Mild strain cross protection against orange stem pitting strains of Citrus tristeza virus (CTV)

Deborah Hailstones
NSW Department of Primary Industries

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Level 1
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Telephone: (02) 8295 2300
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Deborah Hailstones, Nerida Donovan, Aida Ghalayini and

Elizabeth Howard

NSW DEPARTMENT OF
PRIMARY INDUSTRIES

CT03002

Project Leader

Deborah Hailstones
Elizabeth Macarthur Agricultural Institute
PMB 8 Camden
NSW 2570
Ph 02 4640 6442 fax 02 4640 6415

Key personnel

Nerida Donovan
Elizabeth Macarthur Agricultural Institute
PMB 8 Camden
NSW 2570
Ph 02 4640 6450 fax 02 4640 6415

This report summarises the results of a field trial initiated in 2001, funded by HAL as project CT03002 between July 2005 and May 2006

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Media Summary

Citrus tristeza virus (CTV) is an aphid-borne virus that causes the most economically important viral disease of citrus. There are numerous strains of the virus (mild to severe) causing several diseases and exhibiting different symptoms in different citrus species.

One disease caused by CTV is orange stem pitting (OSP) which makes infected trees unproductive. In Australia, the disease has only been found to occur in Queensland. Orange production is a comparatively small sector of the Queensland citrus industry but this disease would have a devastating impact on national orange production if it spread to citrus growing areas in other states.

In most citrus producing areas, losses due to CTV are minimised through the use of tolerant rootstocks such as *Poncirus trifoliata*, citrange and rough lemon. In countries like Australia where CTV is widespread, the only available means of controlling the virus in susceptible scions is by mild strain cross protection (MSCP). Cross protection involves 'pre-immunising' trees with a mild strain of the virus to protect against more severe strains. MSCP has been used to effectively protect grapefruit trees against stem pitting in Australian orchards for the past 40 years.

There is little information on the number and diversity of strains that cause OSP in Queensland. Also, a suitable pre-immunising isolate has not been identified to protect sweet oranges against OSP. There is evidence that the mild CTV isolate used to protect grapefruits against stem pitting is only likely to be effective against one of the two OSP strains known to occur in Queensland.

In 2001, a field trial was established in Queensland to test a number of potential pre-immunising isolates. Each tree was assessed for stem pitting symptoms and sampled for biological and/or molecular analyses. Surveys have also been conducted in OSP affected areas to find new OSP or potential pre-immunising isolates.

Results confirmed a high disease pressure in the area of the field trial, including the two known OSP inducing isolates. There is also evidence that other OSP-inducing isolates are present in the field but are sufficiently different from known isolates to remain undetected by the existing assays. This means that we have no rapid method to detect OSP-inducing CTV, nor a full understanding of the diversity of OSP strains that occur in Queensland.

All field trees inoculated with mild strains were offered greater protection against OSP than the non-inoculated control trees. It is premature to recommend any of the pre-immunising isolates tested in this field trial for use in a cross protection strategy however this project has pinpointed some isolates with potential and some isolates that are unlikely to be of practical use in a field situation against OSP.

This work has provided evidence that the genetic diversity of CTV in the field is greater than previously thought. More work is needed to characterise the isolates collected in this study. A more extensive and structured survey of Queensland orchards is also recommended to identify other OSP-inducing isolates.

Technical Summary

Citrus tristeza virus (CTV) is an aphid-borne Closterovirus that causes the most economically important viral disease of citrus. There are numerous strains of the virus (mild to severe) causing several diseases and exhibiting different symptoms in different citrus species.

The most serious disease resulting from infection of Australian citrus trees with CTV is stem pitting. The introduction of orange stem pitting (OSP) strains of CTV into an area can severely limit or destroy an industry through a significant yield reduction, such as has occurred in Brazil (Salibe, 1966; Muller *et al*., 1968) and Peru (Roistacher, 1988).

In most citrus producing areas, losses due to CTV are minimised through the use of tolerant rootstocks such as *Poncirus trifoliata*, citrange and rough lemon. In countries like Australia where CTV is endemic, the only available means of control in susceptible scions is by mild strain cross protection (MSCP) (Bar-Joseph *et al*., 1981). Cross protection is defined as "the use of a mild virus isolate to protect plants against economic damage caused by infection with a severe challenge strain(s) of the same virus" (Gonsalves and Garnsey 1989). MSCP has been used to effectively protect grapefruit trees against stem pitting in Australian orchards for the past 40 years.

Orange stem pitting (OSP) was found in 1990 in Washington navel orange and Ortanique tangor trees in the Central Burnett area of Queensland (Owen-Turner, 1990; Broadbent *et al*., 1992). Orange production is a comparatively small sector of the Queensland citrus industry but the issue of effective cross protection must be urgently addressed given the impact that OSP could have on national orange production if OSP-inducing isolates become more widespread. However, there is little information on the number and diversity of strains that cause OSP in Queensland. Also, a suitable pre-immunising isolate has not been identified to protect sweet oranges against OSP. There is evidence that the mild CTV isolate used to protect grapefruits against stem pitting is only likely to be effective against one of the two OSP inducing sub-isolates known to occur in Queensland.

In 2001, a field trial was established to test a number of potential pre-immunising isolates. Navelina budwood was pre-immunised with the isolates and top-worked to field trees on CTV-resistant rootstocks in an area under high disease pressure from OSP-inducing strains of CTV. The ability of the isolates to provide protection against orange stem pitting symptoms in the field was evaluated.

Trees were monitored in 2003, 2005 and 2006. Stem pitting symptoms were assessed and each tree was sampled for biological and/or molecular analyses. The field samples were tested for the presence of *Citrus tristeza virus* by biological indexing and direct tissue blot immunoassay (DTBIA) and the viral isolates within them examined by reverse transcription and polymerase chain reaction (RT-PCR) followed by restriction digestion and/or nucleotide sequence analysis, as required. This determined the presence of pre-immunising and super-infecting isolates of CTV in the field samples and characterised those isolates at different levels.

In 2005, surveys were also conducted in OSP affected areas for apparently healthy sweet orange trees in OSP affected orchards. Such trees may contain mild strains of CTV with the potential to serve as new pre-immunising isolates in the future. Preliminary analysis of these samples has been conducted.

Molecular methods that specifically detect the presence of the two known OSP-inducing isolates confirmed a high disease pressure in the area of the field trial. Some field trees showing symptoms of OSP tested negative in both assays, indicating that other OSP-inducing isolates are also present in the field but are sufficiently different from PB155 and 235 to remain undetected by the existing assays. This means that we have no rapid method to detect OSP-inducing CTV, nor a full understanding of the diversity of endemic OSP-inducing isolates. Further work is required to

characterise these other strains and determine the number and type of pre-immunising isolates required to protect against them.

All field trees inoculated with mild strains were offered greater protection against orange stem pitting than the non-inoculated control trees. A greater range of viral variants were also present in the control trees, although each tree in the trial was found to contain a mixture of viral isolates. It is premature to recommend any of the pre-immunising isolates tested in this field trial for use in a cross protection strategy for the protection of sweet orange trees against OSP inducing strains of CTV. However this project has pinpointed some isolates with potential (PB64, PB67, PB61 and B1) and some isolates that are unlikely to be of practical use in a field situation against OSP (PB57).

This work has provided evidence that the genetic diversity of CTV in the field is greater than thought. More work is needed on biological segregation and characterisation of the isolates collected in this study. A more extensive and structured survey of Queensland orchards is also recommended to identify other OSP-inducing isolates. There is also a need to examine the same genomic regions studied by overseas research groups to align our CTV populations to those described elsewhere (Hilf *et al*., 2005; Lopez *et al*., 1998).

1 Introduction

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* within the family *Closteroviridae*, causes the most economically important viral disease of citrus. There are numerous strains of the virus (mild to severe) causing several diseases and exhibiting different symptoms in different citrus species. Spread of the disease is via an aphid vector or through infected propagation material.

In most citrus producing areas, losses due to CTV are minimised through the use of tolerant rootstocks such as *Poncirus trifoliata*, citrange and rough lemon. In countries like Australia, South Africa and Brazil where CTV is endemic, the only available means of controlling the virus in susceptible scions is by mild strain cross protection (MSCP) (Bar-Joseph *et al*., 1981). Cross protection is defined as "the use of a mild virus isolate to protect plants against economic damage caused by infection with a severe challenge strain(s) of the same virus" (Gonsalves and Garnsey 1989).

The most serious disease resulting from infection of Australian citrus trees with CTV is stem pitting. Stem pitting results from the abnormal differentiation of cambium cells into phloem and xylem vessels in sensitive scions and contributes to tree stunting, reduced fruit size and quality and the brittleness of branches. Strains of CTV that cause stem pitting do not affect all scions equally – studies examining the biological properties of Australian strains on indicator plants identified isolates that pit grapefruits but not sweet oranges, and vice versa (Zhou, 2001). Similarly, a recent study of a global collection of 372 CTV isolates found 13 that pitted oranges but not grapefruit and 37 that pitted grapefruit but not sweet orange (Hilf *et al*., 2005).

In Australia, MSCP is used to effectively protect grapefruit trees against stem pitting. The mild, protective isolate, referred to as PB61, that is used to pre-immunise grapefruit trees was originally obtained from a large, healthy and productive Marsh grapefruit tree in an orchard in Griffith NSW where the other trees were declining with grapefruit stem pitting. MSCP has successfully protected the Australian grapefruit industry for the last 40 years, although super-infection by severe isolates (MSCP breakdown) can occur in some environments.

Significantly, severe stem pitting of sweet oranges is considered the worst of the diseases caused by CTV as successful control methods have not been identified (Muller *et al.* 1968). The introduction of orange stem pitting (OSP) strains of CTV into an area can severely limit or destroy an industry through a significant yield reduction, such as has occurred in Brazil (Salibe, 1966; Muller *et al*., 1968) and Peru (Roistacher, 1988). Infected trees become unviable by 20 years of age (Roistacher and Moreno 1991) depending on the stage at which infection occurs. Dramatic costs are incurred due to the reduced yield and subsequent tree removal and replacement.

In Australia, sweet orange varieties were generally tolerant of endemic isolates until 1990 when severe symptoms of orange stem pitting (OSP) were found in Washington navel orange and Ortanique tangor trees in the Central Burnett area of Queensland (Owen-Turner, 1990; Broadbent *et al*., 1992). The initial response to the outbreak was the destruction of ten thousand trees and the restriction of the interstate movement of planting material from Queensland (Broadbent *et al.*, 1992). Since 1990 thousands of trees have experienced reduced yield or have been eradicated due to infection with OSP strains of CTV and quarantine measures remain in place.

Whilst orange production in Queensland is a comparatively small sector of the industry, the issue of effective cross protection must be urgently addressed, given the impact that OSP could have on national orange production if OSP-inducing isolates become more widespread. However, the potential to establish an MSCP strategy for the long-term management of orange stem pitting strains of CTV is complicated by a number of factors.

Firstly, there is little information about the number and diversity of strains that cause OSP. Most field isolates of CTV contain mixtures of strains (Ayllon *et al*., 2001; D'Urso *et al*., 2003; Gillings *et al*., 1993); and this is true of isolates characterised internationally as causing orange stem pitting (Roy *et al*., 2004; Yang *et al*., 1999). Intensive input is required to segregate these mixtures into their components for analysis and monitoring. Similarly, the original orange stem pitting isolates from Australian citrus trees (referred to as PB72 and PB75) were found to be mixtures that were segregated using single aphid transmissions (as described in Broadbent *et al*., 1996) to purify one OSP-inducing sub-isolate from each of these field isolates. The resulting sub-isolates, referred to as PB155 and PB235 respectively, have been characterised using biological and molecular means (eg Zhou, 2001; Connor 2001). Presumptive evidence indicates that one of these may have been introduced from overseas via infected budwood whilst the other may have arisen independently from a local strain. However, any number of other OSP-inducing strains may exist or may have arisen since that time, compromising our ability to detect and monitor the pathogen.

Secondly, there are no rapid means to discriminate OSP-inducing strains from those causing other symptoms. Inoculation to biological indicator plants is effective but time consuming. Serological methods that are implemented successfully overseas to discriminate mild from severe strains of CTV (Permar *et al*., 1995) or more specifically, OSP-inducing strains from others (Nikolaeva *et al*., 1998) are not effective against Australian populations of the virus (Zhou, 2001). No simple molecular marker has ever been identified that specifically correlates with any biological property of CTV (eg Hilf *et al*., 2005).

The failure of discriminatory assays developed overseas to discriminate between Australian isolates of CTV suggests that the genetic variation of local isolates is different to and/or is greater than that found elsewhere. This was recently confirmed by a study that aimed to assign isolates to groups using molecular and biological means but failed to assign 19 of 25 Australian isolates to any group (Hilf *et al.*, 2005). This potentially greater pool of CTV variants means that the Australian industry must never become complacent in terms of its perceived ability to cope with this virus.

More disturbingly for the use of MSCP as a strategy to protect sweet oranges, earlier research conducted by Dr. Hailstones, Mrs Pat Barkley and colleagues during HRDC-funded project CT97009 (Barkley and Hailstones, 2001) indicated that the mild isolate (PB61) used to protect grapefruits against stem pitting, is not likely to afford adequate protection to sweet oranges. Specifically, a series of controlled glass house experiments showed that plants pre-immunised with PB61 were effectively protected against PB235 but not PB155. Additional pre-immunising isolates are therefore required.

In the past, the selection of potential new pre-immunising isolates has been a lengthy and empirical task. However project CT97009 assessed the genetic relationship between CTV variants in a range of CTV isolates from around Australia. Three clades of CTV were identified that are analogous to different "species" or "lineages" of the virus. A number of subgroups were identified within each clade that we refer to as genotypes. Similar studies elsewhere using different collections of CTV have identified two (Hilf *et al*., 1999; Hilf et al., 2005), three (Lopez *et al*., 1998) and five (Roy and Brlansky, 2004) groups of CTV, each with subgroups within them. Importantly for the potential use of MSCP against OSP, other results from CT97009 suggest that protection may be more effective when the pre-immunising and challenge isolates are within the same clade (i.e. share a high level of genetic identity) than when they are more disparate. Establishing effective cross protection therefore requires an understanding of the diversity of field strains in the local area and means to detect and discriminate pre-immunising and challenge strains.

In 2001, Drs Hailstones and Donovan used the information from CT97009 to select seven pre-immunising isolates for use in a field trial. Six CTV isolates were selected for evaluation from the genetic clades identified in the earlier project, including PB61, the mild pre-immunising isolate used to protect grapefruits. A seventh isolate was obtained from a healthy sweet orange tree still standing

after the surrounding trees had succumbed to OSP. Navelina budwood was pre-immunised with the isolates and top-worked to field trees on CTV-resistant rootstocks in an area under high disease pressure from OSP-inducing strains of CTV. The ability of the isolates to provide protection against orange stem pitting symptoms in the field was evaluated.

Trees were monitored in 2003, 2005 and 2006 and were sampled for biological and/or molecular assessment. All field trees inoculated with mild strains were offered greater protection against orange stem pitting than the non-inoculated control trees. Mild symptoms evident in the field and on biological indicator plants indicate three of the isolates (PB64, PB67 and B1) may be suitable for use against orange stem pitting strains of *Citrus tristeza virus*.

The field samples were tested for the presence of *Citrus tristeza virus* by biological indexing and direct tissue blot immunoassay (DTBIA) and the viral isolates within them examined by reverse transcription and polymerase chain reaction (RT-PCR) followed by restriction digestion and/or nucleotide sequence analysis, as required. This determined the presence of pre-immunising and super-infecting isolates of CTV in the field samples and characterised those isolates at different levels.

In 2005, surveys were also conducted in OSP affected areas for apparently healthy sweet orange trees in OSP affected orchards. Such trees may contain mild strains of CTV with the potential to serve as new pre-immunising isolates in the future. Preliminary analysis of these samples has been conducted.

Molecular methods that specifically detect the presence of the two known OSP-inducing isolates were implemented to screen the field for their presence. Some field trees showing symptoms of OSP tested negative in both assays, indicating that other OSP-inducing isolates are also present in the field. Further work would be required to characterise these other strains and determine the number and type of pre-immunising isolates required to protect against them.

2 Materials and Methods

2.1 Field trial

During spring 2001 a field trial was established in Gayndah in the Central Burnett region of Queensland, an area where orange stem pitting strains of CTV are endemic. Gayndah has a temperate to tropical climate with summer dominant rainfall (Figure 1, BOM 2004).

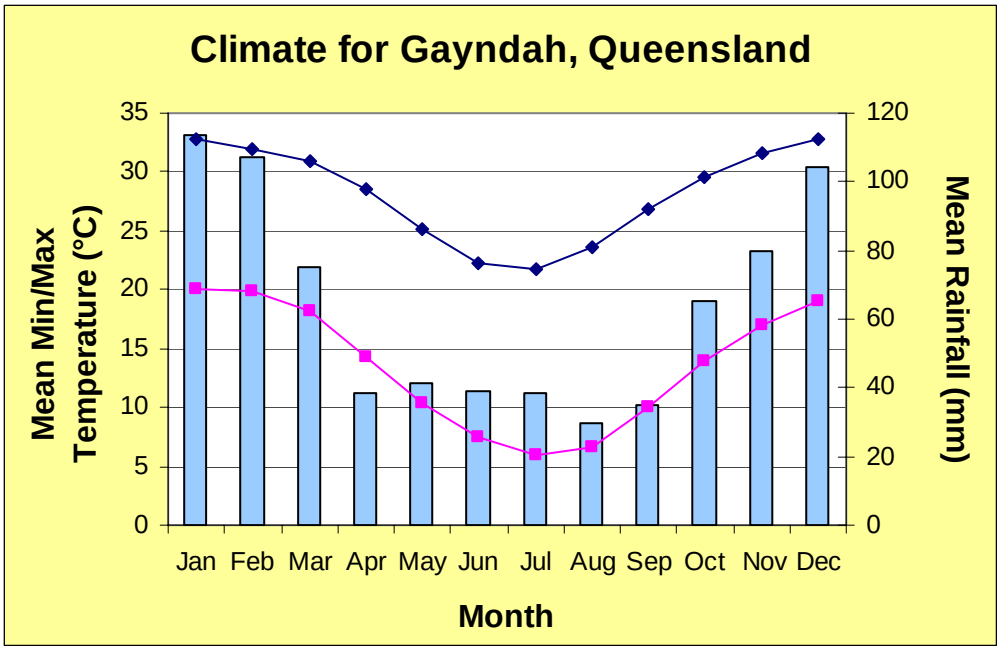

Figure 1: Temperature and rainfall data for Gayndah, Queensland (BOM 2004)

In November 1999, a number of Navelina trees were propagated in the EMAI nursery using buds taken from a virus free mother tree. Each tree was graft inoculated with one of seven PB isolates (3 trees per isolate). These trees were tested for CTV by direct tissue blot immunoassay (DTBIA). Trees that tested negative for the presence of CTV were re-inoculated and re-tested.

The Gayndah orchard block selected for the field trial contained Navelina sweet orange (*Citrus sinensis* cv 'Navelina') trees that were severely affected by orange stem pitting. The field trees were cut back to below the bud union and shoots from the citrange (*Citrus sinensis* x *Poncirus trifoliata* cv 'Troyer') rootstocks were tested in February 2001 for the presence of CTV by DTBIA. No virus was detected.

The rootstocks in the field were top-worked during September 2001 with budsticks of Navelina cut from the inoculated nursery trees. Each budstick was tested by DTBIA and only those that tested positive for the presence of CTV were used for top-working. There were eight field trees per treatment except for PB57 where only 3 trees were top-worked as there was insufficient budwood containing the PB 57 isolate. In addition eight trees were top-worked with virus-free Navelina buds to act as a control "no protection" treatment.

Treatments were not randomly distributed due to production constraints but were in ordered sets of eight for ease of management and original propagation (Figure 2). There were two buffer trees at either end of the row and one buffer tree between each set of eight. The buffer trees were top-worked with Navelina budsticks containing pre-immunising strain B1. All trees in the field trial were exposed to natural CTV infection by the local aphid population.

B1
B1
Virus-free
Virus-free
Virus-free
Virus-free
Virus-free
Virus-free
Virus-free
Virus-free
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PB 8
PB 8
PB 8
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Figure 2: Field plan of the trial established in 2001 (actual field layout – all trees in 1 row). Note that five trees were not topworked at all due to lack of budwood pre-immunised with PB57, these are marked as "-".

The seven treatment isolates were PB 8, PB 50, PB 57, PB 61, PB 64, PB 67 and B1. These were selected for the mild symptoms they induced on biological indicator plants such as West Indian lime (*Citrus aurantifolia*) and/or the genetic clades to which they belong, as determined in earlier analyses (CT97009) (Table 1). The six PB strains were sourced from trees held at EMAI although they originally came from orchards in New South Wales and Victoria (Table 1). B1 is an un-purified isolate that had been obtained prior to 2001 from an apparently-healthy Navelina sweet orange tree in the field trial block. This tree remained standing when other orange trees in the block were

decimated by OSP (Figure 3). This isolate had been maintained in Symons Sweet orange (*Citrus sinensis*) plants at EMAI in the interim.

Table 1: Characteristics of CTV strains - subisolates PB155 and 235 (subisolates known to induce symptoms of OSP) and pre-immunising isolates used in the field trial

Isolate	Origin	Coat protein *Hin*f I RFLP group	Helicase	
			Clade	Genotype
PB155	Ortanique tangor, Qld	3	X	B
PB235	Benyenda navel, Qld	1	Y	F
PB8	Appleby smooth seville, Vic	6	X	A and C
PB50	Victoria grapefruit R18T8, Vic	5	Y	G
PB57	Appleby smooth seville, Vic	6	X	C
PB61	Marsh grapefruit, NSW	5	Y	G
PB64	Victoria grapefruit R18T8, Vic	1 and 8	Y	F
PB67	Marsh Chislett, Vic	3	Y	G
B1	Navelina sweet orange, Qld	3	X, Y, Z	mixed

Figure 3: Navelina sweet orange tree on Troyer citrange rootstock – B1 treatment in field trial. Photo taken May 2002 when the surrounding trees suffering from OSP had been removed below the bud union and top-worked with mandarin varieties.

The field trial was assessed in November 2003, April 2005 and June 2006. Trees were examined for their degree of stem pitting by peeling the bark from branches. The degree of pitting was scored using the following rating system:

0 = no pitting

1 = very mild pitting

2 = mild pitting

3 = moderate pitting

4 = severe pitting

5 = very severe pitting

One pitting score was given for each tree.

Observations concerning tree and fruit size were recorded during each assessment. In 2006 tree height and width were measured using a measuring stick. The data collected was used to calculate canopy volume using the following formula:

$$\text{Canopy surface area} = \{ \pi \times (r^3) / 1.5 \} + \{ 2 \times \pi \times r \times (\text{height} - r) \}$$

where r is the radius of the canopy

Samples were collected for biological and molecular analysis in 2003 and 2005. In 2003 budsticks were sampled from each quadrant of one tree per treatment and in 2005 budsticks were sampled from each quadrant of each tree. In order to avoid cross-contamination, pruning tools were dipped in freshly made bleach solution (3% sodium hypochlorite) between trees.

Biological data from 2005 were statistically analysed using an ordinal logistic regression in the statistical software package GenStat. There was no statistical difference between the treatments with all P-values being greater than the critical value of 0.05. The small scale of the field trial and the lack of treatment randomisation make the credibility of statistical analysis questionable. No further attempts were made to analyse the results by statistical means.

2.2 Grafting and scoring biological indicators

Budsticks collected from the field trees in 2005 were grafted to West Indian lime (*Citrus aurantifolia*) and Symons sweet orange (*C. sinensis*) indicator plants in April 2005. Indicator plants were graft inoculated with approximately 5 mm^2 sections of bark with two grafts made per indicator plant, one plant per field budstick (four indicator plants per field tree) and an un-inoculated plant as a negative control in each pot.

In June 2005 the West Indian lime indicator plants were assessed for their degree of vein clearing. Vein clearing is distinguished by transparent leaf veins leading to chlorosis of the leaves (Figure 4).

Figure 4: Vein clearing on West Indian lime. Left to right: no vein clearing; mild; moderate; and severe.

The vein clearing was scored on the leaves of indicator plants as:

0 = no vein clearing

1 = very mild

2 = mild

3 = moderate

4 = severe

One score was given for each indicator plant resulting in four scores per field tree. These scores were averaged to obtain one score per field tree (Appendix 1).

In October 2005 the Symons sweet orange indicator plants were sampled and assessed for their degree of stem pitting (Figure 5). This was a destructive sampling where stems were boiled in water to soften the bark for peeling. Peeled stems were air dried before scoring.

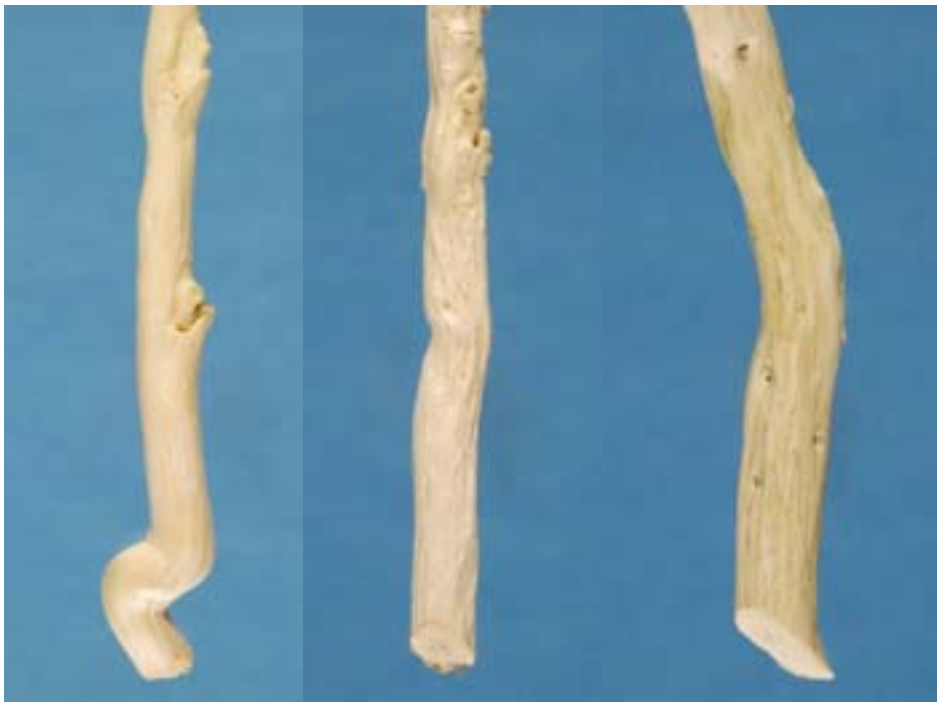


Figure 5: Stem pitting symptoms. Left to right: very mild; moderate; and very severe

The degree of pitting was scored using the same scale as in the field trial:

0 = no pitting

1 = very mild pitting

2 = mild pitting

3 = moderate pitting

4 = severe pitting

5 = very severe pitting

One score was given for each indicator plant resulting in four scores per field tree. These scores were averaged to provide one score per field tree (Appendix 1).

2.3 Serology

The presence of *Citrus tristeza virus* was confirmed in the quadrant samples from the field trip and each of the West Indian lime indicator plants via direct tissue blot immunoassay (DTBIA). This technique uses specific anti-sera to indicate the presence or absence of CTV.

Stem sections from the indicator plants were blotted onto a pure nitrocellulose membrane (*Bio-Rad* Trans-Blot® Transfer Medium 0.45µm). Known positive and negative controls were also included. The blotted membrane was incubated in 10mL 1% bovine serum albumin (BSA) in PBS (8g NaCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCl in 1L distilled water adjusted to pH 7.4) for 1 hour at room temperature. Alkaline phosphatase-conjugated CTV antibodies were then added at half the recommended dilution for ELISA for a 2 hour incubation period. The membrane was washed in PBST wash buffer (PBS plus 0.5g Tween 20 in 1L distilled water adjusted to pH 7.4) before pouring alkaline phosphatase substrate (*Sigma*® Fast™ 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium Tablets, one BCIP/NBT tablet per 10mL deionised water) over the membrane. The reaction was terminated after colour development in the positive control by transferring the membrane to deionised water. The processed membrane was examined under a dissecting light microscope and the presence of viral particles determined by the development of purple colour in CTV infected tissue.

2.4 Nucleic acid extraction

Nucleic acids were extracted from 10-20 mg of leaf tissue using the method developed during Project CT97009 and published in full in Hailstones *et al*, 2000. Briefly, tissue was ground in liquid nitrogen and nucleic acids extracted into 60 µL TES (100 mM Tris-Cl pH 8.0, 2 mM EDTA and 2% SDS) and 60 µL buffer-saturated phenol:cholorofrom:isoamyl alcohol (25:24:1). The extract was incubated at 70°C for 5-10 min, centrifuged and 40 µL of the aqueous phase applied to a home-made mini-column of Sephadex G50-80 in sterile TNE (10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA). The columns were microfuged at 4,500 rpm for 4 min to retrieve the eluate, 1 µL was used directly in Reverse Transcription Polymerase-chain-reaction (RT-PCR).

2.5 RT-PCR and gel analysis

For RT-PCR, 1 µL of each RNA extract was reacted in the Super Script ™ III One Step RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen Pty Ltd), according the manufacturer's instructions. All primer sequences are shown in Table 2.

Table 2: Primers implemented in this project

Primer Name	Sequence (5'-3')	Target	Reference
CP1	ATGGACGACGAAACAAAG	Coat protein	Gillings *et al.*, 1993
CP3	TCAACGTGTGTTGAATTT	Coat protein	Gillings *et al.*, 1993
HEL F2	GTTAYGARGCTCSTCCCGGTGGTG	Helicase	Connor, 2001
HEL R2	AACTCGAGCCGGTTCGTGTARRTTAC	Helicase	Connor, 2001
155 F	GAAATAGGAGTGTGCGTA	PB 155	Connor, 2001
155 R2	AAGTGTCTTCGTTATCACCAACGA	PB 155	Connor, 2001
235 F	GAAAGCAACACAGCGAGCAA	PB 235	Connor, 2001
P23 R	TCAGATGAAGTGGTGTTCAC	PB 235	Connor, 2001
XF 100	GCAAGCRGAGATTTYTCRAA	Clade X	Connor, 2001
YF 466	GGCTTTCTGAGTTTTACCCSC	Clade Y	Connor, 2001
ZF 766	ACAACCACGTWGTYGTRGCG	Clade Z	Connor, 2001
A11R 854	HCGAWYGAAWYTTNKCRARAATYYC	All clades	Connor, 2001
FF 567	CGACAAGGCAGCGAGA	Genotype F	Connor, 2001
GF 496	ACTGCTAATGTCGGRAGGA	Genotype G	Connor, 2001
CF 180	GGTAGAGTATTCACTGTAGGT	Genotype C	Connor, 2001
YR 807	TATCRTCGTACCTCCTACTAG	Genotypes F and G	Connor, 2001
CR 312	GTGAATATATGAATTTGTCTACGG	Genotype C	Connor, 2001

Conditions and profiles for all reactions were re-optimised initially for use under current conditions, with reference to appropriate controls. All reactions used the buffer supplied and final concentrations of other components as per Table 3. All PCR cycles were performed with 15 seconds denaturation, 30 seconds annealing and 45 seconds extension. Reaction products were separated by electrophoresis in 1% agarose gels in TBE buffer and stained in ethidium bromide, viewed under UV light and images captured using a GelDoc 2000 (BioRad).

Table 3: Parameters of RT-PCR using Invitrogen one step RT-PCR kit to targets indicated

	Coat protein	Helicase	p23 PB 155	p23 PB 235	Clade X (helicase)	Clade Y (helicase)	Clade Z (helicase)
Final volume	20 L	20 L	10 L	10 L	10 L	10 L	10 L
Amplified fragment	672 bp	1163 bp	377 bp	596 bp	799 bp	433 bp	131 bp
RT temp, time	45°C 30 min	43°C 40 min	43°C 40 min	58°C 40 min	43°C 40 min	60°C 30 min	43°C 40 min
Annealing temp	TD [1] 51-43 °C	TD 60-52 °C	TD 68-61°C	TD 68-58°C	TD 60-52°C	TD 68-62°C	TD 61-53 °C
No. cycles	40	40	40	35	40	40	40
$MgSO_4$	2 mM	mM	mM	1.6 mM	2 mM	1.6 mM	mM
5' primer	CP1 0.2 M	HELF2 0.2 M	155F 0.2 M	235F 0.2 M	XF100 0.8 M	YF466 0.2 M	ZF766 2 M
3' primer	CP3 0.2 M	HEL R2 0.2 M	155R2 0.2 M	P23R 0.2 M	A11R854 0.8 M	A11R854 0.2 M	A11R854 2 M
RT/ Taq	0.8 L	0.8 L	0.4 L	0.4 L	0.4 L	0.4 L	0.4 L

[1] TD = Touch Down over a number of cycles (4-8) at 1 or 2 °C/cycle.

2.6 Nested PCR

Where the result of an RT-PCR to a particular clade indicated that the clade was present, 1 μL of the reaction was used in subsequent nested PCR to detect the presence of specific genotypes within that clade.

One μL of reaction product from a first round RT-PCR was added to nested PCR using internal primers as per Table 2. One unit of Invitrogen DNA Taq polymerase was used with the buffer supplied in a final volume of 10 L. Final concentration of dNTPs was 250 M and of $MgCl_2$ was 1.25 mM in all cases. Conditions were optimised for each genotype and are shown in Table 4.

Table 4: Reaction parameters for nested PCRs for genotypes F, G and C

	F genotype	G genotype	C genotype
Amplified fragment	280 bp	351 bp	172 bp
Cycle time in seconds - denature/anneal/extend	20/20/40	30/30/60	30/30/60
Annealing temperature	TD [1] 67-61 °C	TD 67-61 °C	TD 62-56°C
Number of cycles	35	40	40
5' primer	FF 567 0.3	GF 496 0.9	CF 18 0.3
3' primer	YR 807 0.3	YR 807 0.9	CR312 0.3

[1] TD = Touch Down over a number of cycles (4-8) at 1 or 2 °C/cycle.

2.7 Digestion

Amplified PCR products to the coat protein gene were digested with *Hin*f I restriction enzyme (Roche). Usually 10 µL of PCR product was incubated at 37°C for 1-2 hours using the recommended buffer in a total volume of 20 µL. Digest products were separated by electrophoresis in 2% agarose gels in TBE.

Restriction fragment length polymorphism (RFLP) profiles of the coat protein gene after digestion with *Hin*f I were assigned to groups based on band sizes. The profiles of the 10 known groups are shown in Figure 6.

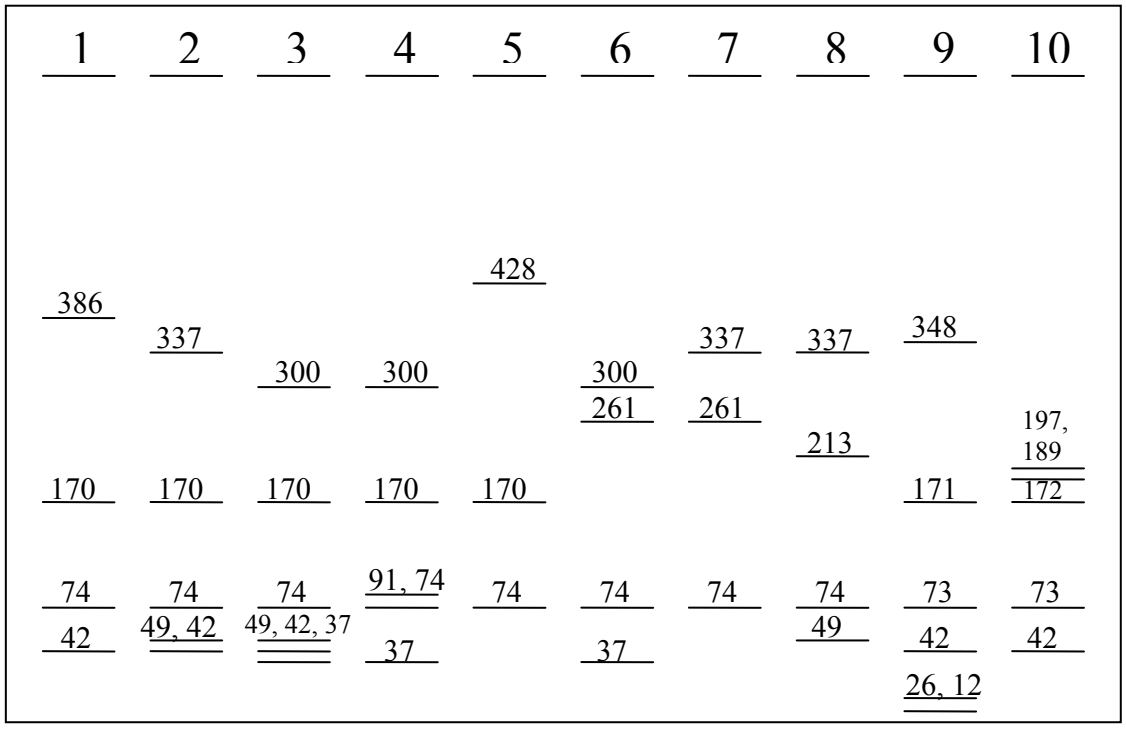


Figure 6: Schematic diagram illustrating ten RFLP groups for *Citrus tristeza virus* coat protein gene following digestion with *Hin*f I and electrophoresis. Numbers above lines indicate the size in base pairs of the resulting fragments. Groups 1 to 7 were identified in Gillings *et al*., 1993, Group 8 was identified by Zhou (2001), Groups 9 and 10 were identified in Hailstones *et al*., 2002.

2.8 Cloning

Amplified fragments from the helicase region were ligated into the Promega pGEM®-T and pGEM®-T Easy plasmids using Promega's Vector systems, according to the manufacturer recommendations. Recombinant colonies were identified using blue/white selection and 30 - 40 colonies from each cloning event were screened by PCR to confirm the presence of the insert. Plasmid inserts were screened for diversity by digestion with *Rsa* I and *Hin*f I (Roche) in different reactions, according to the manufacturer's recommendations. Clones were assigned to groups based on banding profiles, one clone from each of the groups identified was subjected to nucleotide sequence analysis.

2.9 Analysis of nucleotide sequences

The coat protein gene and the 1163 bp band from the helicase region were amplified from CTV cDNA, and then purified using the JetQuick Spin Column Technique (as per the manufacturer recommendations). The nucleotide sequencing reaction was set using the Dye Terminator Cycle Sequencing Kit (Beckman Coulter) and analysed on a CEQ 8000 Genetic Analysis System (Beckman Coulter). Nucleotide sequences were aligned manually using BioEdit (© Tom Hall, California, 1997-2005, available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html) or using the program ClustalW, sourced from the Australian National Genomic Information Service (ANGIS) interface. The sequences of the primers were removed. Phylogenetic analyses were performed using applications from ANGIS and the default parameters. The most parsimonious tree was generated from the aligned nucleotide sets using the application DNApars, distance matrices were assembled using DNAdist and neighbour joining trees were generated from these using the program Neighbour.

2.10 Orchard survey and analysis

Four orchards separate from the site of the field trial were also surveyed in April 2005 - one in Gayndah, two in Mundubberah and one in Bundaberg. Trees appearing to be healthy despite growing in diseased orchards were targeted for sampling. Samples were collected from these trees, tested for the presence of CTV by DTBIA and graft inoculated onto Symons sweet orange and West Indian lime indicator plants. Symptoms of stem pitting were recorded on the Symons sweet orange indicator plants and vein clearing was assessed on leaves of the West Indian lime plants. All methods used for the biological, serological and molecular testing of the orchard survey samples were the same as those used to test the field trial samples collected in 2005.

3 Results

3.1 Assessment of field trial

3.1.1 November 2003

By late November 2003, 6 trees in the trial had either died or the top-working had failed: 2 that had been pre-immunised with PB50, 1 with PB61 and 3 with PB67. These trees were excluded from all analyses.

The control trees that had not been pre-immunised with any viral strain showed moderate to severe stem pitting symptoms (Figure 7, Appendix 1) confirming that OSP-CTV had been transmitted to the trees by aphids. Amongst the trees that had been pre-immunised, symptoms were most severe in those pre-immunised with PB 50. All other surviving trees showed fewer symptoms than the trees not containing a pre-immunising strain. Aphids were present in large numbers on the most recent flush.

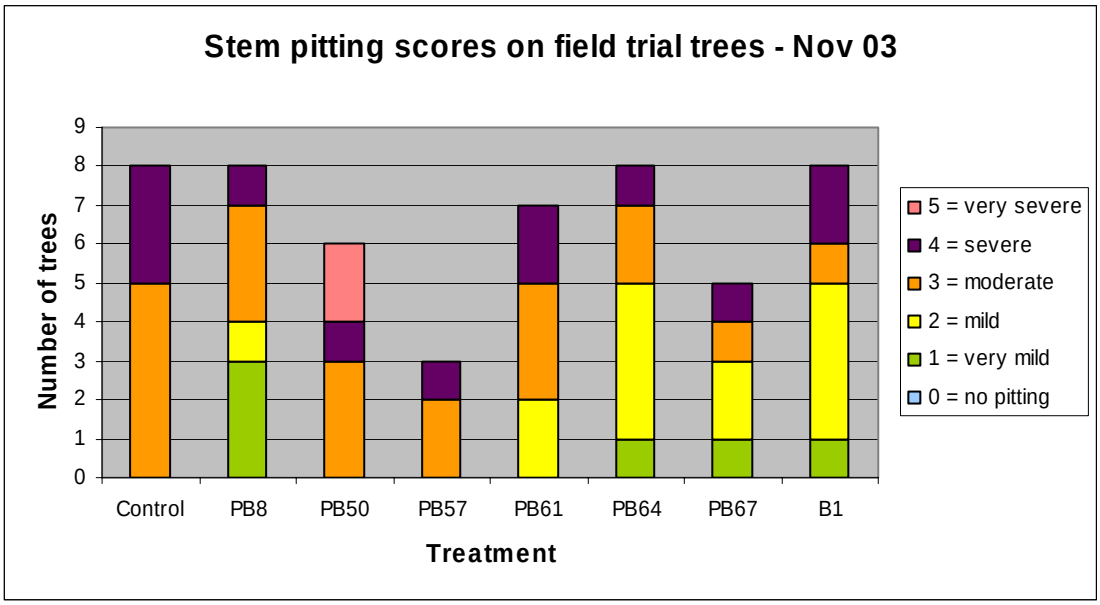


Figure 7: Stem pitting severity scores for field trees assessed in April 2005. The control treatment is where the trees were topworked with virus-free budsticks.

3.1.2 April 2005

By autumn 2005, the symptoms generally appeared to be less severe than those observed in November 2003. The majority of trees exhibited mild to moderate stem pitting (scores 1-3) and eight trees were assessed as having no stem pitting (Figure 8, Appendix 1). Of the treatments in which all eight trees could be assessed, PB64 had the lowest stem pitting severity scores while the control treatment had the highest scores.

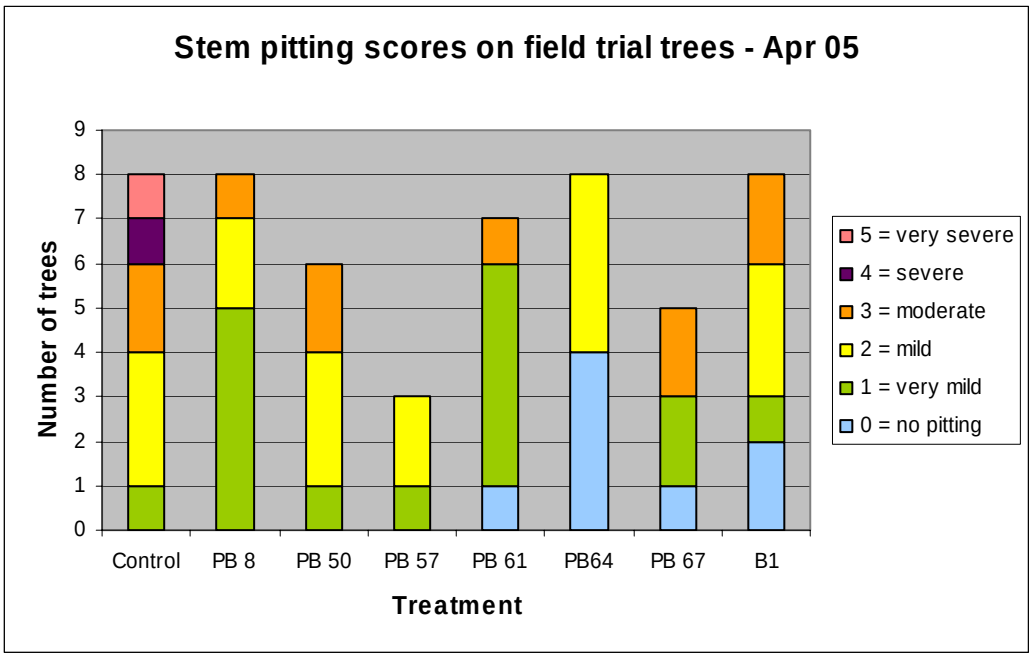


Figure 8: Stem pitting severity scores for field trees assessed in April 2005. The control treatment is where the trees were topworked with virus-free budsticks.

The ordinal logistic regression performed on the pitting data returned the probabilities of achieving each pitting score in each treatment. There was no statistical difference between the treatments with all P-values being greater than the critical value of 0.05.

The assessments of tree and fruit size were consistent across the field trial. One tree was scored as being large while the remaining trees were scored as being small or medium in size. All trees exhibited fruit of a standard size. The amount of fruit on the trees varied throughout the trial and did not appear linked to any particular treatment but no data was recorded for analysis. Aphids were present in large numbers on many of the field trees.

3.1.3 June 2006

In June 2006, the majority of trees exhibited very mild to mild stem pitting symptoms (scores 1-3) with four trees assessed as having no stem pitting (Figure 9, Appendix 1). As in 2005, the control trees had the highest stem pitting scores and only mild symptoms were found in the PB64 treatment. Isolates B1, PB67 and PB61 also performed well. Of the pre-immunising treatments, only PB8 and PB50 had trees exhibiting moderate stem pitting symptoms. Aphids were feeding on the most recent flush.

The severity of stem pitting symptoms exhibited in the field trial trees seems to have decreased over time (from 2003 to 2006) but this could be due to the assessment of trees at different times of the year.

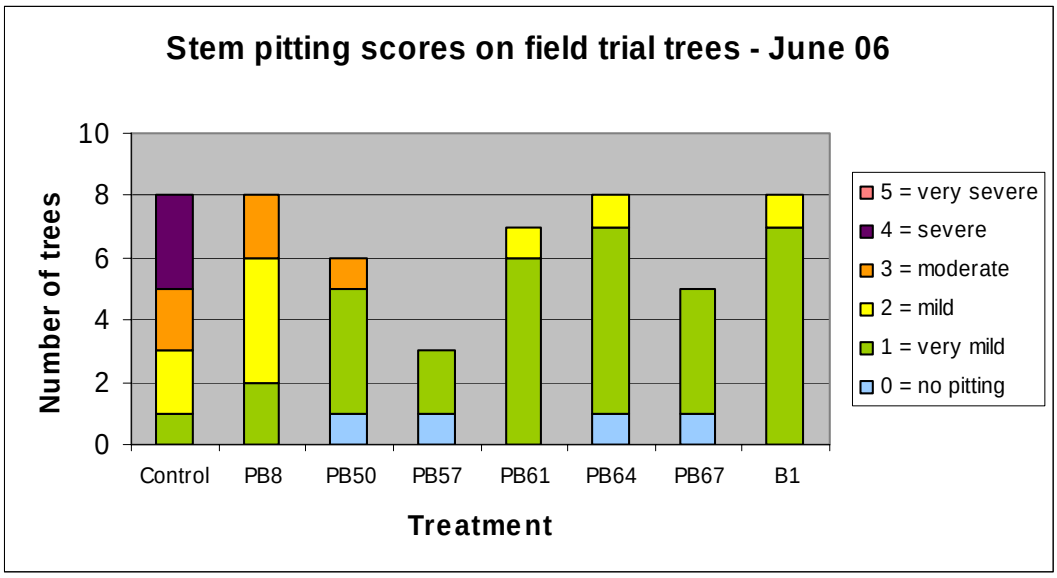


Figure 9: Stem pitting severity scores for field trees assessed in June 2006. The control treatment is where the trees were top-worked with virus-free budsticks.

There was not a significant treatment difference in the height and width of field trees, as measured in June 2006 (Appendix 1). As a consequence there was not much difference in canopy surface area (Figure 10, Appendix 1) and any differences do not correspond to symptom expression. The trees inoculated originally with PB67 had the smallest canopy surface area but relatively mild stem pitting symptoms were found in the branches that were assessed. The most severe stem pitting symptoms were observed in the control trees but stem pitting does not appear to have affected tree size to date.

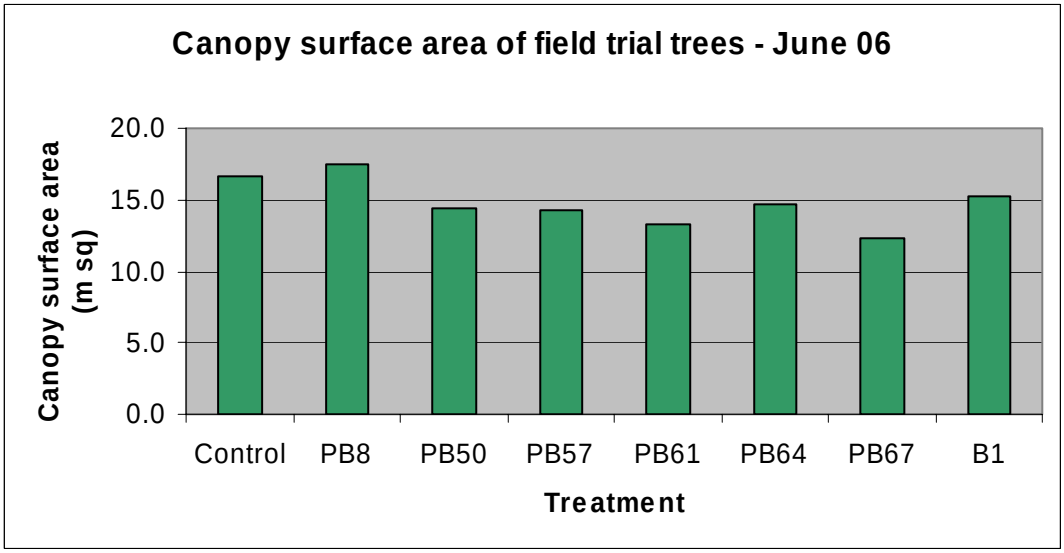


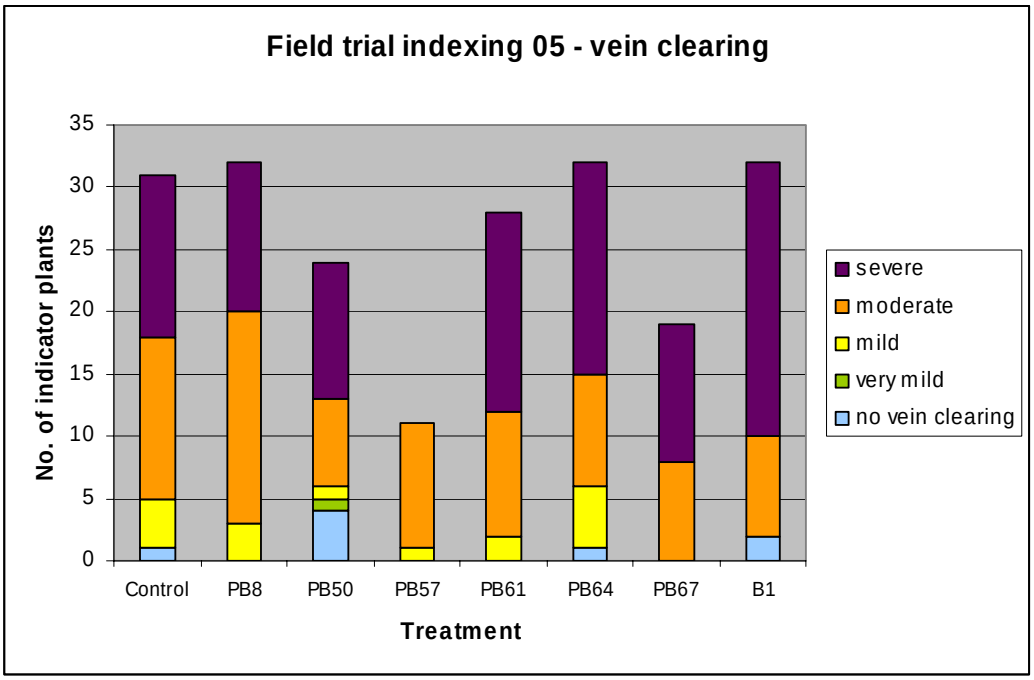
Figure 10: Canopy surface area (m^2) of field trial trees measured in June 2006

The fruit had been picked from the field trial trees in April therefore no observations concerning fruit size or number could be recorded. The grower did comment that this year there appeared to be differences between trees in the number and size of fruit. This data was not recorded but it is planned to record data on fruit size and load from each individual trial tree in April 2007.

3.2 Biological assessment of samples

Almost all field trial samples indexed to West Indian lime plants (*Citrus aurantifolia*) exhibited vein clearing. Vein clearing scores are shown in Figure 11. The ordinal logistic regression performed on the data determined that B1 was the only treatment to have scores significantly lower than the non-inoculated field control (P = 0.027; P > 0.05) (Appendix 1).

A)



B)

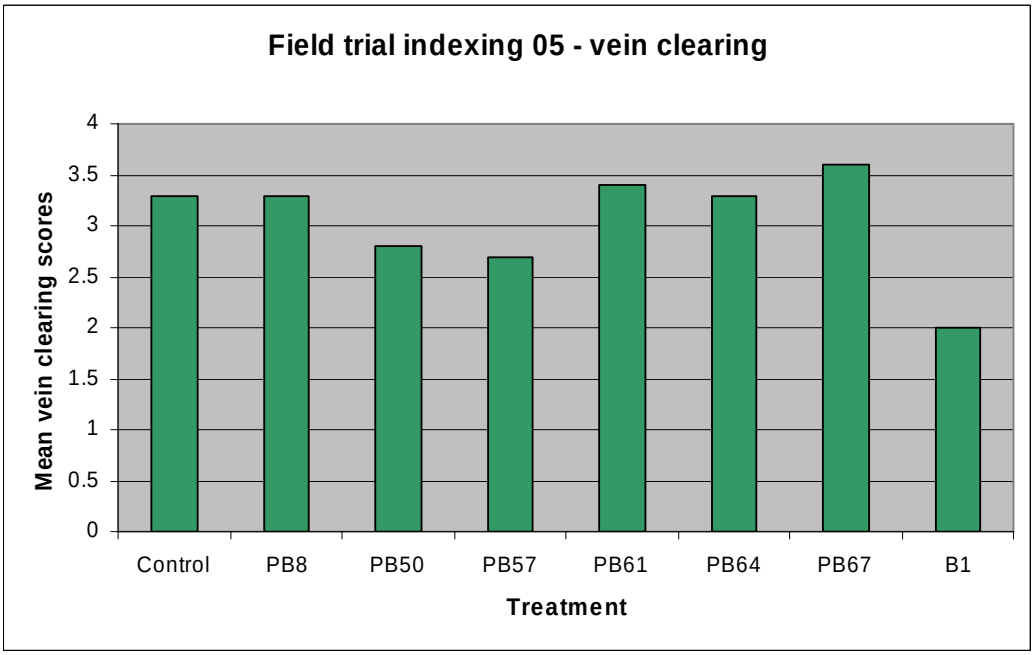
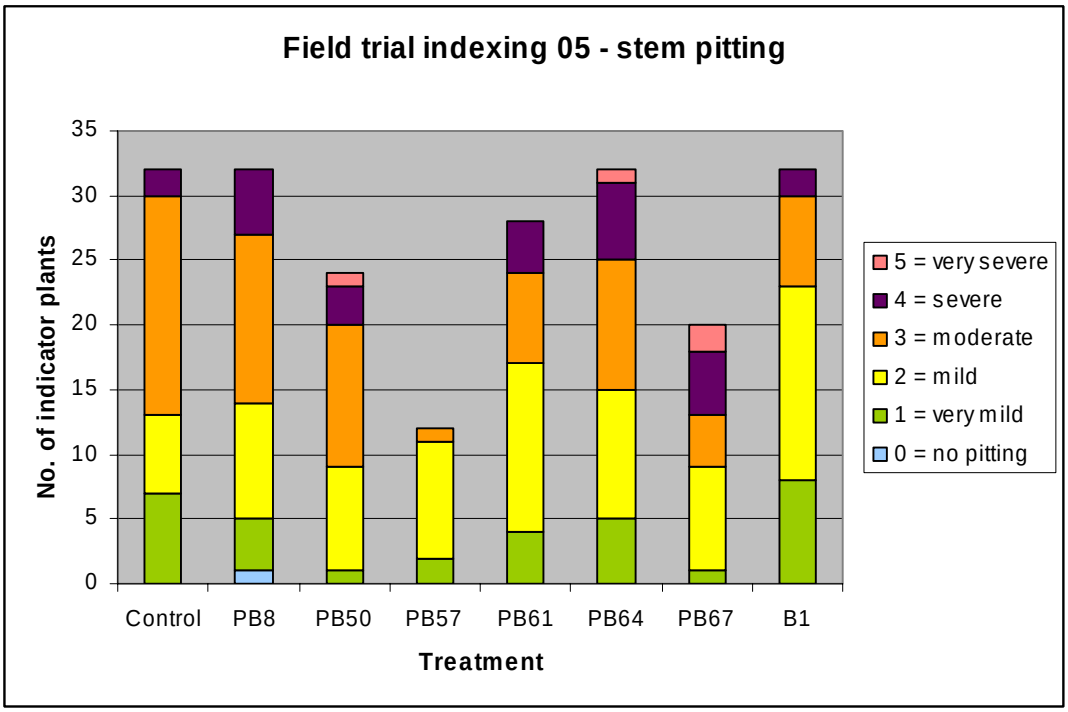


Figure 11: Vein clearing severity scores of field trial samples indexed to West Indian lime (*Citrus aurantifolia*) indicator plants. (A, previous page) scores for each of the indicator plants for each treatment, (B) mean score for each treatment. The control treatment is where trees were top-worked with virus-free budsticks.

Samples from the field trees were also scored based on the extent and severity of pitting in the Symons sweet orange (*Citrus sinensis*) indicator plants. Symptoms varied but severe stem pitting was evident in trees from all treatments (Figure 12, Appendix 1). Very severe stem pitting symptoms were observed on indicator plants inoculated with samples taken from field trees pre-immunised with PB50, PB64 and PB67 (Figure 12).

A)



B)

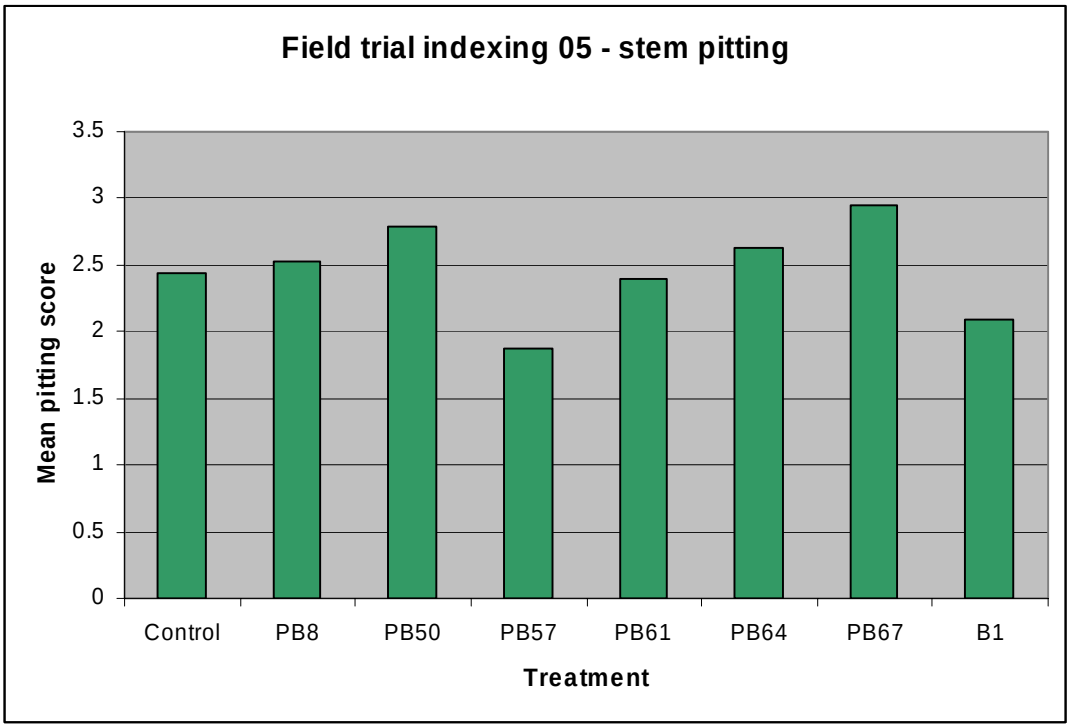


Figure 12: Stem pitting severity scores of field trial samples indexed to Symons sweet orange (*Citrus sinensis*) indicator plants. (A) scores for each of the indicator plants for each treatment, (B) mean score for each treatment. The control treatment is where trees were topworked with virus-free budsticks.

3.3 Serological screening

DTBIA indicates the presence or absence of CTV in a sample cannot differentiate between CTV isolates. All the field samples and West Indian lime (WIL) indicator plants were assessed by DTBIA and all returned positive reactions for CTV. This means that all the field trees in the trial contain at least one CTV isolate but there could be more than one isolate present in each tree, introduced by aphids.

3.4 Molecular analysis

RNA extracts were prepared from all WIL indicator plants inoculated with field material. Extracts were used in a number of different Reverse Transcription-Polymerase Chain Reactions (RT-PCR) to analyse the viral isolates within them.

3.4.1 Coat protein gene

The CTV coat protein (CP) gene was amplified from all extracts and analysed for Restriction Fragment Length Polymorphisms (RFLP) following digestion with the restriction enzyme *Hin*fI (referred to as the CP/*Hin*f RFLP profile). Profiles were compared to digestion patterns that characterise the ten pre-defined groups shown in Figure 3 (Gillings *et al*., 1993; Zhou 2001, Hailstones *et al*., 2002). Results are shown in Appendix 2. Analysis of the CP/*Hin*f RFLP profiles from almost all of the extracts indicated the presence of fragments adding to more than 672 base pairs, indicating mixed isolates of CTV in the field extracts.

Amongst the samples collected in 2003, the CP/*Hin*f RFLP profile of group 3 was detected in all trees (Appendix 2). Profiles 1, 2 and 5 were also detected, though less frequently. The presence of other CP/*Hin*f RFLP profiles was not observed, even where the field trees had been pre-immunised with isolates of other groups. For example T15 and T31 were pre-immunised with PB8 and PB57 respectively, both of which have a CP/*Hin*f RFLP profile of group 6, but a group 6 profile was not observed in either case.

Extracts from indicator plants grafted from samples collected in 2005 from T15, T32 and T40 were negative for CTV despite repeated extraction and reaction, suggesting that inoculation to the indicator plants had been unsuccessful. These trees were excluded from subsequent analyses.

In the extracts from indicator plants inoculated with the other field samples collected in 2005, mixtures of CP/*Hin*f RFLP profiles were again common (Appendix 2). CP/*Hin*f RFLP groups 1 and 3 were found to predominate with rarer instances of groups 2, 5, 6 and 8. Again, very few of the field trees generated profiles that matched those of their pre-immunising treatments and where present these were usually in mixtures with other RFLP groups. In the cases where the field tree samples contained the same profiles as their treatments the profile evident was from CTV coat protein *Hin*f I groups 1 or 3.

Six extracts were selected for nucleotide sequencing of the CP gene, to look for variation that may be masked by restriction analysis alone. One extract was selected from the initially virus free group (T10), one from each of the groups pre-immunised with PB8, 64 and B1 (T14, T55 and T66 respectively) and two from the group pre-immunised with PB50 (T22 and T27). The combined molecular analysis of the extracts from all of the trees pre-immunised with PB57 and PB61 had indicated that these trees all contained too many viral variants within them to allow direct nucleotide sequencing using this means, so none of the trees pre-immunised with PB57 or PB61 were used in this analysis.

The nucleotide sequences obtained for the coat protein genes of isolates from T10, T14, T22, T27 and T66 were subjected to a theoretical analysis to search for *Hin*f I restrictions sites within them. This indicated that all would produce CP/*Hin*f RFLP profile number 3, supporting earlier findings based on the actual digestion of the amplicons from these extracts.

More detailed analysis of the full nucleotide sequence nevertheless confirms that differences do occur between the nucleotide sequences of these isolates in the coat protein gene. However, because the differences occur at locations other than the recognition site for *Hin*f I, they do not impact on the restriction pattern obtained with this enzyme. Overall, the coat protein gene sequences of T22, T55, T14, T27 and T10 are more than 98.5% identical to each other. T66 is more removed from this group, being only 93 to 95% identical to the other field isolates.

The nucleotide sequences of the coat protein genes of these field trees were compared using phylogenetic methods to the sequences of a selection of other isolates obtained from the international database. Results were the same using both Neighbour Joining (NJ) and Maximum Parsimony (MP) methods, the NJ tree is shown in Figure 13.

T22, T55, T14, T27 and T10 cluster together and are separate from T66. The group containing T22, T55, T14, T27 and T10 also includes severe OSP-inducing subisolate PB155, grapefruit stem pitting isolate PB51, an isolate from a mandarin tree at Dareton and JX1-1, a database sequence from China (Figure 13).

T66 clusters in a separate group that includes two clones from PB219 (a severe Australian grapefruit stem pitting isolate), isolate VT (a decline isolate from Israel), NUaga (a seedling yellows strain from Japan), 13C (an isolate from Portugal) and AF249279 from South Korea (no biological information is available on these isolates).

A third group is formed by a collection of mild isolates that includes PB61, T30 from Florida and T385 from Spain, PB246 (a mild isolate derived from PB219) and TAM11, an isolate from Mexico (for which no biological information is available). The second known OSP inducing isolate PB235 also falls within the third group, confirming its close relationship to PB61 despite the fact that it is known to induce symptoms of OSP.

As would be expected, less difference is observed between the isolates on analysis of the amino acid sequences inferred from the nucleotide sequence data. The inferred protein sequences obtained from isolates T10, T22 and T27 show 100% identity to each other and T66 is no more different to any other isolate than they are to each other. Maximum difference between any two isolates at this level is 3.5 %, between T55 and T66.

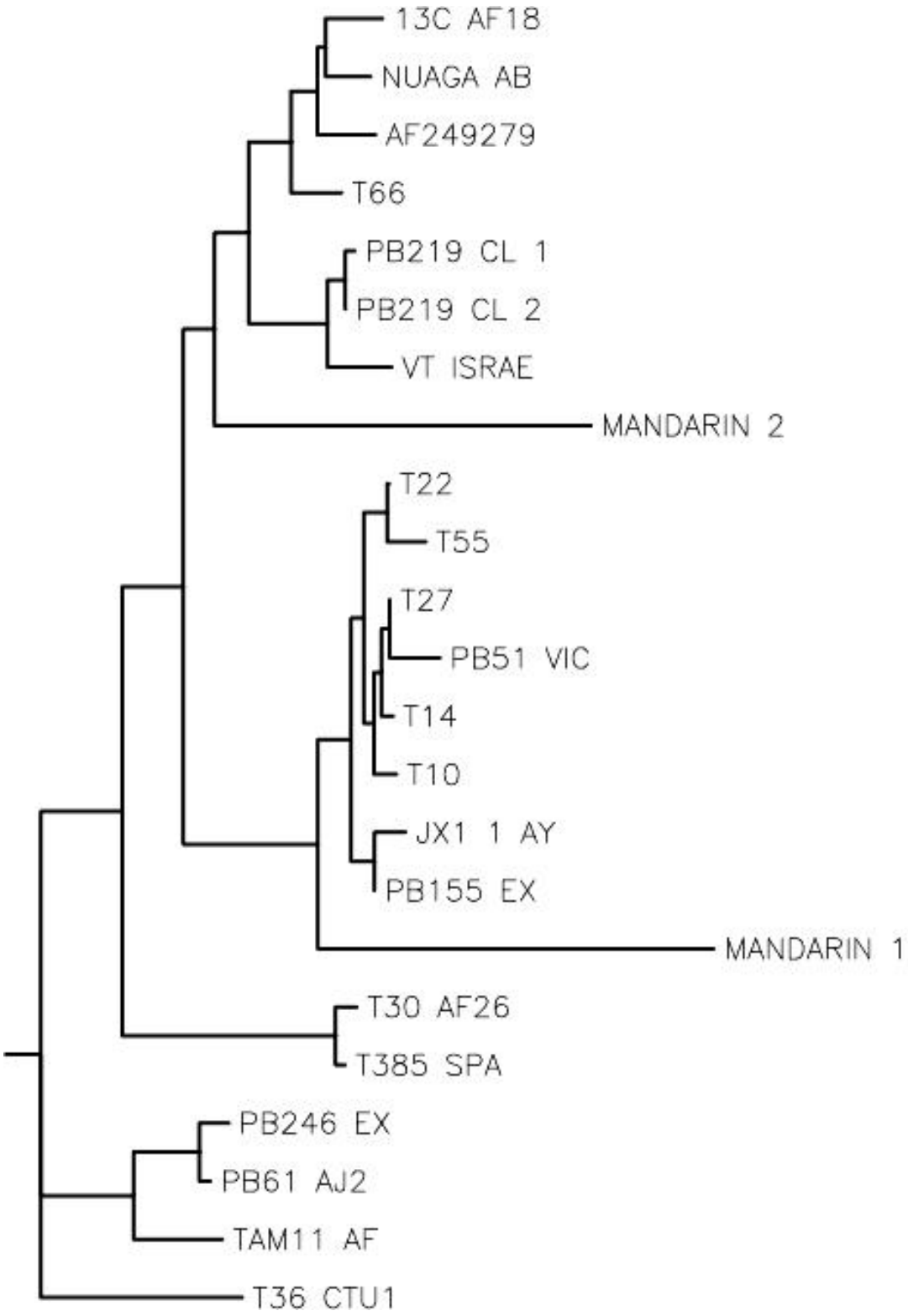


Figure 13: Neighbour Joining tree of coat protein sequences from this project and the international database. Sequences of T10, T14, T22, T27, T55 and T66 are from this study, previously unpublished sequences from our laboratory were used for PB219 clones 1 and 2, PB51, PB155, PB61, PB246 and two clones from Mandarin from Dareton. Sequences for other isolates (13C, NUaga, AF249279, VT, JX1-1, T30, T385, TAM11, T36) were obtained from the international database.

3.4.2 p23 gene

All RNA extracts were screened by RT-PCR to the p23 gene, using the assays designed in CT97009 to specifically identify the two known OSP-inducing subisolates of CTV that are referred to as PB155 and PB235.

Using these assays to test the samples collected in 2003, PB235 was detected only in the sample from an uninoculated tree T7 and in the sample from T43, which had been pre-immunised with PB61. PB155 was detected only in T15, a tree pre-immunised with PB8. The remaining samples were negative for both assays (Appendix 2).

Amongst the samples collected in 2005 (Figure 14, Appendix 2), the known OSP-inducing strains were detected most often in the 8 control trees that were top-worked with virus-free budwood - 4 were positive for both strains, 3 for one or the other and only 1 remained negative for both. Stem pitting symptoms were observed during each field assessment on the tree that tested negative for both known OSP-inducing strains (Appendix 1).

Of the pre-immunised trees, 6 tested positive for PB155, 3 for PB235 and 6 for both PB155 and PB235. The 27 remaining trees were negative for both known OSP-inducing strains despite the fact that many of them showed symptoms of OSP in the field and/or on SSO indicator plants (Appendix 1 and 2).

These results confirm that strains consistent with the two known OSP-inducing subisolates were present in the field at the time of the trial. Significantly, some trees showing field symptoms of OSP were found to be negative for both known strains. Therefore it is likely there are CTV isolates in the field trial trees that are able to cause orange stem pitting in addition to the previously identified PB155 and PB235 isolates.

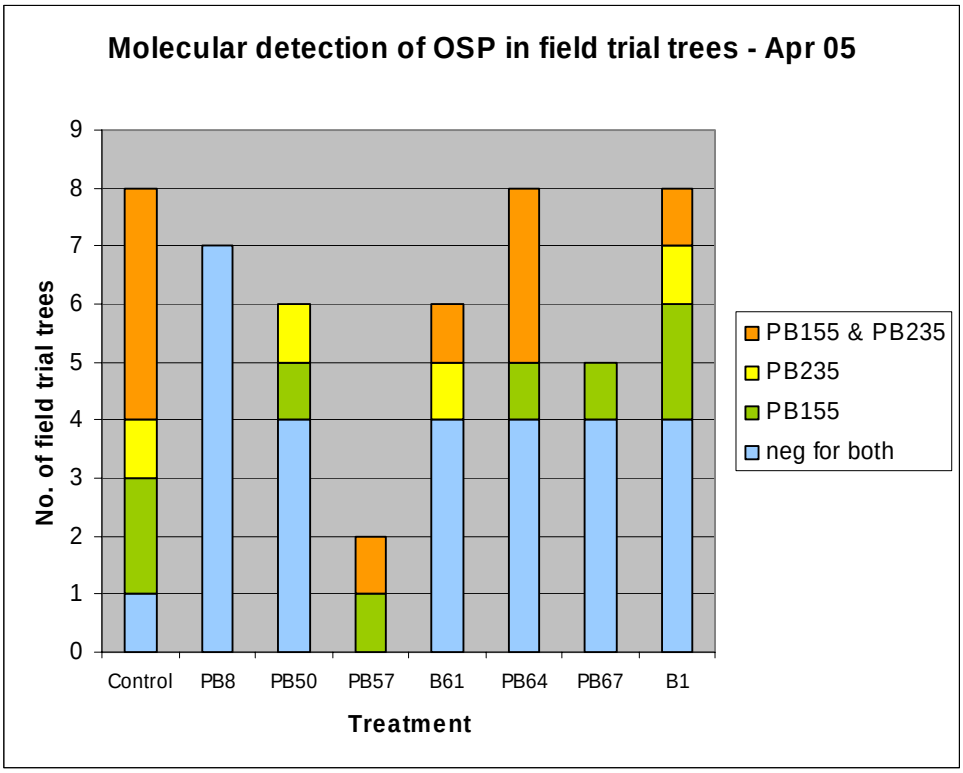


Figure 14: Molecular detection of OSP inducing isolates of CTV (PB155 and PB235) in field trial trees using RT-PCR to the p23 gene.

3.4.3 Helicase region

Project CT97009 had identified three clades of CTV (referred to as X, Y and Z) from analysis of helicase variants within field isolates sourced from within Australia from a range of locations and hosts and causing different symptoms. A number of genotypes were further identified within each clade (A to D in clade X; F, G H1 and H2 in clade Y; E1 and E2 in clade Z). That project also developed molecular reactions to the helicase region to identify the presence of each of the clades specifically, and to the genotypes within them, in RNA extracts derived from samples on indicator plants. Samples are screened initially in each of the three clade-specific RT-PCRs for the presence of variants within that clade. Any sample that returns a positive result for any of the clades can then be analysed separately in series of nested PCRs to determine which of the specific genotypes within that clade is present.

The use of the nested PCR, involving two separate rounds of amplification, provides a more sensitive detection of viral subtypes within a mixture and provides contextual information on how the diversity of the strains present at the site of the field trial fits within the "lineages" of CTV identified earlier. Time constraints on this project prevented a comprehensive analysis of all the specific genotypes present in each field sample. We performed a more limited analysis using RT-PCRs to each of the three clades and then selected nested PCRs corresponding to the genotypes of the pre-immunising isolates used in the field trial. Appendix 2 contains the results of these analyses.

Samples from field trees collected in both 2003 and 2005 generally showed the presence of CTV variants derived from more than one CTV clade and frequently from all three. The C genotype, the genotype of the pre-immunising isolates PB8 (T12 to T19) and PB57 (T30 and 31) was not detected in the field samples at all. Pre-immunising isolate PB64 is of the genotype F which was found to be very widespread in both 2003 and 2005. Three pre-immunising isolates PB50 (T21 to 27), PB61 (T39 to 46) and PB67 (T57 to T62) are of genotype G, which was found to be comparatively widespread amongst all the pre-immunised trees in the trial, not just those pre-immunised with the G-type isolates. Curiously, the G genotype was not evident in any of the trees that were initially virus-free (T3 to T10) when sampled in 2005 and was only detected in one of 9 samples taken in 2003.

Time constraints also prevented an extensive analysis of the diversity of helicase variants present in samples from the field trees after grafting to biological indicators. Two samples collected from trees in 2005 were subjected to full analysis through cloning and sequencing of all of their detectable helicase variants. The trees selected were T55 (pre-immunised with PB64) and T66 (pre-immunised with isolate B1). Both field trees were selected because indicators derived from the field trees showed symptoms of OSP yet they tested negative in the p23 assay for both known OSP-inducing CTV strains (Figure 14, Appendix 2), which may indicate that they contain novel OSP-inducing strains. Also, the results of the combined testing shown in Appendix 2 suggested that the existing helicase assays detected few if any strains within these isolates (F only in T55 and none in T66), implying that they may contain completely novel strains. Isolates from these two trees were also the most disparate on the crude analysis of CP gene sequences described in Section 2.4.1 above.

8 clones obtained from T55 and 9 clones from T66 contained helicase inserts. Digestion of each insert with *Rsa* I and *Hin*f I in different reactions revealed 3 different groups amongst the T55 clones and 2 amongst the T66 clones. These low numbers suggest little genetic diversity within each isolate, consistent with earlier results (see above). One clone from each group was selected (these are referred to as clones 55.1, 55.3, 55.26, 66.14 and 66.20) and subjected to nucleotide sequence analysis. Sequences were compared to those of clones obtained in CT97009 by distance analysis and phylogenetic methods.

Figure 15 shows the combined results for these five clones (plus those obtained from survey trees reported in section 3.3.4).

Clones T55.1 and 66.14 fit neatly within genotype F, clone T55.3 also clusters within F though more loosely. Group F was detected in the specific assay in T55 but not in T66, although analysis of the primer sites indicates that mismatched sequences were not likely to be the cause of this failure to detect. It is possible then that variants of the F genotypes may be only infrequently present in T66. PB235, one of the two known OSP-inducing subisolates is of genotype F but its presence was not detected using the p23 assays.

Clone 66.20 is of genotype D, whereas PB155 is B. The reason why T66 returned a negative result in all assays (except coat protein gene) remains unclear.

Both T55 and T66 showed symptoms of OSP whilst being negative in both p23 assay and most or all helicase assays. In theory any of the variants sequenced might be the causal agent of their observed OSP symptoms.

Clone 55.26 is more interesting, lying separate from all three previously-described clades using both NJ and MP analyses. Undoubtedly this variant would have escaped detection using all our existing assays. A number of explanations can be envisioned for the origin of this variant but the parent isolate certainly warrants further investigation to biologically segregate the components from within this mixture and examine their biological properties.

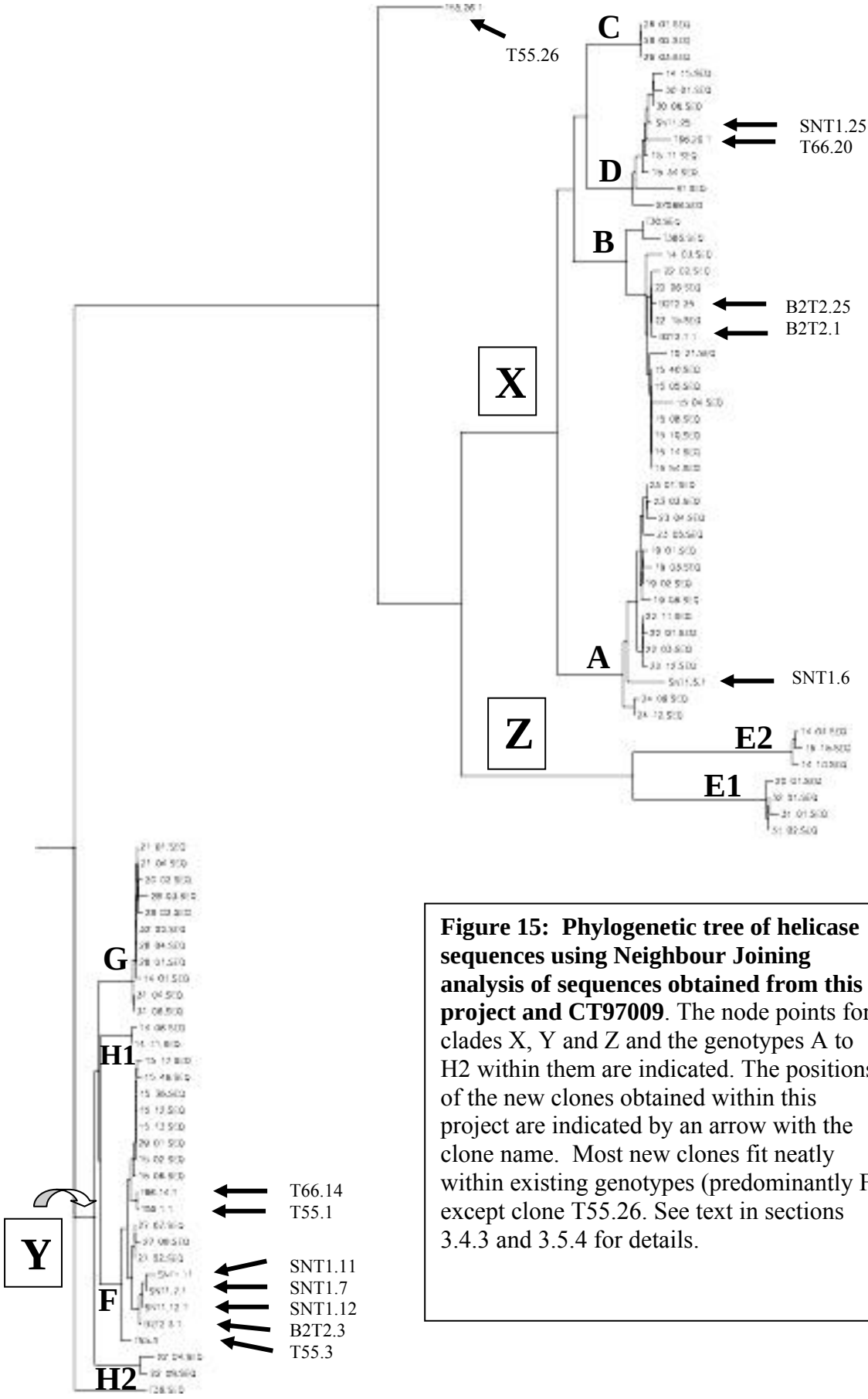


Figure 15: Phylogenetic tree of helicase sequences using Neighbour Joining analysis of sequences obtained from this project and CT97009. The node points for clades X, Y and Z and the genotypes A to H2 within them are indicated. The positions of the new clones obtained within this project are indicated by an arrow with the clone name. Most new clones fit neatly within existing genotypes (predominantly F), except clone T55.26. See text in sections 3.4.3 and 3.5.4 for details.

3.5 Orchard survey, April 2005

3.5.1 Field observations

The survey of citrus orchards in Gayndah, Mundubberah and Bundaberg highlighted the extensive presence of *Citrus tristeza virus* in the Central Burnett region of Queensland. Each surveyed orchard showed evidence of CTV in the form of mild and severe pitting symptoms on peeled stems (Appendix 3).

Six of the trees sampled showed no symptoms of OSP despite growing in heavily diseased orchards, so these may contain strains of CTV that are potentially protective against OSP-CTV. Four trees showing mild stem pitting symptoms and at locations more distant from the field trial were sampled because they may contain strains of OSP-inducing CTV that have not yet been characterised.

3.5.2 Serological screening

All samples collected during the orchard survey tested positive for CTV via DTBIA (Appendix 3).

3.5.3 Biological indexing

When indexed to West Indian lime and Symons Sweet orange indicator plants, the samples from the orchard surveys consistently returned less severe vein clearing and stem pitting scores than those from the field trial. The orchard survey group averaged mild vein clearing and stem pitting symptoms (mean indexing scores are shown in Figure 16).

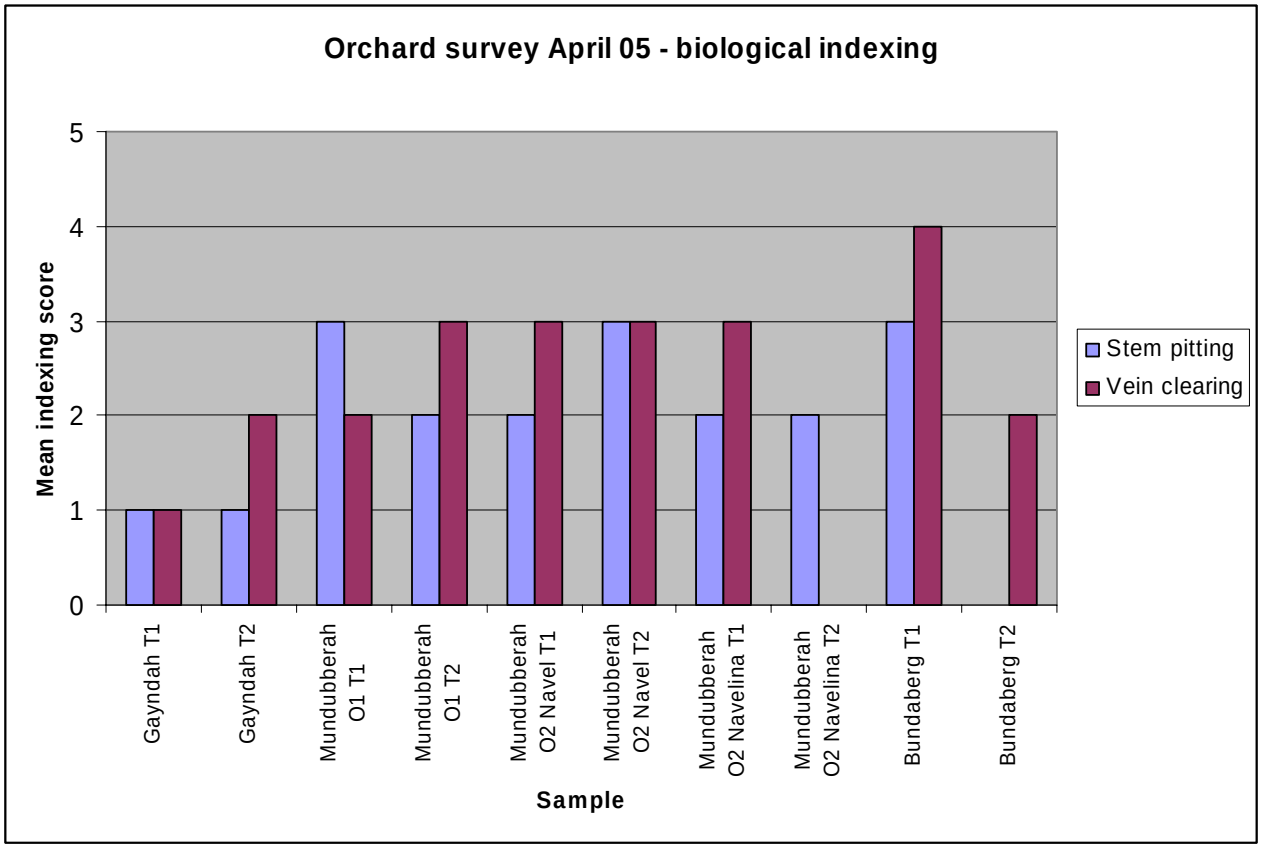


Figure 16: Mean stem pitting and vein clearing scores on Symons sweet orange and West Indian lime indicator plants (respectively), inoculated with samples collected in April 2005 during the orchard survey.

3.5.4 Molecular analysis

Molecular analyses indicated that the majority of trees contained mixed infections, as demonstrated initially by their CP/*Hin*f RFLP profiles (Appendix 4). Seven trees were positive in the p23 assays for both known OSP-inducing strains, two were positive only for PB155 and one was negative for both. The helicase assays indicated all trees contained CTV variants from two or more clades and genotype F was detected in all trees. Genotype G was detected in three of ten trees but genotype C was not detected.

Two trees were selected for detailed analysis of the helicase variants within them using cloning and nucleotide sequencing, as described for the trees in the trial. Gayndah Navel Tree 2 (referred to as B2T2) was selected because it was positive for both known OSP-inducing strains but showed no OSP symptoms in the field – it may potentially harbour naturally protective strains. Navel Tree 2 from Mundubberah Orchard 1 was initially selected for the same reason but no clones of CTV were recovered from the experiment so this tree was not considered further. Bundaberg Navel Tree 1 (referred to as SNT1) was selected because it was negative for both PB155 and PB235 but showed symptoms of OSP, so may contain previously-uncharacterised strains of OSP-inducing CTV.

Amongst the clones screened from B2T2 and SNT1, 17 and 13 clones respectively were positive for helicase inserts. Restriction analysis of these inserts indicated the presence of 4 different groups for B2T2 and 5 for the tree SNT1. One clone from each group was selected for nucleotide sequencing and phylogenetic analysis – these are referred to as B2T2.1, B2T2.3, B2T2.22, B2T2.25, SNT1.6, SNT1.7, SNT1.11, SNT1.12 and SNT1.25. These were subjected to nucleotide sequencing and phylogenetic analysis, results of the Neighbour Joining tree are shown in Figure 15.

Many of the clones analysed (SNT1.7, SNT1.11, SNT1.12 and B2T2.3) fit within the F genotype, although as shown in Figure 15 they (together with clone T55.3 from the field trial) significantly extend the range of variants characterised as belonging to this subgroup. Potentially these clones may be associated with either new OSP-inducing variants (in the case of the clones from SNT1) or (in the case of clone B2T2.3) a variant providing protection against the F-genotype subisolate PB235, that was detected in this tree although it showed no symptoms.

Two clones from tree B2T2 (B2T2.1 and B2T2.25) are of genotype B. The known OSP-inducing subisolate PB155 is also of genotype B, so either of these clones could be associated with a variant providing protection to this asymptomatic tree.

Clone SNT1.6 falls within genotype A and SNT1.25 within genotype D. Either of these might be associated with novel variants inducing the OSP symptoms seen in this tree, which was negative for both PB155 and PB235.

4 Discussion

The results obtained during the course of this field trial indicate that some degree of protection against OSP was offered to each of the pre-immunised trees. This was evident both at the level of field observation and molecular analysis, with the control trees that were initially virus-free showing the most severe stem pitting symptoms and the presence of a greater range of viral variants. However, whilst field observations strongly indicate a reduction in symptom development in pre-immunised trees, the small size of the trial precluded the possibility of detecting any statistically significant differences between treatments. A larger scale field trial would be required to provide statistically accurate results, though this may not be possible given commercial considerations.

Presence of OSP isolates in the field

The control trees were not pre-immunised with any CTV strains but 7 out of 8 of these trees were found to be positive for either or both of the two known OSP-inducing strains of CTV (PB155 and PB235) using the p23-based molecular assays that specifically detect these 2 strains. This confirms there is a high OSP disease pressure in the area of the field trial. In contrast, the trees that had been pre-immunised were less likely to be positive for either or both, regardless of their treatment groups. This suggests that pre-immunisation with any isolate provides some protective advantage against infection with these particular OSP-inducing strains.

Theoretically it is possible that the low frequency with which we have detected the presence of strains PB155 and PB235 in the pre-immunised trees using the p23-based assay is due to shortcomings of the assay itself. This seems unlikely, particularly since the assays have been validated against (natural and blended) RNA extracts of known composition in our laboratory, during both this project and the earlier project CT97009. Evidence accumulating since the completion of project CT97009 suggests that, despite the fact that it has no homolog in other Closteroviruses, the p23 region has a role in CTV pathogenicity. This makes the p23 region potentially a most useful target for molecular detection and discrimination. Firstly, the p23 protein has been shown to control asymmetric accumulation of CTV RNA, indirectly enhancing the expression of particular genes (Satyanarayana *et al*., 2002). Secondly, Mexican lime plants expressing just the p23 protein display symptoms resembling infection with intact virus (Ghorbel *et al*, 2001), an effect that has since been shown to be independent of the viral strain from which the p23 protein is expressed and to be specific to citrus plants (Fagoaga *et al*., 2005). p23 has also been shown to be potent suppressor of intracellular (but not intercellular) gene silencing in transformed tobacco plants (Lu *et al*., 2004).

Other studies of the genetic relationships between p23 genes of a range of isolates indicated three groups of sequences that the authors correlated with symptom severity (Sambade *et a*l., 2003). When we included the p23 sequences of Australian isolates into their data set (results not shown), PB61, PB155 and PB235 all fitted neatly within their groupings, although their placement questions the direct correlation with symptom severity reported by Sambade and colleagues. Nevertheless, the groupings confirm that the primer sequences used in our discriminatory assays would detect all the other members of their respective groups, confirming the robust nature of the assays we have employed here.

In light of these observations, one of the most significant findings from this work is how many of the pre-immunised trees tested negative for both PB155 and 235 but nevertheless showed symptoms of OSP in the field. This indicates that other OSP-inducing isolates are present in the field but are sufficiently different from PB155 and 235 to remain undetected by the existing assays. This is alarming and means that we have no rapid methods to detect OSP-inducing CTV, nor a full understanding of the diversity of endemic OSP-inducing isolates.

Potential pre-immunising strains

In this trial, there appeared to be a decrease in symptom expression in the field trial trees over time, with symptoms observed to be more severe in November 2003 (spring flush) compared with April 2005 and May 2006 (summer flush). This could be explained by the introduction of new CTV strains over time that may afford some protection against OSP strains of CTV.

Trees pre-immunised with PB64 exhibited the mildest stem pitting symptoms. The isolates B1, PB67 and PB61 were also good performers, although the results were not entirely consistent between the three assessments. None of the treatments had an effect on tree size or canopy surface area. More assessments need to be made of fruit load and size. Synergistic reactions (enhancement of symptoms leading to severe tree decline) were not observed between pre-immunising and endemic field strains. It should be recognised that a background presence of minor isolated pits can be detected in sweet orange clones, thought to be caused by CTV isolates but having no effect on tree health or productivity (Broadbent *et al*., 1992). The pitting assessed in this trial was more significant and often associated with gum deposition and brittleness.

Isolate B1 performed well in biological indexing as well as in the field. In contrast to their field results, severe CTV symptoms were observed on indicator plants inoculated with samples from treatments PB64 and PB67. Symptom expression for all isolates was more acute on Symons sweet orange and West Indian lime indicator plants than on the field trees. This reflects the fact that these varieties are chosen for use in biological indexing specifically because they are highly susceptible to particular strains of CTV and conducive to expressing clear symptoms. Once inoculated the indicator plants are also maintained in a controlled environment at a temperature conducive to viral replication (Garnsey *et al*., 1987). The more distinct symptoms seen on indicator plants might also reflect that the method used reveals the underlying tissue more easily and cleanly. In future we will adapt this to the assessment of the field trees, by collecting field samples and then treating them in the laboratory as for indicator plants (boiling, peeling whole stems of even length and then air drying, see Section 2.2). This will allow more reliable comparison between trees and facilitate easier assessment of symptoms.

There are also reports in the literature of variations in CTV populations following transmission of isolates to different hosts (Ayllon *et al*., 1999). The relative composition of variants within mixed isolates may change between field trees and indicator plants, leading to differences in symptom expression depending on which isolates are transferred to the indicator plants via the graft inoculation. The biological indexing results combined with the molecular analyses suggest that all of the field trees contain a mixture of CTV isolates and that stem pitting isolates are present in the field trees as well as potential cross protecting mild isolates.

Potential pre-immunising strains must be practical for use within an MSCP strategy in addition to providing effective protection against severe CTV strains. Our results have demonstrated that PB57 afforded some protection against stem pitting strains of CTV compared with the control trees but this isolate could not be used on a wide scale as it is difficult to introduce the virus to new material. Poor distribution of this isolate in the budwood after inoculation meant that there were insufficient, healthy buds to use to inoculate all of the allocated field trial trees. PB57 is unlikely to be an effective pre-immunising isolate for use in a cross protection strategy for sweet oranges.

Characterisation of CTV isolates

The studies reported here into the characterisation of variants in selected isolates provides some information about the genetic diversity of CTV in the field, although no biological information can be inferred from the molecular data. The finding that clone T55.26 falls definitively outside the CTV clades described to date (Figure 15) provides further evidence that the diversity is greater than previously imagined. The Australian situation may thus be more like the scheme described by Roy and Brlansky (2004), with more than three groups, rather than the two or three suggested by others (Connor, 2001; Hilf *et al*., 1999, Lopez *et al*., 1998). Certainly clone T55.26 represents a "novel" variant that would not be detected by our current assays and it may also be inducing the OSP symptoms seen in this tree. Other clones from trees SNT1 and T66 are of genotypes other than those to which the known OSP-inducing strains belong and these might be the variants inducing the symptoms of OSP seen in these trees. Clones from tree B2T2 extend the range of variants within a known genotype, these may be providing it with protection against the PB155 and PB235-type strains detected within it. The cloning analyses performed here strongly support further work on biological segregation of these isolates, to underpin a cross protection strategy for sweet oranges.

Another significant observation is that the pre-immunising isolates were rarely detected in the field samples, even using the most specific and sensitive helicase assays, although isolates with the same molecular profiles can sometimes be detected in indicator plants. More frequent sampling, particularly in the early period, may help to determine if the pre-immunising isolates are being rapidly overrun by endemic strains. The time that elapsed between the initiation of the trial and the first sampling reflects the difficulties associated with implementing useful and effective methodologies.

If the pre-immunising isolates used in this trial are being rapidly dominated by the aphid-borne strains because of the high level of disease pressure, this may limit their usefulness in a cross-protection strategy. On a more positive note, the pre-immunising isolates may be specifically degraded by the host through gene silencing, reducing their detectable titre but nevertheless establishing the molecular mechanism to protect the host against the introduction of closely related isolates. However, we observed no correlation between the clade of the pre-immunising isolate and the clade of variants subsequently detected in the treatment groups (Appendix 2). For example, T12 to T19 were pre-immunised with PB8, from clade X, but variants from clade X were detected by molecular means in any trees within that treatment group. This is not consistent with within-clade protection being provided through a gene silencing mechanism, although again this may relate to the time between inoculation and the first sampling. A larger scale field trial with more frequent sampling would be required to investigate this further.

The specific helicase assays detected the presence of the F genotype in most trees, and many of the characterised clones also fell within F. No other Y-clades clones were detected at all. Only one of the pre-immunising isolates introduced to the field, PB64 is of genotype F. This suggests that the F genotype was likely to be widespread at the site prior to the trial commencing, though the range of variants detected here is broader than observed in our earlier study (Connor, 2001).

Three pre-immunising isolates PB50, PB61 and PB67 are of genotype G. This genotype was relatively common amongst all the pre-immunised trees (though less common than F), but was only detected in one sample from all the trees that were initially virus-free. This may mean that some G-genotype variants are easily transmitted by aphids, although this is not supported by observations from our previous work that indicated poor transmission of the G-genotype isolate, PB61, by aphids (Zhou, 2001)

Conclusions

All of the pre-immunising isolates appeared to provide some degree of protection against OSP inducing isolates. The combined results of this work mean that it is premature to firmly recommend

any of the isolates tested. Nevertheless, much important information has been obtained. Further work is needed on the isolates collected in this study, in addition to conducting a more structured survey of affected areas to identify additional OSP and potential pre-immunising strains and the establishment of more extensive field trials.

Technology Transfer

The work in this project and the issue of orange stem pitting has been presented at scientific and industry conferences within Australia, prior to project approval and during the project term:

> Donovan NJ, Owen-Turner J, Howard EJ, Hailstones DL, Burgess LW. Evaluating strains of *Citrus tristeza virus* for protection against orange stem pitting in a field trial in Queensland. 15th Biennial Australasian Plant Pathology Conference, Geelong, Australia, 26-29th September 2005 p 314
>
> Hailstones DL, Donovan NJ, Owen-Turner J. Mild strain cross protection against orange stem pitting strains of *Citrus tristeza virus*. 6th Australasian Plant Virology Workshop, Gold Coast Australia, 30th August – 2nd September 2004 p 71
>
> Donovan NJ, Hailstones D, Chambers G. Orange stem pitting. National Citrus Nursery Workshop, Mildura, Australia 19-20th June 2002 pp 21-24

Results of the project have also been presented in an Honours thesis:

> E.J. Howard (2005) Mild strain cross protection against orange stem pitting strains of citrus tristeza virus. Honours thesis submitted to the Faculty of Agriculture, Food and Natural Resources, University of Sydney, November 2005 as partial requirement for the degree Bachelor of Science in Agriculture.

Two extension articles are planned for submission to future editions of Australian Citrus News. One article will serve as a grower's guide to citrus tristeza virus and a second article will outline the findings of this project. Orange stem pitting will be highlighted as a potential problem for the southern orange industry, in addition to building awareness about exotic strains of CTV that occur in Asia like mandarin stem pitting. The need for further research in this area will also be stressed.

An extension article outlining the project findings is also planned for submission to Agriculture Today, a NSW DPI publication distributed in 'The Land' newspaper.

The short term nature of the project means the work alone does not lend itself to publication in a refereed scientific journal. However the knowledge gained during the project will be included in a manuscript reviewing the current status of CTV research in Australia.

Recommendations – scientific and industry

It is premature to recommend any of the pre-immunising isolates tested in this field trial for use in a cross protection strategy for the protection of sweet orange trees against OSP inducing strains of CTV. However this project has pinpointed some isolates with potential (PB64, PB67, PB61 and B1) and some isolates that are unlikely to be of practical use in a field situation against OSP (PB57).

The short-term nature of the funded component of the field trial means that further work is recommended:

It is recommended that data be collected on fruit load and size from the established field trial in April 2007. It should be noted that the grower has agreed to maintain the field trial for at least one more year.

It may be useful to record field observations in November 2006 to establish if the difference in symptom severity between November 2003 and the April assessments was due to the time of sampling or the age of the trial.

This work has provided evidence that the genetic diversity of CTV in the field is greater than previously thought, including other OSP-inducing isolates. More work is needed on biological segregation and characterisation of the isolates collected in this study. A more extensive and structured survey of Queensland orchards is also recommended to identify other OSP-inducing isolates.

There is a need to examine the same genomic regions studied by overseas research groups to align our CTV populations to those described elsewhere (Hilf *et al*., 2005, Lopez *et al*., 1998). It would also be useful to validate the primers described in Sambade *et al*., 2003 against our collection to further investigate the correlation between symptom expression and p23 groupings.

Glossary

Cloning – a group of DNA molecules derived from one original length of DNA sequences and produced by a bacterium or virus into which it was introduced using genetic engineering techniques, often involving plasmids.

Coat protein gene – segment of a virus's genome that, once translated, encodes the coat or capsid protein of that virus.

CTV – *Citrus tristeza virus*. Aphid borne closterovirus pathogenic to citrus. There are numerous strains of the virus causing different disease symptoms in different citrus varieties.

DTBIA - Direct tissue blot immunoassay. A serological technique that detects the presence of absence of citrus tristeza virus but does not determine which viral strain is present.

Grafting – a method of plant propagation by which the cut surfaces of 2 plants are joined to form a living union.

Helicase region – a segment found in the genomes of some viruses that once translated encodes the helicase protein. The region is part of a larger segment referred to as Open Reading Frame 1 that is initially processed as one unit and subsequently cleaved into three component proteins.

Indexing – the testing of plant material for the presence of plant viruses and viroids.

Indicator plant – a plant that is highly susceptible to a specific virus producing distinct symptoms, and is used for the detection and identification of that virus when inoculated with material from a test plant.

Inoculation – the transfer of a pathogen onto a host.

MSCP – Mild strain cross protection. The phenomenon by which plant tissues infected with a mild strain of a virus are protected from infection by more severe strains of the same virus.

Nucleic acid – an acidic substance containing pentose, phosphorous and pyrimidine and purine bases. Nucleic acids determine the genetic properties of organisms (Agrios 1997).

Nucleotide – the building blocks of DNA and RNA.

OSP – Orange stem pitting. Strains of citrus tristeza virus that cause symptoms of stem pitting in sweet oranges but do not induce symptoms in infected mandarin trees.

Pathogen – an organism (usually micro-) that is able to cause disease.

p23 gene – the 3'-terminal region of the *Citrus tristeza virus* genome that when translated encodes a protein of unknown function (see also Discussion) but with a molecular mass of 23 kD.

PCR – Polymerase chain reaction. A technique that allows almost infinite amplification (multiplication) of a segment of DNA for which a primer (short piece of DNA) is available (Agrios 1997).

RNA – Ribonucleic acid. A nucleic acid involved in the synthesis of proteins. The most common nucleic acid of plant viruses.

RT-PCR – Reverse transcriptase polymerase chain reaction.

Stem pitting – a viral disease symptom characterised by depressions on the stem of an infected plant.

Viral isolate – (our definition) all the viruses isolated from a field tree. In the case of CTV is often a mixture of different types of the same virus.

Viral strain –(our definition) A viral population (often quite pure in makeup) that is consistently associated with a particular biological property (eg symptom severity) and that does not appear to change much over time, often despite repeated transmission.

Viroid – small, naked, circular RNA molecule that can infect plant cells, replicate and cause disease.

Virus – a sub-microscopic obligate parasite that is made up of nucleic acid and protein.

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Aida Ghalayini

Elizabeth Howard

John Owen-Turner

Deborah Hailstones / Nerida Donovan

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Appendices

Appendix 1: Biological assessment of field trial trees in November 2003, April 2005 and June 2006

Tree no.	Pre-immunising isolate	Stem pitting score in field trees			Tree Height (m)	Tree width (m)	Canopy surface area (m^2)		Biological indexing	
		Nov-03	Apr-05	Jun-06				Treatment mean (SEM)	SSO	WIL
3	Virus-free	3	2	1	2.97	2.52	19.81		1	3
4	Virus-free	3	2	2	2.87	2.75	21.07		2	4
5	Virus-free	4	4	2	2.4	2.6	15.88		3	4
6	Virus-free	3	3	3	2.13	2.17	11.13		3	4
7	Virus-free	4	3	4	2.42	2.42	14.76		3	3
8	Virus-free	4	5	3	2.49	2.57	16.39		3	3
9	Virus-free	3	1	4	2.37	2.45	14.58		2	3
10	Virus-free	3	2	4	2.7	2.71	19.26	16.61 (0.41)	3	4
12	PB 8	2	1	3	2.73	2.73	19.69		3	3
13	PB 8	1	1	2	2.6	2.68	18.16		3	4
14	PB 8	1	1	3	2.72	2.68	19.17		2	2
15	PB 8	3	1	2	2.93	2.28	17.47		3	3
16	PB 8	3	2	1	2.62	1.97	13.11		3	4
17	PB 8	4	2	1	2.56	2.64	17.50		4	4
18	PB 8	3	3	2	2.38	2.64	16.01	17.44 (0.26)	1	3
19	PB 8	1	1	2	2.41	2.91	18.40		3	4
21	PB 50	3	3	3	2.6	2.68	18.16		3	4
22	PB 50	3	2	1	2.39	2.34	13.99		3	4
23	PB 50	3	2	1	2.29	2.75	16.06		2	3
25	PB 50	5	3	0	2.61	2.31	15.39		4	3
26	PB 50	4	1	1	2.13	2.22	11.40		4	0
27	PB 50	5	2	1	2.09	2.24	11.23	14.37 (0.45)	3	4
30	PB 57	3	1	1	2.29	2.77	16.21		2	3
31	PB 57	4	2	1	2.06	2.46	12.25		2	2
32	PB 57	3	2	0	2.37	2.4	14.24	14.24 (0.66)	2	3
39	PB 61	4	1	1	2.22	2.31	12.56		4	3
41	PB 61	3	1	1	2.22	2.2	11.92		2	4
42	PB 61	2	0	1	2.22	2.28	12.38		3	4
43	PB 61	3	1	1	2.44	2.49	15.40		3	4

Tree no.	Pre-immunising isolate	Stem pitting score in field trees			Tree Height (m)	Tree width (m)	Canopy surface area (m^2)		Biological indexing	
		Nov-03	Apr-05	Jun-06				Treatment mean (SEM)	SSO	WIL
44	PB 61	4	3	2	1.96	2.33	10.78		2	3
45	PB 61	3	1	1	2.15	1.97	10.21		2	4
46	PB 61	2	1	1	2.64	2.82	19.69	13.28 (0.47)	2	3
48	PB 64	4	2	1	2.27	2.59	14.75		3	4
49	PB 64	3	0	1	2.55	2.89	19.50		3	4
50	PB 64	2	0	1	2.42	2.41	14.69		2	3
51	PB 64	2	2	1	2.26	2.09	11.56		1	3
52	PB 64	3	2	1	2.16	2.43	12.84		4	3
53	PB 64	2	0	1	2.16	2.53	13.47		4	3
54	PB 64	1	0	0	2.31	2.53	14.66		2	4
55	PB 64	2	2	2	2.38	2.61	15.79	14.66 (0.30)	3	4
57	PB 67	1	0	0	2.36	2.46	14.57		2	3
59	PB 67	2	1	1	2.1	2.6	13.43		3	4
60	PB 67	4	3	1	1.96	2.47	11.54		4	4
61	PB 67	2	3	1	2.2	2.26	12.12		2	4
62	PB 67	3	1	1	1.93	2.12	9.53	12.24 (0.38)	5	4
66	Benham 1	2	3	1	2.21	2.21	11.90		3	4
67	Benham 1	2	2	1	2.68	2.87	20.50		2	4
68	Benham 1	2	0	2	2.1	2.18	10.98		1	3
69	Benham 1	1	0	1	2.3	2.58	14.92		3	4
70	Benham 1	2	2	1	2.54	2.98	20.21		2	4
71	Benham 1	3	2	1	2.21	2.55	13.99		2	4
72	Benham 1	5	3	1	2.44	2.52	15.62		2	4
73	Benham 1	4	1	1	2.24	2.52	14.04	15.27 (0.44)	3	2

Appendix 2: Molecular analysis of field trial trees sampled April 2005

Year	Tree	Pre-immunising isolate	OSP score	CP/*Hin*f I group :		p23 Detection of:		Helicase region – Detection of :						
								Clade			Genotype			Selected for cloning
				of P/I isolate	Detected	PB155	PB235	X	Y	Z	F	G	C	
2003	2	B1		3 (5)	1, 3	-	-	+	+	-	+	+	-	
	7	Virus-free			1, 3	-	+	+	+	+	-	-	-	
	15	PB8		6	3	+	-	+	+	+	+	-	-	
	25	PB50		5	3 (2, 5)	-	-	+	+	+	+	-	-	
	31	PB57		6	3 (2, 5)	-	-	+	+	+	+	-	-	
	43	PB61		5	3	-	+	+	+	+	+	-	-	
	53	PB64		8	3 (2, 5)	-	-	-	-	-	-	-		
	59	PB67		3	3	-	-	-	-	-	-	-	-	
	69	B1		3 (5)	3	-	-	-	-	-	-	-	-	
2005	3	Virus-free	2		1, 2, 3 , 5	+	+	+	+	+	+	-	-	
	4	Virus-free	2		1, 2, 3	+	-	+	+	+	+	-	-	
	5	Virus-free	4		1, 3 (2)	+	-	+	-	+	+	-	-	
	6	Virus-free	3		1, 2, 3	+	+	+	+	+	+	-	-	
	7	Virus-free	3		1, 2, 3	+	+	+	+	+	+	-	-	
	8	Virus-free	5		3, 5 (1, 2)	-	+	+	+	+	+	-	-	
	9	Virus-free	1		1, 3 (2, 5)	+	+	+	+		+	-	-	
	10	Virus-free	2		3	-	-	-	+	+	+	-	-	
	12	PB8	1	6	3	-	-	+	+	+	-	+	-	
	13	PB8	1	6	3 (5)	-	-	+	+	+	+	+		
	14	PB8	1	6	3 (5)	-	-	+	-	-	-	-	-	
	16	PB8	2	6	3 (1)	-	-	+	+	+	+	-	-	
	17	PB8	2	6	3 (1, 2)	-	-	+	+	+	+	-	-	
	18	PB8	3	6	3 (1)	-	-	+	+	+	+	-	-	
	19	PB8	1	6	1, 3 (2)	-	-	+	+	+	+	-	-	
	21	PB50	3	5	3	-	-	+	+	+	+	-	-	
	22	PB50	2	5	3	-	-	-	-	+	-	-	-	
	23	PB50	2	5	3 (1, 2)	+	-	+	+	+	+	-	-	
	25	PB50	3	5	3 (1)	-	+	+	+	+	+	-	-	
	26	PB50	1	5	1, 3, 8	-	-	+	+	+	+	-	-	

Year	Tree	Pre-immunising isolate	OSP score	CP/*Hinf* I group :		p23 Detection of:		Helicase region – Detection of :						
								Clade			Genotype			Selected for cloning
				of P/I isolate	Detected	PB155	PB235	X	Y	Z	F	G	C	
2005	27	PB50	2	5	3	-	-	-	-	-	-	-	-	
	30	PB57	1	6	1, 3, 8	+	-	+	+	+	+	+	-	
	31	PB57	2	6	3	+	+	+	+	+	+	-	-	
	39	PB61	1	5	3	-	-	+	+	+	+	-	-	
	41	PB61	1	5	3	-	-	+	+	+	-	-	-	
	42	PB61	0	5	3	+	+	-	+	+	-	+	-	
	43	PB61	1	5	3	-	+	+	+	-	+	+	-	
	45	PB61	1	5	3, 8	-	-	+	+	+	+	-	-	
	46	PB61	1	5	3	-	-	+	+	+	-	+	-	
	48	PB64	2	8	3 (1)	+	+	+	+	+	+	-	-	
	49	PB64	0	8	3 (1)	-	-	+	+	+	+	+	-	
	50	PB64	0	8	3 (1)	+	-	+	+	-	+	-	-	
	51	PB64	2	8	2, 3, 6	-	-	+	+	+	+	-	-	
	52	PB64	2	8	3, 8	-	-	+	-	+	-	-	-	
	53	PB64	0	8	1, 3	+	+	+	+	-	+	+	-	
	54	PB64	0	8	2, 3, 6	+	+	+	+	+	+	-	-	
	55	PB64	2	8	2, 3	-	-	-	+	-	+	-	-	✓
	57	PB67	0	3	1, 3	-	-	+	+	+	+	+	-	
	59	PB67	1	3	3	-	-	-	+	-	-	-	-	
	60	PB67	3	3	3	-	-	-	+	+	+	-	-	
	61	PB67	3	3	1, 2, 3	-	-	-	-	+	-	-	-	
	62	PB67	1	3	2, 3	+	-	+	+	+	-	-	-	
	66	B1	3	3 (5)	3	-	-	-	-	-	-	-	-	✓
	67	B1	2	3 (5)	3	-	-	-	+	-	+	+	-	
	68	B1	0	3 (5)	3, 5	+	-	+	+	-	-	+	-	
	69	B1	0	3 (5)	3	+	+	+	+	-	+	+	-	
	70	B1	2	3 (5)	3	-	-	-	-	-	-	-	-	
	71	B1	2	3 (5)	1, 2, 3	-	+	+	+	+	+	+	-	
	72	B1	3	3 (5)	1. 3	+	-	+	+	-	+	+	-	
	73	B1	1	3 (5)	3	-	-	-	-	+	-	-	-	

Appendix 3 – Biological assessment of trees in other orchards

Orchard location	Variety	Tree	OSP	Stem pitting scores on SSO	Vein clearing on WIL	DTBIA for CTV
Gayndah	Navel	1	-	1	1	+
Gayndah	Navel	2	-	1	2	+
Mundubberah - orchard 1	Washington navel	1	-	3	2	+
Mundubberah - orchard 1	Washington navel	2	-	2	3	+
Mundubberah - orchard 2	Navel	1	-	2	3	+
Mundubberah - orchard 2	Navel	2	-	3	3	+
Mundubberah - orchard 2	Navelina	1	+	2	3	+
Mundubberah - orchard 2	Navelina	2	+	2	0	+
Bundaberg	Navel	1	+	3	4	+
Bundaberg	Navel	2	+	0	2	+

Appendix 4 – Molecular analysis of trees in other orchards, sampled April 2005

Orchard location	Variety	Tree	OSP	CP/*Hin*f I group	p23		Helicase regions detected						Selected for cloning
					Detection of:		Clade			Genotype			
					PB155	PB235	X	Y	Z	F	G	C	
Gayndah	Navel	1	-	1, 3	+	-	+	+	+	+	-	-	
Gayndah	Navel	2	-	1, 3	+	+	+	+	+	+	-	-	✓
Mundubberah - orchard 1	Washington navel	1	-	1, 5 (3)	+	+	+	+	+	+	+	-	
Mundubberah - orchard 1	Washington navel	2	-	3 (5)	+	+	+	+	+	+	+	-	✓ (but not successful)
Mundubberah - orchard 2	Navel	1	-	1, 3	+	+	+	+	+	+	-	-	
Mundubberah - orchard 2	Navel	2	-	1. 3	+	+	+	+	+	+	-	-	
Mundubberah - orchard 2	Navelina	1	+	1,2,3,5	+	+	+	+	+	+	-	-	
Mundubberah - orchard 2	Navelina	2	+	1,2,3,5	+	+	+	+	-	+	+	-	
Bundaberg	Navel	1	+	1,3	-	-	+	+	+	+	-	-	✓
Bundaberg	Navel	2	+	1,3	+	-	+	+	+	+	-	-	