Early-season replacement for Imperial mandarin

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Early-season replacement for Imperial mandarin

Malcolm W. Smith et al.

Queensland Department of Agriculture, Fisheries and Forestry

Project CT09014: Early-season replacement for Imperial mandarin

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The purpose of this report is to document the start of a mandarin breeding program aimed at producing new early season varieties. It describes the process and achievements of 5 years of hand-pollinations and the field progeny blocks that now exist as a result of these efforts. Detail of families attempted and generated, disease culling, nursery activities and size and nature of field blocks now established at Bundaberg Research Facility are provided, as a basis and reference for future evaluation and selection.

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May 2014

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Media Summary

The early-season mandarin market in Australia is dominated by Imperial mandarin, an old variety which originated at Emu Plains, NSW in 1890, and presumably SE Asia prior to that. Its early maturity time, ease of peeling, small size and low seed count have made it popular with generations of Australians, and underpinned marketing campaigns focussed on young families for lunch box fruit. Beyond Australia, this variety is of no commercial interest or significance, having poor colour, low Brix, low juice content and short post harvest life. Even within the Australian domestic market, recent fruit quality problems, notably granulation, threaten the future of Imperial as buyers look for alternatives available from overseas. Problems like granulation are exacerbated by periodic oversupply on the domestic market and no option for exports; because of both postharvest deterioration and lack of overseas consumer acceptance of the variety. Research is underway to resolve these quality problems with Imperial, but an additional option is to find new varieties for this early-season timeslot.

Horticultural managers with Queensland DAFF recognised the increasing need for better mandarin genetics in the early time-slot and in 2008 approached their citrus breeding team to commence a hybridisation program. This was quickly backed-up by HAL who co-invested in the breeding work described in this report. The inadequacy of the national citrus R&D levy is widely recognised and it is only through the willingness of the DAFF to invest royalty funds and with matched VC support from HAL that this long-term breeding activity was able to commence.

Controlled pollinations were conducted each flowering season from 2009 to 2013 using the best available parents from the extensive collections at Bundaberg Research Facility, with additional pollinations conducted at Kerikeri Research Station in 2010 aimed at capturing additional genetics not present in Australia. More than 18,000 hand pollinations were performed utilising 91 different mandarin parents. This resulted in close to 30,000 hybrid seed which were individually peeled and raised in the nursery facilities at Bundaberg Research Facility. A disease screening technique was implemented during the nursery phase and resulted in more than one third of the hybrids being culled prior to field planting. This will ensure that any new varieties selected from the program are genetically resistant to Alternaria Brown Spot, the most important citrus fungal disease in Australia.

This project has resulted in more than 15,000 new hybrid trees now growing in field at BRF. These plantings capture a diverse range of parents carefully chosen to maximise the chance of creating commercially desirable mandarins that mature early in the season.

Technical Summary

Imperial mandarin is the main variety grown for the early-season time-slot in Australia. It has been grown commercially in Queensland for more than 50 years, is well known to consumers, wholesalers and supermarkets, and orchardists continue to make new plantings of this important variety. Production of Queensland Imperials is over 2 million 9kg cartons annually, making it the largest single variety of mandarin. However there is significant season variation in production and during the life of this project this production ranged from 2.1 million cartons in 2013 to 2.9 million cartons in 2009. Despite this popularity Imperial has major faults that cause problems for growers, consumers, and at other points along the supply chain. It has major problems with granulation in some seasons which is causing increasing concern for the supermarket trade who are seeking consistent quality in all their fresh produce lines. The variety is also unsuitable for export having a soft thin skin that does not transport well, and besides this, the variety lacks many of the traits sought by most export markets. Indeed, the Imperial mandarin is 'marginal' for many important traits including juice content, sugar, and colour development. This problem with poor colour development may increase if the current upward trend in temperatures in the main production areas continues.

With these issues in mind, and in tandem with current research activities designed to resolve them, DAFF management recognised the need to commence a breeding program focussed on the early part of the mandarin season. This was motivated by the belief that an opportunity exists for a new early-season variety that does not granulate, develops strong external colour under warm autumn conditions, and that is suitable for sea freighting to overseas markets. Such a variety would protect the industry from consumer backlash (and import substitution) during seasons where granulation is severe, and may strengthen the early domestic market by shifting supply to export markets. DAFF invested its own royalty funds to make this happen, and the work was soon matched by HAL as a VC project. The resulting work is reported here.

All germplasm held in the collection at Bundaberg Research Facility was reviewed to identify accessions with useful traits for early-season breeding. Access was also gained to the collection held by Plant and Food NZ at Kerikeri, and all available data was examined. The breeding team met each July to formulate a crossing program and assigned priorities and target numbers. This program changed each season as new parents became available, more information on existing parents was developed, and results from previous pollinations (where available) was reviewed. The implementation of the crossing program was further influenced by climate/seasonal conditions causing changes in flowering intensity, flowering duration, pollen development and fruit set.

Over the five flowering seasons the number of pollinations ranged from 1,262 (2012) to 5,590 (2011) with a total of 18,528 individual pollinations using more than 90 different parents. While only 7% of these pollinations occurred at Kerikeri, it enabled the inclusion of an additional 13 parents not available in Australia.

Fruit set was highly variable, reflecting the wide range of parents chosen and the inclusion of 'difficult' accessions. The desire for seedlessness in mandarins creates huge obstacles for breeders since many parents carrying desirable traits have low fertility; poor/nil pollen production, seed abortion, and/or low seed numbers. None-the-less seedlessness was a major consideration in the crosses performed, such as one example where 370 pollinations resulted in 59 fruit with a total of seven seed from which only four trees made it to field planting. Fruit set averaged 18% but ranged from 8% in 2012, to 33% in 2010 (NZ).

Disease screening was a critical component of this program, with the objective that only hybrids resistant to EBS would be field planted. This was achieved thanks to an efficient inoculation and culling methodology implemented during the nursery phase by the project team. Identification of highly virulent single-spore isolates of the Alternaria Brown Spot (ABS) fungus, vigorous healthy nursery seedlings, and careful control of temperature and humidity were important components of this success. Information on segregation for disease resistance, and genotyping of parents were additional benefits from the work. Although the project generated 29,873 hybrid seed, little more than half of these survived through to field planting largely as a result of disease screening. Compensation for such a large loss of material, often from difficult crosses, will come from having future selections that are genetically resistant to this important disease.

The project has successfully established 15,138 hybrid mandarin trees in the field, with a final planting of 2013 pollinations planned for April 2015. This population is now available as a resource for the selection of superior early-season genotypes that may out-perform existing troublesome varieties and provide expanded commercial opportunities. The work to date has been conducted to ensure a high probability of future success by utilising the best parental material available and generating large progeny populations from which to make selections.

Chapter 1: Background

1.1 Project intent

The aim of this project was to develop large hybrid seedling populations using elite parent material, from which future early-season mandarin varieties could be selected. More particularly, we aimed to produce segregating populations with a high chance of genotypes that would overcome some of the significant problems encountered with Imperial mandarin, especially when grown in warm climates.

The strategy used superior hybrids already developed in the breeding work at Bundaberg Research Facility, as well as existing early-season commercial varieties, and elite material not currently present in Australia but available in New Zealand. We used assortative mating in a pollination program that incorporated parent and family information already gleamed from previous breeding work. Although only a small program, the strategy of targeting the early-season market window helped ensure that the breeding objectives and selection of parents were clearly focused.

Desired outputs of the project were segregating populations derived from elite parent material that had a high probability of generating superior early-season mandarin varieties. The intended outcome of the project was that the citrus industry would have more strategic breeding activity under way aimed at better early-season mandarin varieties. This increased breeding focus on the early time-slot offered the potential of overcoming significant limitations with Australia's most important mandarin variety (Imperial), such as bad granulation, unsuitability for export, and poor colour development in warm production areas.

1.2 Project context

Imperial mandarin is the dominant variety in the early-season production time-slot in Australia. It is well known to consumers, wholesalers and supermarkets, and growers continue to make new plantings of this variety. In 2008 there were 1,646 ha of Imperial mandarin planted in Australia, followed by Murcott at 1,479 ha, then Afourer at 283 ha (SunRISE21Inc. 2008). Queensland produced 49,000 tonnes of mandarins in 2012/13 (HAL 2014) of which Imperial is estimated to be slightly more than half.

Despite this popularity, Imperial has major faults that cause problems for growers, consumers, and at other points along the supply chain. Fruit from this variety can be severely granulated, and this is the main quality problem cited by consumers (McKinna 2003). Studies have been conducted to develop management strategies that reduce granulation in Imperial (Hofman and Smith 2008) though no solution has been found that completely solves the problem in all seasons. We have demonstrated that

granulation is a heritable trait, and it is a characteristic that we select against in current breeding work.

A second problem with Imperial is its unsuitability for export. It is a soft, thin-skinned variety that does not transport well. While small quantities of the variety have been exported in the past, this has mostly been via airfreight. The physical nature of the fruit precludes reliable long-distant sea transport. Even if these transport issues could be resolved, there is some doubt as to whether the variety would meet the expectations of consumers outside of Australia. It is very much a 'provincial' variety that has not attained any commercial significance (either in terms of production or consumption) outside of Australia.

Thirdly, the variety is 'marginal' in many characteristics. It has low juice and sugar content, and develops poor colour under warm conditions. There is evidence that temperatures are on a strong upward trend in the current production area and if this continues then there may be increasing problems with the production of quality Imperial fruit (Smith et al 2008). According to Saunt (2000), "…the colour is never better than yellowish-orange when grown in the Central Burnett District, Queensland. In the more southerly Murray River region with cooler autumn temperatures the colour is much improved."

Based on the above problems, we believe there is an opportunity for a new early-season variety that does not granulate, develops strong external colour under warm autumn conditions, and that is suitable for sea freighting to overseas markets. Such a variety would protect the industry from consumer backlash (and import substitution) during seasons where granulation is severe, and may strengthen the early domestic market by shifting supply to export markets.

1.3 Pre-existing breeding activity

A number of breeding activities were already underway prior to the commencement of this project, and these were carefully considered and reviewed prior to launching into this new breeding work.

Within the National Citrus Scion Breeding program (CT07000) crosses specifically aimed at early-season mandarins had been made. Details are provided in (Sykes and Smith 2007) pg. 16-19. The parents chosen for this work included Imperial, Clementines, and selections with Satsuma, Ellendale, Imperial, sweet orange and Clementine parentage. Progeny from these crosses were being evaluated at CSIRO Merbein, and the work had demonstrated the potential to generate extremely early maturing hybrids (e.g. Hybrid 91-03-04). However climatic conditions during autumn are far cooler in southern Australia and it is possible that selections made under such conditions will not colour sufficiently when grown in Queensland. Also, granulation

is not as severe a problem as it is in Queensland, making it difficult to select against this trait. More significantly, during the course of this project, CSIRO abandoned horticultural research, closed the Merbein facility and their citrus breeder retired. More than 30 years of citrus breeding activity came to an end without any commercially significant varieties being released. An inspection of CSIRO breeding fields at the close of their program revealed nothing of obvious parental value to the Bundaberg-based citrus breeding.

However, it was quite the contrary when the breeding team "discovered" the germplasm held at Kerikeri New Zealand and were able to utilise it in crosses forming part of this project. The purpose of utilizing New Zealand varieties was not to grow this germplasm in Queensland, but to use it as parental material so that useful genes (such as those controlling male sterility, rapid sugar accumulation, large fruit size, early season maturity) are incorporated into new hybrids while selecting against unwanted characters (such as rough skin texture and poor colour). This cool-climate germplasm is only useful within the context of segregating populations assessed under subtropical conditions. Large populations and high selection intensity are necessary to ensure useful genes are captured within environmentally adapted hybrids.

The triploid breeding component of CT07000, now continued in CT11000, also contains crosses that may generate early-season selections. For example, Clementines have been used extensively as seed parents, as has Imperial itself, and hybrids from these parents may prove to be early-season. Details on this crossing activity can be found in (Sykes and Smith 2007) pg. 67-72 and (Sykes *et al.* 2004) pg. 95-117. A problem with this triploid breeding, in relation to early-season breeding, is that it has not solely targeted the early-season production period, and that the recovery rate of this breeding technique precludes the rapid generation of large progeny populations. Crossing at the diploid level using early-season parents would enable the rapid generation of large populations to which high selection pressure could be applied.

Early-season hybrids are also likely to be selected from the Mandarin Hybridisation Project which moved into a commercialisation phase (CT09023 "Commercial development of subtropical mandarin hybrids") around the same time that CT09014 commenced. A number of families aimed at the early-season window were generated in the later years of the hybridisation stage of this project. Some hybrids in these families have recently commenced fruiting. The problem with this program is that no new crosses are being made. Consequently, valuable information gained about the 'worth' of particular crosses is not being utilised, neither are some of the new selections being incorporated as parents.

We therefore conclude that, although there are existing breeding activities capable of delivering an early-season Imperial replacement, these fall short in terms of the parental material being used, the size of the populations being screened, and the environmental conditions under which selections are being made.

1.4 Theoretical foundation

There are a number of requirements that need to be met in order to have a good chance of success with this breeding project. Four key questions were considered prior to the project commencing:

Is the trait heritable?
It is widely accepted that if both parents are early maturing varieties then the majority of progeny will also mature during this time-slot. Curiously, we could not find any citrus reference that demonstrates this heritability definitively, though even some of the oldest breeding efforts in citrus (e.g. Furr, 1963) acknowledge that if you want early maturing progeny then parents should be early maturing. Experience at BRF and in other breeding programs confirms this trend. We can target the early-season maturity window by utilising parents that are themselves early maturing.

Are good parents available?
A particular effort has been exerted at BRF over the previous few seasons to identify early-season hybrids that may have merit as parents. For example we have endeavoured to identify hybrids that can develop strong external colour development prior to the onset of cool temperatures (Smith *et al.* 2008). In breeding an early-season replacement for Imperial we envisage using Imperial itself, other existing early-season varieties (e.g. Satsuma, Clementines, Nova, Fremont), as well as hybrids from the breeding work at BRF. There are at least 10 selections from the existing work at BRF that we believe merit use as parents for breeding early-season mandarins. We have also consulted with overseas breeders concerning early-season breeding and what additional parents might be available. They have urged the use of existing selections from the BRF program rather than relying solely on established early-season varieties. They have also cautioned against relying too strongly on Satsuma, because of its poor performance under warm subtropical conditions. As an example of some of the BRF hybrids that could be used for early-season breeding, "07C004" is a [(Imperial x Murcott) x Fremont] hybrid that is monoembryonic, low-seeded, has flat seeds and matures early in the season. It was given Priority 1 when selected in 2007 on account of its excellent fruit quality. Similarly "08C004" is a [(Aust Clementine x Murcott) x (Ellendale x Kara)] hybrid which is monoembryonic, early maturing, has high Brix levels and excellent appearance. It was also given Priority 1 when selected in 2008. This new project enabled the development of a crossing program that captured these potential parents in combinations likely to generate useful segregants.

What selection pressure is required, and how well can the population be phenotyped for the trait(s) of interest?
Even in the best families, we find a very small percentage warrant first-round selection. Less than 1% of seedlings are selected for progression to Stage Two

testing, and selections at Stage Two are also heavily culled. Consequently, we believe there is a need to generate large populations in order to have any chance of breeding successful new varieties. It will be relatively easy to phenotype the population for early-season maturity, as we will need to see early colour development as well as internal palatability. These characteristics seem to be relatively stable. Characters such as granulation, skin texture, and fruit size may be more difficult to define because of strong seasonal, juvenility, and crop load influences. Advanced selections that are seeded will be irradiated in order to develop low-seeded variants.

What size populations need to be generated?
We have demonstrated an ability to efficiently generate and evaluate large populations. Large populations are considered essential in order to develop mandarins that can achieve acceptable quality early in the season in a subtropical production environment (where few cool night are experienced prior to harvest).

We believe that the objective of breeding an early-season replacement for Imperial mandarin is achievable using a select group of high quality parents, and by applying high selection pressure to a large population of hybrids.

Chapter 2: Crossing program and pollination activity

2.1 Introduction

No two mandarin parents possess the necessary set of traits in such obvious expression that they could be chosen as the sole participants in this program. Instead we must, from a vast array of potential parents, choose a smaller group that can be combined with some probability of identifying useful segregants.

2.2 Materials and Methods

The breeding team, consisting of the breeder and two scientific assistants, met in July each year to develop and document the crosses planned for the flowering season (August/September). All the data on germplasm held at Bundaberg Research Facility and at Kerikeri was reviewed in order to identify parents with characteristics of early-maturity, high Brix, non-puffing skin, colour development under warm conditions, and good fruit size. Data from existing progeny blocks from other breeding programs was also reviewed to identify those potential parents likely to transmit desirable traits. In the four seasons prior to commencing this work, a deliberate effort was directed toward assessing Stage Two selections from past BRF diploid breeding (~360 genotypes) early in the season (March/April). Consequently, this information could be used to find potential new parents that consistently displayed characteristics suitable for this early-season breeding work.

The amount and complexity of information available to the breeding team increased each year and the process of choosing the best crossing program normally took 4-6 days of intense discussion and information checking. There were never the resources, or desire, to choose a set of parents and cross them in every combination; the experienced breeding team knew which crosses were destined to fail and ensured that time was not committed to these. One such example is crossing two parents that both have small fruit regardless of other good trait they may possess. If traits are to be captured from a small fruited parent then it needs to be combined with a parent that has large fruit.

Apomixis (associated with polyembryony) is a significant obstacle in mandarin breeding because it prevents the use of some parents as seed parents. Knowledge of seed type is thus the first consideration when developing a crossing program. It is impractical to cross two polyembryonic parents because the resulting seedlings will be predominantly identical to the seed parent and the occasional zygotic seedling impossible to identify without time-consuming laboratory techniques. Many of the selections from previous breeding at BRF are polyembryonic, because of the heavy

reliance on Murcott as a parent. Consequently these new polyembryonic selections could only be included in the current program as pollen parents.

2.3 Results and Discussion

The crossing programs for 2009 through to 2013 are shown in Tables 2.1 to 2.6, along with the actual number of pollinations performed. Even where some desired crosses could not be made, because of absence of flowers or pollen, the crossing activity was ambitious in every season and both physically and intellectually strenuous to implement. Pollinations commence as soon as the desired parents started flowering and continued until no more flowers were available; a period generally extending 4-6 weeks (Figure 2.1). In 2009, pollinations for 58 different families were performed with families numbers in subsequent years being; 68 families at BRF in 2010, 48 families at Kerikeri in 2010, 141 families in 2011, 21 families in 2012, and 68 families in 2013.

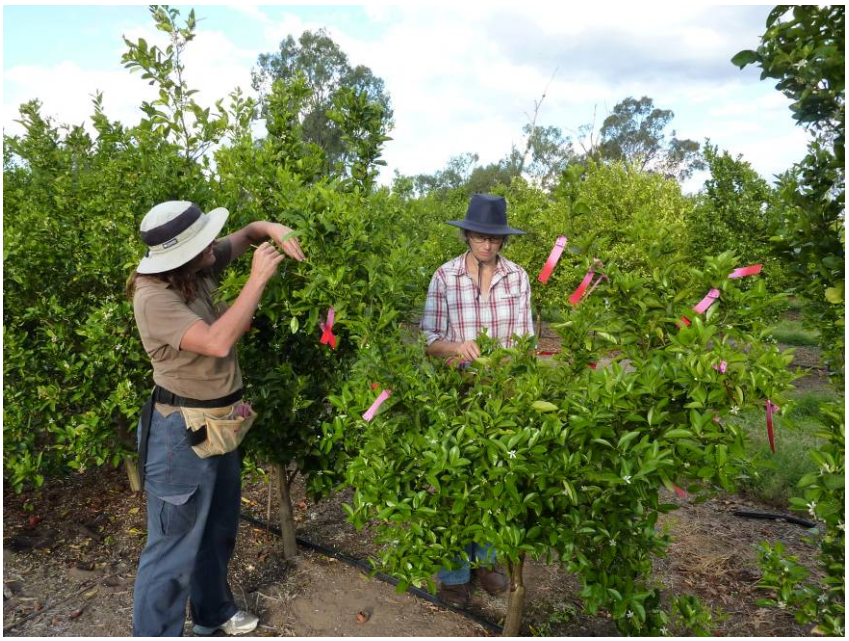

Figure 2.1: Project staff making the most of a brief citrus flowering season to complete a large and complex crossing program.

Not only was 2011 the most complex pollinating year (140 families) but it was also the largest in terms of total pollinations, with 5,590 performed. In this season pollen was collected from 31 mandarin varieties and applied in various combinations to 37 varieties. By contrast, the 2012 flowering season was the least complex with the breeding team having 13 pollen parents and 8 seed parents and performing a total of 1,262 pollinations.

Every flowering season is different for mandarins, with some varieties choosing not to flower, to set poorly, or to produce no pollen. The success of this project in

conducting such a large number of pollinations across a diverse range of parents, is in part due to it being spread across 5 seasons so that variations in parent performance could be compensated.

Table 2.1 Pollination plan and actual pollinations performed during the 2009 citrus flowering season, Bundaberg Research Facility.
"*" indicates crosses that were planned but could not be performed, see text for explanations.

	Pollen Parent																							
Seed Parent	01C011	02C014	02C018	02C065	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clausellina	Corsica1	De Nules	Fremont	Miho Wase	Okitsu	Satsuma Silverhill	Grand Total
01C011		45		30	60	*	*	30	40	*	*	*	*	65	50										320
02C018		70			50		15							20											155
02C065	50	30			50	8	*	40	40	*	*	*	*	24		50	40	45	*	40	40	35	50	40	582
05C016	60	50	50	30		9	42	35	50	*	*	*	*	22	36										384
07C001	8														2										10
07C004	*	*	*	*	*	*		*	*	*	*	*	*	*	*										0
08C002	30			30	30	*	*	*		*	*	*	*	*	*										90
08C004	*			*	*	*	*	*	*	*		*	*		*			*				*	*	*	
08C009	*			*	*	*	*	*	*	*	*		*		*										
09C013	*		*	*	*	*	*	*	*	*	*	*	*		*										
09C018	10				20	*	12	13	20	*	*	*	*	*		*	*	20	*	*		20	20	20	155
Arufatina				48			*								29										77
Aust Clem				257			*								124										381
Corsica1				*			*								*										
De Nules				80			*								15										95
Fallglo					100		*								15										115
Grand Total	158	195	50	475	310	17	69	118	150					131	271	50	40	65		40	40	55	70	60	2364

Table 2.2 Pollination plan and actual pollinations performed during the 2010 citrus flowering season, Bundaberg Research Facility.
"*" indicates crosses that were planned but could not be performed, see text for explanations.

	Pollen Parent																													
Seed Parent	00C018	00C019	01C011	01C030	02C002	02C018	02C065	02C109	02C122	03C055	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clausellina	Corsica 1	De Nules	Fremont	Miho Wase	Okitsu	Satsuma Silverhill	Grand Total
00C019	40			40	40						45													40			40	40	40		325
01C011							40				40	2	9	30		30	*	*	*	*	40										191
01C030		50						50		50																					150
01C044																								40				40	40		120
02C002		40								55																					95
02C018											50		*							*											50
02C065			*								*	*	*	*		*	*	*	*	*		*	*	*	*	*	*	40	*	*	40
02C122		60	*	50		70	40	40		55	30	*	*	20		20	*	*	*	*	25			20				20	20		470
03C066									35						10		17							40				41	38		181
05C014																								40					34		74
05C016		40	29			40	40			40		*	*	40		40	*	*	*	*	55										324
07C001							40		40		40																				120
07C004			*			*	*				*	*		*		*	*	*	*	*	44										44
08C004			*				*				*	*	*	*		*		*	*		*			*				*	*	*	
08C009			*				*				*	*	*	*		*	*	*	*		*										
09C013			*			*	*				*	*	*	*		*	*	*	*		*										
09C018			*								*	*	*	20		*	*	*	*	*		*	*	20	*	*		20	20	*	80
De Nules							60														80										140
Ellendale																								20				20	20	20	80
Grand Total	40	190	29	90	40	110	220	90	75	200	205	2	9	110	10	90	17				244			220			40	221	212	20	2484

Table 2.3 Pollination plan and actual pollinations performed during the 2010 citrus flowering season, Kerikeri Research Station NZ.
"*" indicates crosses that were planned but could not be performed, see text for explanations.

	Pollen Parent																														
Seed Parent	90-1373	90-359	90-397	90-685	90-781	96-267	96-543	96-577	96-582	96-786	99-0410	Afourer	Aoshima	Barl. Ellendale	Caffin	Carte Noir	Clem. x Mly.	Enc. x Mly.	Fremont	Imperial	Kiyomi	Lee	Matsuyama	Michal	Miyagawa	Murcott	Nova	Page	Seto	Shiro	Sunburst	Grand Total
90-1373											*	18	*								*			40	*			30				88
90-397											*	35	*								*				*			22	*			57
90-621												30																60				90
90-685											*	*							*		*	*				*	*	*	*		*	
90-781											*	30	*								*				*			24	*	16		70
91-354																										1	5	2				8
91-418												28							*	*		*				*	*	46	*		*	74
96-267												35							*	*		35				*	11	40	*		20	141
96-407												25														5						30
96-41												80										40		28		25	4	104			7	288
96-543												*							*	*		*				*	*	*	*		*	
96-577												*							*	*		*				*	*	*	*		*	
96-582												5							*	*		*				*	*	*	*		*	5
96-786												*							*	*		*				*	*	*	*		*	
99-0410				*								*	*	*	*				*			*			*	*	*	*	*		*	
Barl. Ellendale		13				*	*	*	*	*	*		*			3	*	*				22	*	30	*				*	20		88
Bay Sweetie												40										40						40				120
Caffin											*		*												*				*	11		11
Carte Noir													*	30											*				*			30
Corsica1													*												*				*			
H22Tangor												33												23				40				96
Imperial													*												*				*			
Kiyomi	11		*	*	*							13	*	30	*				*			*			*	19	*	*	*	5	*	78
SRA89													*												*				*			
Grand Total	11	13										372		60		3						137		121	0	50	20	408		52	27	1274

Table 2.4 Pollination plan and actual pollinations performed during the 2011 citrus flowering season, Bundaberg Research Station. "*" indicates crosses that were planned but could not be performed, see text for explanations.

	Pollen Parent																																						
Seed Parent	00C013	00C025	00C029	01C011	01C030	01C033	02C002	02C109	02C122	03C046	03C066	05C020	06C008	06C016	06Q006	07C004	07C007	08C001	08C002	08C004	08C008	08C013	09C009	09C012	09C013	09C017	09C021	10Q084	11C014	11C015	11C016	11C028	11C033	11Q024	Clausellina	Miho Wase	Okitsu	Satsuma silverhill	Grand Total
00C018																																			50	131	103	*	284
00C019					60									50			*						40	*			*			60	40				46	13	150		459
00C029																																			80	43	50	*	173
01C011									50	50		50		40			*			46				*			*	12		30	30			*					308
01C030																	*				40																		40
01C033																	*																						
01C046		50																									*						*						50
02C002										50							*																						50
02C014																																			*	*	*	*	
02C022																											*												
02C059																											*												
02C063																																			*	*	14	*	14
02C065																																			55	*	44	*	99
02C109																																			*	*	60	*	60
02C110													25			25																			25	35	25		135
02C112		90					58														20								*	30	*		*		35	50	50	*	333
02C122										50												50	30		40			*	*					*	*	50	50	*	270
03C022																																55			74	92	70	*	291
03C035																					10														*	*	50	*	60
03C055																																			*	*	20	*	20
03C066													50	50			*				10		*							20	20	35			50	50	50	*	335
03C069																																33							33
05C003								30			40								30	40			10				*												150
05C016													60	60						40		40	7		8					40	32				50	50	50	*	437
05C020													*	*		*								*		*	*				*				*	*	50	*	50
06C008																		46	45	40		45			27							37							240
06Q006		6												7										*		*				*	*		*						13
06Q010		10												7										*		*				*	*		*						17
07C001		50	50										50		39										30				*				*		47	64	40	*	370
07C004									35				35										15			30	*												115
07C007																																			30	*	40	*	70
08C002																																35							35
08C004		20											10	70							17			*		15					20				30	30	85		297
08C009		25		30	35	21			20	20			20																										171
09C012																									*				*										
09C013										25																													25
09C017																									61				*										61
09C018																																			34	55	50	*	139
09C021																																			*	*	*	*	
10C001													40	25									9							22	31								127
11C014										26														*		22		*						*					48
11C033	50	50									20		50							20			11																201
Daisy																																					10		10
Grand Total	50	301	50	30	95	21	58	30	105	221	60	50	340	309	39	25		46	75	186	97	135	122		166	67		12		202	173	195			606	663	1061		5590

Table 2.5 Pollination plan and actual pollinations performed during the 2012 citrus flowering season, Bundaberg Research Facility.
"*" indicates crosses that were planned but could not be performed, see text for explanations.

	Pollen Parent													
Seed Parent	00C025	00C029	02C063	05C014	05C016	06C007	06C016	08C006	09C009	09C012	11Q024	C. wakonai	Okitsu	Grand Total
01C011	30									30				60
01C030													60	60
05C014			50											50
05C016	50		42											92
06C007	40	40						40	40				50	210
07C001		110		100	100		100				*	120		530
08C006		60				56			70				60	246
09C021													14	14
Grand Total	120	210	92	100	100	56	100	40	110	30	0	120	184	1262

Table 2.6 Pollination plan and actual pollinations performed during the 2013 citrus flowering season, Bundaberg Research Facility.
"*" indicates crosses that were planned but could not be performed, see text for explanations.

	Pollen Parent																					
Seed Parent	00C018	00C025	01C011	02C063	05C014	05C015	05C016	05C028	06C016	07C004	08C016	09C009	09C012	09C014	09C017	11C016	12C024	Clauselina	Encore	Miho Wase	Okitsu	Grand Total
01C011		100		**50**	**50**	**50**			200			100	100		100			100	20	165	100	1135
01C046																*		100			100	200
01C049																					42	42
01C050						**200**														50	70	320
02C059																		100			100	200
02C122									26													26
03C022																		50		20	139	209
03C066									100									102			101	303
05C014	**200**		**200**	**200**					100									108			100	908
05C015																		100			100	200
05C016																		50		81	100	231
05C018									60											100	100	260
05C028						**100**			30		**100**		52							23	35	340
06C015																		120			100	220
07C001					30		37															67
07C004	**100**		**105**	**98**											40		*	40			80	463
07C007			**60**	**55**					30	**60**		32			30	*		53			60	380
08C016								*												*	*	
09C002																				10	10	20
Encore			30																			30
Grand Total	300	100	395	403	80	350	37		546	60	100	132	152		170	0		923	20	449	1337	5554

Values in bold are crosses from which the seed will be irradiated prior to planting

Chapter 3: Fruit numbers and percentage fruit set

3.1 Introduction

Fruit set following hand pollination in citrus is affected by climatic conditions, tree health, and the genetic make-up of the two parents. Large variations can occur between seasons such that the same cross performed in one year can not be guaranteed to give the same result in subsequent years. Although it is reasonably accurate to predict the number of fruit required to produce a certain population size of hybrids (based on seed numbers for fruit being reasonably stable) it is the 'set' of fruit which most easily disrupts the development of target population numbers.

3.2 Materials and Methods

Fruit resulting from the hand pollinations were harvested when the seed parent was nearing its normal maturity time. Each flower was tagged using a colour-coded strip tag at the time of pollination and these tags were removed with any fruit set so that both parents could be identified. In the case of Kerikeri pollinations, the project leader travelled to New Zealand and harvested the fruit and extracted and possessed the seed over a five day period predicted as being closest to fruit maturity for most of the seed parents used.

The number of fruit set from each pollen parent on each seed parent was recorded.

3.3 Results and Discussion

Numbers of fruit set, and the associated percentage of pollinations that set fruit, are shown for 2009-2012 pollinations in Tables 3.1-3.5. The largest number of fruit resulting from controlled pollinations was harvested in 2011 when 1,085 fruit were picked. Fruit numbers in the other years of the project were 400 fruit in 2009, 319 fruit in 2010 (BRF), 420 fruit in 2010 (NZ), and 98 fruit in 2012. All pollinations in each year of the project were performed by the same team of people using identical techniques.

The best overall fruit set from hand pollinations occurred at the NZ site in 2011 where 33% of pollinations resulted in fruit (Figure 3.1). At Bundaberg, the best year was 2011 when 19% of pollinations set, while in contrast only 8% set from 2012 pollinations. However, these overall figures mask the important effect of individual parents as can be clearly seen for both pollen and seed parents in each of the years described in Tables 3.1-3.5. For example in Table 3.3 the seed parent 96-267 gave

low fruit set with four of the five pollens used whilst 96-41 had good fruit set on all seven of the pollen parents used on it. There was also large variation from season to season as illustrated by 01C011 whose overall set from 2009 to 2012 was 23%, 4%, 13% and 12% respectively. Even an identical cross made in two different seasons often resulted in distinctly different set rates. For example, 01C011 x 08C001 gave 43% set from 2009 pollinations (Table 3.1) but only 3% in the following year (Table 3.2).

Figure 3.1: Fruit set and seed extraction at Kerikeri, NZ: **a.** Kiyomi tree and **b.** fruit from pollinations; **c.** sample of additional parent discovered and accessed as a result of the collaboration; **d.** seed extracted and treated in NZ ready for transport to Australia; **e.** seed after passing through AQIS quarantine treatments and ready for sowing at Bundaberg Research Facility

Table 3.1 Number of fruit set, and percentage of pollinations that set fruit, for crosses performed in 2009, Bundaberg Research Station.

		Pollen Parent																									
Seed Parent	Data	01C011	02C014	02C018	02C065	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clausellina	Corsica1	De Nules	Fremont	Miho Wase	MihoWase	Okitsu	Satsuma Silverhill	Grand Total
01C011	Fruit		14			3			13	7					13	23											73
	%set		31		0	5			43	18					20	46											23
02C018	Fruit		11			8		1																			20
	%set		16			16		7							0												13
02C065	Fruit	1	3			1	0		6	6					1		3	5	11		3	11	5		9	8	73
	%set	2	10			2	0		15	15					4		6	13	24		8	28	14		18	20	13
05C016	Fruit	7	6	5	5			8	9	10					1	4											55
	%set	12	12	10	17		0	19	26	20					5	11											14
07C001	Fruit	0														0											
	%set	0														0											
07C004	Fruit																										
	%set																										
08C002	Fruit	9			3	4																					16
	%set	30			10	13																					18
08C004	Fruit																										
	%set																										
08C009	Fruit																										
	%set																										
09C013	Fruit																										
	%set																										
09C018	Fruit					1													1				1				3
	%set	0				5		0	0	0									5				5		0	0	2
Arufatina	Fruit				8											2											10
	%set				17											7											13
Aust Clem	Fruit				67											54											121
	%set				26											44											32
Corsica1	Fruit																										
	%set																										
De Nules	Fruit				15											5											20
	%set				19											33											21
Fallglo	Fruit					8										1											9
	%set					8										7											8
Total of Fruit Set		17	34	5	98	25		9	28	23					15	89	3	5	12		3	11	6		9	8	400
Average of % set		11	17	10	21	8	0	13	24	15					11	33	6	13	18		8	28	11		13	13	17

Table 3.2 Number of fruit set, and percentage of pollinations that set fruit, for crosses performed in 2010, Bundaberg Research Station.

		Pollen Parent																														
Seed Parent	Data	00C018	00C019	01C011	01C030	02C002	02C018	02C065	02C109	02C122	03C055	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clauselina	Corsica 1	De Nules	Fremont	Miho Wase	Okitsu	Satsuma silverhill	Grand Total
00C019	Fruit	13			10	9						8													20			10	13	9		92
	%set	33			25	23						18													50			25	33	23		28
01C011	Fruit							1				4	0	0	1		2					1										9
	%set							3				10	0	0	3		7					3										4
01C030	Fruit		3						1		2																					6
	%set		6						2		4																					4
01C044	Fruit																								5				1	6		12
	%set																								13				3	15		10
02C002	Fruit		11								7																					18
	%set		28								13																					20
02C018	Fruit											2																				2
	%set											4																				4
02C065	Fruit																												15			15
	%set																												38			38
02C122	Fruit		0		2		11	0	10		0	9			9		2					6			0				5	7		61
	%set		0		4		16	0	25		0	30			45		10					24			0				25	35		16
03C066	Fruit									0						0		1							1				0	0		2
	%set									0						0		6							3				0	0		1
05C014	Fruit																								0					0		0
	%set																								0					0		0
05C016	Fruit		8	1			2	6			7				6		11					9										50
	%set		20	3			5	15			18				15		28					16										15
07C001	Fruit							0		0		0																				0
	%set							0		0		0																				0
07C004	Fruit																					13										13
	%set																					30										30
08C004	Fruit																															
	%set																															
08C009	Fruit																															
	%set																															
09C013	Fruit																															
	%set																															
09C018	Fruit														0										2				2	0		4
	%set														0										10				10	0		5
De Nules	Fruit							16														16										32
	%set							27														20										23
Ellendale	Fruit																								1				0	1	1	3
	%set																								5				0	5	5	4
Total Sum of Fruit		13	22	1	12	9	13	23	11	0	16	23	0	0	16	0	15	1				45			29			10	36	23	1	319
Total Average of %set		33	13	3	15	23	10	9	14	0	9	12	0	0	16	0	15	6				18			11			25	15	11	5	12

Table 3.3 Number of fruit set, and percentage of pollinations that set fruit, for crosses performed in 2010, Kerikeri Research Station, NZ.

		Pollen Parent																														
Seed Parent	Data	90-1373	90-359	90-397	90-685	90-781	96-267	96-543	96-577	96-582	96-786	99-0410	Afourer	Aoshima	Barl. Ellendale	Caffin	Carte Noir	Clem. x Mly.	Enc. x Mly.	Fremont	Imperial	Kiyomi	Lee	Matsuyama	Michal	Miyagawa	Murcott	Nova	Page	Seto	Shiro	Sunburst	Grand Total
90-1373	Fruit												6												12				9				27
	%set												33												30				30				31
90-397	Fruit												14																9				23
	%set												40																41				40
90-621	Fruit												20																50				70
	%set												67																83				78
90-685	Fruit																																
	%set																																
90-781	Fruit												11																8		4		23
	%set												37																33		25		33
91-354	Fruit																										1	1	0				2
	%set																										100	20	0				25
91-418	Fruit												5																9				14
	%set												18																20				19
96-267	Fruit												3										6					2	6			6	23
	%set												9										17					18	15			30	16
96-407	Fruit												5														1						6
	%set												20														20						20
96-41	Fruit												42										18		8		5	2	62			3	140
	%set												53										45		29		20	50	60			43	49
96-543	Fruit																																
	%set																																
96-577	Fruit																																
	%set																																
96-582	Fruit												1																				1
	%set												20																				20
96-786	Fruit																																
	%set																																
99-0410	Fruit																																
	%set																																
Barl. Ellendale	Fruit		4														0						3		9						1		17
	%set		31														0						14		30						5		19
Bay Sweetie	Fruit												20										14						18				52
	%set												50										35						45				43
Caffin	Fruit																														6		6
	%set																														55		55
Carte Noir	Fruit														9											0							9
	%set														30																		30

Corsica1	Fruit																																
	%set																																
H22Tangor	Fruit												0												0				1				1
	%set												0												0				3				1
Imperial	Fruit																																
	%set																																
Kiyomi	Fruit	0											2		4												0				0		6
	%set	0											15		13												0				0		8
SRA89	Fruit																																
	%set																																
Total of Fruit		0	4										129		13		0						41		29	0	7	5	172		11	9	420
Average of %set		0	31										35		22		0						30		24		14	33	42		21	33	33

Table 3.4 Number of fruit set, and percentage of pollinations that set fruit, for crosses performed in 2011, Bundaberg Research Station.

		Pollen Parent																																						
Seed Parent	Data	00C013	00C025	00C029	01C011	01C030	01C033	02C002	02C109	02C122	03C046	03C066	05C020	06C008	06C016	06Q006	07C004	07C007	08C001	08C002	08C004	08C008	08C013	09C009	09C012	09C013	09C017	09C021	10Q084	11C014	11C015	11C016	11C028	11C033	11Q024	Clausellina	Miho Wase	Okitsu	Satsuma silverhill	Grand Total
00C018	Fruit																																			16	23	18		57
	%set																																			32	18	17		20
00C019	Fruit					29									6									2							26	1				24	0	69		157
	%set					48									12									5							43	3				52	0	46		34
00C029	Fruit																																		9	10	14		33
	%set																																		11	23	28		19
01C011	Fruit									6	9		3		4						11								2		5	0								40
	%set									12	18		6		10						24								17		17	0								13
01C030	Fruit																					19																		19
	%set																					48																		48
01C033	Fruit																																							
	%set																																							
01C046	Fruit		1																																					1
	%set		2																																					2
02C002	Fruit										16																													16
	%set										32																													32
02C014	Fruit																																							
	%set																																							
02C022	Fruit																																							
	%set																																							
02C059	Fruit																																							
	%set																																							
02C063	Fruit																																					2		2
	%set																																					14		14
02C065	Fruit																																			10		14		24
	%set																																			18		32		24
02C109	Fruit																																					9		9
	%set																																					15		15
02C110	Fruit													1			5																			2	4	4		16
	%set													4			20																			8	11	16		12
02C112	Fruit		3					1														0								4					0	0	2		10
	%set		3					2														0								13					0	0	4		3
02C122	Fruit										8												8	0		7										17	10		50
	%set										16												16	0		18										34	20		19
03C022	Fruit																																31			16	12	10		69
	%set																																56			22	13	14		24
03C035	Fruit																					1																16		17
	%set																					10																32		28
03C055	Fruit																																					13		13
	%set																																					65		65
03C066	Fruit													15	1							5								1	0	8			4	9	13		56
	%set													30	2							50								5	0	23			8	18	26		17
03C069	Fruit																															4							4
	%set																															12							12
05C003	Fruit								9			13								14	4			0															40
	%set								30			33								47	10			0															27
05C016	Fruit													21	8						8		11	1		3					19	1				7	18	18		115
	%set													35	13						20		28	14		38					48	3				14	36	36		26
05C020	Fruit																																					10		10
	%set																																					20		20

06C008	Fruit																		12	12	13		6			6							16							65
	%set																		26	27	33		13			22							43							27
06Q006	Fruit		0												2																										2
	%set		0												29																										15
06Q010	Fruit		1												0																										1
	%set		10												0																										6
07C001	Fruit		1	1										1												0									15	27	14		59
	%set		2	2										2		0										0									32	42	35		16
07C004	Fruit									15				12										2			3													32
	%set									43				34										13			10													28
07C007	Fruit																																		4		15		19
	%set																																		13		38		27
08C002	Fruit																															11							11
	%set																															31							31
08C004	Fruit		4											0	12							3					0					1			13	8	36		77
	%set		20											0	17							18					0					5			43	27	42		26
08C009	Fruit		7		8	10	2			3	5			3																										38
	%set		28		27	29	10			15	25			15																										22
09C012	Fruit																																								
	%set																																								
09C013	Fruit										14																														14
	%set										56																														56
09C017	Fruit																									4														4
	%set																									7														7
09C018	Fruit																																		1	0	0		1
	%set																																		3	0	0		1
09C021	Fruit																																								
	%set																																								
10C001	Fruit													1	0									0								0	0							1
	%set													3	0									0								0	0							1
11C014	Fruit										1																0													1
	%set										4																0													2
11C033	Fruit	1	0									0		0							0			0																	1
	%set	2	0									0		0							0			0																	0.5
Daisy	Fruit																																						1		1
	%set																																					10		10
Total of Fruit		1	17	1	8	39	2	1	9	24	53	13	3	54	33		5		12	26	36	28	25	5		20	3		2		55	3	70			121	128	288		1085
Average of %set		2	6	2	27	41	10	2	30	23	24	22	6	16	11	0	20		26	35	19	29	19	4		12	5		17		27	2	36			20	19	27		19

Table 3.5 Number of fruit set, and percentage of pollinations that set fruit, for crosses performed in 2012, Bundaberg Research Station.

		Pollen Parent													
Seed Parent	Data	00C025	00C029	02C063	05C014	05C016	06C007	06C016	08C006	09C009	09C012	11Q024	C. wakonai	Okitsu	Grand Total
01C011	Fruit	4									3				7
	%set	13									10				12
01C030	Fruit													16	16
	%set													27	27
05C014	Fruit			1											1
	%set			2											2
05C016	Fruit	2		11											13
	%set	4		26											14
06C007	Fruit	2	11						13	0				8	34
	%set	5	28						33	0				16	16
07C001	Fruit		5		7	2		0				0	4		18
	%set		5		7	2		0				0	3		3
08C006	Fruit		1				6			0				2	9
	%set		2				11			0				3	4
09C021	Fruit													0	0
	%set													0	0
Sum of Fruit set		8	17	12	7	2	6	0	13	0	3	0	4	26	98
Average of %set		7	8	13	7	2	11	0	33	0	10	0	3	14	8

Chapter 4: Hybrid seed recovery and nursery establishment

4.1 Introduction

Rapid extraction, processing and germination of mandarin seed is important to ensuring the resulting plants gain sufficient size in the nursery to survive field planting in autumn. If this does not occur then plants are either field planted too small and suffer during the winter, or they must be held-over in the nursery which creates a bottle-neck for other research projects. The experienced breeding team have shown that seed extracted from immature fruit germinates poorly, even though it may look morphological mature long before the fruit is ripe. Consequently all hybrid seed generated in this project was only extracted once each seed parent had mature fruit.

4.2 Materials and Methods

Fruit from individual crosses were partially cut equatorially and the seed squeezed from the two halves. These were briefly washed in tap water then placed on paper towel to dry for 3 to 7hrs. Seed numbers were recorded for each individual fruit. Once dry, the seed coat was peeled from each individual seed using fine forceps and working from the chalazal end of the seed (Figure 4.1a-b). Peeled seed were sown into steam pasteurised potting mix based on composted pine bark and 4mm blue metal. Stryofoam boxes (internal dimensions 290mm x 450mm) were filled to a depth of 150mm with the potting mix and the hybrid seed sown at approximately 60 per stryo (460 seeds/m^2). A 50mm layer of perlite was used to cover the seed (Figure 4.1c).

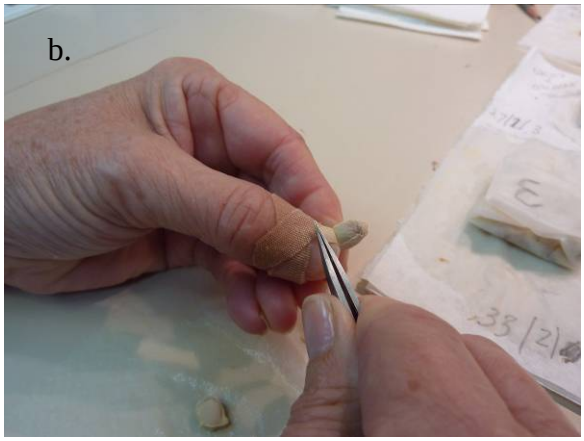

Figure 4.1: Hybrid seed processing after extraction from fruit: **a.** & **b.** seed being peeled; **c.** evenly spacing in styros filled with potting mix then covered with a layer of vermiculite.

The stryos were kept in an enclosed nursery which was hand-watered and fertilised monthly to maximise growth (and prevent problems associated with under/over watering).

Processing seed from the Kerikeri pollinations was slightly more complex because of transport issues and quarantine restrictions. In this case, seed was extracted in the field laboratory at Kerikeri Research Station, washed and surface sterilised before being packed into pre-prepared mesh bags. These bags were then brought back to Brisbane airport with the necessary quarantine permit (IP11007838 valid 16th May 2011 to 16th May 2012) that had been obtained some months earlier. Seed was then hot water dipped at 50°C for 20 minutes followed by a 1% sodium hypochlorite dip for 10 minutes under AQIS supervision using the mesh bags to ensure seed lots were not confused or cross-contaminated (see Figure 3.1d-e). After release from quarantine the seed were transported to Bundaberg Research Facility where they were individually peeled and sown using the same procedures described above.

4.3 Results and Discussion

Tables 4.1-4.5 show the number of hybrids seeds obtained from each cross in each of the 5 seasons. A total of 29,873 seed were produced, not including the flat and poorly formed seed often obtained in mandarin hybrids. The biggest year was from 2011 pollinations when 16,169 hybrid seeds were obtained. This placed significant strain on the nursery facilities at BRF which was alleviated when the breeding team negotiated to use glasshouse facilities at the nearby sugarcane research centre (Figure 4.2). Without the use of these additional facilities it would not have been possible to accommodate the large numbers of hybrids generated in this most successful year. Other years of the project saw more manageable numbers of hybrid seed produced, with 5,059 in 2009, 6,364(BRF) + 886(NZ) in 2010, and 1,395 in 2012. Fruit harvesting was staggered to provide sufficient time to hand-peel each of the nearly 30 thousand seed generated.

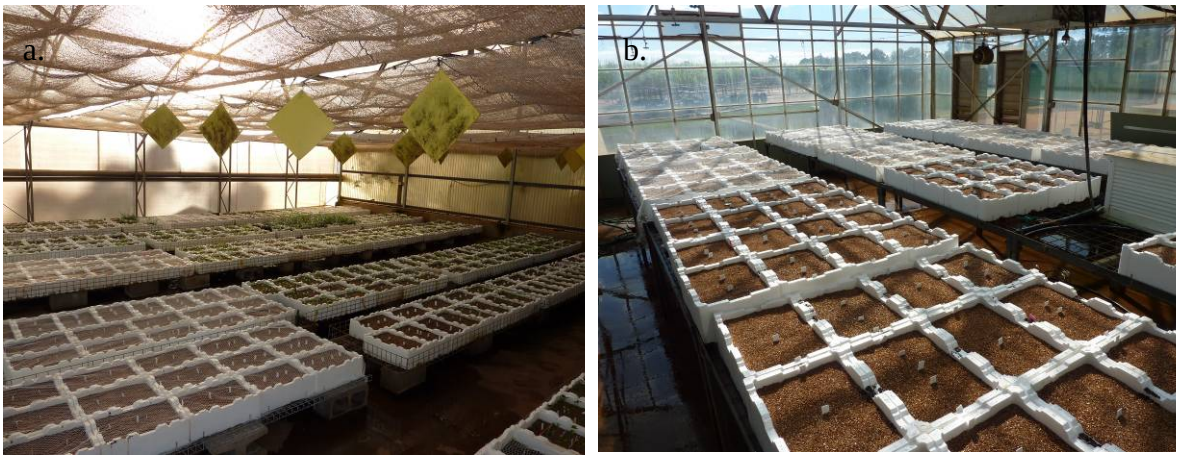

Figure 4.2: Seedling styros soon after sowing: **a.** in the nursery at BRF; **b.** in a nearby glasshouse made available by the sugarcane breeders (formerly BSES) to alleviate the bottleneck created by a particularly successful pollinating season in 2011.

High growth rates were maintained in the nursery which enabled field planting to occur in autumn each year, less than 10 months after sowing. The potting media and fertigation technology developed by the breeding team facilitated this rapid seedling growth, as did hand watering using a regularly monitored chlorinated water supply.

Table 4.1 Number of hybrid seed obtained from pollinations performed in 2009, Bundaberg Research Facility.

	Pollen Parent																								
Seed Parent	01C011	02C014	02C018	02C065	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clauselina	Corsica1	De Nules	Fremont	Miho Wase	MihoWase	Okitsu	Satsuma Silverhill	Grand Total
01C011		293			64			298	142					226	474											1497
02C018		114			102		30																			246
02C065	6	31			7			94	69					12		58	63	138		37	111	70		100	95	891
05C016	121	101	102	77			126	159	231					19	72											1008
07C004																										
08C002	124			31	53																					208
08C004																										
08C009																										
09C013																										
09C018					21													19				13				53
Arufatina				39											22											61
Aust Clem				152											152											304
Corsica1																										
De Nules				389											106											495
Fallglo					256										40											296
Grand Total	251	539	102	688	503		156	551	442					257	866	58	63	157		37	111	83		100	95	5059

Table 4.2 Number of hybrid seed obtained from pollinations performed in 2010, Bundaberg Research Facility.

	Pollen Parent																													
Seed Parent	00C018	00C019	01C011	01C030	02C002	02C018	02C065	02C109	02C122	03C055	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clauselina	Corsica 1	De Nules	Fremont	Miho Wase	Okitsu	Satsuma silverhill	Grand Total
00C019	310			205	237						186													456			168	328	238		2128
01C011							12				41			16		35					18										122
01C030		11						22		13																					46
01C044																								75				20	114		209
02C002		297								166																					463
02C018											45																				45
02C065																												193			193
02C122				29		188		184			165			164		37					95							80	64		1006
03C066																	11							5							16
05C014																															
05C016		169	20			32	84			139				87		182					139										852
07C001																															
07C004																					256										256
08C004																															
08C009																															
09C013																															
09C018																								37				52			89
De Nules							425														422										847
Ellendale																								40					24	28	92
Grand Total	310	477	20	234	237	220	521	206		318	437			267		254	11				930			613			168	673	440	28	6364

Table 4.3 Number of hybrid seed obtained from pollinations performed in 2010, Kerikeri Research Station, NZ.

	Pollen Parent																														
Seed Parent	90-1373	90-359	90-397	90-685	90-781	96-267	96-543	96-577	96-582	96-786	99-0410	Afourer	Aoshima	Barl. Ellendale	Caffin	Carte Noir	Clem. x Miy.	Enc. x Miy.	Fremont	Imperial	Kiyomi	Lee	Matsuyama	Michal	Miyagawa	Murcott	Nova	Page	Seto	Shiro	Sunburst	Grand Total
90-1373												16												15				28				59
90-397												84																27				111
90-621												1																4				5
90-685																																
90-781												145																52		10		207
91-354																										0	0	0				0
91-418												2																2				4
96-267												4										10					8	13			17	52
96-407												8														0						8
96-41												45										27		0		0	0	112			2	186
96-543																																
96-577																																
96-582												0																				0
96-786																																
99-0410																																
Barl. Ellendale		36														0						13		58						0		107
Bay Sweetie												47										26						32				105
Caffin																														1		1
Carte Noir														6																		6
Corsica1																																
H22Tangor												0												0				2				2
Imperial																																
Kiyomi	0											9		24												0				0		33
SRA89																																
Grand Total	0	36										361		30		0						76		73		0	8	272		11	19	886

Table 4.4 Number of hybrid seed obtained from pollinations performed in 2011, Bundaberg Research Facility.

	Pollen Parent																																								
Seed Parent	00C013	00C025	00C029	01C011	01C030	01C033	02C002	02C109	02C122	03C046	03C066	05C020	06C008	06C016	06Q006	07C004	07C007	08C001	08C002	08C004	08C008	08C013	09C009	09C012	09C013	09C017	09C021	10Q084	11C014	11C015	11C016	11C028	11C033	11Q024	Clausellina	Miho Wase	Okitsu	Satsuma silverhill	Grand Total
00C018																																			p	p	p		
00C019					609									64									4							568	23				581		1484		3333
00C029																																			p	p	p		
01C011									110	170		55		68						226								45		87									761
01C030																					278																	278
01C033																																							
01C046		15																																					15
02C002										326																													326
02C014																																							
02C022																																							
02C059																																							
02C063																																					p		??
02C065																																			82		125		207
02C109																																					p		
02C110													18			145																			45	71	83		362
02C112		44					19																							73							36		172
02C122										130												103			112											276	139		760
03C022																															600			239	187	137		1163
03C035																					21																141		162
03C055																																					152		152
03C066													204	2							117									5		104			46	138	135		751
03C069																																71							71
05C003								207			406								390	110																			1113
05C016													347	63						138		238	4		46					334	4				106	240	237		1757
05C020																																					169		169
06C008																		185	149	214		74			68							201							891
06Q006														23																									23
06Q010																																							
07C001		1	0										0																						1	1	4		7
07C004									367				191										17			21													596
07C007																																			91		345		436
08C002																															212							212
08C004		75												104							69									2				231	179	671		1331

08C009		155		132	225	42			53	104			16																										727
09C012																																							
09C013										293																													293
09C017																									1														1
09C018																																		13					13
09C021																																							
10C001													13																										13
11C014										21																													21
11C033	2																																						2
Daisy																																					14		14
Grand Total	2	290	0	132	834	42	19	207	530	1044	406	55	789	324		145		185	539	688	485	415	25		227	21		45		1067	29	1188			1435	1092	3909		16169

p=polyembryonic seed parent, subject to toxin treatment.

Table 4.5 Number of hybrid seed obtained from pollinations performed in 2012, Bundaberg Research Facility.

	Pollen Parent													
Seed Parent	00C025	00C029	02C063	05C014	05C016	06C007	06C016	08C006	09C009	09C012	11Q024	C. wakonai	Okitsu	Grand Total
01C011	37									28				65
01C030													348	348
05C014			14											14
05C016	24		190											214
06C007	35	198						220					152	605
07C001		0		1	0							0		1
08C006		23				98							27	148
09C021														
Grand Total	96	221	204	1	0	98		220		28		0	527	1395

Chapter 5: Screening for Brown Spot resistance

5.1 Introduction

In less than 20 years, Alternaria Brown Spot disease (*Alternaria alternata*) has become the most important fungal disease of Australian citrus resulting in increased production costs through fungicide applications, downgrading of fruit because of lesions, and ongoing headaches with MRLs (maximum residue levels) in export markets. Genetic resistance is seen as the only long-term solution. Fortunately resistance is under simple genetic control and genotypes can be readily separated into resistant and susceptible categories.

5.2 Materials and Methods

The breeding team worked closely with pathologists to refine a method for inoculating young hybrids on a 'commercial scale' necessary to handle the large number of hybrids being generated in this project. Details of this work are in the paper attached.

5.3 Results and Discussion

Early failures pointed to the need to refine the screening technique to get it working reliably and efficiently. Spraying spores onto seedlings growing in the nursery resulted in only a few hybrids developing symptoms, and so the styros containing seedlings were temporarily transferred to a modified cool room set at 25°C and with domestic humidifiers positioned to create a water-laden fog in which leafs remained constantly moist but not dripping (Figure 5.1). Spore inoculations under these conditions often resulted in excellent disease symptom development, but the occasional poor performance of various batches prompted a re-examination of the inoculum source.

Figure 5.1: A batch of hybrid seedlings inside a humidified cool room operating at 25°C. Spore suspensions were spray applied to the seedlings and left to incubate for 4 days.

To improve the pathogenicity of the inoculum, single spore isolates were developed from symptomatic field material collected at BRF. These single spore isolates were then individually tested using a detached leaf assay. This process revealed that many of the isolates were non-pathogenic even though spores germinated well. More importantly, it identified an isolate that produced severe symptoms very soon after inoculation, and this isolate was then used for all future screening of hybrids (Figure 5.2). By combining young vigorously growing hybrid seedlings, moderate temperature and high humidity, and a highly virulent isolate of the fungus, the team were able to remove susceptible seedlings just a few months after sowing. Completing the process so early in the growth of the hybrids made the seedlings very easy to remove from the styros, and created extra space for the remaining resistant hybrids to grow for the next few months in the nursery.

Figure 5.2: Pathogenicity testing of single spore *Alternaria alternata* isolates. Top row, susceptible Murcott mandarin leaves, bottom row resistant Rough Lemon leaves. This particular isolate (AKM452) produced rapid and severe symptoms and was used subsequently throughout the screening and culling processes.

The success of this screening process is illustrated by the fact that only 1 tree out of 15, 053 field grown hybrids has shown symptoms, even though the disease is prevalent at BRF and these hybrid progeny blocks are not sprayed with fungicides.

Success in implementing the screening process on such a large scale is detailed in the attached manuscript. The manuscript is enclosed in full because it arises from a poster presentation by the principal investigator at the International Citrus Congress, Valencia, and it may be some time before publication occurs. It also represents one of the few publishable opportunities arising from CT09014 and documents processes and outcomes made possible by this project.

Commercial-Scale Alternaria Brown Spot Resistance Screening as the First Step in Breeding New Mandarins for Australia

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ABSTRACT

Rapid screening tests and an appreciation of the simple genetic control of Alternaria brown spot (ABS) susceptibility have existed for many years, and yet the application of this knowledge to commercial-scale breeding programs has been limited. Detached leaf assays were first demonstrated more than 40 years ago and reliable data suggesting a single gene determining susceptibility has been emerging for at least 20 years. However it is only

recently that the requirement for genetic resistance in new hybrids has become a priority, following increased disease prevalence in Australian mandarin production areas previously considered too dry for the pathogen. Almost all of the high-fruit-quality parents developed so far by the Queensland-based breeding program are susceptible to ABS necessitating the screening of their progeny to avoid commercialisation of susceptible hybrids. This is done effectively and efficiently by spraying 3-6 month old hybrid seedlings with a spore suspension derived from a toxin-producing field isolate of *Alternaria alternata*, then incubating these seedlings in a cool room at 25°C and high humidity for 5 days. Susceptible seedlings show clear disease symptoms and are discarded. Analysis of observed and expected segregation ratios loosely support the hypothesis for a single dominant gene for susceptibility, but do not rule out the possibility of alternative genetic models. After implementing the routine screening for ABS resistance for three seasons we now have more than 20,000 hybrids growing in field progeny blocks that have been screened for resistance to the ABS disease.

Keywords: *Alternaria alternata*, disease, citrus, genetics, susceptibility

INTRODUCTION

Alternaria brown spot disease (ABS) is caused by a pathotype of the fungus *Alternaria alternata* (Fr.) Keissl. (Pegg, 1966) affecting certain mandarins (e.g. 'Emperor') (*Citrus reticulata* Blanco), tangors and tangor hybrids (e.g. 'Murcott') (*C. reticulata* × *C. sinensis* (L.) Osb.). Advanced leaf symptoms are typically large necrotic areas, surrounded by a chlorotic halo and often associated with vein darkening, premature senescence and entire shoot death (Pegg, 1966; Swart et al., 1998; Timmer et al., 2000). Symptoms on fruit are expressed as sunken, brown lesions, observed reaching up to 5 mm in diameter. A chlorotic halo often surrounds lesions on green fruit, but becomes indistinguishable on coloured fruit. Worldwide, the disease now causes significant problems in almost all areas where susceptible varieties are grown. In Australia, the economic cost of ABS is estimated to be more than USD$3,000 per hectare in fruit losses and control costs (Miles et al., 2011).

Although the ABS disease cycle is relatively simple, it is very challenging to disrupt through management practices such as the application of fungicides. The ability of the fungus to induce symptoms in the plant tissue and sporulate within a period of only a few days makes the pathogen highly damaging under suitable environmental conditions. *Alternaria* is a necrotrophic fungus and conidia are produced on dead tissues in the tree canopy and on abscised leaves and twigs on the orchard floor (Timmer et al., 1998). Production of conidia requires periods of leaf wetness, before they are dislodged and dispersed by wind (Timmeret al., 1998). When conidia germinate on the surface and infect a susceptible host, cell necrosis occurs within 30 hours, and before any host penetration occurs (Pegg, 1966). Cell necrosis is related to the production of a host specific toxin (HST) by the fungus (Kohmoto et al., 1991). This necrosis is the result of leakage of electrolytes from host cells after exposure to the HST (Kohmoto et al., 1979). Only young leaves are susceptible to the fungus, becoming resistant once the leaf cuticle is sufficiently developed (Pegg, 1966). In Australia, fruits are susceptible to the disease regardless of age (Miles et al., 2005). Completion of the disease cycle occurs through the

production of conidiophores and conidia on infected tissue (Timmeret al., 1998). Control using protectant fungicides is reliant upon achieving thorough coverage of rapidly expanding leaves and fruit, before pathogen attack; coverage of expanding plant parts is known to be difficult to achieve and maintain (Timmer et al., 1998). Cultural practices, such as pruning to improve air movement and removal of dead tissues, have proven ineffective under commercial conditions in Australia.

A more reliable and long term sustainable approach to controlling ABS would be the development of cultivars that are resistant to the disease. The susceptibility of mandarins and tangors to ABS is determined by the sensitivity of the cultivar to the HST produced by the `tangerine' pathotype of *A. alternata* (Kohmotoet al., 1991). The inheritance of sensitivity to the HST is hypothesised to be controlled by a single dominant gene (Dalkilic et al., 2005). This simple genetic control creates an opportunity to breed resistant cultivars via conventional hybridisation. Due to the susceptibility of young plants a rapid bioassay of seedlings through direct inoculation may provide excellent results due to the fact that the pathogen: i) grows quickly and readily produces conidia in culture; ii) symptoms are expressed on leaves within very short time periods; and iii) toxin sensitivity under these conditions is an unambiguous trait. Despite these favourable genetic and practical characteristics, breeding for ABS resistance has only recently been considered a priority in Australia. This prioritisation follows a steady increase in ABS disease pressure and fruit losses in production regions traditionally considered too dry for serious ABS epidemics.

Breeding for resistance to ABS is a highly desirable and achievable goal for commercial breeding programs providing screening methods are effective, efficient and low cost. The aim of the research described in this paper is to develop a commercial-scale method for breeding for resistance to ABS in the mandarin breeding program based in Queensland, Australia. The specific aims were to: i) identify and test highly virulent isolates of *A. alternata* for use as an inoculum source; ii) develop a bioassay enabling screening of large numbers of hybrid seedlings; and iii) confirm the genetics of inheritance of resistance to ABS. The methods and findings of this study will assist our citrus breeding program, as well as others, to contribute to the control of this highly damaging disease. The production, evaluation and commercial release of citrus cultivars resistant to ABS will greatly improve the profitability of citrus production in humid production areas where ABS occurs. Furthermore, resistant cultivars will break the reliance on fungicides for control of ABS.

MATERIALS AND METHODS

Source of Isolates

In order to identify highly virulent isolates of *A. alternata* for use as an inoculum source in breeding activities, isolates were obtained from fresh ABS leaf specimens. Leaves with typical ABS lesions were collected from trees of 'Daisy' mandarin (*Citrus reticulata* Blanco) and ‘Wekiwa’ tangelo (a complex hybrid involving *C.* × *paradisi* Macf.) in the Bundaberg region of Queensland. Leaves were briefly surface sterilised by

swabbing both sides with 70% ethanol, then allowing the ethanol to evaporate. Small pieces of leaf tissue were excised from the margins of the lesions and plated onto Petri dishes containing half strength potato dextrose agar (PDA). The plates were incubated at 25°C under near ultra violet light for 3-5 days. Mono-conidial isolates of any *A. alternata* colonies that grew were obtained using standard techniques (Smith, 2002). The mono-conidial isolates were immediately stored at -80°C as spore suspensions in 15% glycerol.

Confirmation of Pathogenicity

In order to confirm the pathogenicity of the isolates obtained above, detached leaf assays were performed using leaves of 'Murcott' mandarin and 'Lockyer' rough lemon (*C. jambhiri* Lush). Leaves were prepared for detached leaf assay based largely on the methods of Timmer et al., (1996). Cultures of the isolates above were established on PDA from under glycerol storage and incubated at 25°C under near ultra violet light for 5 days. Conidia were harvested from the colonies by flooding the Petri dish with sterile distilled water, and lightly scraping the colony surface with a sterile spatula. The resulting spore suspensions were then adjusted to 1×10^5 conidia per ml. For each isolate three 20 µl droplets of spore suspension were placed evenly onto the underside of each of three replicate leaves of each citrus cultivar. The detached leaves were then incubated at 25°C for 5 days to allow lesions to develop. Lesion sizes were measured and compared to evaluate the virulence of the isolates.

Large-Scale Bioassay

In order to develop hybrid cultivars resistant to ABS, large numbers of hybrid seedlings need to be screened each year for resistance to ABS using a direct seedling bioassay. A colony of a highly virulent, toxin producing isolate of *A. alternata* was established from under glycerol storage onto PDA. Within 5 days, the colony was subcultured onto 80 PDA plates for large-scale multiplication of conidia. The plates were incubated at 25°C under black light for 5 days. Spore suspensions were prepared as above, and adjusted to 1×10^5 conidia per ml, resulting in a total of 3-4 l of spore suspension. Each year large populations of hybrid seedlings were produced from the corresponding year of hand pollinations. Seedlings were raised in polystyrene produce boxes (500 × 320 × 280 mm) containing potting mix at approximately 60 seedlings per box. Boxes of seedlings were transferred to shelving in a refrigerated cold room programmed to operate at 25°C. A domestic humidifier (Euky Bear Steam Vaporiser, Extralife, Australia) was added to the cold room to create saturated air capable of maintaining constant leaf wetness without causing runoff. Seedlings were sprayed with the spore suspension to just before run-off using a hand-operated mister. The seedlings were then incubated in the cold room at 25°C and high humidity for 5 days. Plants remained wet with the spore suspension for the entire 5 days of incubation. After incubation the seedling boxes were returned to a shadehouse and visually inspected for disease symptoms. When clear ABS symptoms were observed on susceptible seedlings, the results were recorded, and the diseased seedlings discarded. Following inspection for

ABS, the remaining resistant seedlings were grown for a further 6 months in a shadehouse before field planting at high-density (10,000 trees per ha) for horticultural evaluation.

Genetics of Resistance

In order to confirm if the genetics of resistance was following the segregation ratios expected for a single recessive allele for resistance, as observed by Dalkilic et al. (2005), the segregation ratios from the large-scale bioassay were subjected to chi-square analysis.

RESULTS AND DISCUSSION

Confirmation of Pathogenicity

Of the 13 mono-conidial isolates retrieved from the lesions on leaves of 'Daisy' and 'Wekiwa', only 5 produced symptoms on 'Murcott' leaves in the detached leaf assay. The remaining 8 isolates failed to produce any symptoms. None of the isolates from 'Daisy' and 'Wekiwa' produced symptoms on the 'Lockyer' leaves, whilst symptoms were produced on these leaves by control isolates cultured from symptomatic rough lemon leaves. Based on these results it was concluded that the 5 isolates from 'Daisy' and 'Wekiwa' were of the tangerine pathotype of *A. alternata*. The relatively low recovery of pathogenic isolates from diseased tissue suggests a high frequency of saprophytic *A. alternata* colonisation of symptomatic tissue. Furthermore, differences in lesion size (data not shown) indicate putative differences in virulence between the 5 pathogenic isolates that produced symptoms. These observations highlight the need for thoroughly characterised isolates to be used in the screening process.

Large-Scale Bioassay

Symptoms of ABS were first observed 24-48 hours after inoculation, and continued to develop during incubation. In 2010, 2011 and 2012 totals of 5,843, 7,083 and 17,089 hybrid seedlings, respectively, were inoculated with *A. alternata* and inspected for ABS symptom development. Out of these 30,015 seedlings, 9,038 were culled due to the formation of ABS lesions after inoculation. The effectiveness of inoculation was consistent between years, based on the proportions of susceptible progeny resulting from 24 crosses that were repeated in multiple years. For example, 05C016 × 02C018 resulted in 27% and 33% susceptible progeny in 2011 and 2012, respectively. 05C016 × 02C065 resulted in 39% and 38% susceptible progeny in 2011 and 2010, respectively. DeNules × 09C018 resulted in 0% susceptible progeny in both 2011 and 2010. Some inconsistencies were observed, but only in 7 of the 24 cases where the same cross was made in multiple years.

Genetics of Resistance

The inheritance of susceptibility to *A. alternata* being hypothesised to be controlled by a single dominant gene (Dalkilicet al., 2005) is only partially supported by our crosses, as

shown in Table 1. The examples in Table 1 were chosen objectively on the basis of being the crosses with the largest population sizes, and/or include a parent of a well-known cultivar. In most examples there is a trend towards observing fewer susceptible offspring than expected. Crosses between two resistant parents produced almost no susceptible hybrids as expected. However, crosses between a resistant and a susceptible parent generally resulted in only ~30% susceptible hybrids, when the expected value was 50%. Similarly, crosses of two susceptible parents generally resulted in less than 60% susceptible hybrids when 75% was expected. Deviations from expected segregation ratios can occur due to a number of reasons including; i) a tendency for disease escapes during the inoculation procedure; ii) incubation conditions being suboptimal for *A. alternata*; iii) human error in detecting symptomatic plants and; iv) genetic control being more complex than a single gene. Disease escapes may be the result of incomplete coverage of all plants with spore suspension and/or the absence of young susceptible leaves on particular seedlings at the time of treatment. Incubation conditions being suboptimal for *A. alternata* infection and ABS development is considered unlikely. The ideal conditions for ABS are prolonged periods of leaf wetness at 25°C (Canihos et al., 1999). The inoculation conditions in the selection procedure mirrored these conditions, with clear ABS symptoms developing on successfully inoculated susceptible seedlings. Human error in detecting symptoms after inoculation cannot be ruled out, even though seedlings were assessed by experienced operators. Indeed, human error might be expected to overestimate disease susceptibility (rather than the underestimate we have observed) considering reports of susceptible reactions on small leaves taken from resistant accessions (Reis et al., 2007). Disease escapes, poor incubation conditions and human error in detection would result in susceptible hybrids inadvertently being field planted. Some of these hybrids could reasonably be expected to later develop disease symptoms in the field; particularly when considering that these field plantings receive no fungicide applications. Of the >20,000 screened hybrids planted in the field, only one plant has shown ABS symptoms to date.

While errors in phenotyping cannot be dismissed, the absence of large numbers of diseased hybrids appearing in field plantings, and the consistent 'underestimation' of susceptible progeny across a range of screenings with different parents, in different years, suggests that a single gene model may not always be sufficient to explain segregation when heterozygous parents are used.

Dalkilic et al., (2005) suggest that cytoplasmic genes may explain distorted segregation seen in their reciprocal backcross. To further test this possibility, we identified 5 parental combinations where reciprocal crosses had been made. Using the susceptible parent as the female or male did not consistently increase or decrease the percentage of susceptible hybrids produced (Table 2). We therefore conclude that cytoplasmic genes do not satisfactorily explain distorted segregation ratios for ABS susceptibility.

Although homozygous susceptible cultivars are known, such as 'Minneola' and 'Orlando' (Dalkilicet al., 2005), none of these have featured heavily in our breeding

program because of fruit quality problems, and all were removed from the program before ABS screening commenced. Instead, the crossing and ABS screening have demonstrated that all susceptible parents in the program are heterozygous and capable of producing disease resistant hybrids. This has important practical implications because all susceptible hybrids can be discarded without concern of loosing desirable traits from ABS-susceptible parents in the breeding program.

Breeding mandarins resistant to ABS is an achievable goal that will pay dividends for citrus producers, consumers, and the environment. In the case of ABS, genetic resistance is expected to be highly robust and unlikely to breakdown under field conditions. This is largely due to resistance being the result of an absence in the host of a receptor site for the toxin, as has been demonstrated specifically for toxin sensitivity in rough lemon (Tsuge et al., 2013), rather than the presence of single or multiple resistance genes which tend to exert selection pressure upon the pathogen to overcome resistance (Poland et al., 2009). Testament to the robust nature of this form of resistance is the long-standing resistance to ABS of 'Imperial' mandarin (*C. reticulata*) and other cultivars in Queensland despite growing alongside highly diseased varieties such as ‘Murcott’.

The distorted segregation ratios, assuming a single gene model, require further investigation to determine whether they are an artefact of the screening methodology, or have a genetic basis. If these consistently distorted ratios are related to methodological problems then very substantial numbers of ABS susceptible hybrids will have been field planted. These populations in the field will be monitored over their life for signs of susceptibility to ABS under field conditions.

Even with the possibility that some susceptible hybrids have escaped the screening process, the methods described herein have removed nearly 10,000 ABS susceptible hybrids at a very early stage in the program. Practicality has come from an inoculation system that uses equipment already used by the program, or that is of very low cost and readily available (e.g. a standard cold room and domestic humidifier vs. a dedicated controlled environment facility). Adopting simple, cost-effective screening methods has been critical to the routine implementation of ABS resistance screening in the breeding programme.

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Table 1. Tests for segregation of *Alternaria alternata* resistance in citrus hybrids subject to large scale bio-assay.

Crosses	Model	Sus[z]	Res[z]	Chi-square 0:1	Chi-square 1:1	Chi-square 3:1
00C019 (‘Ellendale’ × ‘Murcott’) × ‘Clausellina’	ss × ss	1	327	1.00[y]		
00C019 (‘Ellendale’ × ‘Murcott’) × ‘Miho Wase’	ss × ss	0	179	0.00		
00C019 (‘Ellendale’ × ‘Murcott’) × ‘Okitsu’	ss × ss	1	170	1.00[y]		
00C019 (‘Ellendale’ × ‘Murcott’) × 11C015 (‘Imperial’ × ‘Nova’)	ss × ss	0	512	0.00		
‘Daisy’ × 09Q035 (06Q011 × Ellendale)	Ss × ss	32	12	50.28	4.79	13.89
03C024 (‘Fina’ × ‘Murcott’) × 10Q033 (‘Encore’ × 06Q006)	Ss × ss	193	330	236.67	18.26	482.07
03C024 (‘Fina’ × ‘Murcott’) × 10Q055 (‘Ellendale’ × 01C007)	Ss × ss	179	339	216.39	25.31	503.88
03C024 (‘Fina’ × ‘Murcott’) × 11Q034 (‘Ellendale’ × 06Q010)	Ss × ss	139	368	161.08	54.50	229.73
‘Fallglo’ × 05C016 (‘Ellendale’ × ‘Murcott’)	ss × Ss	51	164	57.86	31.90	113.09
07C004 (IM111 × ‘Fremont’) × 02C122 (‘Ellendale’ × ‘Murcott’)	ss × Ss	144	188	183.88	2.93[y]	68.74
00C019 (‘Ellendale’ × ‘Murcott’) × 10Q046 (‘Encore’ × 06Q010)	ss × Ss	8	252	8.12	142.82	282.57
02C002 (‘Aust Clem’ × ‘Murcott’) × 09Q029 (‘Ellendale’ × 06Q006)	ss × Ss	52	100	62.73	7.77	51.01
‘Daisy’ × 09Q028 (Ellendale × 06Q008)	Ss × Ss	7	8		0.03[y]	2.53[y]
03C022 (‘Ellendale’ × ‘Murcott’) × 11C028 (‘Ellendale’ × 01C028)	Ss × Ss	315	232		6.33	37.11
05C007 (‘Aust Clem’ × ‘Murcott’) × 09Q032 (IM111 × 06Q008)	Ss × Ss	253	187		4.98	30.13
05C003 (‘Imperial’ × ‘Murcott’) × 03C066 (‘Ellendale’ × ‘Murcott’)	Ss × Ss	214	153		5.1	23.00

[z]Sus = susceptible to *A. alternata*, Res = resistant to *A. alternata*

[y]$P > 0.05$

Table 2. Tests for segregation of *Alternaria alternata* resistance in reciprocal crosses of citrus hybrids subject to large scale bio-assay.

Crosses	Model	Sus[z]	Res[z]	Chi-square 1:1
00C019 (‘Ellendale’ × ‘Murcott’) × 05C016 (‘Ellendale’ × ‘Murcott’)	ss × Ss	16	90	29.41
05C016 (‘Ellendale’ × ‘Murcott’) × 00C019 (‘Ellendale’ × ‘Murcott’)	Ss × ss	34	64	4.70
02C018 (‘Oroval’ × ‘Imperial’) × 05C016 (‘Ellendale’ × ‘Murcott’) 2010	ss × Ss	30	69	7.99
05C016 (‘Ellendale’ × ‘Murcott’) × 02C018 (‘Oroval’ × ‘Imperial’) 2010	Ss × ss	33	68	6.25
02C018 (‘Oroval’ × ‘Imperial’) × 05C016 (‘Ellendale’ × ‘Murcott’) 2011	ss × Ss	12	12	0.00[y]
05C016 (‘Ellendale’ × ‘Murcott’) × 02C018 (‘Oroval’ × ‘Imperial’) 2011	Ss × ss	8	22	3.45[y]
02C065 (‘Ellendale’ × ‘Murcott’) × 05C016 (‘Ellendale’ × ‘Murcott’)	ss × Ss	4	2	0.34[y]
05C016 (‘Ellendale’ × ‘Murcott’) × 02C065 (‘Ellendale’ × ‘Murcott’)	Ss × ss	27	44	2.06[y]
02C065 (‘Ellendale’ × ‘Murcott’) × 08C002 (‘Imperial’ × ‘Nova’)	ss × Ss	15	55	12.44
08C002 (‘Imperial’ × ‘Nova’) × 02C065 (‘Ellendale’ × ‘Murcott’)	Ss × ss	14	15	0.02[y]
09C018 (‘Ellendale’ × 01C028) × 05C016 (‘Ellendale’ × ‘Murcott’)	ss × Ss	4	7	0.42[y]
05C016 (‘Ellendale’ × ‘Murcott’) × 09C018 (‘Ellendale’ × 01C028)	Ss × ss	16	37	4.33

[z]Sus = susceptible to *A. alternata*, Res = resistant to *A. alternata*

[y]$P > 0.05$

Chapter 6: Field planting and establishment

6.1 Introduction

It is important to establish hybrids under field conditions as quickly as possible in order to maximise vegetative growth and minimise the juvenile period. Citrus commonly take 7 to 10 years in the field before they commence fruiting and then the first few years of fruiting can provide a false impression of the true phenotype of the hybrid. Therefore it was essential that this project demonstrate how effectively a large population of hybrids can be field deployed in just a 5 year period, as a way of reinforcing support for future tree-crop breeding endeavours.

6.2 Materials and Methods

Hybrid seedlings of approximately 300mm height were transferred in their stryo from the protected nursery to an outdoor area to "harden-off" two to four weeks prior to expect field planting. After this period they were bare-rooted from the stryo boxes and directly planted onto plastic mulch in newly prepared paddocks. Irrigation was via long-life drip tape, and seedlings were established in a twin-row on each bed of plastic mulch, with spacing of 0.5m within each row (Figure 6.1).

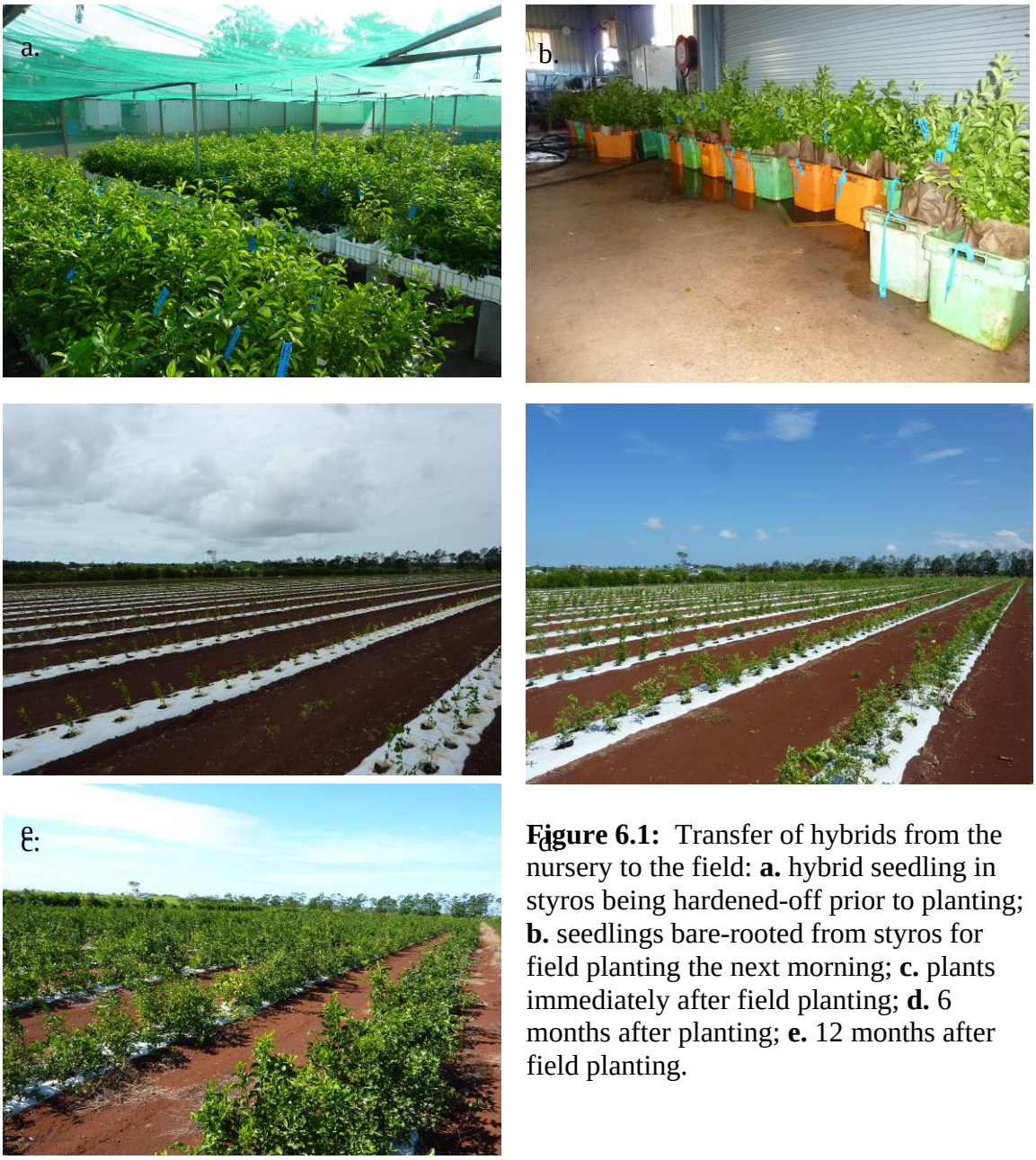

Figure 6.1: Transfer of hybrids from the nursery to the field: **a.** hybrid seedling in styros being hardened-off prior to planting; **b.** seedlings bare-rooted from styros for field planting the next morning; **c.** plants immediately after field planting; **d.** 6 months after planting; **e.** 12 months after field planting.

Planting generally occurred in April of each year to avoid the summer heat while also giving the plants time to establish before the onset of winter. Prior to planting, the number of hybrids in each family was determined, so that half would be planted together as one replicate and the other half planted in a different part of the paddock as the second replicate. This system enables an assessment of family value with validation across the two replicates. More importantly it avoids making an already complex collection of families impossible to keep track of as the trees mature.

6.3 Results and Discussion

Field planting occurred with the loss of very few hybrids and all blocks are now well established. The earliest of these plantings occurred in June 2011 with some of these trees now close to 3m tall (Figure 6.2).

Figure 6.2: Hybrids in "M block". Field planted June 2011: **a.** November 2011, five months after planting; **b.** same rows May 2014, 3 years after planting.

Details of the hybrids that have been field established in each year of the project are described in Tables 6.1-6.5. Slightly more than the 15,000 hybrids are already field planted (Figure 6.3). Trees from 2013 pollinations are expected to be planted in April 2015.

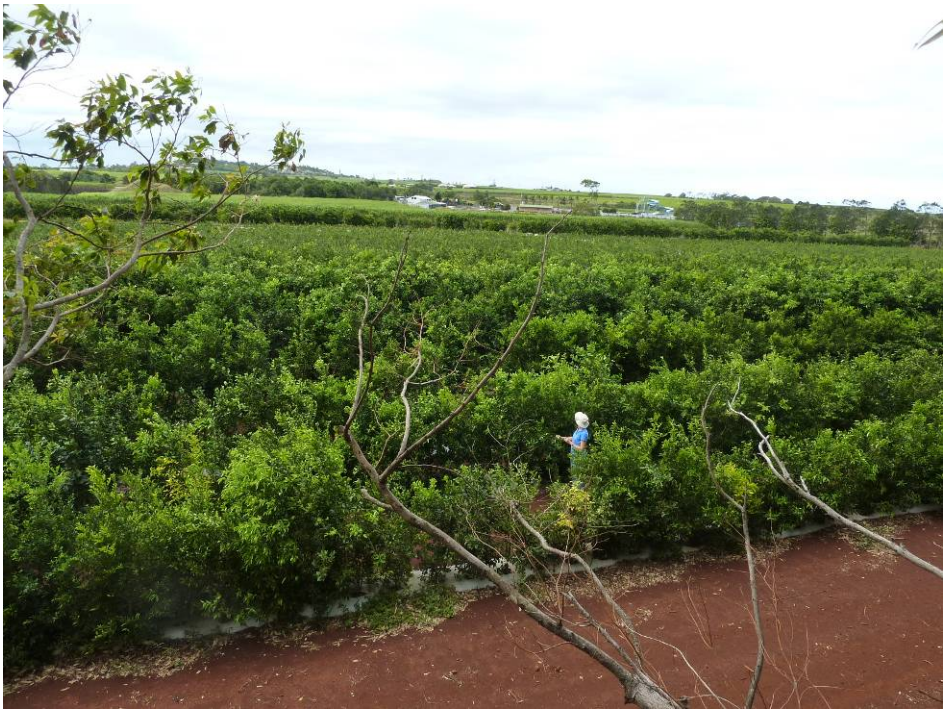

Figure 6.3: Project staff member inspecting some of more than 15,000 mandarin hybrids created and field established during this project.

The largest planting occurred in April 2013 (2011 pollinations) when 8,579 trees went into the ground. Amongst these, the largest families are from 00C019 parentage with crosses like 00C019 x Okitsu satsuma having 1,248 hybrids. This same seed parent also resulted in families of over 400 hybrids when combined with 01C030, 11C015 and Clausellina satsuma. Almost all families are of sufficient size to be able to estimate their genetic worth, which may guide future hybridisation activity.

Table 6.1 Number of field planted trees from pollinations performed in 2009.
Established in "M Block" Bundaberg Research Facility June 2011.

	Pollen Parent																								
Seed Parent	01C011	02C014	02C018	02C065	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clausellina	Corsica1	De Nules	Fremont	Miho Wase	MihoWase	Okitsu	Satsuma Silverhill	Grand Total
01C011		83			25			136	57					104	207											612
02C018		34			57		23																			114
02C065	4	19			2			42	48					12		43	43	121		34	77	59		90	74	668
05C016	30	24	60	44			38	41	65					11	32											345
07C004																										
08C002	30			14	12																					56
08C004																										
08C009																										
09C013																										
09C018					6													10				5				21
Arufatina				29											17											46
Aust Clem				104											88											192
Corsica1																										
De Nules				259											60											319
Fallglo					123										19											142
Grand Total	64	160	60	450	225		61	219	170					127	423	43	43	131		34	77	64		90	74	2515

Table 6.2 Number of field planted trees from Bundaberg pollinations performed in 2010.
Established in "M, O & P Blocks" Bundaberg Research Facility March/April 2012.

	Pollen Parent																														
Seed Parent	00C018	00C019	01C011	01C030	02C002	02C018	02C065	02C109	02C122	03C055	05C016	07C001	07C004	08C001	08C002	08C003	08C004	08C009	08C013	09C013	09C018	Arufatina	Aust Clem	Clauselina	Corsica 1	De Nules	Fremont	Miho Wase	Okitsu	Satsuma silverhill	Grand Total
00C019				93	116						74													278			65	174	139		939
01C011							7				11			9		22					9										58
01C030		2						7		1																					10
01C044																								39				18	76		133
02C002		189								58																					247
02C018											11																				11
02C065																												84			84
02C122				9		81	2	63			78			51		17					26							124	41		492
03C066																	2							3							5
05C014																															
05C016		57	12			18	41			40				22		51					72										313
07C001																															
07C004																					186										186
08C004																															
08C009																															
09C013																															
09C018																								24				38			62
De Nules							152														236										388
Ellendale																								25				15	13	19	72
Grand Total		248	12	102	116	99	202	70		99	174			82		90	2				529			369			65	453	269	19	3000

Table 6.3 Number of field planted trees from NZ pollinations performed in 2010.
Established in "M, O & P Blocks" Bundaberg Research Facility March/April 2012.

	Pollen Parent																														
Seed Parent	90-1373	90-359	90-397	90-685	90-781	96-267	96-543	96-577	96-582	96-786	99-0410	Afourer	Aoshima	Barl. Ellendale	Caffin	Carte Noir	Clem. x Mly.	Enc. x Mly.	Fremont	Imperial	Kiyomi	Lee	Matsuyama	Michal	Miyagawa	Murcott	Nova	Nove	Page	Seto	Shiro	Sunburst	Grand Total
90-1373												6												9					17				32
90-397												43																	15				58
90-621												1																	1				2
90-685																																	
90-781												88																	27		6		121
91-354																																	
91-418												2																					2
96-267												1										6					5		8			9	29
96-407												6																					6
96-41												34										7							85			2	128
96-543																																	
96-577																																	
96-582																																	
96-786																																	
99-0410																																	
Barl. Ellendale		20																				5		18									43
Bay Sweetie												16										15							13				44
Caffin																																	
Carte Noir														6																			6
Corsica1																																	
H22Tangor																													1				1
Imperial																																	
Kiyomi												8		22																			30
SRA89																																	
Grand Total		20										205		28								33		27			5		167		6	11	502

Table 6.4 Number of field planted trees from pollinations performed in 2011.
Established in "Q & R Blocks" Bundaberg Research Facility April & May 2013.

	Pollen Parent																																						
Seed Parent	00C013	00C025	00C029	01C011	01C030	01C033	02C002	02C109	02C122	03C046	03C066	05C020	06C008	06C016	06Q006	07C004	07C007	08C001	08C002	08C004	08C008	08C013	09C009	09C012	09C013	09C017	09C021	10Q084	11C014	11C015	11C016	11C028	11C033	11Q024	Clausellina	Mino Wase	Okitsu	Satsuma silverhill	Grand Total
00C018																																							
00C019					447									39									3							435	18				407	0	1248		2597
00C029																																							
01C011									28	93		18		20						93									40									292
01C030																					159																	159
01C033																																							
01C046		7																																					7
02C002										123																													123
02C014																																							
02C022																																							
02C059																																							
02C063																																							
02C065																																			50		91		141
02C109																																							
02C110													13			89																			36	45	47		230
02C112		26					16														13									54							23		132
02C122										61												43			28										176	97		405
03C022																															233			77	129	31		470
03C035																					6															43		49
03C055																																				55		55
03C066													70								48									2		39			18	92	70		339
03C069																																18							18
05C003								37			149								72	26																			284
05C016													105	22						55		102	1		20					127	2				74	178	141		827
05C020																																					124		124
06C008																		59	35	98		42			35							75							344
06Q006																																							
06Q010																																							
07C001																																							
07C004									155				55										7			11													228
07C007																																			50		247		297

08C002																																44							44
08C004		30												45							35											1				186	140	491		928
08C009		103		19	93	11			15	61			14																										316
09C012																																							
09C013										141																													141
09C017																									1														1
09C018																																			10				10
09C021																																							
10C001													1																										1
11C014										9																													9
11C033	1																																						1
Daisy																																					7		7
Grand Total	1	166		19	540	11	16	37	198	488	149	18	258	126		89		59	107	272	261	187	11		84	11				658	21	409			908	760	2715		8579

Table 6.5 Number of field planted trees from pollinations performed in 2012.
Established in "R Block" Bundaberg Research Facility April 2014.

	Pollen Parent													
Seed Parent	00C025	00C029	02C063	05C014	05C016	06C007	06C016	08C006	09C009	09C012	11Q024	C. wakonai	Okitsu	Grand Total
01C011	15									17				32
01C030													263	263
05C014			3											3
05C016	8		36											44
06C007	1	24						50					69	144
07C001														
08C006		10				22							24	56
09C021														
Grand Total	24	34	39			22		50		17			356	542

Chapter 7: Conclusion and Recommendations

This project has successfully achieved everything that it was set up for. In terms of outputs it has used elite parental material to generate populations of mandarin hybrids from which superior new early-season commercial varieties can be selected. These populations of hybrids have been designed to have the highest possible probability of offering segregants that will overcome significant consumer and market outlet problems with Imperial mandarin.

All the desired project Outcomes have been exceeded, with nearly twice the anticipated 15,000 hybrids being generated, such that even with severe culling for disease resistance the final field population still exceeds the original target. It was anticipated that the crossing program would include at least five existing early-season varieties, 10 superior selections from previous breeding work at Bundaberg, and three mandarin genotypes available in New Zealand (but not present in Australia). In practice, the project has exceeded all these targets. Eleven existing early-season varieties were incorporated along with more than 50 selections from existing breeding work at Bundaberg. A further 14 genotypes not present in Australia were incorporated into the program by the Bundaberg breeding team operating in New Zealand.

The two agreed criteria by which the outcomes of this project were to be assessed when it commenced in 2009 were:

1. field establishment of progeny blocks from the desired parental combinations
2. health and vigour of these progeny blocks.

The existence at BRF of more than 15,000 disease-resistant vigorously growing hybrids, derived from a well constructed and documented crossing program, points to the success of the project.

It is recommended that a new project be developed to maintain the established progeny blocks through until fruiting. It is only then that any industry and commercial value can be extracted from the previous 5 years of work and investment from DAFFQ and HAL. Any new project should consider the option of continued hybridisation to lift the assessment populations beyond 15,000. While this may already seem like a large number of hybrids, it has been generated efficiently by an experienced breeding team well positioned to continue such work. It is a good lesson to remember that Australia's most successful fruit tree breeding program owes its achievements to John Cripps, who recognised from the outset that he would need 50,000 hybrids in order to stand a chance of finding a world-beating apple. Although we have generated large numbers of hybrids, and already screened them for disease resistance, we are still well short of the population size likely to guarantee success in

tree crops like apple and citrus where few segregants are edible and far fewer possess the large number of traits required for commercial success.

Chapter 8 Extension activities, Project publicity, Acknowledgements and References

8.1 Extension activities and Project publicity

Malcolm W. Smith (2010) New funding for citrus breeding work. Oral presentation. H&FS Management Team University of Central Queensland, Bundaberg, 25th March 2010.

Malcolm W. Smith (2010) Longer term strategies. Oral presentation. Imperial quality/marketing workshop. Citrus Australia Ltd Grower Conference. Golden Orange Hotel Gayndah 26th August 2010.

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Andrew K. Miles, Malcolm W. Smith and Andre Drenth (2011) Breeding for disease resistance in citrus: The uncommon commonsense solution. Oral presentation, Australasian Plant Pathology Society Seminar Series, Maroochy Research Facility, Nambour 23rd June 2011.

Andrew Miles, Malcolm Smith and Andre Drenth (2011) Fighting brown spot in mandarins, Poster presentation. Citrus Australia Ltd National Conference Barossa, South Australia, 25th to 26th October 2011.

Malcolm W. Smith, Debra Gultzow and Toni Newman (2012) New genetics for the citrus industry. Oral presentation. H&FS Management Team. 28th March 2012,

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