maintained this departmental commitment to the work. **Bruce Boucher** was instrumental in establishing better nursery facilities at BRS. **Alok Kumar** and **Brad Wells** from Hort Innovation were quick to recognise the merits of the project proposal and ensured it was supported as a Voluntary Contribution project and with sufficient run-time (5 years) to generate useful industry outcomes. **Vino Rajandran** has continued to provide this support.

Queensland citrus growers and business managers have made it possible to conduct a large and diverse rootstock improvement program by providing trial sites and covering all associated costs. They have never received or asked for payment, motivated only by the desire to find new technology that will improve the future viability of their industry. We particularly thank **Russell Baker**, **Ian, Judy and Zach Shepherd**, **Craig and Bindi Pressler**, **Will Thompson** and **Grant Schrader**, **Jose and Debbie Caamano**, and **Nick and Deb Ulcoq**.

Carola Parfitt is thanked for her involvement and efforts during the first few years of the work, including negotiating access to much needed nursery space from a nearby institution. Casual labour has been supplied by Jesse Thomas, Tim Caflisch, Saba Sinai-Mameghang, Daniel Hall, Joseph Loveday, Jessie Roberts, Shallan Herrig and Jane Rose. Yan Diczbalis and Matt Weinert are thanked for their efforts with the NQ pomelo trial. Advice from colleagues has been instrumental in some of the innovative ideas developed during the project, and we particularly thank Andrew Miles, Patricia Barkley, Mark Hoult, Graeme Sanderson, Matt Webb, Nerida Donovan, Andre Drenth, Jay Anderson, Andrew Manners, Kathy Grice, Tony Cooke, Joanne DeFaveri, Alison Kelly, Yu Pei Tan and Tim Herrmann for sharing their knowledge and experience.

NSW Department of Primary Industries and the University of California supplied some of the germplasm tested in this project and we thank Graeme Sanderson, Sandra Hardy, Myles Parker, Claire Federici and Mikeal Roose for their efforts.