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RESEARCH ARTICLE

Occurrence and molecular characterization of *Potyvirus* present in the garlic crop in the Mediterranean ecosystem of the central valley of Chile

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ABSTRACT

The garlic (*Allium sativum* L.) cultivated in the O'Higgins Region, the main garlic-producing area in Chile, is the commercial type known as pink garlic, which presents a low yield and a high prevalence of symptoms associated with viral infections. A survey was carried out in the three localities of the region that cultivate this vegetable to identify, by reverse transcription polymerase chain reaction (RT-PCR), the presence of *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV). The results indicated a prevalence of 99.5% and 54.0% respectively. In addition, a molecular and phylogenetic analysis of the amplified genome fragments for both viruses was performed. Using the nucleotide BLAST (BLASTn) tool, nucleotide sequences were compared with sequences available in the National Center for Biotechnology Information (NCBI) database. The Chilean sequences were aligned with complete sequences available at NCBI and a phylogenetic tree was constructed using the Neighbor-Joining method. The results showed genetic variability among Chilean isolates.

Key words: Garlic, LYSV, OYDV, Potyvirus.

INTRODUCTION

Garlic (*Allium sativum* L.) is a vegetable cultivated since ancient times, being highly valued as a condiment. This is because of the bulb's organoleptic, nutritional, nutraceutical, and cultural characteristics (D'Archivio et al., 2019). Currently, garlic is the tenth most-produced vegetable in the world with 28 million tons, and the sixth most traded between countries (Rabobank Group, 2018). Worldwide this plant commercial production is not based on the botanical seed, but through the vegetative propagation of bulbs, which makes garlic cultivation especially vulnerable to viral diseases as the main route of dissemination of these viruses is through the vegetative propagation of the bulb, in addition to the spread by vectors and the absence of agrochemicals for this type of diseases control. These types of infections are considered chronic worldwide and are of great economic importance, due to the high prevalence, decrease in weight, and loss of commercial quality of the garlic bulbs (Simon and Jenderek, 2003; Gimenez et al., 2016).

Several viral species are described in the world infecting the garlic crop, which are distributed in four different genera (Celli et al., 2019). These genera are: *Potyvirus*, *Carlavirus*, *Allexivirus*, and *Tospovirus*. In the genus *Potyvirus*, the *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) are the species that produce the most severe decrease of the garlic bulb commercial yield, in both caliber and weight by 69% and 54%, respectively (Lot et al., 1998; Lunello et al., 2007). The identification of these viruses in a garlic-producing region is important for the development of control strategies (Santosa et al., 2023).

Nowadays, despite the impact of potyviruses on garlic production, there is still a significant knowledge gap regarding the prevalence of populations of these viruses in worldwide garlic crops (Cremer et al., 2021).

In Chile, garlic is currently the first fresh export vegetable, which together with onions represents 95% of Chilean exports of fresh vegetables. Traditionally is the Libertador General Bernardo O'Higgins Region (from now on O'Higgins Region) the main garlic-producing territory in Chile, concentrating 48% of the garlic surface cultivated by multiple small-holder farmers, who historically have cultivated traditional garlic, the commercial type known as pink garlic, characterized for its organoleptic and post-harvest characteristics (ODEPA, 2021). In these traditional garlic plantations, the small-holders farmer reports low yields and a high prevalence of symptoms that can be associated with viral infections such as decreased yield, plant dwarfism, diffuse mottles, chlorotic striations, and leaf deformation. Plus, in recent years the displacement of the traditional garlic cultivated in the O'Higgins Region by foreign Chinese garlic, a type of commercial garlic with better yields, risks the *in-situ* conservation and social history of the traditional garlic of the O'Higgins Region. Therefore, the present work aimed to identify the presence of OYDV and LYSV in the cultivation of garlic in the O'Higgins Region, estimate the prevalence of each virus, and molecularly characterize a region of the 3' end of the genome of these viruses.

MATERIALS AND METHODS

Plant material

During the 2017, 2018, and 2019 growing seasons, a collection of 189 samples of leaves of garlic plants (*Allium sativum* L.) commercial type pink garlic, was carried out, distributed in nine properties located in the localities of Rengo, Quinta de Tilcoco, and Requínoa (Figure 1), the main garlic producing territories in the Libertador General Bernardo O'Higgins Region (from now on O'Higgins Region). In each field, between 15 and 25 randomly distributed samples were collected, which were individualized in properly labeled plastic bags and then stored in a cooler with an ice pack until they arrived at the Virology Laboratory of INIA La Platina, Santiago, Chile, where they were processed immediately. In each sample, the presence of *Onion yellow dwarf* (OYDV) and *Leek yellow stripe* (LYSV) viruses was analyzed by reverse transcription polymerase chain reaction (RT-PCR) analysis.

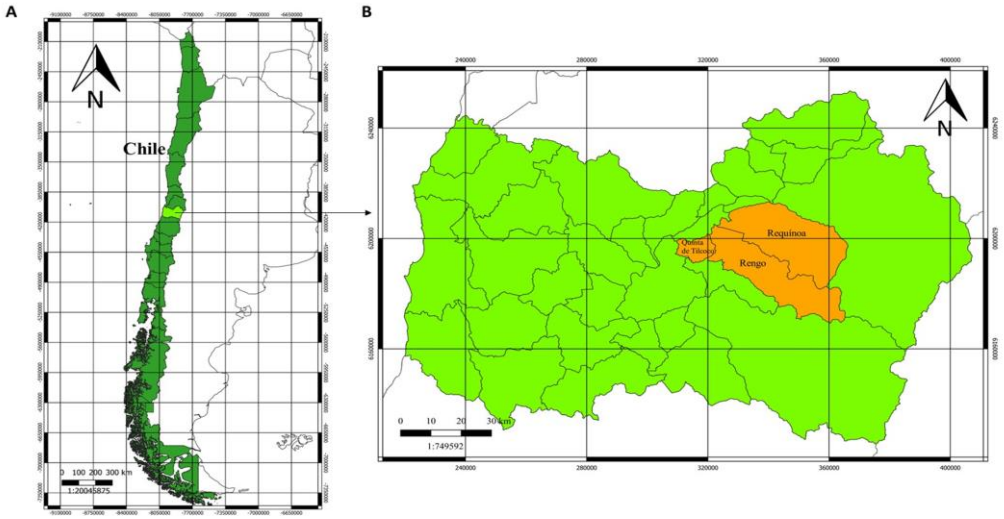

Figure 1. Map of the garlic sampling. A: The location of the Libertador General Bernardo O'Higgins Region is highlighted in light green. B: Location of the three localities where the samples were taken (orange): Rengo, Requínoa and Quinta de Tilcoco. The scale bar is shown in the lower left corner of both images and corresponds to 300 km for A and 30 km for B.

RNA extraction and RT-PCR amplification

The RNA extraction was performed using the TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA), following the manufacturer's instructions. The purity and concentration of the extracted RNAs were measured through a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA), accepting a value close to 2 in the 260/280 ratio. Subsequently, the RNAs obtained were treated with DNAsa (RQ1 RNase-Free DNase; Promega, Madison, Wisconsin, USA), to eliminate all the DNA from the sample and only preserve the RNAs, considering that OYDV and LYSV have a genome composed of RNA.

The RNA was used as template for the synthesis of a complementary strand of DNA (cDNA), which was carried out using the enzyme reverse transcriptase (M-MLV Reverse Transcriptase, Promega), following the protocol: Treated RNA was mixed with 1.0 µL random primers (200 ng μL^{-1}, Invitrogen) and completed at a volume of 13.5 µL with nuclease-free water (Molecular Biology Grade Water, Corning, Glendale, Arizona, USA). This mixture was incubated at 70 °C for 5 min and then put on ice for 5 min, to avoid the formation of secondary structures and prepare the sample for the reverse transcription (RT) reaction. Subsequently, 4µL M-MLV 5X reaction buffer, 1 µL dNTPs (10 mM), 1 µL M-MLV RT (200 u μL^{-1}), and 0.5 µL RiboLock RNase Inhibitor (40 u μL^{-1}, Thermo Scientific) was added, the thermal scheme used was 25 °C for 5 min, 37 °C for 60 min and 70 °C for 15 min.

To evaluate the quality of the cDNA obtained, a PCR reaction was performed to amplify a fragment of the 18S subunit of the plant's rRNA (Du et al., 2006), using the enzyme DNA polymerase (GoTaq Flexi DNA Polymerase, Promega), by the following reaction mixture: 5 µL 5x GoTaq Flexi Buffer, 2 µL $MgCl_2$ solution (25 mM), 1 µL dNTPs (10 mM), 0.5 µL forward primer 18S-F, 0.5 µL reverse primer 18S-R (Table 1), 0.13 µL GoTaq DNA Polymerase (5 u μL^{-1}) and 2 µL cDNA. A volume of 25 µL was completed with nuclease-free water (Corning). As a negative control, the temperate volume was replaced by nuclease-free water. The thermal scheme described in Table 2 was followed. The 255 bp amplified was observed in a 1.5% agarose gel, using GeneRuler 100 bp (Thermo Scientific) as the molecular weight standard.

Table 1. Primers for molecular analyses.

Organism	Id Primer	Sequence 5´-3´	Fragment size	Reference
Onion yellow dwarf virus	OYDVF-10214 OYDVR-10539	CGTTTGTTTGGCCTGGATGGTAACG GTCTCYGTAATTCACGC	318 pb	Majumder and Baranwal, 2014
Onion yellow dwarf virus	IR-OYDV-F2 IR-OYDV-R	CGRTTGGARGCYATATGTGC AARCGGTTAGTTGCCCCTCT	936 pb	This work
Leek stripe yellow mosaic virus	1-LYSV 2-LYSV	TCACTGCATATGCGCACCAT GCACCATACAGTGAATTGAG	1 kb	Fajardo et al., 2001
Gene 18S	18S-F 18S-R	GAGAAACGGCTACCACATCCA CGTGCCATCCCAAAGTCCAAC	255 pb	Du et al., 2006

Table 2. Thermal schemes used for each primer PCR reaction.

		Primers			
Stage	No of cycles	OYDVF-10214 OYDVR-10539	IR-OYDV-F2 IR-OYDV-R	1-LYSV 2-LYSV	18S-F 18S-R
Initial denaturation	1	95 °C × 3 min	94 °C × 3 min	95 °C × 2 min	94 °C × 3 min
Denaturation	35	95 °C × 45 s	94 °C × 1 min	95 °C × 1 min	94 °C × 30 s
Annealing		56 °C × 30	50 °C × 1 min	50 °C × 2 min	58 °C × 30 s
Extension		72 °C × 1 min	72 °C × 1 min	72 °C × 1 min	72 °C × 30 s
Final extension	1	72 °C × 5 min	72 °C × 10 min	72 °C × 7 min	72 °C × 5 min

After verifying the quality of the cDNA, a PCR was performed to determine the presence of OYDV and LYSV; in both cases, specific primers described by the literature capable of amplifying a fragment of the gene coding for the coat protein were used (Fajardo et al., 2001; Majumder and Baranwal, 2014) (Table 1). The amplification of each fragment was carried out using the same DNA polymerase above, in the following reaction mixture: 4 µL 5x GoTaq Flexi Buffer, 1.6 µL $MgCl_2$ solution (25 mM), 1 µL dNTPs (10 mM), 0.5 µL forward primer and 0.5 µL reverse primer, 0.1 µL GoTaq DNA Polymerase (5 u μL^{-1}) and 2 µL cDNA. A volume of 20 µL was completed with nuclease-free water (Corning). As a positive control, cDNA was used from a sample that had previously been identified as positive for this virus, and as a negative control, the temperate volume was replaced by nuclease-free water. The thermal diagrams used are detailed in Table 2. The amplified was observed in a 1.5% agarose gel, using GeneRuler 100 bp (Thermo Scientific) as the molecular weight standard.

Cloning, PCR product sequencing, and sequence analysis

For the molecular characterization of OYDV, we designed a set of primers capable of amplifying a fragment of the region 3' of the genome of the virus that was larger than that amplified by the primers described in the literature. For this, 55 OYDV sequences stored in the National Center for Biotechnology Information database (NCBI, Bethesda, Maryland, USA; https://www.ncbi.nlm.nih.gov/) were used, privileging isolates found in America and Asia. Using the MAFFT tool (Katoh et al., 2019), a multiple alignment analysis was carried out to determine the areas of greatest consensus and, within them, considering the characteristics that the primers must possess, the IR-OYDV-F2 and IR-OYDV-R primers were designed to amplify a fragment of 936 bp (Table 1). For the molecular characterization of LYSV, the same 1-LYSV and 2-LYSV primers described above were used, amplifying a fragment of the gene coding for viral coat protein (CP).

From the total samples analyzed, at least three of them were chosen for each virus for cloning and sequencing. The procedure consisted of inserting the amplicon into a pGEM-T Easy vector (Promega), which was cloned into chemically competent *Escherichia coli* (DH5α Competent Cells, Invitrogen). The insertion of the sequence of interest was verified by PCR using the specific primers for each virus (Table 1). For each virus, three clones were chosen for each isolate, whose plasmids were purified using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sent to Macrogen Inc. (Seoul, Korea) for sequencing.

The sequences of the amplified fragments for the OYDV and LYSV were compared with other sequences published in the NCBI database, using the Basic Local Alignment Search Tool (BLAST available at https://blast.ncbi.nlm.nih.gov/Blast.cgi). For the sequences obtained from each virus, a phylogenetic tree was generated with complete genome sequences of OYDV and LYSV found in NCBI, using the Neighbor-Joining method with 1000 repeats. All nucleotide sequences were translated into amino acid sequences and an alignment was performed to estimate the percentage of identity between the Chilean OYDV and LYSV sequences and those obtained from NCBI. All sequence analyses were performed with the MEGA 7 program (Kumar et al., 2016).

RESULTS AND DISCUSSION

Identification and prevalence of OYDV and LYSV

The results of this work showed a high prevalence of viruses. In 99.5% of the samples analyzed using OYDVF-10214 and OYDVR-10539 primers, the expected fragment of 318 bp corresponding to the partial sequence of the OYDV CP was obtained. While in 54% of the samples studied, using the 1-LYSV and 2-LYSV primers, the expected fragment of 1 kb corresponding to the complete sequence of the CP of LYSV was amplified (Figure 2). None of the three localities presented a massive infection of LYSV. Nonsignificant differences were found between the percentage of infection between the sectors of Rengo (61%) and Quinta de Tilcoco (50%), but Requínoa (30%) was significantly lower (Figure 3). While in the case of OYDV, the prevalence rates obtained were similar between Rengo (99%), Quinta de Tilcoco (100%), and Requínoa (100%) (Figure 4). Considering that sampling was carried out randomly in the main sectors of the O'Higgins Region where this vegetable is grown, these data suggest that the infection with OYDV is widespread on a larger scale in the crop. This result is expected, since the commercial production is based on the vegetative propagation of garlic without phytosanitary considerations, as farmers use their cultural practices for their own production of the bulbs that will

be used for the establishment of the next season crop, causing the virus to easily spread among the field due to vectors. On the other hand, the observed prevalence of LYSV suggests that it is an infection that has recently affected the garlic crop, in an infection event posterior to OYDV.

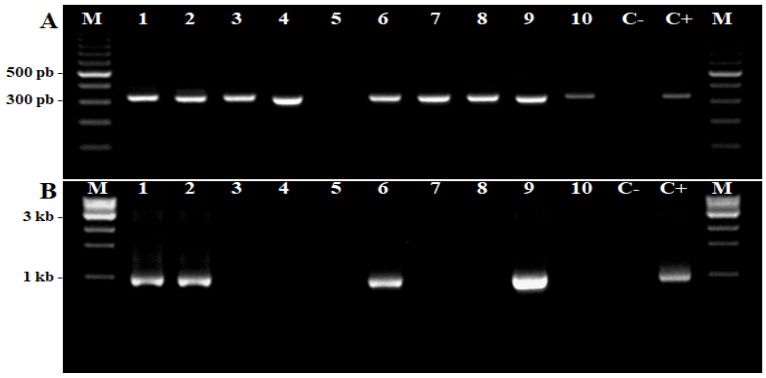

Figure 2. Electrophoresis in 1.5% agarose gel. 1-10: Different samples of garlic. M: Molecular weight marker; C-: negative control; C+: positive control. A: *Onion yellow dwarf virus* (OYDV) PCR. Expected 318 bp fragment observed in lanes 1 to 4 and 6 to 10. B: *Leek yellow stripe virus* (LYSV) PCR. Expected 1 kb fragment seen in lanes 1 and 2, 6 and 9.

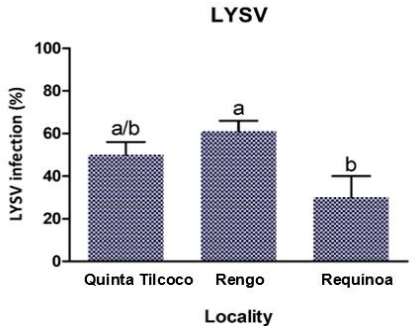

Figure 3. Percentage of infection by *Leek yellow stripe virus* (LYSV) in three localities. Statistical analysis was performed in the Infostat program using a mixed generalized linear model for binary variables. We use a logit link and a Fisher LSD comparison test (alpha = 0.05).

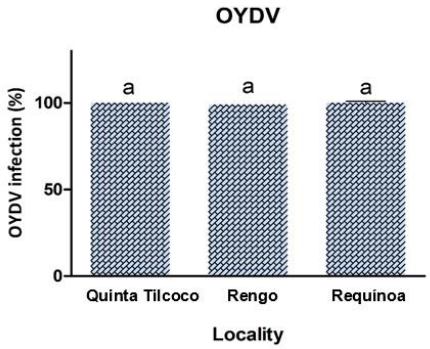

Figure 4. Percentage of *Onion yellow dwarf virus* (OYDV) infection in three localities. Statistical analysis was performed in the Infostat program using a mixed generalized linear model for binary variables. We use a logit link and a Fisher LSD comparison test (alpha = 0.05).

Cloning, PCR product sequencing and sequence analysis

Sequencing was performed for the molecular characterization of each virus's amplified region. Of the total positive samples obtained, at least three of them were randomly chosen for each virus, which were cloned and sequenced.

The OYDV sequencing was carried out using IR-OYDV-F2/IR-OYDV-R primers that amplified a fragment of 936 bp corresponding to the replicase (nuclear inclusion b, NIb)-coat protein (NIb-CP) region from the virus genome (Table 1). The efficiency and quality of these primers were verified through PCR with both positive and negative samples using OYDVF-10214/OYDVR-10539 primers (data not shown). The resulting amplicons were cloned and sequenced, the sequences obtained are available in the NCBI database with the codes OQ557506, OQ557507, and OQ557508. In the case of LYSV, sequencing was performed with the same primers, 1-LYSV/2-LYSV, used to identify the virus. The sequences obtained are available in the NCBI databases with the codes OQ557509, OQ557510, OQ557511, OQ557512, and OQ557513.

Using the BLASTn tool, the sequences obtained from the amplified fragment of the LYSV genome from five Chilean isolates were compared with other sequences available in the NCBI database, showing an identity percentage between 99.8% and 98.8% and a Query cover of 100%, with the sequence MN059480.1 from China. While another of the Chilean sequences had a 95.97% identity with an Argentine isolate (KF597284.1). When the Chilean sequences were translated to amino acids and compared with the aforementioned sequences, homology between 100.00% and 97.17% was obtained (Table 3).

Table 3. Identity percentage of partial viral coat protein (CP) sequences from Chilean isolates of the *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) against sequences available at NCBI.

Sequence ID (Gene bank code)	Chilean isolate ID	Virus	Nucleotide identity percentage	Amino acid's identity percentage	Sequence ID (Gene bank code)	Isolate origin
OQ557509	6793-2 Chile	LYSV	98.80	100.00	MN059480.1	China
OQ557513	L1-1 Chile	LYSV	99.60	98.94	MN059480.1	China
OQ557512	94-6 Chile	LYSV	99.80	99.65	MN059480.1	China
OQ557511	96-5 Chile	LYSV	99.70	99.29	MN059480.1	China
OQ557510	87-4 Chile	LYSV	95.97	97.17	KF597284.1	Argentina
OQ557506	7401 Chile	OYDV	90.95	95.82	MN059636.1	China
OQ557507	OY1-6 Chile	OYDV	89.24	94.21	MN059632.1	China
OQ557508	OY94-1	OYDV	89.94	95.82	MN059630.1	China

The phylogenetic analysis of the five Chilean sequences of LYSV was performed by aligning them with 33 isolates obtained from the NCBI database, which resulted in six main groups or clades (Figure 5). Of the five Chilean sequences, four were assigned to the same group. The fifth sequence was assigned to a different and distant clade from the previous one (OQ557510), despite being obtained from the same vegetable farm as the sequence (OQ557511), which shows the genetic variability that may exist within the same crop. The phylogenetic tree clustered most Chilean isolates along with isolates from China, which may be because most of the sequences found in NCBI come from China, being the largest producer and most sampled crops. Plus, approximately 99% of Chile's garlic imports come from that country (ODEPA, 2020). This imported garlic is used for consumption and vegetative propagation, thus facilitating the virus population to easily mutate and recombine with other viruses that coexist in the garlic crops (Bereda et al., 2017; Taglienti et al., 2017). While the clustering of the Chilean isolates with Argentinian isolates is expected considering that are neighboring countries, making the probabilities of germplasm movement between both countries higher.

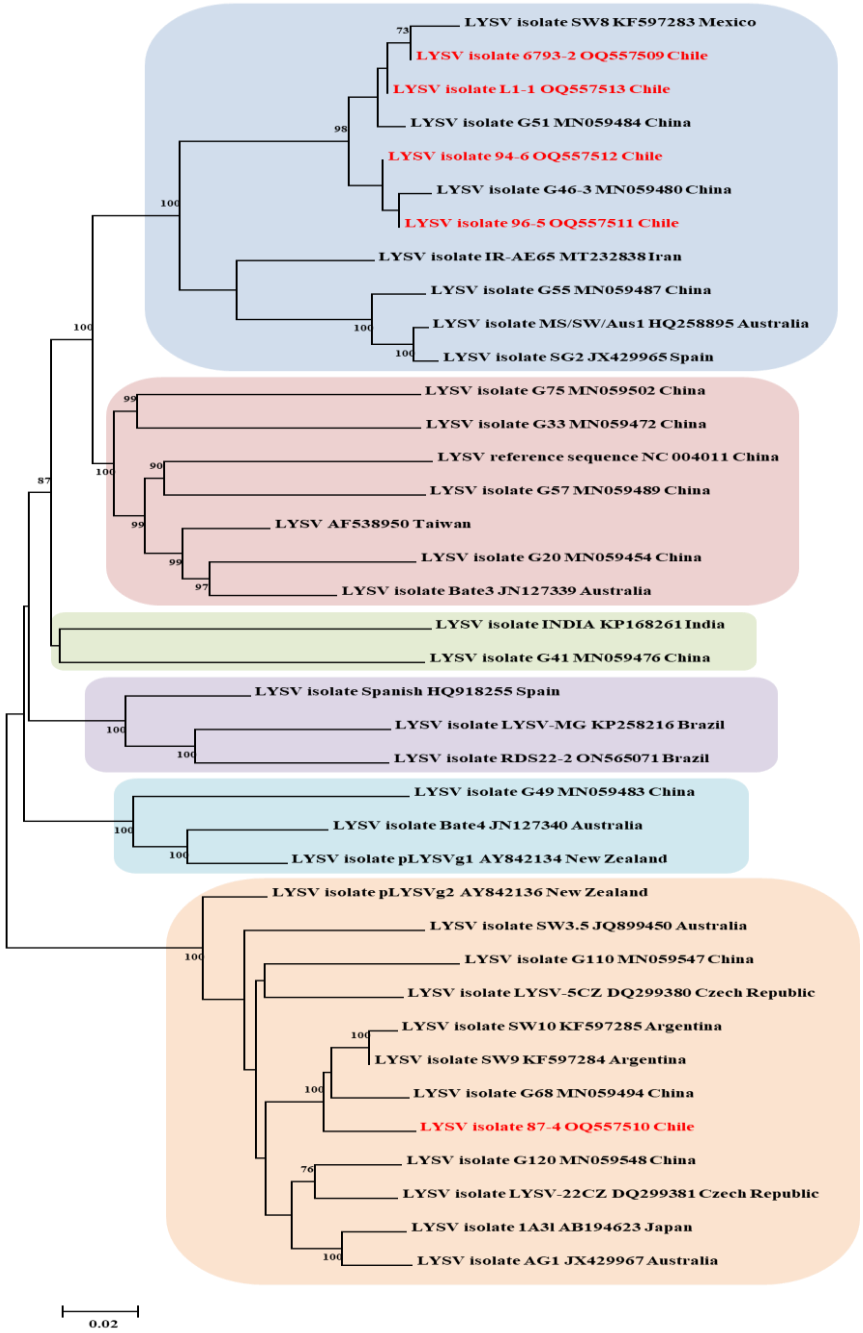

Figure 5. Consensus phylogenetic tree constructed with Mega software, version 7 (Kumar et al., 2016). The Neighbor-Joining method was used based on the complete sequence of *Leek yellow stripe virus* (LYSV) from 33 isolates obtained from the NCBI database and five Chilean isolates (red highlight). Values in the nodes indicate the Bootstrap values (%) with 1000 replicates.

The same BLASTn analysis was performed for the three partial nucleotide sequences of the CP of the Chilean isolates of OYDV. The results indicated identities between 90.95% and 89.24% and a Query Cover of between 100% and 99% with three sequences available in the NCBI of isolates from China. When translated the Chilean sequences into amino acids sequence and comparing them with the previous

sequences, homology between 95.82% and 94.21% was observed (Table 3). In the case of OYDV, the phylogenetic analysis was performed by aligning the Chilean sequences with the other 30 sequences obtained from the NCBI database. This analysis resulted in five well-defined groups, with Chilean isolates clustering along with isolates from Argentina in two groups (Figure 6). The fact that Chilean sequences are found in different clades may be indicative of the high genetic variability that, as in the case of LYSV, is found in garlic cultivation in the same region.

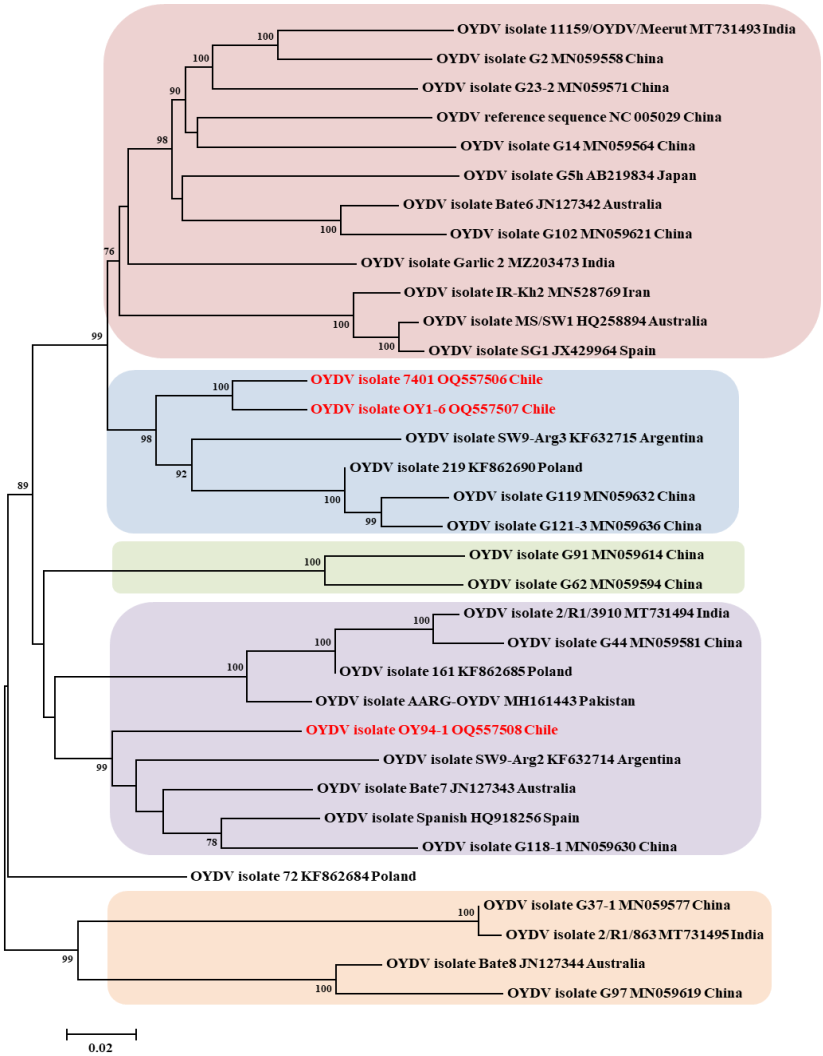

Figure 6. Consensus phylogenetic tree constructed with Mega software, version 7 (Kumar et al., 2016). The Neighbor-Joining method was used based on the complete sequence of *Onion yellow dwarf virus* (OYDV) from 30 isolates obtained from the NCBI database and three Chilean isolates (red highlight). Values in the nodes indicate the Bootstrap values (%) with 1000 replicates.

Small-scale agriculture has an important role in food production, more sustainable agriculture, and in situ conservation of crops. The traditional cultivation of garlic in Chile is maintained mainly by peasant agriculture, which due to cultural practices has its crops highly affected by viral infections as reported in this work. Our results reflect this situation, showing that in the localities studied in the O'Higgins Region the infection produced by OYDV and LYSV corresponds to approximately 100% and 54%, respectively.

It is relevant to mention that the low quality of the garlic that is typically produced in the O'Higgins Region causes producers to leave this crop aside and prefer the production of Chinese garlic, which offers better yields but lacks the historical, social, and organoleptic characteristics of traditional garlic. According to scientific reports, viral diseases generate losses that can exceed 50% in garlic crops (Conci et al., 2003; Lunello et al., 2007; Pérez-Moreno et al., 2014, Mang et al., 2022), since there is still no chemical control over them, the only way to establish a virus-free and sustainable crop over time is through in vitro sanitization of plant material. This technique has been implemented in several countries such as Argentina and India, which are leaders in garlic production (Conci et al., 2005; Pramesh and Baranwal, 2015). However, before a sanitation system can be established, it is necessary to know the prevalence of the virus, among other aspects. Thus, this work becomes an important contribution to this problem, since it is the first research in Chile that studies the prevalence and molecular characterization of potyvirus present in garlic crops in the O'Higgins Region. However, it is only the beginning, since further studies are required to know the OYDV and LYSV pathogenicity and estimate the impact that these viruses have on Chilean garlic crops. As well as is important to start phylogeny and evolution studies to resolve the relationship of Chilean virus isolates with others present in the world.

CONCLUSIONS

According to the results obtained in this work, the garlic production in the localities of the Libertador General Bernardo O'Higgins Region: Quinta de Tilcoco, Rengo, and Requínoa is widely infected by *Onion yellow dwarf virus* (OYDV; 99.5%) and to a lesser extent by *Leek yellow stripe virus* (LYSV; 54.0%).

The molecular characterization of LYSV and OYVD genome amplified from the Chilean isolates shows genetic variability among crop fields since distant isolates of the same virus were found in the same locations.

Author contribution

Conceptualization: M.M. Methodology: I.R., N.N., V.T. Validation: M.M., I.R. Formal analysis: M.M., I.R. Investigation: M.M. Resources: M.M. Data curation: M.M., I.R. Writing-original draft: M.M. Writing-review & editing: M.M., R.L., A.D. Visualization: M.M. Supervision: M.M. Project administration: M.M. Funding acquisition: M.M. All co-authors reviewed the final version and approved the manuscript before submission.

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