

## Research Article

## First comprehensive sanitary report of citrus viruses and viroids in Uruguay

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### Abstract

Citrus is the highest-value fruit crop in terms of international trade. However, citrus species are susceptible to several diseases caused by different pathogens which directly cause a decrease in production leading to economic losses. In the last half-century, the citrus industry in Uruguay has had a strong socio-economic impact and is also constantly evolving to stay competitive in world markets, by introduction of new varieties and improvement of production practices to obtain high yielding orchards. Nevertheless, despite the existence since 2014 of the Uruguayan National Citrus Sanitary and Certification Program, scarce information is available regarding the virus and viroid status of commercial citrus in Uruguay. The incidence of citrus tristeza virus (CTV), citrus psorosis virus (CPsV), satsuma dwarf virus (SDV), citrus exocortis viroid (CEVd), hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bark cracking viroid (CBCVd) and citrus bent leaf viroid (CBLVd) was investigated in this study, as well as CTV genotypes prevalent in the country. Molecular diagnostic assays were used to test 1175 samples including Valencia and Navel sweet oranges, Mandarin hybrids, Clementines and lemons, which were randomly collected from seven citrus-producing provinces. Only 6% of the samples were negative for the pathogens screened, while 93% of them were CTV positive. SDV, CBLVd and CBCVd were not detected. Co-infections were frequently detected, finding plants with up to four simultaneous pathogens, including CTV in all co-infected plants. This is the first comprehensive survey of several citrus-infecting viruses and viroids in Uruguay, as well as a determination of the CTV genotypes prevalent in the country.

**Keywords:** Citrus viruses, citrus viroids, CTV genotypes, survey, Uruguay, molecular detection

### Introduction

Citrus is one of the most important fruit crops worldwide, but it is susceptible to several insect-borne and graft-transmissible pathogens and efficient disease management is required for competitive and sustainable production (Tennant et al. 2009; Donkersley et al. 2018; Spreen et al. 2020). Citrus growers worldwide are constantly challenged to introduce new varieties to increase competitiveness in export markets. One of the main approaches to replace old varieties with new ones in established commercial fields is by means of top-working (Albrecht et al. 2017). Although this practice shortens the production period compared to planting a new orchard, it should only be applied on healthy trees for long-term sustainability of the orchard (Sanderson et al. 2007). Thus, it is crucial to monitor the sanitary status of the crop as well as the certification of planting material regarding

different pathogens (Lee 2015). Besides, with the threat of the global citrus industry facing Huanglongbing (HLB), a clear picture of the disease status of a country is extremely important to reinforce good management practices of the crop, especially in countries that still remain HLB-free (da Graça et al. 2016; Gabriel et al. 2020).

In Uruguay, citrus has become one of the main non-traditional export commodities after rice and dairy, generating foreign exchange earnings of about 80 million dollars per year. In the last five years, citrus production has been on average around 242 thousand tons annually, half of which is exported to fresh fruit markets and the remainder is consumed by the domestic market or sent for processing (DIEA 2019, 2020). Citrus producing areas covered around 14700 hectares with 7.2 million trees, 82% of which are productive (DIEA 2020). Produced citrus types include 40.5% oranges, 36% mandarins,

23.2% lemons and 0.3% grapefruits (DIEA 2020). There are two main production regions in the country. The northern region, that includes Artigas, Salto, Paysandú and Río Negro provinces, mainly produces mandarins and oranges which accounts for 88% of total citrus production of the country. Canelones, Colonia, San José and Montevideo provinces belong to the southern citrus region, which mainly produces lemons (DIEA 2019). There are approximately 400 citrus farms, however, 75% of the production originates from a few large sized farms (DIEA 2019).

*Citrus* spp. are vulnerable to infection by several economically important citrus-infecting viruses and viroids that affect tree health and impact production. Pathogens of citrus include CTV (*Closteroviridae*, *Closterovirus*), CPsV (*Aspiviridae*, *Ophiovirus*), SDV (*Secoviridae*, *Sadwavirus*), as well as viroids of the *Pospiviroidae* family: HSVd (*Hostuviroid*), CEVd (*Pospiviroid*), CBCVd (*Cocadviroid*), CBLVd (*Apscaviroid*) and CDVd (*Apscaviroid*) (Zhou et al. 2020). CTV is the causal agent of the most important viral disease of citrus namely tristeza disease and has been reported in all citrus producing areas worldwide (Moreno et al. 2008; EPPO 2019). The virus is responsible for three different syndromes affecting citrus including: tristeza or quick-decline, seedling yellows and stem pitting (Moreno et al. 2008; Roistacher et al. 2010). Disease expression depends on both CTV strains present, and the scion-rootstock combination (Harper et al. 2015). Currently, eight strains have been characterised (Harper 2013, Yokomi et al., 2018). CPsV is the causal agent of psorosis disease and symptoms include bark-scaling of the trunk and main branches (Garcia 2012). The disease was reported from many citrus growing areas of the world (Roistacher 1993). Satsuma dwarf disease, caused by SDV, was reported in Turkey, Iran, China, Japan, and North and South Korea (EPPO 2019). Trees affected by SDV become stunted, develop boat-, or spoon-shaped leaves, and fruit quality and yield are reduced (Iwanami 2010). Viroid-infected citrus, grafted onto sensitive rootstocks such as trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) or the citranges (*Citrus sinensis* (L.) Osb x *P. trifoliata*), show symptoms of bark cracking, scaling, stunting and yield loss depending on the viroid (Bani-Hashemian et al. 2009). HSVd, CEVd, CBLVd, and CDVd are distributed worldwide, whereas CBCVd has a seemingly limited distribution (Singh et al. 2003; Malfitano et al. 2005; Murcia et al. 2009; Cao et al. 2010; Cook et al. 2012).

The presence of some of these graft-transmissible pathogens was previously reported in Uruguay and CTV and its most efficient vector, *Toxoptera citricida* Kirkaldii, were shown to be endemic (Koch de Brotos and Boasso, 1955). More recent studies reported the molecular and biological characterization of CTV field isolates and described the circulation of VT, T3, T36, RB and HA16-5 CTV genotypes in the country either as single or mixed infections (Benítez-Galeano et al. 2015; 2018; Hernández-Rodríguez et al. 2017; Rubio et al. 2019).

CTV, CPsV and CEVd were reported in Salto province based on field symptoms (Tucci et al. 1965) and the presence of HSVd, CDVd, CEVd and CBLVd were reported for the first time 20 years ago (Pagliano et al. 1998). A recent analysis describes the presence and distribution of HSVd, CDVd, CEVd and CBLVd at rates of 92%, 50%, 23% and 21%, respectively (Pagliano et al. 2013). Contaminated graft and rootstock material leads to both quality and quantity decrease, as well as longevity, estimating losses of 30% of the national production (Bertalmío et al. 2012). The National Citrus Sanitary and Certification Program (PNSCC, by its acronym in Spanish) provides certified budwood and rootstocks to growers, free of pathogens such as CTV, CPsV, HSVd and CEVd. Since 2014, the use of certified material by the PNSCC has been mandatory (Bertalmío et al. 2012).

Despite the limited size of the citrus production-areas of Uruguay, the current diversity and distribution of virus and viroids affecting citrus is uncertain. In the present study the distribution and incidence of CTV, CPsV, SDV, CEVd, HSVd, CBLVd, CBCVd and CDVd, as well as the main CTV genotypes prevalent in the country, were investigated to contribute to an integrated pathogen management strategy development.

## Materials and Methods

### Sampling design

For sampling design the number of productive trees per geographic region and citrus type were taken into account based on an official citrus survey strategy of the Ministry of Livestock, Agriculture and Fisheries. We implemented a stratified sampling scheme with proportionate allocation being the strata defined to consider all possible combinations of citrus type and provinces. Thus, the sample size associated with each stratum was defined by  $n_i = n \cdot N_i / N$ , where  $n$  is the total sample size,  $N_i$  is the number of productive trees in the stratum  $i$  and  $N$  is the number of productive trees in the country. Concerning oranges and mandarins, productive trees are strongly grouped in Salto and Paysandú provinces. Productive lemon trees are highly grouped in San José province.

To be able to extrapolate results to the rest of the country, the number of collected samples was calculated based on a 95% confidence interval and an error tolerance up to 3%. Samples were collected from commercial groves over 26 months, from November 2014 to December 2016, with a simple random sampling method. A total of 1175 samples were collected across seven of the 19 provinces of Uruguay, including Artigas, Salto, Paysandú, Río Negro, San José, Montevideo and Canelones (Figure 1).

The geographic location (geo-referenced coordinates) of every sample was recorded. Sampling sites, as well as the number of samples collected in each province, were selected based on production volume, plant age and citrus variety. Four tree age categories were defined; group 1, trees up to 5 years old; group 2, 6 to 14 years; group 3, 15

to 20 years; and group 4, trees over 20 years old. Citrus types sampled included sweet oranges (*Citrus sinensis*), both Valencias and Navels, Mandarin hybrids (*Citrus reticulata* Blanco), Clementines (*Citrus clementina* Hort. Ex tan.) and lemons (*Citrus limon* (L.) Burm. f.). Only the

Afourer cultivar was sampled for the mandarin hybrid group. All samples were grafted onto *P. trifoliata* rootstocks. A summary of total collected samples per geographic region, citrus variety and plant age is shown in table 1.

**Table 1**

Number of samples per citrus type collected from November 2014 to December 2016 listed per geographic region and plant age. The total number of samples collected at each province is in brackets.

		Citrus varieties					
		Valencia	Navel	Clementine	Mandarin hybrid	Lemon	
Geographic region	North	Artigas (n=15)	5	0	8	0	2
		Salto (n=509)	219	133	55	82	20
		Paysandú (n=450)	117	120	104	97	12
		Río Negro (n=26)	7	5	8	6	0
	South	San José (n=61)	0	0	8	32	21
		Montevideo (n=32)	0	0	0	10	22
		Canelones (n=82)	0	0	0	7	75
Plant age*	Group 1 (n=194)	53	36	21	47	37	
	Group 2 (n=327)	82	67	37	128	13	
	Group 3 (n=251)	64	54	66	54	13	
	Group 4 (n=399)	148	100	59	4	88	

\*Four samples were not assigned to any age group.

#### Isolation of total RNA and RT-PCR

Leaf tissue was taken from 5 different sites of the canopy of each tree and pooled as one sample. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. As all the targeted pathogens have an RNA genome, first-strand cDNA synthesis was performed with random primers and the RevertAid first strand cDNA synthesis kit (Thermo Scientific Inc., Hanover, MD, USA) according to manufacturer's instructions (Supplementary materials for details). Detection of viruses and viroids was performed using previously published conventional and real-time PCR assays (Supplementary file for details). For detection of SDV, and viroids CEVd, HSVd, CBLVd, CDVd and CBCVd conventional PCR was used (Bernad and Durán-Vila 2006; Iwanami 2010). A real time PCR assay with SensiFAST™ SYBR® No-ROX kit (Meridian Bioscience, USA) was used for CPsV detection (De Francesco et al. 2015). For CTV detection, a real-time PCR with a hydrolysis probe as described by Bertolini et al. (2008) was used. For CTV characterization, a conventional PCR targeting p25 gene was used (Iglesias et al. 2008).

#### CTV characterization

For CTV genotypes determination circulating in the country, a total of 451 CTV positive samples were bi-directionally Sanger sequenced (Macrogen, Seoul, South Korea). CTV sequences of 555 nucleotides in length encompassing the p25 gene were aligned with ClustalW in MEGA 6.0 (Tamura et al. 2011). The model of nucleotide substitution that best fit the dataset (HKY) was selected using the jModelTest program according to the Akaike Information Criterion (AIC) (Akaike 1974; Posada 2008). Maximum Likelihood (ML) phylogenetic trees were reconstructed with PhyML program using an online web server (Guindon and Gascuel 2003; Guindon et al. 2010). The heuristic tree search was performed using the SPR branch-swapping algorithm and branch support was calculated with the approximate likelihood-ratio (aLRT) SH-like test (Anisimova and Gascuel 2006). Complete genome sequences were retrieved from GenBank for reference sequences of eight CTV genotypes (T36: AY340974, U16304; T30: AF260651, Y18420; T3: KC525952; VT: EU857538, EU937519, U56902; T68: EU076703, JQ965169; RB: FJ525431, FJ525434; HA16-5: GQ454870; S1: KU589212, KU589213). Uruguayan sequences generated

were deposited in Genbank database with the following accession numbers: KX257284-KX257350, MN721464 - MN721811.

### Statistical analysis

In order to compare infected trees rate according to pathogen, geographic region, citrus type or plant age, a multiple hypothesis testing was performed considering Holm-Bonferroni method (Holm, 1979). Since Bonferroni's bound for family-wise error is quite conservative, p-values up to  $4e-3$  were considered to declare significance. R software version 3.3.3 (<https://www.r-project.org>) and the proportion test (`prop.test()`) which approximates the binomial distribution by the normal for large samples, were used for statistical calculations.

### Results

In the present study 1175 samples collected in the main Uruguayan citrus growing areas were analysed for the presence of eight graft-transmissible citrus pathogens. CTV was the most frequently detected virus. It was detected in 1096 samples corresponding to 93.3% prevalence. CPsV was identified in 529 samples, with a detection rate of 45%. HSVd and CDVd were less prevalent, with detection rates of 3.6% and 15.6% respectively. SDV, CBLVd and CBCVd were not detected, whereas CEVd was detected only in one sample. No pathogens were detected in 6% of all collected samples and less pathogen-free plants were detected, the older the trees were.

#### Pathogen prevalence by geographic region

The two main citrus growing regions of Uruguay differ in citrus types planted, soil composition and climatic conditions. Due to these regional differences samples were representatively collected as per the sampling strategy and 1000 samples were collected from the north and 175 from the south. The prevalence of CTV, CPsV and CDVd differed between the northern and southern regions. The prevalence of CTV was significantly higher in the northern region at 94.4% (p-value:  $4.4e-4$ ) compared to 89.6% in the samples collected from the southern region. The presence of CDVd was also higher in the north, at 17.3% compared to 5.7% in the south (p-value:  $1.5e-4$ ). Conversely, the prevalence of CPsV was significantly higher in the southern region, with a detection rate of 60% compared to a prevalence of 42.4% (p-value:  $2.3e-5$ ) in the north. HSVd was detected at similar rates in the two regions, with no significant differences. In the northern region 3% of the samples were positive for HSVd, whereas in the south a prevalence of 6.9% was determined. Figure 1 is a graphic representation of the detection of each pathogen per region.

CTV and CDVd were significantly less prevalent in San José province compared to the other six provinces (p-values:  $4.8e-8$  and  $4.0e-3$ , respectively). CTV was

detected in 75% of samples in San José and in other provinces detection ranged between 91% and 100% (Table 2). CDVd

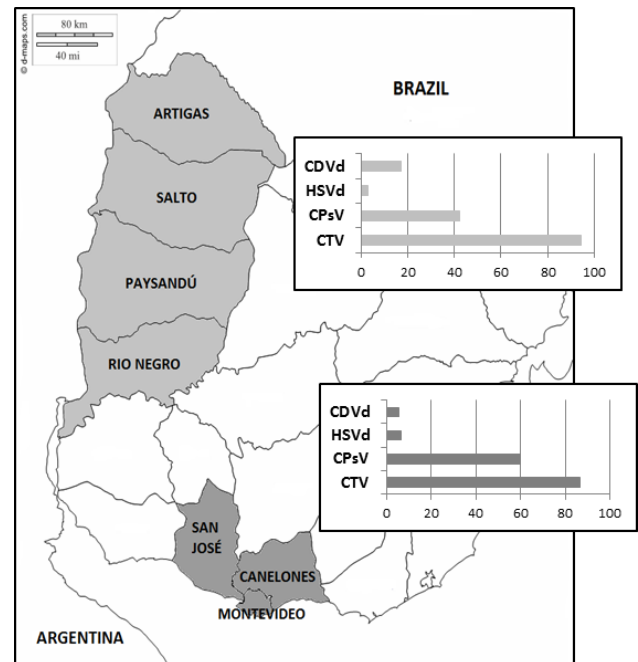


Fig. 1. Percentage of positive samples for the analysed pathogens in the northern (light grey) and southern (dark grey) regions of the country.

was detected in 2% of samples collected from San José whilst in the rest of the country detection ranged between 6% and 24% (Table 2). The detection of CPsV and HSVd was lower in San José compared to the other provinces but this difference was not statistically significant.

#### Pathogen prevalence per citrus type

Differences in pathogen prevalence between citrus types were analysed and results are presented in Table 2. CTV was significantly more prevalent (97%) in sweet oranges compared to mandarins (p-value:  $9.3e-11$ ) and lemons (p-value:  $1.7e-3$ ). Despite relatively high detection rates of CTV in mandarins and lemons (over 85%), no statistically significant differences were found between these citrus types. Conversely, CPsV was more frequently detected in lemons compared to sweet oranges with detection in 74% of samples (p-value:  $1.1e-8$ ). The detection of CPsV in sweet oranges was higher than in mandarins (p-value:  $3.2e-7$ ). Between the mandarin types, Clementines and Mandarin hybrids (Afourer), the detection of CPsV was significantly lower in the latter at 23% (p-value:  $8.5e-5$ ). HSVd was not detected in Afourer and only in a single Navel orange sample. The detection rates for HSVd in the various citrus types ranged from 0% to 11%. CDVd was more prevalent than HSVd in all the citrus types ranging between 11% and 21%. Nevertheless, no significant differences were found for CDVd between citrus types.

Overall, pathogen detection was lowest in Afourer (mandarin hybrid) at 85% for CTV, 23% for CPsV, 11% for CDVd and no detection of HSVd.

**Table 2**

Numbers and percentages of positive samples for CTV, CPsV, HSVd and CDVd per geographic region, citrus type and plant age. Red squares highlight statistically significant results.

		Collected samples	CTV		CPsV		HSVd		CDVd	
			Total	%	Total	%	Total	%	Total	%
Geographic region	Artigas	15	15	100	8	53	0	0	0	0
	Salto	509	478	94	211	41	12	2	61	12
	Paysandú	450	426	95	185	41	13	3	107	24
	Rio Negro	26	25	96	20	77	5	19	5	19
	San José	61	46	75	23	38	1	2	1	2
	Montevideo	32	31	97	24	75	4	13	4	13
	Canelones	82	75	91	58	71	7	9	5	6
Citrus varieties	Valencia	348	343	99	177	51	7	2	51	15
	Navel	258	249	97	110	43	1	0	54	21
	Clementine	183	165	90	76	42	17	9	37	20
	Afourer	234	199	85	54	23	0	0	25	11
	Lemon	152	140	92	112	74	17	11	16	11
Plant age (years)	Group 1 (up to 5)	194	168	87	92	47	4	2	28	14
	Group 2 (6 to 14)	327	303	93	88	27	3	1	52	16
	Group 3 (15 to 20)	251	239	95	92	37	6	2	41	16
	Group 4 (over 20)	399	385	96	257	64	29	7	62	16

#### *Pathogen prevalence and tree age*

The influence of tree age on pathogen prevalence was investigated. No significant differences were found among age groups 1 to 3 for any of the pathogens. However, trees older than 20 years had higher rates of CTV (p-value: 4.0e-3), CPsV (p-value: 2.2e-16) and HSVd (p-value: 2.5e-6).

#### *Prevalence of pathogen co-infections*

Co-infection of pathogens was frequent. Fifty-one percent of the samples were co-infected with at least two pathogens (597 co-infected trees). Forty percent of samples were infected with two pathogens (463 samples) and the most common pathogen combination was CTV and CPsV (84%). Ten percent of samples were co-infected with three pathogens (120 samples) and the most frequent trio was CTV, CPsV and CDVd (80%). Fourteen samples were co-infected with the four pathogens (CTV, CPsV, CDVd and HSVd). The only sample infected with CEVd, a lemon tree from Salto

province, additionally contained CTV, CPsV and HSVd. CTV was present in all co-infected plants. No co-infections of CDVd and HSVd or in combination with CPsV were detected.

More co-infected trees were detected in the northern region compared to the southern region (p-value: 1.7e-3). More pathogen combinations were found in the northern region, e.g. Clementines from Paysandú province had seven different pathogens combinations and lemons from Salto province were infected with six different pathogens combinations (data not shown). Pathogen combinations detected in the south were less complex with only four pathogen combinations (data not shown).

Regarding citrus types, lemons had the highest frequency of co-infections (p-value: 5.5e-5) and mandarins had significantly less co-infections than sweet oranges (p-value: 1.8e-9).

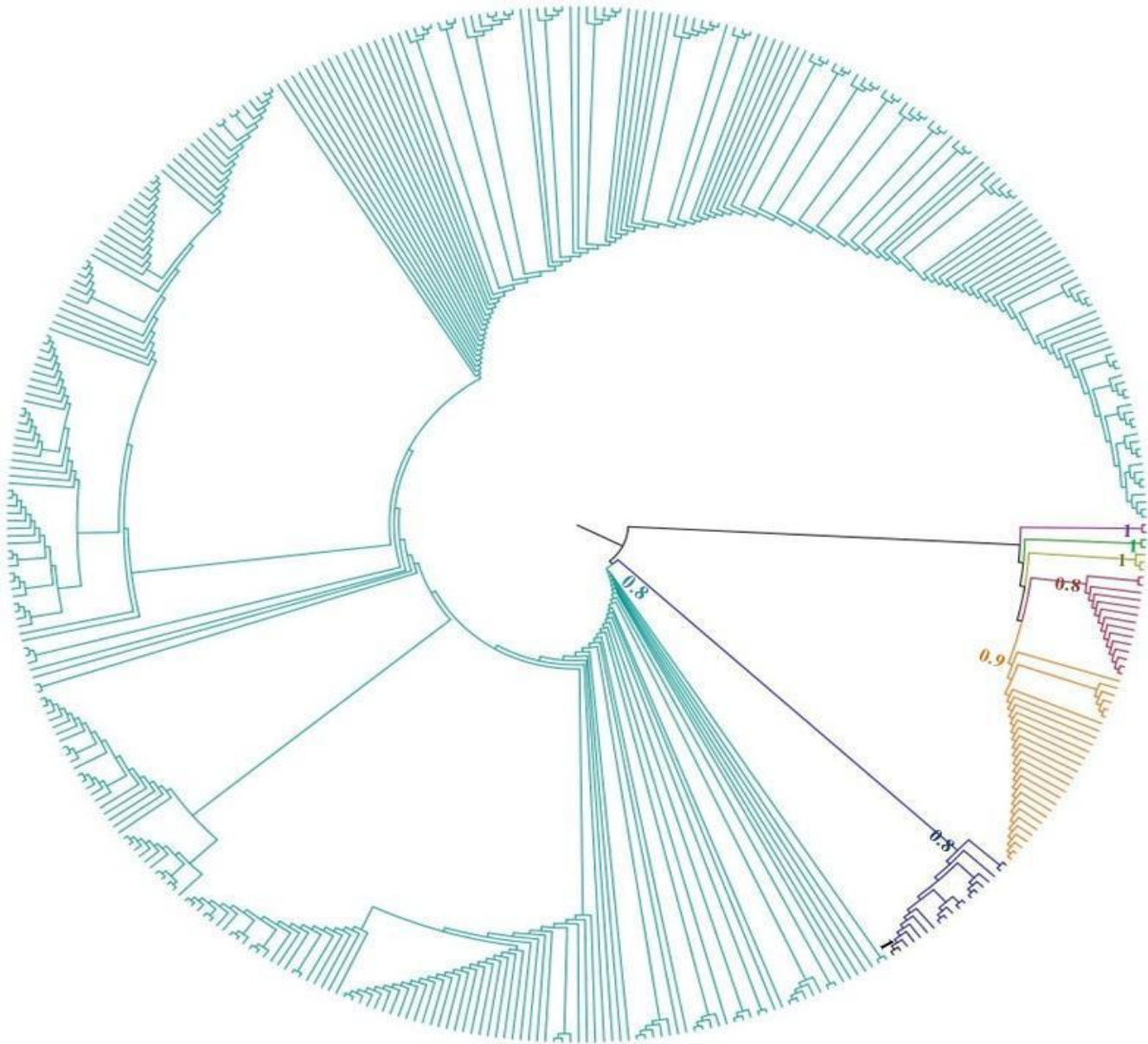
### CTV genotype prevalence

A maximum likelihood phylogenetic tree was constructed with 416 nucleotide sequences of the p25 gene region to determine the prevalence of CTV genotypes. Five genotypes were identified and 87% of the samples grouped into the HA16-5-genotype with an aLRT support of 0.8 (Figure 2).

Twenty-six samples (6.3%) clustered with T68 of which 19 were Afourer samples. Genotypes RB and VT were detected in 3.6% and 2.6% of the analysed samples, respectively. The RB genotype was detected in all the citrus types, but VT was only detected in lemons. Two sequences clustered with T3 genotype. The S1, T30 and T36 genotypes were not detected in any of the samples.

No statistically significant differences were found between the RB, T3 and VT genotypes in plants of different age groups.

However, genotype T68 was significantly more prevalent in plants of age group 2 (p-value:  $7.8e-12$ ), whereas genotype HA16-5 was significantly more prevalent in plants older than 20 years old (p-value: 0.0001). The HA16-5 genotype was more prevalent in sweet oranges compared to other citrus types (p-value:  $1.1e-9$ ), but was significantly less in the Afourer cultivar (p-value:  $2.2e-16$ ).



**Fig. 2.** Cladogram of CTV-p25-gene sequences. Coloured branches represent the seven described genotypes of the virus (T30, green; T36, violet; T3, yellow; VT, red; T68, orange; RB, blue; S1, black; HA16-5, turquoise). Principal nodes' aLRT support values are given.

## Discussion

The aim of this study was to determine the sanitary status of citrus orchards in Uruguay for specific graft-transmissible viruses and viroids. The occurrence and distribution of eight viral and viroid pathogens, as well as the main CTV genotypes prevalent in Uruguayan citrus were determined. Despite regional surveys reporting the presence of CTV, CPsV and citrus viroids including HSVd, CEVd, CDVd and CBLVd, a national survey was not previously conducted (Tucci et al. 1965; Pagliano et al. 1998; 2013). SDV, CBLVd and CBCVd were not detected and CEVd was not widely detected in this study whilst CTV, CPsV, HSVd and CDVd were found widespread in Uruguay.

CTV was the most prevalent virus detected and was present in all the regions sampled and in all citrus types. CTV was however more prevalent in the north of the country in sweet oranges, which is the main citrus type produced in the region. Also, CTV was more prevalent in trees older than 20 years, probably due to the longer exposure to the CTV vector, *T. citricida*. Previous studies reported the presence of CTV in Uruguay, and various CTV genotypes, including the RB genotype, which was found on trifoliolate rootstocks previously thought to be CTV resistant (Garnsey et al. 1987; Benítez-Galeano et al. 2015, 2018; Hernández-Rodríguez et al. 2017, 2019; Rubio et al. 2019). Results also confirmed the previously reported, widespread distribution of the HA16-5 genotype in all the citrus growing regions of the country (Benítez-Galeano et al. 2015, 2017; Rubio et al. 2019).

This multi-causal scenario is a product of biological characteristics of the virus and management strategies. The endemic distribution of CTV in Uruguay can be attributed to the presence of the most efficient vector of CTV and the existence of older orchards which acquired CTV over time. The PNSCC stated in 2014 the compulsory use of certified budwood and rootstocks, but the benefits of the sanitary scheme were not observed in older orchards established prior to the scheme. These results support the long-term goals of the National Research Program for Citrus Production for implementing cross protection for CTV management, previously demonstrated to minimize damage by CTV severe strains in Brazil and South Africa (Roistacher et al. 2010). However, the implementation of cross-protection could be challenging due to the diversity and population complexity of CTV strains found in the country (Benítez-Galeano et al. 2015, 2017; Hernández-Rodríguez et al., 2019; Rubio et al. 2019). As cross-protection is genotype specific, it would be reasonable to implement cross-protection for HA16-5 genotype due to its widespread distribution, as well as for RB genotype since 90% of Uruguayan citrus trees are grafted onto trifoliolate rootstocks (Folimonova 2013; Bergua et al. 2016).

After the destruction of citrus groves by tristeza in Uruguay in the 1940s, an increased incidence and disease

severity of psorosis was reported (Tucci et al. 1965). This spread of CPsV could be explained by an indiscriminate use of diseased budwood (Tucci et al. 1965). Natural spread of the virus has also been suggested in Uruguay, Argentina and in Texas, USA, but no vector has been found yet (Campiglia et al. 1976; Pujol and Beñatena 1965; Timmer and Garnsey 1980; Garcia et al. 2017; Hernandez-Rodriguez et al. 2020; Zhou et al. 2020). CPsV was the second most prevalent pathogen detected in this study and was also present in all regions sampled. This is the first quantitative survey for CPsV which detected the virus in 45% of the samples. This result, in combination with the high CTV and CPsV co-infection rate, could suggest the presence of a common vector for both viruses. Despite Hernández-Rodríguez et al. (2020) could not find evidence of CPsV transmission by *T. citricida*, further experiments with other aphid species are necessary to test this hypothesis.

Although the limited distribution of SDV around the world, SDV infects nearly all of the citrus and citrus relatives and the most sensitive citrus type is mandarin (Iwanami 2010). The introduction of infected material to Uruguay is possible due to commercial relationships with countries that have reported the presence of the virus. SDV was included in the present study since spoon-shaped leaves were observed in Uruguayan citrus groves and 36% of the citrus planted are mandarins. The virus was not detected, suggesting that the observed symptoms could have been due to physiological effects, such as thermal shock.

Pagliano et al. (2013) reported the occurrence and distribution of HSVd, CDVd, CEVd and CBLVd with detection rates of 92%, 50%, 23% and 21%, respectively. Results of this study however, showed lower incidences of HSVd, CDVd and CEVd and no detection of CBLVd. Viroids are transmitted by infecting budwood and contaminated tools. Appropriate management strategies such as removal of symptomatic plants and tool disinfection, as well as the use of certified plant material over time could explain the low detection of these pathogens.

A focus of the National Research Program for Citrus Production to gain greater access to international citrus markets by varietal replacement has led to the planting of new orchards such as was done for Afourer since 2007. The use of imported certified plant material at that time could explain the lower prevalence of CTV, CPsV and CDVd and the lack of detection of HSVd in this cultivar. Younger orchards also have a shorter exposure time for CTV infection to have occurred.

The widespread distribution of CTV and its vector *T. citricida*, changes on rootstocks usage over time, the lack of knowledge about the sanitary condition of plants used as bud sources, and the absence of a plant material sanitation and certification program set the perfect scenario for dissemination of citrus virus and viroids. Therefore, co-infections of viruses and viroids were found more frequently in older orchards. However, the implementation of the PNSCC has seemingly led to a

decrease in the incidence of citrus viruses and viroids. The prevalence of HSVd, CEVd and CDVd was shown to be lower than previously reported and CBLVd was not detected again. Additionally, this study is informative for growers and they should be encouraged to test for graft-transmissible pathogens prior to top-working older orchards to new varieties.

Besides, the information gathered here about CTV genotypes circulating in the country is extremely important to support the national efforts to develop cross-protection to manage the virus.

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