Survey of *Citrus tristeza virus* (CTV) diversity in pigmented *Citrus x paradisi* (Macfad.) (Grapefruit) trees in north-western Argentina

Read, D.A.[2,4], Palacios, M.F.[1], Kleynhans, J.[2],. Figueroa, J.[1], Foguet, L[1,] Stein, B.[1], and G. Pietersen[2,3]

[1]Estacion Experimental Agroindustrial Obispo Colombres (EEAOC), Tucumán, Argentina. [2]Department of Microbiology, University of Pretoria, Pretoria, South Africa.

[3]Agricultural Research Council- Plant Protection Research Institute, South Africa.

[4] Centre for Microbial Ecology and Genomics, University of Pretoria, Pretoria, South Africa.

Abstract

Citrus tristeza virus (CTV) is the most severe viral pathogen of citrus and is responsible for eliciting a wide range of devastating disease symptoms. Grapefruit cultivars (*Citrus x paradisi*) are the most sensitive among citrus to the effects of CTV infections. Grapefruit is an important crop within the north-western Argentine citrus industry; however, production has been affected by CTV stem-pitting. In general, CTV diversity within South America is poorly studied, with data on grapefruit CTV populations being particularly limited. In this study, 50 samples were collected from Star Ruby, Henninger's Ruby and Ruben Pink cultivars, within the provinces of Tucumán, Salta and Jujuy in north-western Argentina. The CTV p33 gene was PCR amplified and the resulting amplicons sequenced with Sanger sequencing. A subset of these amplicons was sequenced with Illumina MiSeq sequencing. AT-1-like sequences were dominant within the majority of populations, as determined by Sanger sequencing, followed by sequences clustering within the unresolved Kpg3/SP/T3 and RB clades. Sequencing by Illumina MiSeq confirmed this, as well as detecting minor sequence types within the HA 16-5, VT, B165 and A18 clades.

Keywords: *Citrus tristeza virus*, grapefruit, Argentina, Illumina sequencing

Introduction

Citrus tristeza virus (CTV) is responsible for the most devastating viral diseases of citrus (Bar-Joseph et al. 1989) and is present in the majority of citrus production areas around the world (Niblett et al. 2000). A number of aphid species such as *Aphis gossypi* and *Aphis spiraecola* spread the virus, however *Toxoptera citricida* (Brown citrus aphid) is the most efficient vector (Yokomi et al. 1994) and is also the most important vector for CTV in Argentina (Valiela 1959). In addition to the potential for causing severe disease symptoms, CTV spreads rapidly with an infection rate of up to 95% within two years of planting, depending on the vector species and identity of endemic viral strains (Gottwald et al. 1996).

CTV strains elicit a range of disease phenotypes depending on the host cultivar and infecting strain (Moreno et al. 2008), of which stem pitting and decline are most important to commercial citriculture (Harper 2013). Grapefruit cultivars are among the most sensitive hosts to CTV, especially strains causing stem-pitting (SP) (van Vuuren and Manicom 2005), which also leads to reductions in plant productivity and reduced fruit size. In order to reduce the negative effects of infections by endemic CTV strains, the citrus industries in a number of countries such as South Africa and Brazil practice mild strain cross protection (Moreno et al. 2008). CTV is endemic to citrus production areas in Argentina (Valiela 1959; Foguet 1961) and, coupled with a lack of a commercial cross-protection scheme has led to CTV becoming a major threat to the Argentine citrus industry, especially in the production of pigmented grapefruits.

Grapefruit production has been an important part of the citrus industry in North-West Argentina, especially in the provinces of Salta and Jujuy. The range of cultivars planted has been limited, based on their apparent tolerance to severe CTV strains, with Heninnger's Ruby, Redblush, Ruben Pink, Star Ruby and Henderson Ruby being most planted. Heninnger's Ruby is the oldest pigmented seedless grapefruit cultivar introduced by the

Estación Experimental Agroindustrial Obispo Colombres (EEAOC) in 1939 (Foguet and Foguet 1990). Ruben Pink is a local cultivar selected in Jujuy in 1963 as a bud sport from Foster Seedless (Foguet 1983). In the past three decades, grapefruit trees in these regions have been severely affected by stem-pitting symptoms, leading to reduced productivity and death of trees (Foguet and Foguet 1990; Foguet et al.1999).

Of the commonly grown cultivars, Star Ruby is the most susceptible to CTV infection, with trees showing stem-pitting symptoms in as little as 2-3 years after planting. Trees may also be stunted, with twig dieback, and reduced fruit size. The productive lifespans of Star Ruby orchards have been reduced to as few as 5-6 years and plantings are no longer commercially viable in North-West Argentina. Henderson and Redblush are also very susceptible to CTV-SP. Henninger's Ruby and Ruben Pink both show symptoms of stem-pitting in the field, although fruit size and yield are generally commercially acceptable (Foguet and Foguet 1990; Foguet and Gonzalez 1992; Foguet et al. 1999). Populations of CTV within grapefruit cultivars in Argentina have been poorly studied. A number of field isolates have been characterised through single strand conformation polymorphism (SSCP) (Iglesias et al. 2005b; 2008) and polymerase chain reaction (PCR) and sequence analysis of clones (Iglesias et al. 2005a; 2008).

During a recent study, Read and Pietersen (2016) demonstrated the usefulness of targeted next generation sequencing for the analysis of the genotype composition of CTV populations using primers that amplify the p33 gene. This gene was demonstrated to be important in superinfection exclusion of CTV genotypes from mixed infections (Folimonova et al. 2010; Folimonova 2013) and a determination of the variability in p33 gene sequences found in any geographical location is an important first step in understanding the disease aetiology, epidemiology and ultimately control.

The aim of this study was to determine the genotype composition of CTV populations in grapefruit growing regions of Tucumán, Salta and Jujuy provinces in Argentina by doing a analysis of fifty samples from a survey of this region, using direct Sanger- and next generation sequencing of p33 gene amplicons.

Materials and Methods

Collection of material

Fifty grapefruit (*Citrus x paradisi* Macfad.) samples were collected from five sites within the Tucumán, Salta and Jujuy provinces, Argentina during August 2014 and April 2015. Leaf material was collected from various parts of each tree. Sampled cultivars included Star Ruby, Henninger´s Ruby and Ruben Pink. Four of the Star Ruby samples and one of the Ruben Pink samples collected from the EEAOC site had been pre-immunised in 1995, with the GFMS12 population from South Africa.

Biological characterisation of selected isolates

In 2015, four isolates, namely 14-4005, 14-4008, 14-4013 and 14-4017 (*Table 1*) were selected, to represent the three cultivars from this study, as well as the production region of Tucumán and the more northerly regions of Jujuy and Salta. Four biological replicates of *Citrus x aurantifolia* cv. Mexican lime, *Citrus x aurantium* (Sour orange), *Citrus x paradisi* cv. Duncan grapefruit and *Citrus x sinensis* cv. Pineapple sweet orange indicators (Garnsey et al. 1987) were used for graft-inoculations. Each plant was inoculated in triplicate, using individual bark patches from each source plant and maintained under greenhouse conditions. After inoculation, each plant was cut back to stimulate the production of new growth. Vein clearing, leaf cupping and stunting were evaluated at one, two, three, four and seven months

post-inoculation (mpi). Stem pitting was only determined at seven mpi due to the destructive peeling away of bark tissue. Symptoms were rated according to the following scale: 0 = no reaction; 0.5 = very mild reaction; 1 = mild reaction; 2 = moderate reaction; and 3 = severe reaction.

RNA isolation, reverse transcription and PCR amplification

RNA extractions were carried out with a GeneJET plant RNA isolation kit (Thermo, Vilnius, Lithuania) using 100mg of plant material macerated in liquid nitrogen. The amplification of the p33 gene of CTV from each sample was carried out using a two-step reverse-transcriptase polymerase chain reaction (RT-PCR) as described by Read and Pietersen (2015), using the following primer pair: p33-F forward primer (5′ GATGTTTGCCTTCGCGAGC 3′) and the p33-R reverse primer (5′ CCCGTTTAAACAGAGTCAAACGG 3′). Amplicons were then shipped in 200µl 70% ethanol to South Africa. Samples were then precipitated, using a standard ethanol precipitation protocol. Each purified PCR amplicon was re-amplified using the same PCR amplification system, in case of DNA degradation during shipping.

Direct sequencing of p33 gene amplicons

To remove single stranded DNA from PCR products, 0.5µl of 10 U exonuclease (Thermo, Vilnius, Lithuania) and 2µl of 2U FastAP® (Thermo, Vilnius, Lithuania) was added to the amplification products and reaction was carried out as per manufacturer's instructions. The amplicons were subjected to direct Sanger sequencing by adding 1µl BigDye® Terminator mix v3.1 (Applied Biosystems, Foster City, CA, USA), 2.25µl 5x BigDye® v3.1 sequencing buffer, 0.75µl 2µM Univ-p33-F primer and molecular grade water to a total volume of 10µl, to 2µl of the purified PCR products. A single cycle of 94°C for 1 minute, 30 cycles of 94°C

for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes was utilised for the sequencing reaction. Sequencing products were purified using ethanol precipitation, according to Sambrook (2001). The purified sequencing products were submitted to the African Centre for Gene Technologies (ACGT), Automated Sequencing Facility, Department of Genetics, University of Pretoria, South Africa and sequenced using an ABI Prism® 3500xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences not conforming to a quality criterion of a minimum *phred* score of 30 were omitted from further analysis.

Phylogenetic analysis of the direct sequence data

Initial taxonomic identities of each direct Sanger sequence was determined using the online version of the BLASTn algorithm (www.ncbi.nlm.nih.gov/BLAST) with the non-redundant nucleotide (nt) database. Chromas Lite 2.1 (Technelysium, Brisbane, Australia) was used to edit and correct errors in chromatograms. Alignments of sequences were carried using the CLUSTAL W alignment software (EBI, Cambridgeshire, England) within the BioEdit Sequence alignment editor 7.1.3 (Hall 1999). The cognate p33 gene region was trimmed from 45 full-genome reference sequences accessed from GenBank (www.ncbi.nlm.nih.gov/genbank). These were (accession number with strain names in brackets): NC_001661 (T36); AY 340974 (Qaha); U16304 (T36); DQ272579 (Mexico); AY170468 (T36); EU937521 (T36); KC517485 (FS674-T36); KC517486 (FS701-T36); KC517487 (FS703-T36); KC517488 (FS577); JX266713 (Taiwan-Pum/M/T5); AF001623 (SY568); AF260651 (T30); Y18420 (T385); KC517489 (FS701-T30); KC517490 (FL278-T30); KC517491 (FS703-T30); JF957196 (B301); FJ525432 (NZRB-G90); GQ454869 (HA 18-9); FJ525435 (NZRB-M17); JX266712 (Taiwan-Pum/SP/T1); FJ525431 (NZRB-M12); FJ525433 (NZRB-TH28); FJ525434 (NZRB-TH30); JQ798289 (A18); KC525952 (T3); HM573451 (Kpg3); EU857538 (SP); GQ454870 (HA 16-5); DQ151548 (T318A);

AB0463981 (NUagA); JQ911664 (CT11A); KC517493 (FL202-VT); U56902 (VT); KC517492 (FS703-VT); EU937519 (VT); KC517494 (FS701-VT); KC262793 (L192GR); JQ911663 (CT14A); FJ525436 (NZ-B18); JQ965169 (T68); EU076703 (B165); JQ061137 (AT-1) and KC333869 (CT-ZA3). Neighbour-joining phylogenetic trees were constructed for each alignment, using MEGA 6 (Tamura et al. 2013) and the Maximum Composite Likelihood substitution model with a 1000 bootstrap replicates.

Illumina MiSeq sequencing

A subset was selected from the samples with p33 gene amplicons that had been sequenced directly with Sanger technology, and subjected to Illumina sequencing (Illumina, San Diego, CA, USA). These samples were selected in a way that would allow for the greatest combinations of cultivar and geographical location, within the financial constraints of the number of amplicons that could be sequenced with Illumina technology. Paired-end DNA libraries were prepared using the Nextera V2 sample kit (Illumina, San Diego, CA, USA). The samples were sequenced at the Agricultural Research Council (ARC), Biotechnology Platform, Pretoria, South Africa, using an Illumina MiSeq instrument.

Illumina MiSeq data analysis

All trimming and analyses of the Illumina MiSeq datasets was carried out using CLC Genomics workbench 5.5.1 (Qiagen Bioinformatics, Aarhus, Denmark). Data was imported as pair-end reads with a distance range of 180-300. Adapter and quality trimming was performed using the default program settings with Nextera V2 transposase adapter sequences (Transposase1: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG; Transposase2: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG). Quality control was carried out using the Fast QC function. Datasets were mapped to the cognate p33 region of a

set of reference sequences and the following reference assembly parameters: Length fraction: 0.9; similarity fraction: 0.9; global alignment off; non-specific match handling with map randomly on (Read and Pietersen 2015). Closely related reference sequences were grouped into the following clades and MiSeq reads mapping to these references were assigned to their respective clades: **RB**: F957196 (B301); FJ525432 (NZRB-G90); GQ454869 (HA 18-9); FJ525435 (NZRB-M17); JX266712 (Taiwan-Pum/SP/T1); FJ525431 (NZRB-M12); FJ525433 (NZRB-TH28); FJ525434 (NZRB-TH30). **Kpg3/SP/T3**: HM573451 (Kpg3); EU857538 (SP); KC525952 (T3). **HA 16-5**: GQ454870 (HA 16-5). **VT**: JQ911664 (CT11A); KC517493 (FL202-VT); U56902 (VT); KC517492 (FS703-VT); EU937519 (VT); KC517494 (FS701-VT); KC262793 (L192GR); DQ151548 (T318A); AB0463981 (NUagA); KC333869 (CTZA3). **AT-1**: JQ061137 (AT-1). **T36**: NC_001661 (T36); AY 340974 (Qaha); U16304 (T36); DQ272579 (Mexico); AY170468 (T36); EU937521 (T36); KC517485 (FS674-T36); KC517486 (FS701-T36); KC517487 (FS703-T36); KC517488 (FS577). **Taiwan-Pum/M/T5**: JX266713 (Taiwan-Pum/M/T5). **T30**: AF001623 (SY568); AF260651 (T30); Y18420 (T385); KC517489 (FS701-T30); KC517490 (FL278-T30); KC517491 (FS703-T30). **B165**: JQ911663 (CT14A); JQ965169 (T68); EU076703 (B165); FJ525436 (NZ-B18), **A18:** JQ798289 (A18).

Results

Biological characterisation of selected isolates

Isolates from Tucumán, Salta and Jujuy, yielded variable reactions on their respective indicator hosts (Table 1). Those from Tucumán yielded milder reactions than those from Jujuy and Salta in almost all the indicator plants.. Stem pitting in sour orange indicator plants was moderate to severe for isolates from Salta and Jujuy while moderate to no reaction was observed for those from Tucumán. Furthermore, leaf symptoms and stunting in Duncan grapefruit and Mexican lime indicator plants were more severe for isolates from Salta and Jujuy. None of the biological replicates showed any sweet orange stem-pitting reaction.

Direct Sanger sequencing of p33 gene amplicons

Data relating to the cultivar, rootstock, collection site, date of sampling and year of planting are listed in *Table 2*. In addition to this, sequence relatedness information for amplicons sequenced only with Sanger sequencing, is also listed in *Table 2*. For amplicons sequenced with both direct and Illumina MiSeq technologies, data are listed in *Table 3*. A total of forty-four sequences of the fifty amplicons sequenced with the Sanger method met the quality criterion of a mean *phred* score of 30. A representative dendrogram from which the phylogenetic information was derived is shown in *Figure 1*.

Table 1: *Symptom rating observed on various citrus indicator hosts inoculated with CTV isolates from grapefruit from this study 7th month post-inoculation.*
Symptom rating scale: 0 = no reaction; 0.5 = very mild reaction; 1 = mild reaction; 2 = moderate reaction; and 3 =severe reaction.
Values provided for each replicate separately.

Cultivar/Rootstock	Sample collection site	Sample number	Symptom rating on the each of the biological replicates of various indicator hosts							
			Mexican lime			Duncan grapefruit			Sour orange	Sweet orange
			Leaf symptoms	Stunting	Stem pitting	Leaf symptoms	Stunting	Stem pitting	Stem pitting	Orange Stem pitting
Star Ruby/Swingle	Tucumán	14-4005	2-2-3-3	1-2-2-3	1-2-2-3	1-3-3-3	1-2-2-3	0,5-2-2-3	0-0-0-0	0-0-0-0
Ruben Pink /Cleopatra	Tucumán	14-4008	2-2-3-3	0-0-0-2	1-1-1-3	0-0-0-0	0-0-0-2	0,5-0,5-1-3	0-0-2-2	0-0-0-0
Ruben Pink / Cleopatra	Jujuy	14-4013	3-3-3-3	2-3-3-3	1-1-1-2	3-3-3-3	2-3-3-3	1-1-1-3	2-2-3-3	0-0-0-0
Henninger´s /Cleopatra	Salta	14-4017	3-3-3-3	1-3-3-3	0-1-2-3	3-3-3-3	0-1-3-3	0-1-2-3	2-2-2-2	0-0-0-0

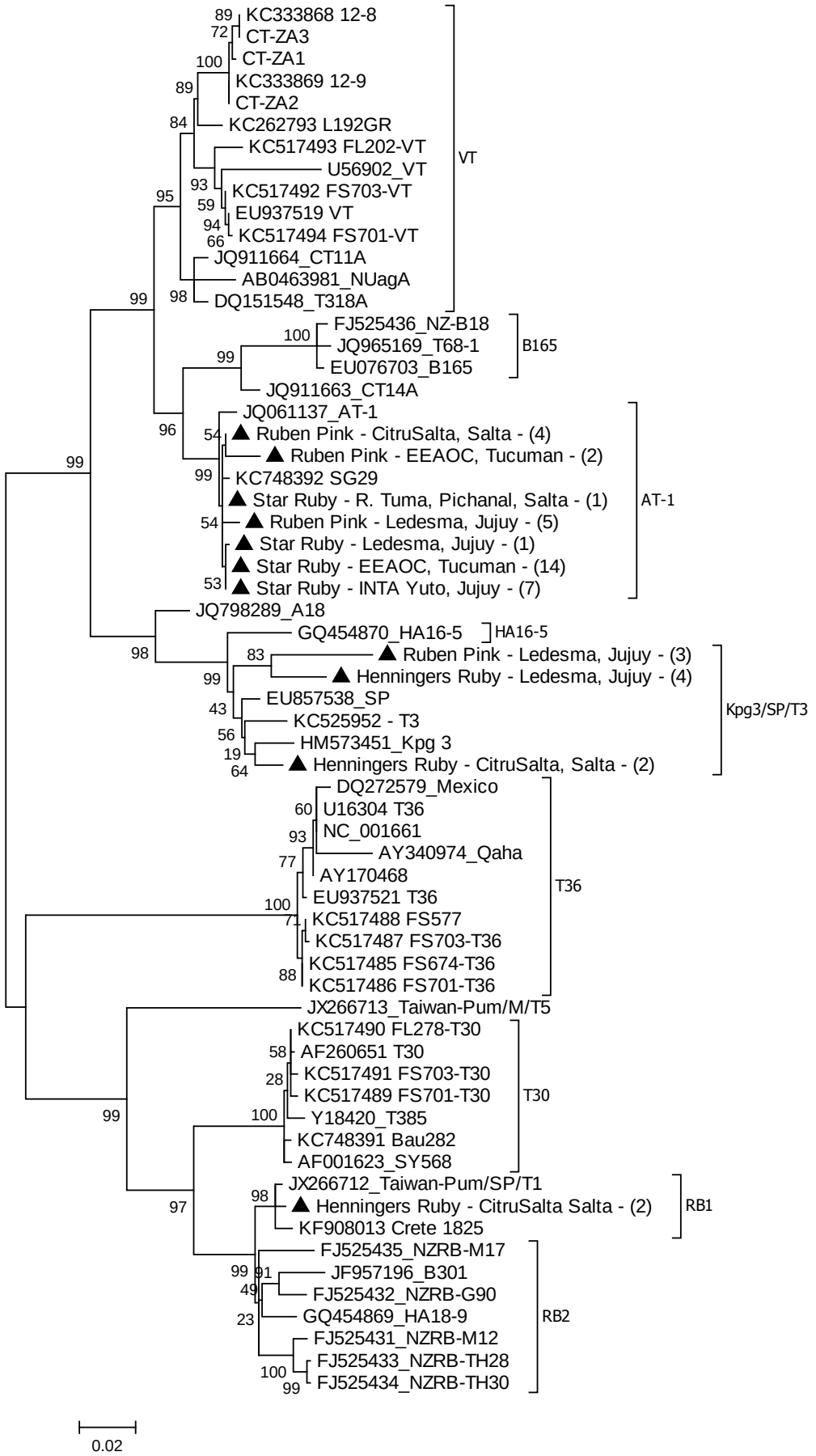

Figure 1: *Dendrogram representing the direct Sanger sequences (population consensus sequence) derived from pigmented grapefruit cultivars collected from the major grapefruit production areas within Argentina. The dendrogram was produced using a neighbour-joining phylogeny with 1000 bootstrap replicates. The branches marked with a ▲ symbol represent a collapsed group of almost identical sequences with the number of sequences represented indicated in brackets, these were derived from the same cultivar and grown within the same production area.*

Table 2: *CTV population consensus sequence obtained by direct Sanger sequencing of the p33 gene of grapefruit samples from Argentina.*

Cultivar	Rootstock	Sample collection site	Sample number	Date sampled	Year of planting	Closest isolate (BLAST)	Genotype (dendrogram)
Star Ruby	*Swingle citrumelo (C. trifoliata X C. paradisi)*	EEAOC Tucumán	14-4000 Pre-immunised with GFMS12	08-2014	1995	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4001 Pre-immunised with GFMS12	08-2014	1995	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4002	08-2014	1995	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4003	08-2014	1995	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4004	08-2014	1995	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4005	08-2014	1995	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	EEAOC Tucumán	14-4006 Pre-immunised with GFMS12	08-2014	1992	AT-1 (JQ061137)	AT-1
	79AC, Cleopatra X Swingle citrumelo (C. reshni) X (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4007 Pre-immunised with GFMS12	08-2014	2007	AT-1 (JQ061137)	AT-1
	Citrus volka-meriana	EEAOC Tucumán	14-4010	08-2014	1982	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	15-4000	04-2015	1995	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	EEAOC Tucumán	15-4001	04-2015	1992	AT-1 (JQ061137)	AT-1
	79AC, Cleopatra X Swingle citrumelo(C. reshni) X (C. trifoliata X C. paradisi)	EEAOC Tucumán	15-4002	04-2015	2007	AT-1 (JQ061137)	AT-1
	Citrus volkameriana	EEAOC Tucumán	15-4004	04-2015	1982	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	15-4063	04-2015	1995	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	Ledesma, Jujuy	14-4016	08-2014	2003	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	R.Tuma, Pichanal,Salta	14-4022	08-2014	1988	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	14-4023	08-2014	1987	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	14-4024	08-2014	1987	AT-1 (JQ061137)	AT-1

	Cleopatra (C. reshni)	INTA Yuto, Jujuy	14-4025	08-2014	1996	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	14-4026	08-2014	1996	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	15-4076	04-2015	1987	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	15-4077	04-2015	1987	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	15-4078	04-2015	1987	AT-1 (JQ061137)	AT-1
Henninger's Ruby	*Cleopatra (C. reshni)*	Ledesma, Jujuy	14-4011	08-2014	1994	SP (EU857538)	Kpg3/SP/T3
	Cleopatra (C. reshni)	Ledesma, Jujuy	14-4012	08-2014	1994	SP (EU857538)	Kpg3/SP/T3
	Cleopatra (C. reshni)	Ledesma, Jujuy	15-4073	04-2015	1994	Taiwan-Pum/SP/T1 (JX266712)	Kpg3/SP/T3
	Cleopatra (C. reshni)	Ledesma, Jujuy	14-4018	08-2014	1976	Kpg3 (HM573451)	Kpg3/SP/T3
	Cleopatra (C. reshni)	CitruSalta,Salta	14-4017	08-2014	1976	Taiwan-Pum/SP/T1 (JX266712)	RB
	Cleopatra (C. reshni)	CitruSalta,Salta	15-4069	04-2015	1976	Taiwan-Pum/SP/T1 (JX266712)	RB
	Cleopatra (C. reshni)	CitruSalta,Salta	15-4070	04-2015	1976	Kpg3 (HM573451)	Kpg3/SP/T3
Ruben Pink	*Cleopatra (C. reshni)*	EEAOC Tucumán	14-4008	08-2014	1989	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	EEAOC Tucumán	14-4009 Pre-immunised with GFMS12	08-2014	1992	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	Ledesma, Jujuy	14-4013	08-2014	1994	SP (EU857538)	Kpg3/SP/T3
	Cleopatra (C. reshni)	Ledesma, Jujuy	14-4014	08-2014	1994	AT-1 (JQ061137)	AT-1
	Swingle citrumelo (C. trifoliata X C. paradisi)	Ledesma, Jujuy	14-4015	08-2014	2003	AT-1 (JQ061137)	AT-1
	Troyer citrange (C. sinensis x P. trifoliata)	Ledesma, Jujuy	14-4019	08-2014	1989	AT-1 (JQ061137)	AT-1
	Troyer citrange (C. sinensis x P. trifoliata)	Ledesma, Jujuy	14-4020	08-2014	1989	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	Ledesma, Jujuy	14-4021	08-2014	1989	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	Ledesma, Jujuy	15-4074	04-2015	1994	Taiwan-Pum/SP/T1 (JX266712)	Kpg3/SP/T3
	Cleopatra (C. reshni)	Ledesma, Jujuy	15-4075	04-2015	1994	Taiwan-Pum/SP/T1 (JX266712)	Kpg3/SP/T3
	Troyer citrange (C. sinensis x P. trifoliata)	CitruSalta, Salta	15-4065	04-2015	1992	AT-1 (JQ061137)	AT-1
	Troyer citrange (C. sinensis x P. trifoliata)	CitruSalta, Salta	15-4066	04-2015	1992	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	CitruSalta, Salta	15-4067	04-2015	1992	AT-1 (JQ061137)	AT-1
	Cleopatra (C. reshni)	CitruSalta, Salta	15-4068	04-2015	1992	AT-1 (JQ061137)	AT-1

Table 3: *Results of Illumina MiSeq read mapping to various CTV strains for representative samples from pigmented grapefruit trees collected in Tucumán, Salta and Jujuy provinces Argentina.*

						Sanger sequencing		Illumina MiSeq sequencing										
Cultivar	Rootstock	Sample collection site	Sample number	Date sampled	Year of planting	Closest isolate (BLAST)	Genotype (dendrogram)	Total number of reads mapping to refs	RB (%)	Kpg3/SP/ T3 (%)	HA 16-5 (%)	VT (%)	AT-1 (%)	T36 (%)	Taiwan-Pum/ M/T5 (%)	T30 (%)	B165 (%)	A18 (%)
Star Ruby	*Swingle citrumelo (C. trifoliata X C. paradisi)*	Ledesma, Jujuy	14-4016	08-2014	2003	AT-1 (JQ061137)	AT-1	22 798	15.6	22.3	1.3	0.4	52.1	-	-	-	6.7	1.2
	Cleopatra (C. reshni)	INTA Yuto, Jujuy	15-4076	04-2015	1987	AT-1 (JQ061137)	AT-1	543 629	6.1	-	-	4.5	82.9	-	-	-	6.6	-
Henninger's Ruby	*Cleopatra (C. reshni)*	Ledesma, Jujuy	14-4011	08-2014	1994	SP (EU857538)	Kpg3/SP/T3	25 989	45	51.1	2.8	-	0.1	-	-	-	-	-
	Cleopatra (C. reshni)	CitruSalta,Salta	15-4069	04-2015	1976	Taiwan-Pum/SP/T1 (JX266712)	RB	630 503	87.7	11.9	0.3	-	-	-	-	-	-	-
Ruben Pink	*Cleopatra (C. reshni)*	EEAOC Tucumán	14-4008	08-2014	1989	AT-1 (JQ061137)	AT-1	44 431	40.8	0.5	-	-	51.2	-	-	-	6.9	-
	Cleopatra (C. reshni)	Ledesma, Jujuy	14-4013	08-2014	1994	SP (EU857538)	Kpg3/SP/T3	71 334	22.2	66.7	2.3	-	5	-	-	-	0.7	2.8
	Cleopatra (C. reshni)	Ledesma, Jujuy	15-4074	04-2015	1994	Taiwan-Pum/SP/T1 (JX266712)	Kpg3/SP/T3	11036122	35	44.7	0.7	1.4	16.6	-	-	-	1.5	-

The majority of CTV population consensus sequences (from direct sequencing) (n = 34) clustered within a branch containing the AT-1 (JQ061137) cognate reference sequence. While, this sequence type was prevalent within all of the geographic regions sampled, this was the only sequence type detected in the EEAOC collection site in Tucumán. AT-1-like sequences were detected in two of the three cultivars sampled, namely Star Ruby and Ruben Pink but not in Henninger's Ruby. The second most prevalent sequence type was Kpg3/SP/T3 (so called, as the p33 gene sequence is unable to resolve between the Kpg3, SP and T3 reference sequences). Kpg3/SP/T3-like sequences appeared confined to the Salta and Jujuy collection sites in the Henninger's Ruby, and Ruben Pink, with the majority of these sequences observed on Henninger's Ruby (6). RB-like (Resistance-breaking) sequences were the least prevalent of the three genotypes detected by direct Sanger sequencing, being present within the CitruSalta collection site in Salta, from two samples collected from Henninger's Ruby.

Illumina sequencing of a subset of p33 gene amplicons

The number of reads per sample varied from between 22 798 to more than eleven million. The percentages of reads mapping to their corresponding genotypes are listed in *Table 3* along with information regarding the cultivar, rootstock, sample collection site, date sampled and year planted for each sample. The dominant genotype according to the Illumina MiSeq data in all cases matched the CTV population consensus sequence obtained with direct sequence as expected. The only genotype detected in all seven populations was RB with levels varying between 6.1% and 87.7% of mapped reads, albeit with only one sample showing dominance for this genotype (Henninger's Ruby from Salta). AT-1 and Kpg3/SP/T3 were present in all but one isolate each and were also the dominant genotype in three isolates. The levels of Kpg3/SP/T3 varied widely across cultivar/collection site combinations from

0.5% to 67% of total reads, with the highest levels observed in samples from Jujuy. The HA16-5 genotype was detected as a minor component in five of the seven datasets, at very low levels between 0.7% and 2.8% of total mapped reads. The VT, B165 and A18 genotypes were detected sporadically within populations of different cultivars and collection sites, at levels of 0.4 – 4.5%, 0.7 – 6.9% and 1.2 – 2.8% of total mapped reads, respectively.

Discussion and conclusions

The purpose of this study was to examine the diversity of CTV populations, associated with pigmented grapefruit cultivars from the growing regions of Tucumán, Salta and Jujuy provinces, in north-western Argentina. These cultivars included Star Ruby, Henninger's Ruby and Ruben Pink. This was done through the amplification of the p33 gene (Read and Pietersen 2015), followed by an initial estimation of the dominant CTV strain in the population through phylogenetic analysis of direct Sanger sequences of the resulting amplicons. A selected subset of samples was sequenced using an Illumina MiSeq platform to confirm the CTV population consensus sequence obtained by direct sequencing. The presence of minor CTV strain components in each population was also determined in this manner.

In general, CTV diversity is poorly studied in South America (Benítez-Galeano et al. 2015; Read et al. 2017). The study by Iglesias et al. (2008) has until now been the most recent, regarding genetic diversity of CTV within Argentinean grapefruit. That study focussed on using the SSCP analysis of the p23, p25 and p27 gene regions, showing that the major component of the majority of CTV populations grouped within a unique branch close to the VT reference sequence. Similar results were obtained (Iglesias et al. 2005b) for the p20 gene. These sequences were probably similar to the AT-1-like sequences observed in this

study but could not be identified by Iglesias et al. (2005b; 2008) due to the limited number references available.

The current study, with a sample size of forty-four, represents a significant contribution to the volume of data available for CTV diversity in grapefruits from Argentina and to our knowledge is one of the largest to date. While direct Sanger sequencing is unable to provide the identities of all the components of a CTV population, it generally provides the identity of the dominant component (Read and Pietersen 2016). All 23 of the direct Sanger sequences associated with the Star Ruby samples collected in this study, grouped with the AT-1 reference within the neighbour-joining dendrogram. The Illumina MiSeq sequencing of two Star Ruby samples confirmed that the dominant component of these populations was an AT-1-like strain present as 52% and 83% of the population respectively. The dominant components of CTV populations from the Henninger's Ruby cultivar were different from that of Star Ruby, being dominant for either Kpg3/SP/T3 or RB-like sequences. Interestingly, Iglesias et al. (2005a) characterized a Henninger´s Ruby isolate from Jujuy by the analysis of p20 and p23 genes and found that one group of sequence variants clustered with severe reference isolates (VT and SY568) and the other group clustered with mild reference isolates (T30 and T385) in relatively equal proportions. Further analysis of the Illumina MiSeq data for the Henninger's Ruby CTV populations, shows that AT-1 associated sequences were almost undetectable. These apparent differences in affinity for particular components may have contributed to the improved performance of Henninger's Ruby over Star Ruby in field trials (Foguet and Foguet 1990; Foguet and Gonzalez 1992). The CTV population profiles derived from the Henninger's Ruby samples in this study, are generally comparable to those observed in Star Ruby in South Africa, where either Kpg3/SP/T3 and RB-like sequences were dominant in the majority of populations analysed with the same technique (Read and Pietersen 2016). All of the samples collected from the study by Read and Pietersen (2016)

were from pre-immunized trees, many of which were planted 23 years prior to collection and still producing large fruit. Foguet et al. (1999) also showed experimentally that Star Ruby pre-immunized with a South African cross-protecting population performed better than non-pre-immunized Star Ruby trees during the first 5 years after planting in a field trial in Tucumán province. However, after 8 years, the performance between pre-immunised and non-pre-immunised trees appeared the same (Foguet, personal communication). The sequences derived from the CTV populations of pre-immunized and non-preimmunized Star Ruby trees, in Argentina grouped with the AT-1 reference.

The direct sequencing results for samples collected from the Ruben Pink cultivar showed that eleven of the populations were dominant for AT-1 and three for Kpg3/T3/SP. The three Ruben Pink samples sequenced using Illumina MiSeq reflected the results obtained for their corresponding direct sequences. The Illumina MiSeq data also showed that the Ruben Pink populations were composed of a significant proportion of RB-like sequences. The levels of RB-like sequences (22 – 41% of mapped reads) were greater than observed for Star Ruby (6-16% of mapped reads). This apparent increased tolerance of Ruben Pink to stem-pitting could be the result of a reduced affinity for certain CTV strains resulting in these symptoms.

The biological indexing experiment showed that the isolates from Salta and Jujuy generally elicited more severe symptoms on their respective hosts than those from Tucumán. AT-1 was shown to be dominant for both isolates from Tucumán and Kpg3/SP/T3 and RB were the dominant sequence types within each respective isolate from Salta and Jujuy. Analysing Ruben Pink isolates from both provinces, different genotype proportion of Kpg3/SP/T3 and AT-1 was observed. The one from Jujuy had 66.7% and 0.5% respectively, while Tucuman isolate had inverse ratio (5% and 51.2%). The results of this study appear to uncover an affinity for AT-1-like sequence types by the Star Ruby cultivar in Argentina, which could be one of the factors driving the failure of Star Ruby plantings in this region.

However, until further research is performed, such as whole-genome sequencing and the isolation and empirical analysis of individual components, implicating any strains in symptom expression remains speculative. Ruben Pink has been shown to perform better than Star Ruby under field conditions with only some trees developing stem-pitting symptoms (Foguet and Foguet 1990), nevertheless, trees that were sampled for this study all showed symptoms of stem-pitting and the majority showed varying degrees of decline. This once again suggests that the AT-1-like component could be contributing to the stem-pitting symptoms seen in sensitive grapefruit cultivars in Argentina.

In the study of CTV diversity on pre-immunized Star Ruby plants in South Africa (Read and Pietersen 2016), AT-1-like sequences were not detected in the majority of populations and was only dominant in four of the 92 populations analysed. As for the South African Star Ruby populations, RB-like sequences were observed in all of the Argentinian samples subjected to Illumina sequencing. Further parallels between the populations from Argentina and South Africa analysed with Illumina MiSeq sequencing are found, with the Kpg3/SP/T3-associated sequences present in the majority of populations, and the virtual absence of T30, T36 and Taiwan-Pum/M/T5-like sequences, with a small number of exceptions. This could indicate the movement of CTV strains between these two countries through the historical trade of plant material.

When compared with a previous study of CTV diversity on *Citrus x limon* (Lemon) cultivars in Tucumán province (Read et al. 2017), strain profiles of the pigmented grapefruit cultivars from northwestern Argentina appear to be more diverse as well as more erratic in terms of the relative abundances of reads mapping to their references. The populations associated with lemon cultivars showed a much greater homogeneity with a relatively stable abundance of reads mapping to RB, Kpg3/SP/T3 and HA16-5 reference sequences. Other, minor components within the CTV populations associated with lemon were below or at the

limit of detection. Interestingly, greenhouse analyses of CTV populations associated with lemon cultivars from Tucumán province showed that they generally induced only mild stem-pitting symptoms on a limited number of experimental Duncan grapefruit plants (Read et al. 2017). Therefore, CTV populations associated with lemon cultivars are probably not responsible for the stem-pitting and decline of grapefruit cultivars in northwestern Argentina.

The results of this study have significantly contributed to the body of knowledge regarding CTV population diversity among pigmented grapefruit cultivars from Argentina. The dissimilarity between dominant strains within CTV populations from lemon and grapefruit cultivars corroborate that there is a CTV strain host-specificity (Albiach-Marti 2013; Harper et al. 2015; Zanutto et al. 2013) and suggests that those from lemons do not pose a threat to the grapefruit production. The correlation between the increased presence of the AT-1-like sequences and stem-pitting symptoms has suggested that this component may be responsible for the failure of sensitive cultivars. Further research will be required to confirm this, such as the isolation of individual strains and their inoculation onto indicator hosts, followed by the sequencing of their complete genomes.

Acknowledgements

We would like to acknowledge funding from the National Research Foundation (South Africa) and Ministerio de Ciencia, Tecnologia e Innovación Productiva (Argentina) via the South Africa-Argentina Bilateral agreement programme. We also acknowledge Ledesma S.A.A.I , which allowed samples to be collected from their orchards.

References

Albiach-Marti, M.R. (2013). The Complex Genetics of Citrus tristeza virus. In V Romanowski (Ed.), *Current Issues in Molecular Virology - Viral Genetics and Biotechnological Applications* (pp. 1 – 26). Rijeka, Croatia: InTech

Bar-Joseph, M., Marcus, R., & Lee, R.F. (1989). The continuous challenge of Citrus tristeza virus control. *Annual Reviews in Phytopathology*, 27, 291-316.

Benítez-Galeano, M.J., Rubio, L., Bertalmío, A., Maeso, D., Rivas, F., & Colina, R. (2015). Phylogenetic studies of the three RNA silencing suppressor genes of South American CTV isolates reveal the circulation of a novel genetic lineage. *Viruses,* 7, 4152-4168

Foguet, J.L. (1961). Presencia del virus de la tristeza de los citrus en Tucumán. *IDIA Suplemento,* 6, 80-86

Foguet, J.L. (1983). Origen y características del pomelo Foster seedless cultivado en el Noroeste Argentino. San Miguel de Tucumán, Argentina: Estación Experimental Agroindustrial Obispo Colombres.

Foguet, J.L.& Foguet, A. (1990). Reacción de algunos cultivares de pomelos rosados y rojos a las estirpes de tristeza presente en el Noroeste Argentino. *Revista Avance Agroindustrial*, 40, 2-3.

Foguet, J.L., & Gonzalez, J.L. (1992). Observaciones sobre el estado actual de la tristeza en los pomelos de Noroeste Argentino. *Revista Avance Agroindustrial*, 49, 33-34.

Foguet, J.L., Blanco, A.,Gonzalez, J.L., & Vinciguerra, H. (1999). Variedades de pomelos rojos en el Noroeste Argentino. *Revista Avance Agroindustrial*, 77, 7-12

Folimonova, S.Y., Robertson, C.J., Shilts, T., Folimonov, A.S., Hilf, M.E., Garnsey, S.M., & Dawson, W.O. (2010). Infection with strains of Citrus tristeza virus does not exclude superinfection by other strains of the virus. *Journal of Virology*, 84, 1314–1325.

Folimonova, S.Y. (2013). Developing an understanding of cross-protection by Citrus tristeza virus. *Frontiers in Microbiology*, 4, 76

Garnsey, S.M., Gumpf, D.J., Roistacher, C.N., Civerolo, E.L., Lee, R.F., Yokomi, R.K., & Bar-Joseph, M. (1987). Toward a standardized evaluation of the biological properties of citrus tristeza virus. *Phytophylactica*, 19(2), 151-157

Gottwald, T.R., Garnsey, S.M., Cambra, M., Moreno, P., Irey, M. & Borbón, J. (1996). Differential effects of Toxoptera citricida vs. Aphis gossypii on temporal increase and spatial patterns of spread of citrus tristeza. In: da Graça, J.V., Moreno, P., & Yokomi, R.K. (Eds.), *Proceedings of the 13th Conference of the International Organization of Citrus Virologists*. (pp. 120 -129). Riverside, California: IOCV

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.

Harper, S.J. (2013). Citrus tristeza virus: evolution of complex and varied genotypic groups. *Frontiers in Microbiology*, 4, 93

Harper, S.J., Cowell, S.J. & Dawson, W. O. (2015). Finding balance: Virus populations reach equilibrium during the infection process. *Virology*, 485, 205–212

Iglesias, N. G., Marengo, J., Riquelme K., Costa N., Plata M. I. & Semorile L. (2005a). Characterization of the population structure of grapefruit isolate of *Citrus tristeza virus* (CTV) selected for pre-inmunization assays in Argentina. In: Hilf, M.E., Duran-Vila, N., & Rocha-Peña, M.A. (Eds.), *Proceedings of the 16th Conference of the International Organization of Citrus Virologists*. (pp. 150–158). Riverside, California: IOCV.

Iglesias, N. G., Riquelme K., Marengo, J., Costa N., Plata M. I. & Semorile L. (2005b). Genetic structure of *Citrus tristeza virus* (CTV) populations from Field argentinian grapefruit isolates. . In: Hilf, M.E., Duran-Vila, N., & Rocha-Peña, M.A. (Eds.), *Proceedings of the 16th Conference of the International Organization of Citrus Virologists*. (pp. 143–149). Riverside, California: IOCV.

Iglesias, N.G., Gago-Zachert, S.P., Robledo, G., Costa, N., Plata, M.I., Vera, O., Grau, O., & Semorile, L.C. (2008). Population structure of Citrus tristeza virus from field Argentinean isolates. *Virus Genes*, 36, 199–207

Moreno, P., Ambrós, S., Albiach-Martí, M.R., Guerri, J., & Peña, L. (2008). *Citrus tristeza virus*: A pathogen that changed the course of the citrus Industry. *Molecular Plant Pathology*, 9, 251-268.

Niblett, C.L., Genc, H., Cevik, B., Halbert, S., Brown, L., Nolasco, G., Bonacalza, B., Manjunath, K.L., Febres, V.J., Pappu, H.R. & Lee, R.F. (2000). Progress on strain differentiation of *Citrus tristeza virus* and its application to the epidemiology of citrus tristeza disease. *Virus Research*, 71, 97 - 106

Read, D.A., & Pietersen, G. (2015). Genotypic diversity of Citrus tristeza virus within red grapefruit, in a field trial site in South Africa. *European Journal of Plant Pathology*, 142, 531-545.

Read, D.A., & Pietersen, G. (2016). Diversity of Citrus tristeza virus populations in commercial grapefruit orchards in Southern Africa, determined using Illumina MiSeq technology. *European Journal of Plant Pathology*, 148, 379 - 391

Read, D.A., Palacios, M.F., Figueroa, J., Foguet, L., Kleynhans, J., Stein, B., & Pietersen, G. (2017). Survey of Citrus tristeza virus (CTV) strains in Citrus x limon (L) Burm f. (lemon) in Tucumán Province, Argentina. *European Journal of Plant Pathology*, doi: 10.1007/s10658-017-1245-2

Sambrook, J. (2001). *Molecular Cloning: 'A laboratory manual'*. New York: Cold Spring Harbor Laboratory Press.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, 30, 2725-2729

Valiela, M.V.F. (1959). The present status of Tristeza in Argentina. In: Wallace J.M (Ed.), Proceedings of the Conference on Citrus Virus Diseases, Riverside,California: University of California, Division of agricultural sciences

van Vuuren, S. P., & Manicom, B. Q. (2005). The response of star ruby grapefruit to different Citrus tristeza virus isolates. In: Hilf, M.E., Duran-Vila, N., & Rocha-Peña, M.A. (Eds.), *Proceedings of the 16th Conference of the International Organization of Citrus Virologists*. (pp. 112–116). Riverside, California: IOCV.

Yokomi, R.F., Lastra, R., Stoetzel, M.B., Damsteegt, V.D., Lee, R.F., Garnsey, S.M., Gottwald, T.R., Rocha-Peña, M.A., & Niblett, C.L. (1994). Establishment of the Brown Citrus Aphid (Homoptera: Aphididae) in Central America and the Caribbean Basin and Transmission of Citrus tristeza virus. *Journal of Economic Entomology*, 87, 1078-1085.

Zanutto, C. A., Corazza, M. J., de Carvalho Nunes, W. M. & Müller, G. W. (2013). Evaluation of the protective capacity of new mild *Citrus tristeza virus* (CTV) isolates selected for a preimmunization program. *Scientia Agricola*, 70, 116-124