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RESEARCH PAPER

Aggressive *Citrus tristeza virus* isolates in Chile are MCA13-positive and VT type, while mild isolates are MCA13-negative and T30 type

Ximena Besoain¹, Edson Bertolini², Rocío Camps¹, Fiona Ramella¹, María Teresa Gorris², Margarita Torres¹, and Mariano Cambra²

¹Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Casilla 4-D, Quillota, Chile.

²Instituto Valenciano de Investigaciones Agrarias, Apdo. Oficial, 46113, Moncada, Valencia, Spain.

Abstract

X. Besoain, E. Bertolini, R. Camps, F. Ramella, M.T. Gorris, M. Torres, and M. Cambra. 2015. Aggressive *Citrus tristeza virus* isolates in Chile are MCA13-positive and VT type, while mild isolates are MCA13-negative and T30 type. Cien. Inv. Agr. 42(2):251-262. *Citrus tristeza virus* (CTV) was reported in the 1960's to affect Meyer lemon trees in Chile, but no field symptoms were observed. This study performed a complete biological, serological and molecular characterization of one hundred CTV isolates obtained from different hosts and geographical origins. Decline symptoms (DI) were not found on trees grafted onto sour orange. In Pica Oasis in northern Chile, stem pitting (SP) was found to affect grapefruit and Mexican lime trees. Most isolates present in the central area were considered mild (MCA-13-negative), while in the northern area aggressive isolates were observed and detected. Some of these isolates were capable of causing SP on grapefruit under field and greenhouse conditions and on sweet orange under greenhouse conditions. Almost all of these isolates were MCA13-positive and had nucleotide sequences associated with the VT genotype.

Key words: Biological indexing, CTV, molecular characterization, stem pitting, seedling yellows.

Introduction

Citrus tristeza virus (CTV) is most likely present in all citrus-producing countries worldwide (CABI, 2010), although its prevalence is highly variable. It has been detected in isolated trees and is not yet considered an epidemic in many countries in the Mediterranean, Near East, and Central and South America (Cambra and Moreno, 2000;

Moreno *et al.*, 2008), including Chile (Weathers and Sánchez, 1970).

There are three major syndromes associated with this pathogen: i) tristeza caused by CTV isolates that are decline-inducing (DI) on citrus trees that have been grafted onto sour orange (*Citrus aurantium* L.) or lemon (*C. limon* (L.) Burm.f.) trees; ii) stem pitting (SP), which is produced in grapefruits (*C. paradisi* Macf.) or sweet oranges (*C. sinensis* (L.) Osbeck); and iii) seedling yellows (SY), which affects sour oranges, lemons and

grapefruits and normally occurs under greenhouse conditions (Moreno *et al.*, 2008).

Garnsey *et al.* (1987) proposed a standardized evaluation for the biological characterization of CTV isolates by assessing biological characteristics (SHR) of the CTV isolates on five different hosts that are especially sensitive to the virus. This approach would allow the characterization of the biological properties of the CTV isolates analyzed to date.

For the serological characterization, Vela *et al.* (1986) were the first to produce monoclonal antibodies (MAbs) specifically for the detection of CTV and demonstrated that the combination of 3DF1 and 3CA5 was capable of detecting all of the CTV isolates found at that time (Cambra *et al.*, 1990). The CTV coat protein contains multiple epitopes; this characteristic allowed Permar *et al.* (1990) to develop a monoclonal antibody (MCA13) capable of reacting to isolates that were considered aggressive in Florida (USA), isolates capable of inducing DI in sweet orange grafted onto sour orange trees, SY in sour oranges, and SP in grapefruits. In contrast, MCA-negative isolates were only capable of inducing symptoms on Mexican limes or *C. macrophylla*. Permar and Garnsey (1991) found a 90 to 95% correlation between the aggressiveness of the CTV isolates and their reaction with MCA13. However, in Spain the reaction with MCA13 was not totally correlated with the presence of DI symptoms (Cambra *et al.*, 1993).

For the molecular characterization of CTV, Hilf and Garnsey (2000) and Hilf *et al.* (2005) proposed a classification based on the use of 11 pairs of oligonucleotide primers for RT-PCR. This study involved the use of a universal primer T-36 (T36CP) that targeted the coat protein of the isolate, plus 10 primer sets. Three of the primer sets targeted the polymerase region, three the K17 region, three the extreme 5' of the T-36, T-30 and VT isolates, and one the K17 zone of the T-3 isolate. The isolates were classified into 14 groups

in this study, although the majority consisted of variants of the T3, T30, T36 and VT isolates. Recent phylogenetic studies have determined that these isolates show clear divergence (Moreno *et al.*, 2008; Biswas, 2010; Brlansky *et al.*, 2011; Harper 2013; Wang *et al.*, 2013).

In Chile, the importance of the citrus industry has been increasing and has oriented towards the export of fresh fruit, especially lemons, sweet oranges, and mandarins (ODEPA, 2013). In the late 1960's, the presence of CTV was reported in nearly 2000 Meyer lemon trees throughout Chile (Weathers and Sánchez, 1970). In a more recent study by Besoain *et al.* (2008), CTV was detected in sweet orange, tangelo, Mexican lime, and grapefruit trees in orchards from the Tarapacá Region to the O'Higgins Region through the use of biological indexing and serology. The CTV prevalence was 0.38% (Besoain, 2008). However, neither the DI nor SP symptoms were observed throughout all of the citrus orchards studied by the researchers.

The objective of this study was to completely characterize 100 Chilean CTV isolates collected during a previous survey using biological and serological approaches and multiple molecular markers (MMM).

Materials and methods

CTV isolates

A collection of Chilean CTV isolates was established through citrus orchard sampling. The isolates were maintained in Mexican lime and/or sweet orange plants in the Citrus Germplasm Virus and Viroids Collection at the Estación Experimental La Palma of the Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso. From this collection, 100 representative CTV isolates were selected from different geographic locations and citrus host species (Besoain, 2008).

Biological characterization of the Chilean CTV isolates

The biological characterization of the Chilean CTV isolates was accomplished using the SHR protocol (Garnsey *et al.*, 1987; Wang *et al.*, 2013). Four plants of five indicator species were used per isolate: Mexican lime, sour orange, Duncan grapefruit, Madame Vinous sweet orange, and Madame Vinous sweet orange grafted onto sour orange. In this last group, plants found to be CTV-positive after the testing were grafted onto the sweet orange scion with a healthy bud of sour orange to determine which isolates were capable of inducing DI (Pina *et al.*, 2004). Then, the trees were maintained for one year under greenhouse conditions with temperatures between 18–26 °C (Roistacher, 1991). The presence or absence of symptoms was evaluated every six months. The grapefruits were grown under temperatures that oscillated between 32 and 38 °C after the second year, as suggested by Broadbent *et al.* (1996), to stimulate the symptoms of severe stem pitting and the development of deep depressions in the wood.

Serological characterization

The serological characterization of the 100 Chilean CTV isolates was performed using DASI-ELISA (Cambra *et al.*, 1991) with 12 MAbs: 3DF1, 3CA5 and 3BH6 from Spain (Vela *et al.*, 1986), MCA13 from Florida, USA (Permar *et al.*, 1990), C1F10 from Cuba (Besoain, 2008), ECTV176 and ECTV178 from Florida, USA (Garnsey, not published), 3E10 and 4H6H from Taiwan (Tsai *et al.*, 1993), and α CTV4E5-a1, α CTV4B1-3 and α CTVF3-a1 from Morocco (Besoain, 2008).

The following isolates were used as the positive CTV controls: the mild Mediterranean T-300 isolate (Ballester-Olmos *et al.*, 1993); the aggressive T-388 isolate; and the T-397-P isolate, which has a defective epitope recognized by the antibody 3DF1 (Cambra *et al.*, 1993). Healthy plants were used as negative controls. The extracts were

prepared by homogenizing samples of vegetative material (1:10 p/v; sample/extraction buffer) in 0.14 M PBS (pH 7.2 to 7.4) supplemented with 0.2% sodium dimethyl dithiocarbamate (DIECA) (Cambra *et al.*, 1991). The DASI-ELISA was performed according to the method of Cambra *et al.* (1993). The ELISA plates (Nunc Polisorp) were coated with 1 μ g mL⁻¹ of polyclonal immunoglobulins. The MAbs diluted in PBS (pH 7.2–7.4) included: 3DF1 (0.05 μ g mL⁻¹); 3CA5 (0.5 μ g mL⁻¹); MCA13 (1/10,000 ascites fluid); 3BH6 (0.25 μ g mL⁻¹); C1F10 (0.25 μ g mL⁻¹); 4H6H (0.5 μ g mL⁻¹); ECTV-176 (1.0 μ g mL⁻¹); ECTV-178 (1/1,000 ascites fluid); 4F3 α 1 (0.2 μ g mL⁻¹); 3E10 (1/5,000 ascites fluid); α CTV4E5-a1 (0.1 μ g mL⁻¹) and α CTV4B1-34F3 (0.1 μ g mL⁻¹). The optical density (DO₄₀₅) was measured after 60 minutes using a Titrek Multiskan (Flow). The samples were considered positive if the DO₄₀₅ exhibited a two-fold increase over the negative control.

Molecular characterization

The 100 CTV isolates were characterized using the methodology described by Hilf *et al.* (2005). A total of 0.5 g of shoot tissue was collected for each sample analyzed. The CTV extraction was performed by combining the lyophilized sample with 4 ml of extraction buffer (PBS (pH 7.2 to 7.4) supplemented with sodium diethyl dithiocarbamate (DIECA)) and homogenizing in individual polyethylene bags (Bioreba) using the Homex 6000 (Bioreba). Viral RNA extraction was performed using the RNeasy® Plant Mini Kit (Qiagen).

One-step RT-PCR was performed using the primers Pin1/Pin2 (Olmos *et al.*, 1999) and the 11 primers pairs described by Hilf *et al.* (2005). The reaction cocktail was composed of 25 μ L mix for RT-PCR (10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, and 0.3% Triton X100 (w/v)), 1 μ M of each primer, 250 μ M dNTPs, 0.25 units of AMV-RT (Promega), 0.5 units of *Taq* Polymerase (Promega) and 5 μ L of RNA template. The conditions for

RT-PCR were: one cycle of retrotranscription at 42 °C for 45 min, followed by 40 amplification cycles at 94 °C for 30 seconds, 56 °C for 1 min and 72 °C for 1 min, and a final cycle at 72 °C for 10 min. Electrophoresis was performed using a 2% agarose gel stained with ethidium bromide, and the bands were observed using ultraviolet light.

Results

Biological characterization of isolates

Biological groups obtained through the biological characterization of the 100 Chilean CTV isolates are shown in Table 1. Eleven groups were estab-

lished (including the 5' and 8' groups), and another four groups were not completely biologically characterized (ND₁ to ND₄). Based on the results of the biological characterization, the majority of the CTV isolates collected in Chile (56%) showed characteristics of mild or moderate isolates (i.e., only caused a reaction in Mexican limes and/or DI in the combination sweet orange/sour orange plants; Symptoms profiles 7, 13, and ND₄).

For the rest of the CTV isolates, only 17% were capable of causing SP. This included 11% in grapefruits, 8% of which were found in Pica and Matilla, Tarapacá Region (Figure 1A and B) and 3% in Ovalle, Coquimbo Region. Six isolates were demonstrated to be capable of causing SP

Table 1. Symptom profiles obtained from the biological characterization of one hundred Chilean *Citrus tristeza virus* isolates.

Symptom Profiles	Mexican lime	DI ¹ Sweet orange/ sour orange	SY on Sour orange	SP on Sweet orange	SP on Grapefruit cv. Duncan	N° of Chilean CTV isolates/Group ²
1	+	+	+	+	+	0
2	+	+	+	+	-	2
3	+	+	+	-	+	2
4	+	+	+	-	-	18
5	+	+	-	-	+	4
5'	+	+	-	+	-	2
6	+	+	-	+	+	0
7	+	+	-	-	-	33
8	+	-	+	+	+	0
8'	+	-	+	+	-	1
9	+	-	+	-	+	1
10	+	-	+	-	-	5
11	+	-	-	+	+	0
12	+	-	-	-	+	3
13	+	-	-	-	-	20
14	-	-	-	-	-	0
ND ₁	+	ND	+	-	-	4
ND ₂	+	ND	-	+	-	1
ND ₃	+	ND	-	-	+	1
ND ₄	+	ND	-	-	-	3
Total	100	61	33	6	11	100

¹DI biological test proposed by Pina *et al.* (2004).

²Symptom profiles for CTV isolates proposed by Hilf *et al.* (2005), modified by adding the 05' profile.

DI= Decline inducing, SY= Seedling yellows, SP = Stem pitting, ND= Not determined (the DI test proposed by Pina *et al.* (2004) was not performed).

Figure 1. A. Symptoms of severe stem pitting on Marsh grapefruit trees, where deformation of the trunk and branches can be observed. B. Star Ruby grapefruit tree with severe stem pitting depressions in the wood and overgrown, thick bark. C. Left, symptoms of seedling yellows developing on a sour orange seedling tree. D. Duncan grapefruit indicator plant inoculated with a CTV isolate capable of inducing stem pitting in the wood. E. Stem pitting observed on *C. macrophylla* rootstock grafted with lemon in Ovalle.

in sweet oranges; all of these isolates were from the Tarapacá Region (Pica Oasis and Camiña). Symptoms of SY in sour oranges were produced by 33% of the Chilean isolates studied (Table 1, Figure 1C). Additionally, some isolates were capable of causing vein corking in sour oranges and/or Mexican limes.

Generally, there was agreement between the reaction or severity of symptoms observed in the indicator plants and the symptoms observed in the field. Thus, the isolates 42-4, 92-2, 507, 509 and 510 (Table 2) obtained from grapefruit trees with SP symptoms yielded symptoms of this type on the Duncan grapefruit indicator plants (Figure 1D). However, the isolates 494, 495, and 497 obtained from an orchard of Eureka lemons grafted onto *C. macrophylla* in Ovalle caused SP in grapefruits. The trees were asymptomatic at

the time these isolates were collected, but four years later it was possible to observe the development of symptoms on the wood of the rootstocks (Figure 1E). Finally, SP was observed in sweet orange trees grown in a greenhouse, denoting the presence of aggressive CTV isolates capable of causing damage independent of the rootstock used. However, this syndrome was not observed on later samples of CTV-infected sweet orange trees grown in the field.

Serological characterization of CTV isolates

Eight serogroups were established from the 100 CTV isolates through the use of 12 different MAbs (Table 2). The majority (57%) of the isolates from this study reacted to the entire panel of MAbs; these were classified as sero-

Table 2. Biological, serological and molecular classification of *Citrus tristeza virus* isolates obtained from orchards in eight Chilean citrus producing regions.

Region	Locality	Host	Isolate	Biological Group	Serological Group	MCA 13	Molecular ¹ Characterization
Atacama	Vallenar	Sweet orange	281-1	7	5	Neg	VT-VTPol+T30Pol
Tarapacá	Comiña	Sweet orange	101-3	7	1	Pos	VT-VTPol
Tarapacá	Matilla	Grapefruit	179-1	7	1	Pos	VT-VTPol
Tarapacá	Matilla	Mexican lime	96-5	7	1	Pos	VT-VTPol
Tarapacá	Pica	Grapefruit	42-4	12	1	Pos	VT-VTPol
Tarapacá	Pica	Grapefruit	92-2	12	1	Pos	VT-VTPol
Tarapacá	Pica	Grapefruit	511	4	1	Pos	VT-VTPol
Tarapacá	Pica	Mexican lime	95-5	7	1	Pos	VT-VTPol
Tarapacá	Pica	Mexican lime	95-6	13	1	Pos	VT-VTPol
Tarapacá	Pica	Mexican lime	106-5	7	1	Pos	VT-VTPol
Tarapacá	Pica	Sweet orange	89-2	7	1	Pos	VT-VTPol
Tarapacá	Pica	Sweet orange	88-2	3	1	Neg	VT-VTPol
Tarapacá	Pica	Grapefruit	42-3	12	4	Pos	VT-VTPol
Tarapacá	Matilla	Sweet orange	240-1	7	1	Pos	VT-VT5'
Tarapacá	Pica	Mexican lime	327	7	1	Pos	VT-VT5'
Tarapacá	Pica	Mexican lime	521	4	1	Pos	VT-VT5'
Tarapacá	Pica	Mexican lime	524	2	1	Pos	VT-VT5'
Tarapacá	Pica	Sweet orange	337	7	3	Pos	VT-VT5'
Tarapacá	Pica	Grapefruit	510	12	1	Pos	VT-K17
Tarapacá	Pica	Grapefruit	514	4	1	Pos	VT5'
Arica y Parinacota	Azapa	Sweet orange	232-2	4	1	Pos	VT+T30K17
Arica y Parinacota	Azapa	Sweet orange	231-1	ND ₁	1	Pos	VT+T30K17
Tarapacá	Matilla	Mexican lime	301-21	4	1	Pos	VT+T30K17
Tarapacá	Pica	Mexican lime	84-1	4	1	Pos	VT+T30K17
Tarapacá	Pica	Mexican lime	83-3	4	1	Pos	VT+T30K17
Tarapacá	Pica	Sweet orange	87-5	4	1	Pos	VT+T30K17
Tarapacá	Comiña	Sweet orange	101-2	7	1	Pos	VT
Tarapacá	Comiña	Sweet orange	101-4	7	1	Pos	VT
Tarapacá	Comiña	Sweet orange	101-5	4	1	Pos	VT
Tarapacá	Comiña	Sweet orange	101-6	7	1	Pos	VT
Tarapacá	Comiña	Sweet orange	301-34	5'	1	Pos	VT
Tarapacá	Comiña	Sweet orange	301-34B	ND ₂	1	Pos	VT
Tarapacá	Matilla	Mexican lime	96-3	4	1	Pos	VT
Tarapacá	Matilla	Mexican lime	96-1	7	1	Pos	VT
Tarapacá	Matilla	Mexican lime	301-7	7	1	Pos	VT
Tarapacá	Matilla	Mexican lime	301-25B	13	1	Pos	VT
Tarapacá	Matilla	Mexican lime	301-30B	13	1	Pos	VT
Tarapacá	Matilla	Mexican lime	96-6	5	1	Pos	VT
Tarapacá	Matilla	Sweet orange	244-1	4	1	Pos	VT
Tarapacá	Matilla	Tangelo	246-2	7	1	Pos	VT
Tarapacá	Pica	Grapefruit	508	5'	1	Pos	VT
Tarapacá	Pica	Mexican lime	301-2	4	1	Pos	VT
Tarapacá	Pica	Mexican lime	301-6	4	1	Pos	VT
Tarapacá	Pica	Mexican lime	95-3	7	1	Pos	VT
Tarapacá	Pica	Mexican lime	106-3	7	1	Pos	VT
Tarapacá	Pica	Mexican lime	106-4	4	1	Pos	VT
Tarapacá	Pica	Mexican lime	85-1	4	1	Pos	VT
Tarapacá	Pica	Mexican lime	301-5	8'	1	Pos	VT
Tarapacá	Pica	Mexican lime	301-9	13	1	Pos	VT
Tarapacá	Pica	Mexican lime	301-11	13	1	Pos	VT
Tarapacá	Pica	Sweet orange	301-41B	7	1	Pos	VT

Continuation Table 2

Tarapacá	Pica	Sweet orange	301-44B	7	1	Pos	VT
Tarapacá	Pica	Sweet orange	334	3	1	Pos	VT
Tarapacá	Pica	Mexican lime	301-10	10	8	Neg	VT
O'Higgins	Lolol	Sweet orange	490	7	2	Neg	T30Pol
O'Higgins	Perailillo	Limonero	491	13	7	Neg	T30Pol
O'Higgins	Nancagua	Sweet orange	462	7	2	Neg	T30K17+T30Pol
O'Higgins	Nancagua	Sweet orange	464	7	2	Neg	T30K17+T30Pol
Valparaíso	Limache	Sweet orange	305-1	7	1	Pos	T30K17
Valparaíso	Limache	Sweet orange	305-1B	13	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-8B	13	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-15B	7	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-3	3	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-7	7	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-8	ND ₁	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-14	7	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-16	ND ₄	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-5B	4	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-6B	10	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-4	13	2	Neg	T30K17
Metropolitana	Buin	Lemon	366	13	2	Neg	T30K17
Metropolitana	Melipilla	Sweet orange	351	13	2	Neg	T30K17
Metropolitana	Melipilla	Sweet orange	349	7	2	Neg	T30K17
Metropolitana	Paine	Sweet orange	368	13	2	Neg	T30K17
Valparaíso	Limache	Sweet orange	305-2	13	8	Neg	T30K17
Tarapacá	Pica	Grapefruit	509	5	1	Pos	Only T36CP ²
Tarapacá	Pica	Grapefruit	507	5	1	Pos	Only T36CP
Tarapacá	Pica	Grapefruit	512	10	1	Pos	Only T36CP
Tarapacá	Pica	Grapefruit	516	10	1	Pos	Only T36CP
Tarapacá	Pica	Grapefruit	517	10	1	Pos	Only T36CP
Tarapacá	Ovalle	Lemon	495	ND ₃	1	Pos	Only T36CP
Tarapacá	Pica	Grapefruit	525	7	1	Pos	Only T36CP
Atacama	Vallenar	Sweet orange	502	13	2	Neg	Only T36CP
Valparaíso	Limache	Sweet orange	305-11	13	2	Neg	Only T36CP
Valparaíso	Limache	Sweet orange	305-12	ND ₁	2	Neg	Only T36CP
Valparaíso	Limache	Sweet orange	305-18	7	2	Neg	Only T36CP
Valparaíso	Limache	Sweet orange	305-19	ND ₄	2	Neg	Only T36CP
Valparaíso	Limache	Sweet orange	305-19B	7	2	Neg	Only T36CP
Metropolitana	Paine	Sweet orange	369	13	2	Neg	Only T36CP
O'Higgins	Doñihue	Sweet orange	492	7	2	Neg	Only T36CP
O'Higgins	Las Cabras	Sweet orange	467	7	2	Neg	Only T36CP
O'Higgins	Nancagua	Sweet orange	461	7	2	Neg	Only T36CP
O'Higgins	Nancagua	Sweet orange	463	7	2	Neg	Only T36CP
O'Higgins	Peumo	Sweet orange	389	7	2	Neg	Only T36CP
O'Higgins	Peumo	Sweet orange	503	ND ₄	2	Neg	Only T36CP
O'Higgins	Peumo	Sweet orange	504	ND ₁	2	Neg	Only T36CP
O'Higgins	Sta. Cruz	Sweet orange	460	13	2	Neg	Only T36CP
Coquimbo	Ovalle	Lemon	494	5	3	Pos	Only T36CP
Coquimbo	Ovalle	Lemon	497	9	3	Pos	Only T36CP
Coquimbo	Ovalle	Lemon	496	13	6	Neg	Only T36CP

¹The abbreviations express the amplified markers VT (complete), VT5' (only VT5'), T30POL (only T30 Pol marker), T30K17 (only marker K17 of T30 isolate), (-) absence of a band for the corresponding marker, (+) presence of a band for the corresponding marker.

²Only one band obtained corresponds to the universal T36 CP marker.

Pos, MCA13-positive reaction (Permar *et al.*, 1990).

Neg, MCA13-negative reaction.

Table serological group check Besoain (2008).

ND, Not determined.

group 1. The second largest group (serogroup 2) was composed of the CTV isolates (34%) that reacted to all of the MABs with the exception of MCA13.

Sixty percent of the CTV isolates in this study were MCA13-positive (serogroups 1, 3 and 4), almost all of which came from the Tarapacá Region, with the exception of three isolates from Ovalle (Coquimbo Region) and one from Limache (Valparaíso Region). Alternatively, almost all of the isolates from the central zone were MCA13-negative (serogroups 2, 5, 6, 7 and 8) (Table 2). However, 10 of the 11 isolates that caused the SP reaction in Duncan grapefruits were MCA13-positive, and all 6 isolates that caused SP in sweet oranges were MCA13-positive (Tables 1 and 2).

Molecular characterization

Based on the analysis of the RT-PCR amplification with the 11 different combinations of primer pairs (Table 2), it was possible to infer that the Chilean CTV isolates tended to be grouped as follows: VT type, total and partial (47%); T30 type, total or partial (21%); those that showed zones of the genome of T30 plus the VT (7%); and finally a group of isolates that were only recognized by the pair of universal primers T36CP (25%). There was a correlation between the type of isolate and geographic location. For example, the VT type was located primarily in the Tarapacá area, which was the zone where the greatest losses and deterioration associated with severe stem pitting of trees occurred (Figure 1 A and B). Only one isolate of the VT type was found in the Atacama Region; this isolate was specifically located in an old-fashioned variety garden, which agreed with the information gathered in the field that grapefruit cuttings had been taken for new plantings in the oases of Pica and Matilla in the Tarapacá Region (unpublished data).

Global analysis

Table 2 shows the description of the 100 Chilean CTV isolates selected for this study based on their biological, serological and molecular characteristics. Of the 58 CTV isolates from the Tarapacá Region, 56 reacted with MCA13 and were catalogued as the VT genotype and 52 provoked noticeable symptoms on the indicator plants (DI on sour/sweet oranges and SY or SP) (Table 1 and 2). This clear correlation indicates that an integral approach (biological, serological, and molecular) should be used to classify CTV isolates. Nevertheless, isolates were also found in the oases of Pica and Matilla in the Tarapacá Region that were considered to be aggressive in the biological indexing but caused no apparent symptoms in the infected orchard-grown trees after a repeat visit four years later in 2006 despite the fact that some of them demonstrated an MCA13-positive reaction (isolates 301-34, 301-34B and 301-44B).

The Chilean CTV isolates that produced amplicons whose sequences were associated with the T30 genotype were mainly from the central zone. These isolates were observed to be less aggressive in the biological characterization and were MCA13-negative, although some were capable of causing SY in sour oranges (isolates 305-3, 305-8, 305-5B and 305-6B) (Table 2).

Finally, there were a total of 25 CTV isolates that were recognized only by T36CP. Many of these isolates were from the Tarapacá Region and central Chile, but could not be grouped with any known type of isolate (Table 2). Additionally, the Spanish isolates considered to be mild that were used in this study (T-300 and T302) also amplified only the T36CP region. However, some of these isolates (509, 512, 516, 517 and 495) were capable of producing symptoms associated with aggressive isolates in indicator plants under greenhouse conditions (profiles 2, 3, 5, 5', 8', 9, 10 and 12) (Table 1 and 2, Figure 1C and D).

Discussion

Biological characterization

Based on the results obtained from the biological characterization, the aggressive isolates detected in Chile corresponded to isolates primarily from the Tarapacá Region. These isolates exhibited a positive reaction to the MCA13 monoclonal antibody (Permar *et al.*, 1991) and a positive amplification of some of the markers of the VT genotype (Hilf *et al.*, 2005). All of the CTV isolates from this region had positive results for at least one of these two reactions.

Ninety percent of the CTV isolates from the central zone were found to be not very aggressive biologically, were MCA13-negative, and had no amplification of the bands associated with the VT isolates. However, 50% of these isolates caused a DI reaction according to the test proposed by Pina *et al.* (2004). Nevertheless no DI symptoms were observed in the previous survey associated with sweet orange trees grafted on sour orange (Besoain, 2008).

The absence or decline of orchards with sweet orange trees grafted onto sour oranges under field conditions could be attributed to a lack of transmission of these isolates by aphids. These orchards were located in similar conditions to orchards in California or the Mediterranean area where severe DI-epidemics have occurred (Moreno *et al.*, 2008).

Serological characterization

One hundred percent of the isolates were recognized by the mix of the MAbs 3DF1 + 3CA5 that were described as universal (Cambra *et al.*, 1991). The MCAs that could detect all of the Chilean isolates were 3DF1 and 3BH6 from Spain (Vela *et al.*, 1986) and α CTV4E5-a1 and α CTV4B1-3 from Morocco (Besoain, 2008). Nevertheless, two isolates (496 from a lemon tree in Ovalle

(Coquimbo Region) and 281-1 from a sweet orange from Vallenar (Atacama Region)) did not react to the MAb 3CA5, which is considered to be a wide spectrum MAb. Very few CTV isolates that are defective in the 3CA5 epitope have been described (Cambra *et al.*, 1993).

The isolates belonging to groups 3 to 8 possibly contain more simple populations than those in groups 1 and 2. For the characterization of the reference isolates, strain T-300 exhibited the behavior described by Cambra *et al.* (1993) and was classified among group 2. In contrast, strains T-388 and T-397 reacted positively to all of the antibodies and were serologically classified as group 1, while isolate T-397-P with the defective 3DF1 epitope reacted with neither 3DF1 nor 3BH6. Finally, MCA 3E10 was not capable of discriminating between the aggressive and mild isolates (Table 1). This finding did not agree with the observations of Tsai *et al.* (1993), who found it to have a behavior similar to MCA13.

Molecular characterization

The universal primers Pin 1 and Pin 2 (Bertolini *et al.*, 2008), the T36CP primers (Hilf and Garnsey, 2000) and those designed by Bertolini *et al.* (2008) for real-time PCR were capable of recognizing 100% of the Chilean CTV isolates used in this study.

The majority of the Chilean isolates (33%) (Table 2) were included in biological group 7 (capable of causing DI in sweet orange/sour orange) but were grouped into different serogroups (up to 5), suggesting serological diversity. Moreover, these isolates were genotyped by RT-PCR as belonging to four groups (although they were primarily VT), indicating variability. The second relatively frequent group of CTV isolates in Chile was cataloged biologically as typical of group 13, which was only capable of inducing symptoms in Mexican limes. Nevertheless, some of these isolates reacted to MCA13 and their molecular

profile was grouped with VT, T30 + VT, or only CPT36, all of which denoted variability. None of these isolates caused important symptoms in their original hosts in the field. This reaffirms the importance of isolate characterizations that are not based only on biological characteristics (as suggested by Garnsey *et al.* (1987)) but also include serological and molecular characteristics (Wang *et al.*, 2013).

The third most frequent group in Chile (18% of the CTV isolates studied) also showed variability; these isolates belonged to serogroups 1 or 2 and were grouped by RT-PCR as T30, VT and VT + T30. These isolates were grouped biologically in group 4 (capable of inducing DI in sour/sweet and SY in sour oranges). Nevertheless, none of these isolates caused significant symptoms in their original hosts in the field.

In this study, there was agreement between the geographical location of the CTV isolates, their serological reaction to MCA13, and the molecular isolate-type to which they belonged. For example, the isolates originating in the Tarapacá Region

were classified as MCA13-positive and VT. These isolates caused symptoms of extreme stem pitting in the wood to similar extents in the field and in the biological tests. In contrast, a low incidence of isolates with aggressive characteristics was detected in central Chile where the principal commercial orchards are located. However, this situation could change in the future, thereby demonstrating the importance of producing propagation material free of CTV and introducing new improvements in the detection of viruses and viroids.

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Resumen

X. Besoain, E. Bertolini, R. Camps, F. Ramella, , M.T. Gorris, M. Torres y M. Cambra. 2015. Aislados agresivos de *Citrus tristeza virus* en Chile son MCA13 positivo y tipo VT mientras que los aislados leves son MCA13 negativo y tipo T30. Cien. Inv. Agr. 42(2):251-262. El virus de la tristeza de los cítricos fue reportado el año 1960 afectando a árboles de limonero Meyer pero no se observaron síntomas a nivel de campo. Esta investigación tuvo como objetivo realizar una completa caracterización biológica, serológica y molecular de 100 aislados Chilenos de *Citrus tristeza virus* (CTV), previamente obtenidos desde diferentes hospederos y regiones geográficas. No se encontró síntomas de decaimiento (DI) en árboles injertados sobre naranjo amargo. En el Oasis de Pica, localidad de la zona norte de Chile, fue encontrada tristeza causando Stem pitting (SP) en árboles de pomelo y lima Mexicana. La mayoría de los aislados presentes en la zona central se consideraron leves (MCA-13 negativos), mientras que en la zona norte se observaron y se detectaron aislados agresivos, algunos capaces de causar SP en pomelo en condiciones campo e invernadero, y algunos capaces de causar SP en naranjo dulce bajo condiciones de invernadero, de los cuales casi todos fueron MCA-13 positivos y la secuencia de nucleótidos asociada al genotipo VT.

Palabras clave: Acanaladura de la madera, amarillez de plantas, caracterización molecular, CTV, Prueba biológica.

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