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Rev. FCA UNCUYO. 2017. 49(1): 143-156. ISSN impreso 0370-4661. ISSN (en línea) 1853-8665.

Distribution and frequency of *tomato ringspot virus* (ToRSV) in different varieties of *Rubus idaeus* in the Maule Region, Chile

Distribución y frecuencia de *tomato ringspot virus* (ToRSV) en diferentes variedades de *Rubus idaeus* en la Región del Maule, Chile

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Originales: *Recepción:* 07/09/2016 - *Aceptación:* 15/03/2017

Abstract

The raspberry (*Rubus idaeus*) is one of the most important fruit for production in the Maule Region, Chile. Raspberries are affected by the *tomato ringspot virus* (ToRSV), which causes decreased yield and deformed fruit. The objective of this work is to study ToRSV spread in different raspberry varieties in the Maule Region, Chile. The virus was detected using the ELISA test and RT-PCR in the Heritage, Meeker, Chilliwack, Amity and Coho varieties. Bayesian analysis determined the relationship between the percentage of ToRSV incidence in the cultivated varieties and the locations in the different provinces of the Maule Region. It was observed that the Linares province showed the highest levels of the virus in the different varieties: Amity (70%), Meeker (39%) and Heritage (26%), compared to other provinces in the region. These results suggest a high spread of ToRSV through the Maule Region. Nei distance analysis suggests that 14 of the virus isolates coming from the Talca and Linares Provinces would show differences with the ToRSV accessions deposited in the global gene bank (NCBI).

Keywords
ToRSV • *Rubus idaeus* • disease • spread • incidence • Maule • Chile

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Resumen

La frambuesa (*Rubus idaeus*) es un frutal menor de gran importancia productiva en Chile. Una de las enfermedades que lo afectan es *tomato ringspot virus* (ToRSV). Este estudio provee información sobre la dispersión de ToRSV en diferentes variedades de frambueso en la Región del Maule. El virus fue detectado mediante el uso de la prueba ELISA y RT-PCR en las variedades Heritage, Meeker, Chilliwack, Amity y Coho. Mediante un análisis Bayesiano se determinó la relación entre porcentaje de incidencia de ToRSV en las variedades cultivadas y las localidades, de las distintas Provincias de la Región. Se observó que la provincia de Linares presentó la más alta incidencia viral en Amity (70%), Meeker (39%) y Heritage (26%), con respecto a otras provincias de la Región. Estos resultados sugieren que existe una alta dispersión de ToRSV a través de la Región del Maule. El análisis de distancia de Nei propone que los 14 aislados del virus provenientes desde las Provincias de Talca y Linares, mostrarían diferencias con las accesiones de ToRSV depositadas en el banco de genes mundial (NCBI).

Palabras claves
ToRSV • *Rubus idaeus* • dispersión • incidencia • Maule • Chile

Introduction

In the 1980's, cultivation of raspberry (*Rubus idaeus*) began in Chile, with a distribution ranging from the the Valparaiso Region to the Los Lagos Region (7). At present, the crops of this species occupy an area of 10,000 hectares, ranking second nationally among berries being grown in Chile (43%), and positioning the raspberry as the fruit with the most progress, represented predominantly by varieties such as: Heritage (81%), Meeker (15%), and Chilliwack (3%) (11).

Located in the central zone of the Chile, the Maule Region is characterized as an excellent agricultural area, ranking first nationally in the production of apples, kiwis, tomatoes, beets, rice, hazelnut, and smaller fruits such as raspberries, representing 81% of the total area, with 57% of the Maule Region (9, 22). Despite the productivity of this crop, the production has been affected due to the plantations being established with etiolated shoots, which facilitate the spread of pathogens and have lowered raspberry yield to 4 t/ha in Chile. These results are very low in comparison to other countries such as Mexico, the United States and England-Scotland, where the yields rise up to 15 t/ha which may suggest that there are viral pathogenic factors, which may be affecting the production of this fruit (11, 18).

Certain pathogens, such as viruses, viroids, and phytoplasmas, can spread easily through these types of plants, resulting in a high degree of infection in raspberry plants. These infections represent one of the main problems causing decreased production, affecting farmers economically and, consequently, the quality of the harvested fruit (18).

Globally, it has been reported that more than 30 viruses and probable viral agents are infecting the *Rubus* species, including the major *Rubus idaeus* varieties (15). One of the leading viral diseases that affect raspberry plants is the *tomato ringspot virus* (ToRSV), a member of the genus *Nepovirus*, and *Secoviridae* family (24). Characteristic symptoms of the disease are marked rings and chlorotic designs. Whitening of veins that usually occurs during the spring and tends to disappear during the summer is common in plants that often don't show symptoms, but have low vigor or performance. As well as this the presence of deformed fruit is also observed (12).

Medina *et al.* (2006), more than a decade ago, samples were collected from the Maule Region that indicated that the virus is present in orchards from the Linares Province. The high incidence detected, is probably associated to a certain extent with the *Xiphinema* species (29).

The territory of Chile is divided into three different administrative levels: regions, provinces or communes corresponding to the first-, second- and third-level administrative divisions. Currently there is little information about the dispersion across regions, provinces, communes and cities in the Maule Region as well as the frequency of ToRSV occurrences in the different varieties that are currently grown. It is for this reason that the main aim of this research is to study the distribution and frequency of the virus, in the different varieties of *Rubus idaeus* that are currently cultivated in the Maule Region, and at the same time, observe the genetic distance between the local ToRSV nucleotide sequences, var. Heritage, with those already annexed in the global gene bank.

Materials and methods

Sampling

The collection of plant material was carried out in three provinces of the Maule of Región: Curicó, Talca, and Linares in zones that are recognized as *Rubus idaeus* production zones. Collection took place during the 2011-2014 seasons, at different commercial orchards ranging from the La Montaña sector in the north of the study zone, (34°58'34.905 N, 70°52'13.13" W) to the Andean zone of Parral in the south of the study zone (36°09'50.98 S, 71°44'05.16" W).

The sampling process was completely random, during the months of October and December of each season. The samples were collected from plants that had actively growing sprouts, with ages fluctuating between 3 months to 15 years old. Approximately 37 ha were visited across the region, with Heritage, Meeker, Chilliwack, Amity, and Coho being the most common regional varieties.

The number of samples collected was 30 (4 replicates) per hectare. Sample selection was focused on tissue with characteristic symptoms of infection, but mostly (98%) asymptomatic plant material with viral presence was also collected. Once collected, the samples were put on ice and transported in plastic bags, which were subsequently stored at -80°C until processing was carried out.

Viral Identification

ELISA Testing

A double-antibody sandwich ELISA (DAS-ELISA) was used to detect tomato ringspot virus (ToRSV), using the manufacturer's recommended protocol (Agdia, USA). Samples were measured with the RT-2100C Rayto Microplate reader from Germany. A reading was considered positive when the OD_{405} optical density values were greater than twice the value obtained in the negative control. To determine levels of incidence of ToRSV infection at a location, the total number of positive samples with respect to the total number of samples collected was calculated.

RT-PCR for determination of ToRSV

Extraction of RNA from Rubus idaeus

Total nucleic acids were extracted from 200 mg of leaves of *Rubus idaeus*, which were placed in 2.0 ml Labcon Petaluma, California (USA) microtubes and supplemented with a Bertin Technologies (France) 1.4 mm Zirconium bulk bead. The extraction was performed based on the protocol suggested by Chang *et al.* (1993). For grinding, Precellys 24, Bertin Technologies (France) equipment was used at 5500 rpm, with three cycles of 30 seconds and a Cryollys Bertin (France) cooling system. The visualization of RNA was performed over 1% (w/v) agarose gels in TAE 1X buffer, with each well containing 2 μl of the samples. Loading buffer supplemented with Biotium (USA) GelRed™ was then added to the wells. Each RNA sample was treated with DNase digestion enzyme (Invitrogen) according to manufacturer's recommendations.

RT-PCR for determination of ToRSV

The first strand of cDNA thermoscrip was synthesized by SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen), carried out according to the manufacturer's recommendations. Testing of primers was performed with 18S internal control (6). Subsequently, ToRSV detection was performed using U1 and D1 primers, as described by Griesbach (1995). PCR reactions were carried out in the Life Express (China) thermal cycler. The reaction products were determined by agarose gel electrophoresis (2%, w/v) using a 1X TAE buffer supplemented with Biotium (USA) GelRed ™ in order to view them under ultraviolet light.

Statistical Analysis

In the collated data, the presence or absence of the virus was indicated by incidence percentaje (%). Kruskal Wallis Bayesian analysis was performed using the 2014 InfoStat biometric statistical software from the National University of Cordoba (FCA-UNC), Argentina (4). The analysis determined relationships between the independent variables of province, commune, and town with respect to the incidence of ToRSV in different raspberry varieties (Coho, Meeker, Heritage, Chilliwack, and Amity) in the Maule Region of Chile. The level of significance was determined to be ($p \leq 0.05$).

Sequencing and genetic distance

DNA amplicons obtained by RT-PCR were cloned using a StrataClone PCR Cloning kit in accordance with the manufacturer's instructions, and sequenced bidirectionally by Macrogen Company Inc. (Seoul, Korea). The nucleotide sequences obtained were analyzed with the program Geneious R6 (Biomatters LTd., New Zealand) along with the BLAST 2,225 Basic Local Alignment Search Tool from the National Center for Biotechnology Information (NCBI) (USA) in order to determine the sequence homology, when compared with the NCBI. Cluster analysis was carried out using the Tamura Nei genetic distance and the Dendrogram was built with Neighbor-Joining method, using a sequence outgroup for comparison. For both previously mentioned analyses, the same program was used.

Results

Disease symtoms

Of all the collected varieties in the field, only a small percentage (1.25%) of the sampled plants presented viral symptoms. In plants between 9-15 years of age, an increase of fused interveinal yellow regions (figure 1A), and non-commercial deformed fruits (figure 1B) was observed. In the orchards, the Heritage variety plant with an age of between 12 and 15 years old presented more intense symptoms, with less foliage and decreased plant height (Figure 1B). In the case of asymptomatic plants, which constituted the largest percentage of the sample (98.75%), there were no observed differences between them and the plants that tested and were found to be ToRSV free (figure 1C).

ELISA and RT-PCR for viral identification

For all the *Rubus idaeus* samples analyzed by ELISA for the detection of ToRSV in Heritage, Meeker, Amity, Chilliwack, and Coho varieties, it was observed that the values of optical density (DO_{405mn}) in the positive and negative controls were 2.9 and 0.5 respectively, while the plant material with positive response to infection presented a DO_{405nm} 2 ± 0.7, after 1 hour of the hydrolyzed substrate. Among the collections, most of the raspberry plants that presented as asymptomatic with a positive test for ToRSV had the same DO_{405mn} as the positive control. This was the case for all plants in the age range between 3 months to 15 years old. The presence of ToRSV was confirmed by RT-PCR in all varieties that were tested by ELISA. The resulting amplicons showed a DNA fragment of 449 bp, with a fragment of 860 bp corresponding to the internal control 18S rRNA (6, 8).

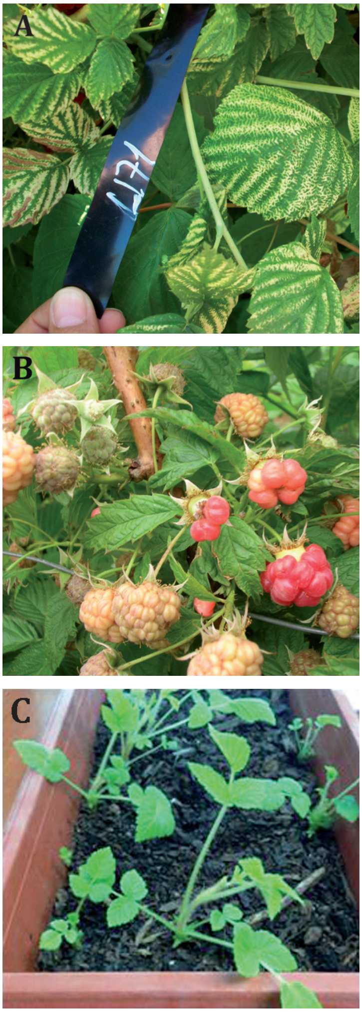


Figure 1. Symptoms ToRSV in raspberry leaves and fruits. Yellowing presence of primary and secondary veins in 3 and 12 year old plants of the Meeker (1A) and Heritage (1B) varieties. 1C, regrowth asyntomatic plant of 5 year old, variety Meeker.

Figura 1. Síntomas de ToRSV en hojas y frutos de frambueso. Presencia de amarillamiento en venas secundarias y terciarias en plantas de 3 y 12 años de edad, variedades Meeker (1A) yd Heritage (1B). 1C, rebrotes asintomático en plantas de 5 años, variedad Meeker.

ToRSV incidence analysis in *Rubus idaeus,* Maule Region

ToRSV incidence by town and commune

The incidence of ToRSV studied throughout the Maule Region showed significant differences (p ≤ 0.001) between the towns dedicated to raspberry cultivation in the various provinces of the region.

In those located in the north of the region, the significant differences are reflected in figure 2A (page 149), noting that 59% of viral infection corresponds to the Amity plant variety located in the town of La Montaña, Teno commune, Curico Province. In the same town the occurrence of the virus is the lowest with a 2% in the Heritage variety.

The towns of Buena Paz and Pichingal (Molina commune) located further south in the Curico Province, showed that Buena Paz had an incidence of 33% for both Coho and Heritage varieties, while in Pichingal the Heritage variety reached 11%.

On the other hand, the villages of the San Clemente and Rio Claro communes (Talca Province) demonstrate significant differences when being compared (p≤0.005). These differences occur in the towns of La Calor and Corralones (San Clemente commune), where viral incidence reached 31% and 20% respectively in the Heritage variety. Chilliwack presented 27% viral presence in Corralones, but the lowest percentage was in the town of Cumpeo (Rio Claro commune), with 9% incidence in the Heritage variety (figure 2B, page 149).

The towns of Bramadero (San Clemente commune) and Santa Rosa (Rio Claro commune) did not show viral infection in the Heritage variety (figure 2B, page 149). A similar pattern is observed when analyzing the towns belonging to the Linares commune (Linares Province), where significant differences (p≤0.001) were established in the town of St. Victor Álamo. Here, the Heritage variety had the highest viral incidence at 80%, while Meeker showed an incidence of 52%, followed by Coho with 7% (figure 2C, page 150).

In the town of San Antonio Encina the incidence reached 70% in the Amity variety, unlike the Meeker and Heritage varieties that only reached 27% and 10%, respectively (figure 2C, page 150). Within the same province, the town of Santa María de Arquén, (Yerbas Buenas commune), and the town of Los Carros (Parral commune) reached only 7% and 8%, respectively, in the Heritage variety. The town of Santa Elena showed no viral incidence in the Heritage variety (figure 2C, page 150).

Analysis of viral incidence (%) in the Maule Region

The *Rubus idaeus*-ToRSV analysis carried out for the data from various provinces of the Maule Region presented significant differences (p ≤ 0.001) between the varieties studied. This significance was set according to what was found in the Linares Province, where the Amity variety presented a remarkable viral incidence of 70%, while in Curicó, only 53% of the sampled plants presented the virus (figure 3, page 150). Within the Talca Province, no plantations or remnants of this variety were found.

The Heritage variety present in the Linares, Talca, and Curicó provinces show a 26%, 11% and 9% incidence, respectively. The Chilliwack variety is only present in the Talca Province, and shows an incidence of 27%.

The Coho variety has a lower incidence in the province of Linares (7%), contrasted with the Curicó Province, where viral incidence was higher (33%).

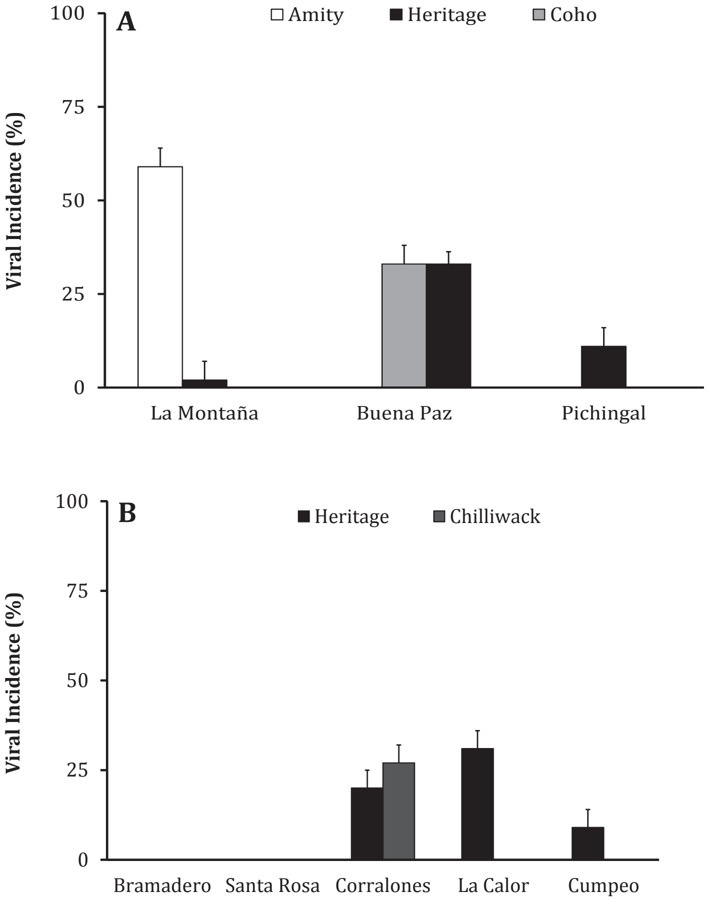


Figure 2. Incidence Percentage (%) of ToRSV in different varieties of *Rubus idaeus* in towns in the Maule Region of Chile. **A:** Tows La Montaña, Buena Paz and Pichingal, Teno and Molina communes, Curicó Province **B:** Towns Santa Rosa, Cumpeo, Bramadero, Corralones and La Calor, Río Claro and San Clemente communes, Talca Province. **C:** San Antonio Encina, San Victor Álamo, Santa María de Arquén, Santa Elena, Los Carros, Yerbas Buenas, Parral and Linares communes, Linares Province.

Figura 2. Porcentaje de incidencia (%) de ToRSV en diferentes variedades de *Rubus idaeus*, Región del Maule, Chile. A: localidades de Comunas La Montaña, Buena Paz y Pichingal de Teno y Molina, Provincia de Curicó. B: localidades de Santa Rosa, Cumpeo, Bramadero, Corralones y La Calor, Comuna de Río Claro y San Clemente, Provincia de Talca. **C:** localidades de San Antonio Encina, San Victor Álamo, Santa María de Arquén, Santa Elena, Los Carros, Yerbas Buenas y Parral, Comuna de Linares, Provincia de Linares.

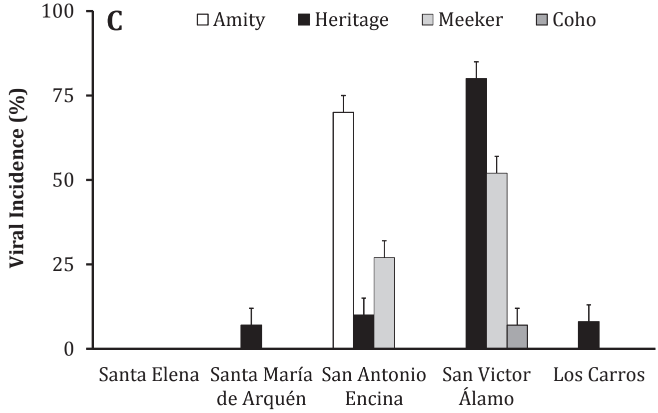


Figure 2 (cont.). Incidence Percentage (%) of ToRSV in different varieties of *Rubus idaeus* in towns in the Maule Region of Chile. **A:** Tows La Montaña, Buena Paz and Pichingal, Teno and Molina communes, Curicó Province **B:** Towns Santa Rosa, Cumpeo, Bramadero, Corralones and La Calor, Río Claro and San Clemente communes, Talca Province. **C:** San Antonio Encina, San Victor Álamo, Santa María de Arquén, Santa Elena, Los Carros, Yerbas Buenas, Parral and Linares communes, Linares Province.

Figura 2 (cont.). Porcentaje de incidencia (%) de ToRSV en diferentes variedades de *Rubus idaeus*, Región del Maule, Chile. A: localidades de Comunas La Montaña, Buena Paz y Pichingal de Teno y Molina, Provincia de Curicó. B: localidades de Santa Rosa, Cumpeo, Bramadero, Corralones y La Calor, Comuna de Río Claro y San Clemente, Provincia de Talca. **C:** localidades de San Antonio Encina, San Victor Álamo, Santa María de Arquén, Santa Elena, Los Carros, Yerbas Buenas y Parral, Comuna de Linares, Provincia de Linares.

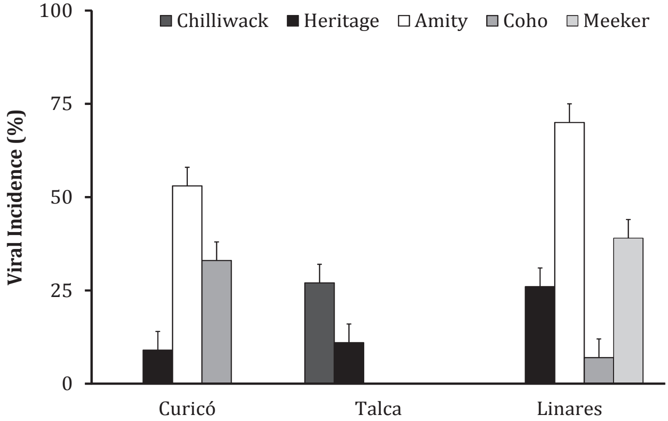


Figure 3. Incidence of ToRSV in different varieties of *Rubus idaeus* in the Province of Curico, Talca and Linares, in the Maule Región, Chile.

Figure 3. Incidencia de ToRSV en distintas variedades de *Rubus idaeus* en la Provincia de Curicó, Talca y Linares de la Región del Maule, Chile.

The orchards with the Meeker variety were found only in the Linares Province, and reached 39% viral incidence (figure 3, page 150).

Analysis of genetic distance

The ToRSV isolates in this study had 82-94% sequence identity, compared with other accessions of NCBI.

The genetic distance analysis based on a matrix of Nei's compared 14 accessions of ToRSV of *Rubus idaeus,* Heritage variety in the Maule Region (figure 4, page 152). KP759300.1, KX507374 and KX507375 belonging to the locality of La Calor, San Clemente; KP759299.1, KX529874, KX529883, KX529881, KX529862, KX529864, KX529861, KX529863, KX529875, KX529867 and KX529865 found in the locality of San Victor Alamo, in the commune of Linares with 7 accessions KM083894.1, DQ641947.1 and GQ141526.1 (*tomato ringspot virus* isolates, raspberries host), GQ141528.1 and GQ141527.1 (*tomato ringspot virus* isolate, blueberry host), KM083892.1 and AF135407.1 (*tomato ringspot virus* isolate, grapewine host).

Two differentiated clusters were observed, the first (A) closer to the majority of the NCBI sequences and the accessions KP759300.1 and KP759299.1 (this study). It is also observed that in A, there is a 31.7% of distance between the accessions KM083892.1 and AF135407.1 regarding the rest of the cluster, while KP50300.1 takes distance in a 21.1% from the accessions DQ641947.1, GQ141528.1, KM083894.1 and KP759299. These last two accessions have a 14% of distance among them, with strawberry plants being the host of both of them.

The second cluster (B), gathers 12 ToRSV sequences coming from the localities of La calor and San Victor Alamo. Within the cluster, the accessions KX529862, KX529864, KX529861, KX529863, KX529875, KX529867, KX529865, KX507374 and KX507375 present a closeness of 91%, regarding KX29883 and KX529881.

In the main node of cluster B, the accession KX529874 was observed with a 15% of distance regarding the others.

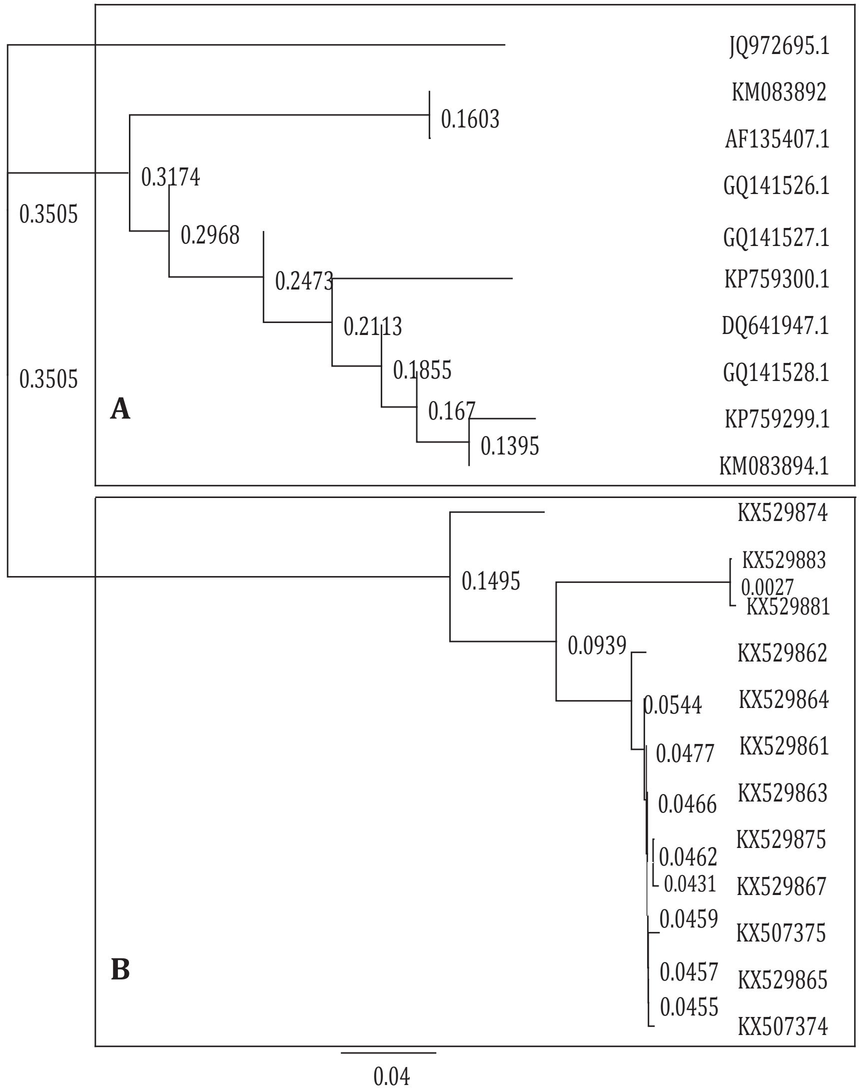
Discussion

ToRSV is one of the viruses that were detected in Chile in the eighties, which presence was found to be associated with both the leaves and seeds of the red raspberry crop (1). Subsequent surveys found the virus to be present in the south-central part of the country, in smaller fruits such as raspberries, red currants, and blueberries (17).

In raspberries, the presence of a viral infection is reflected in a number of characteristic symptoms that range from a decrease of the total growth in infected plants, to leaves with interveinal yellowing and chlorotic rings, and fruit presenting a shelling of the berry (16).

The presence of this final symptom at the time of harvest determines if the final product will be used for juice production or frozen, therefore decreasing the probability of obtaining a market price as high as that received for a fresh product (10, 12).

In this study, it was found that 98.75% of the plants were asymptomatic to the viral presence, and plants with viral signs were consistent with those found in other studies, such as Medina *et al.*, (2006).



Let A and B indicate differentiated clusters.

Letras A, B indican agrupamientos diferenciados.

Figure 4. Neighbor-joining dendrogram built with accessions KM083894.1, DQ641947.1, GQ141526.1, GQ141528.1, GQ141527.1, KM083892.1 and AF135407.1 and tomato ringspot virus (ToRSV) local sequences, from *Rubus idaeus* plant var. Heritage, of the Maule Region.

Figura 4. Dendograma Neighbor-joining construido con accesiones KM083894.1, DQ641947.1, GQ141526.1, GQ141528.1, GQ141527.1, KM083892.1 y AF135407.1 y secuencias locales de tomato ringspot virus ToRSV, obtenidas desde plantas de *Rubus idaeus* var. Heritage, de la Región del Maule.

An interesting field observation made during this study was the presence of plants with bright yellow interveins that faded when approaching the venal sectors of raspberry leaves. In this study, it was observed that the RT-PCR technique was efficient for detection of the virus in the different varieties studied (Heritage, Meeker, Coho, Amity, and Chilliwack), but it's important to consider more sensitive techniques due to the possibility that other isolates of the virus could be found present in the region but have not been included in this study (23, 30). In this sense, qRT-PCR, or TaqMan RT-PCR techniques are a necessary alternative to increase the sensitivity and efficiency of identification in future studies (8, 14, 19, 25, 26, 27).

The presence of ToRSV has been found to be related to multiple species, one of them being the raspberry (5, 20).

In the Maule Region, the manifestation of the virus has been associated with the infection of *Rubus idaeus* throughout the Andes, reaching towns that are in the far north of the region (the communes of Teno and Molina in the Curicó Province), all of which presented ToRSV.

In the town of La Montaña, there was a marked increase of the virus in the Amity variety but not in the Heritage variety, even though both samples belonged to the same orchard.

In the villages of the Rio Claro and San Clemente communes (Talca Province) the predominant variety was again Heritage, but in this case two locations showed no infection (Santa Rosa and Bramadero), which is also similar to the town of Santa Elena for the same variety (Yerbas Buenas commune).

In the southernmost communes of the region, such as Yerbas Buenas, Linares, and Parral, there is a high viral incidence for most of the sampled localities, a condition that has increased over time from previous studies. Medina (2006) obtained similar incidence levels to those found in this study, but did not describe the varieties evaluated. In a similar way, observations from marked plants were collected in the town of St. Victor Álamo during the 2012-2013 season.

In this paper, the progress of ToRSV infection was detected in raspberry leaves (Heritage), showing a 73.1% incidence during the first season, and reaching 100% incidence in the second season evaluated (date not presented). Despite its age (12 years), the orchard studied, like most visited orchards across the region, did not show the characteristic symptoms of infection, remaining 98.75% asymptomatic. This finding makes managing disinfection in the field more difficult, due to the high spread of the virus and the uncertainty conditions in the detection of symptomatic plants.

Periodic checks would allow one to determine whether the orchard is infected with the virus and help control the infection. This is different from previous studies, which observe reduced growth in infected raspberry plants and a very slow dispersion in the field limited to a few meters of plants adjacent to the focal point of the infection (21).

Such results reveal that the spread of the virus is very dynamic in the field, which could be due to everyday maintenance tasks for fruit management, such as weeds that can be a reservoir of the virus, crop cleanup tools that carry land with potential nematodes, poor water management which, added to a (primary and secondary) woody perennial root system, easily allows the attraction of virus vectors since they don't have to travel long distances to feed (20).

One of the vectors that transmits ToRSV is *Xiphinema americanum*, which attaches to the roots of the raspberry plant without causing major damage (5). It is probable that this vector is not present, or the prevalence is very low in the localities that are in the far north of the region such as in the Teno and Molina communes in the Curicó Province. This is different to the southern most communes in areas such as Linares, where it is possible that populations of *Xiphinema* sp exist. This agrees with our results because these localities present the highest percentage of infection by ToRSV. In the Maule Region, *Rubus idaeus* has been presented as one of the fruits affected by ToRSV (17). In this study, varieties collected in various provinces showed that Coho, Meeker, Chilliwack, Heritage, and Amity all possess the virus.

The Amity variety having the highest viral incidence throughout the region, followed by Meeker, Coho, Chilliwack, and Heritage. This situation is interesting because looking at the total planting areas of the varieties presented, Heritage covers the largest area both regionally and nationally (11).

Furthermore, in the samples collected, Heritage is always the predominant variety in the various towns, which raises two possible questions: 1) Is Amity more susceptible to ToRSV infection, or is Heritage more tolerant? Or, 2) are the soils from the sampled orchards infested? Infection assays could give us an answer to this question in the future. On the other hand, the regional analysis of ToRSV-raspberry interaction shows that the province with the highest viral presence is Linares, followed by Curicó, and finally, Talca. It is likely that the presence of the virus and its prevalence is due to the fact that most older orchards belong to the Linares Province, so the fields were infested many years ago with nematode vectors that added to the ancient practices of self-propagation of plant material, and could have generated a vicious circle of transfer continuous raspberry-nematode, nematode-raspberry, which ultimately increased the viral load in the region.

Raspberry production in the Maule Region is linked to small farmers where mechanization is difficult to access.

For years the practice of self-propagation of contaminated material and/or buying plants from nurseries with no certificates was a reality within of the Region. In recent years, this situation has changed marginally due to regional development through programs such as INDAP, which allow the gradual acquisition of new plants, leading to a slow renewal of orchards. However, even these initiatives have not had a sufficiently high influence to control viral dispersion (10). Most raspberry producers of the Maule Region get virus-free plant material originating from different nurseries in the region but, with checks made over time, it was found that the plants eventually acquired the virus.

In this research, we detected plantations that at one year of orchard establishment, with the varieties of Heritage and Coho, presented a ToRSV incidence of 3.2% to 6%, respectively. This suggests that the spread of the virus is probably due to inefficient propagation techniques, making it necessary for meristems *in vitro* cultures to be checked with greater persistence using molecular tools such as qRT-PCR and Taqman probes, which would provide a more reliable certification system in the early stages of the explants (13, 25, 26).

On the other hand, a different alternative is also feasible. Since there is a more pronounced and widespread presence of what is believed to be group *Xiphinema americanun* throughout the soils of the Chile, for this reason, it is suggested that a study be carried out on the presence of the vector (2).

The isolates obtained from putative ToRSV viral polymerase of this study with the primers described by Griesbach (1995) showed high efficiency in detection for all varieties of raspberry analyzed in the Maule Region.

The verification of the information obtained through the cloning of the fragments by RT-PCR, was observed when comparing the amplicons with accessions deposited in the gene bank.

The accessions found in this study, possess a high genetic distance with those deposited in the gene bank, whereas cluster B is the one that presents a greater difference with respect to all the other accessions studied. Is it possible that these differences are due to some type of mutation or adaptation to the country? In this regard, further studies would be required to elucidate the genetic variability of ToRSV isolates that involve gene encodings for RNA2, RNA-1, or other sectors, in order to facilitate a study that can help better understand the nature of the virus both in the Maule Region in Chile, and throughout the world (5, 27).

Conclusions

Finally, *Rubus idaeus*, one of the most important berries in the Maule presented ToRSV infection in the different varieties studied, but most plants were asymptomatic of the virus, with the Linares Province presenting the higher viral incidence percentage. Our results suggest a need to test routinely in *Rubus idaeus* orchards, because there is an increase of the virus in the Maule Region from past studies. Furthermore, sequence analysis of ToRSV indicate that differences exist in some isolates found in Chile compared with other ToRSV isolates from other parts of the world.

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Acknowledgements

We would like to give thanks for the support provided for FONDECYT project No. 11121394 for the financing provided in order to carry out this research.