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SHORT COMMUNICATION

Molecular characterization and survey of viruses and viroids infecting apple and pear trees in southern Brazil

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Abstract

Apple and pear cultivation plays a crucial role in the Brazilian economy, particularly in the states of Rio Grande do Sul (RS) and Santa Catarina (SC). Viruses and viroids pose a significant threat, affecting tree health, fruit quality, and orchard productivity. This research aimed to identify both established and novel etiological agents contributing to the decline of apple and pear trees in SC and RS. A total of 53 samples were collected from nurseries and commercial orchards in the municipalities of Lages, Fraiburgo, São Joaquim, and Painel (SC), as well as Vacaria (RS). Viral infection was assessed via total RNA extraction. Fourteen of these samples underwent high-throughput sequencing (HTS), revealing the presence of citrus concave gum-associated virus (CCGaV) and apple hammerhead viroid (AHVd), two pathogens recently identified in Brazil. Phylogenetic analysis was performed using a global dataset incorporating the sequences generated in this study. Given the potential for viral and viroid infections across different orchards, all 53 samples (43 apple and 10 pear) were also screened by RT-PCR using specific primers for apple stem pitting virus (ASPV), apple stem grooving virus (ASGV), apple mosaic virus (ApMV), apple chlorotic leaf spot virus (ACLSV), CCGaV and AHVd. CCGaV was detected in 16 samples, 13 of which originated from SC. Co-infections of ASPV and ASGV were prevalent, observed in 23 samples, and 18 samples exhibited infections with three or more pathogens, highlighting the complex nature of viral infections impacting orchard management. This constitutes the first detection of CCGaV in SC and the first global report of of AHVd in pear trees.

Keywords Apple hammerhead viroid · Citrus concave gum-associated virus · High throughput sequencing

The apple industry holds considerable global economic importance, ranking second in production volume only to bananas (Korban 2021). Pears, while produced in smaller quantities than apples, are nonetheless a significant pome fruit crop cultivated worldwide (Sawant et al. 2021). These crops are vulnerable to a variety of pathogens (Bragard et al. 2019; Wright et al. 2020), among which viruses have been of particular concern for decades due to the difficulties associated with their diagnosis and control. The development of fruit production in Brazil from the 1960s onward, combined with inadequate regulations governing the introduction of substantial amounts of planting and propagating material, resulted in the introduction of several viral pathogens, including apple stem grooving virus (ASGV, *Capillovirus mali*), apple chlorotic leaf spot virus (ACLSV, *Trichovirus mali*), apple stem pitting virus (ASPV, *Foveavirus mali*) and apple mosaic virus (ApMV, *Ilarvirus ApMV*) (Nickel et al. 2001; Nickel and Fajardo 2021).

Latent viruses, such as ASGV, ASPV, and ACLSV, often do not induce visually detectable symptoms in most commercial apple cultivars. Consequently, they can persist undetected and propagate indefinitely through vegetative and clonal propagation at an industrial scale (Nickel and Fajardo 2009; Várallyay et al. 2022). These latent infections can become more pronounced or exacerbate diseases caused by other pathogens, particularly in mixed infections, under

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environmental stress, or when grafted cultivars are more susceptible (Nickel and Fajardo 2009; Várallyay et al. 2022).

Sustainable apple and pear production relies on the rapid and efficient screening of propagation material (Várallyay et al. 2022). Reliable virus and viroid detection is therefore critical. Traditional plant virus diagnostic methods can be categorized as specific (serological/molecular tests) and non-specific (indicator plants and electron microscopy) (Pecman et al. 2017). High-throughput sequencing (HTS) has revolutionized virus discovery, offering a generic approach to virus identification that does not require prior knowledge of the target pathogen but yields species-specific results (Adams et al. 2009, 2013). HTS has overcome the limitations of traditional plant virology methods, such as transfer to indicator plants or purification of virus particles (Massart et al. 2017; Olmos et al. 2018).

Citrus concave gum-associated virus (CCGaV, *Conguvirus citri*) was initially identified in citrus (*Citrus sinensis* L.) fruit and subsequently shown to be the causal agent of "blind pocket" disease, characterized by concave gum symptoms on citrus fruit, a disease known for decades but of previously unknown etiology (Navarro et al. 2018). Affected trees exhibit deep trunk and chlorotic leaf spots. However, not all CCGaV positive citrus samples displayed symptoms (Navarro et al. 2018). CCGaV was first detected in apple trees in the United States (from material imported from France), and subsequently in China, Brazil, and Italy (Liu et al. 2018, 2021; Wright et al. 2018; Nickel et al. 2020; Minutolo et al. 2021). Within Brazil, the virus has been previously reported in Rio Grande do Sul (Nickel et al. 2020).

Apple hammerhead viroid (AHVd, *Pelamoviroid malleusmali*), a subviral plant pathogen, was first reported to infect apple trees in China (Zhang et al. 2014). Its secondary structure, modeled a "hammer conformation," exhibits stabilizing "kissing loop" interactions (Serra et al. 2018). Initial studies reported no distinct symptoms in AHVd-positive trees (Zhang et al. 2014; Serra et al. 2018). The detection of AHVd in symptomatic trees, which also harbored common apple viruses (Messmer et al. 2017; Szostek et al. 2018), complicated to link viroid infection to observed symptoms. AHVd has since been detected in apple trees in Africa (Hamdi et al. 2022), North America (Szostek et al. 2018), Japan, New Zealand, Brazil, and Europe (Szostek et al. 2018; Chiumenti et al. 2019; Nickel and Fajardo 2021; Fontdevila Pareta et al. 2022). Beyond apples, AHVd has also been detected in loquat (*Eriobotrya japonica*) (Canales et al. 2021).

Here, we report the molecular characterization of CCGaV and AHVd isolates detected in apple (*Malus domestica* B.) and pear (*Pyrus communis* L.) trees in southern Brazil. Additionally, we conducted a survey of the major viruses and AHVd impacting apple and pear production in key municipalities across southern Brazil.

Forty-three apple trees cuttings, representing the cultivars "Fuji Suprema", "Gala Select", "Imperial Gala", "Fuji Kiku", "Gala", "Purple Gala", "Fuji," and "Gala Brookfield", along with the rootstocks "M9", "Marubakaido", "CAT 16" and the Geneva® series "G.213", "G.202", "G.814", "G.210", "G.41," and "G.222", were collected from nurseries and commercial orchards in the municipalities of Fraiburgo (27° 01′ 34″ S, 50° 55′ 17″ W), Lages (27° 48′ 57″ S, 50° 19′ 33″ W), Painel (27º 55' 44" S, 50º 06' 18" W), São Joaquim (28° 17′ 38″ S, 49° 55′ 55″ W) (Santa Catarina; SC), and Vacaria (28° 30′ 43″ S, 50° 56′ 02″ W) (Rio Grande do Sul; RS). Ten pear tree cuttings of the cultivars "Santa Maria", "Rocha," and "Carmen," and the rootstocks "BA-29" and "CAV 03", were also collected in municipality of São Joaquim (SC). All samples were stored at 4°C.

Total RNA was extracted from bark samples (100 mg) collected from young branches of scions and rootstocks cultivars. The tissue was macerated in liquid nitrogen, and RNA extraction was performed using a Qiagen RNeasy Plant Mini Kit, following the manufacturer's protocol. RNA was eluted by adding twice the column volume 55 µl of sterile water and centrifuged at 13,200 rpm for 1 min. RNA quality and concentration were assessed spectrophotometrically using a NanoDrop 2000 (Thermo Scientific). Fourteen samples were selected from the total collection and pooled into four groups: the first comprised "Fuji Suprema" and "Gala Select" apple cultivars; the second, the "Rocha", "Santa Maria", and "Carmen" pear cultivars; the third, the "M9" and "CAV 03" rootstocks; and the fourth, the Geneva series rootstocks "G.202", "G.210," and "G.213". These RNA samples were placed in RNA stable tubes (Biomatrica) and dried in a Speed Vac system (V-AQ mode) for 1 h and 30 min. The dried RNA was then sent for sequencing (Proteimax). HTS reads were generated from a complementary DNA (cDNA) library using the Illumina HiSeq 2500 platform. cDNA library quality was assessed using FastQC software, and adapters and low-quality reads were removed. Sequences were initially analyzed and processed with BBduk tool and assembled using the Tadpole tool (Geneious software v. 2022). The resulting contigs were compared to public databases to identify sequences matching known viral genomes. Finally, contigs and singletons were aligned to NCBI databases using BLASTn and BLASTx with default parameters, and aligned to known viral sequences.

RT-PCR was carried out using the M-MLV Reverse Transcriptase and GoTaq® Flexi DNA polymerase enzyme (Promega) following the manufacturer's recommendations, employing specific primers for the detection of ASPV, ASGV, ApMV, ACLSV, CCGaV and AHVd (Supplementary Table 1). The PCR cycling were implemented according to established protocols (Jelkmann 1994; Candresse et al. 1995; Nickel et al. 2001; Menzel et al. 2002), except

to ACLSV, CCGaV, and AHVd, that the PCR cycling is indicated in Supplementary Table 1.

RT-PCR products were separated by electrophoresis on a 1.0% agarose gel, visualized under UV light, and photographed. Following verification of the RT-PCR product, positive samples were submitted for Sanger sequencing (ACTGene Análises moleculares LTDA) using the corresponding primers for each amplified fragment (Supplementary Table 1). The resulting sequences were compared to those in GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Open reading frames (ORFs) within the contigs were predicted using NCBI's ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), enabling the identification of conserved and functional protein domains using SMART (http://smart.embl-heidelberg.de/) (Letunic et al. 2015). The complete genomes of the characterized viral and viroid isolates were deposited in GenBank. Nucleotide identity was determined using SDT v.1.3 (Muhire et al. 2014), which generates an evolutionarily informative sequence similarity matrix, facilitating precise identification of the most closely related sequences group.

Maximum likelihood phylogenetic trees were constructed using MEGA X (Kumar et al. 2018). Alignments for phylogenetic analysis were generated using the MUSCLE algorithm, also implemented in MEGA X. Nucleotide and amino acid sequence identities were analyzed using the complete genome and coding regions sequences of the closest related viral species identified in this study.

RT-PCR using specific primers confirmed CCGaV infection in three of the 14 sequenced samples. The positive samples originated from the "Gala Select" cultivar (isolates one and two) and the "G.210" rootstock (isolate three). Isolates one and two were from RS and isolate three was from SC. This study focused on characterizing the "G.210" isolate. HTS generated 45 million raw reads (approximately 151 nt each) for this sample. Two contigs were assembled and aligned with the NCBI viral genome database using BLASTn and BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi), revealing high nucleotide identity with CCGaV. Processed sequences were mapped to CCGaV RNA1 (NC_035759) and RNA2 (NC_035454) reference sequences using BBmap (Bushnell 2014). A total of 17,977 reads mapped to RNA1, yielding the complete sequence of CCGaV RNA1 (isolate G.210, 6,681 nt, PQ156135), which shared 96%–100% nucleotide identity with Brazilian and global isolates. The complete CCGaV RNA2 sequence (isolate G.210, 2,703 bp, PQ156132) was assembled from 43,124 reads showing 96%–100% nucleotide identity with Brazilian and global isolates.

The complete genome of the isolate comprised two RNA segments: RNA1 (6,681 nt) and RNA2 (2,703) (Fig. 1). The Weihai CCGaV isolate in turn has RNA1 (6,674 nt) and RNA2 (2,706 nt) (Liu et al. 2021), while the CE-c3 CCGaV isolate has RNA1 (6,674 nt) and RNA2 (2,704 nt) (Minutolo et al. 2021). The RNA1 segments of these isolates range from 6,674 to 6,681 nt and the RNA2 segments range from 2,703 to 2,706 nt, indicating near identical genome sizes. The isolate characterized in this study share conserved 5' (ACACA) and 3' (UGUGU) terminal regions on both RNA1 and RNA2, a characteristic of viruses belonging to the family *Phenuiviridae*, demonstrating the conservation of these genomic elements (Liu et al. 2021). RNA1 contains an ORF on the viral complementary strand, spanning from position 6,644 nt to 90 nt, encoding RdRp of 2,184 amino acids (aa) with an estimated molecular weight of 250 kDa (Fig. 1). The CCGaV-Weihai isolate's RdRp has the same number of amino acid and a similar molecular weight. The RdRp encoded by RNA1 in both isolates is similar in size and molecular weight to the RdRp of the first citrus isolate characterized by Navarro et al. (2018). The RdRp characterized here shared 99.82% (GenBank QDK54398), 99.77% (GenBank QDK54399) and 99.68% (GenBank WBB27578) identity with RdRps from other Brazilian CCGaV isolates associated with "Gala", "Mishima" and "Fuji" cultivars, respectively.

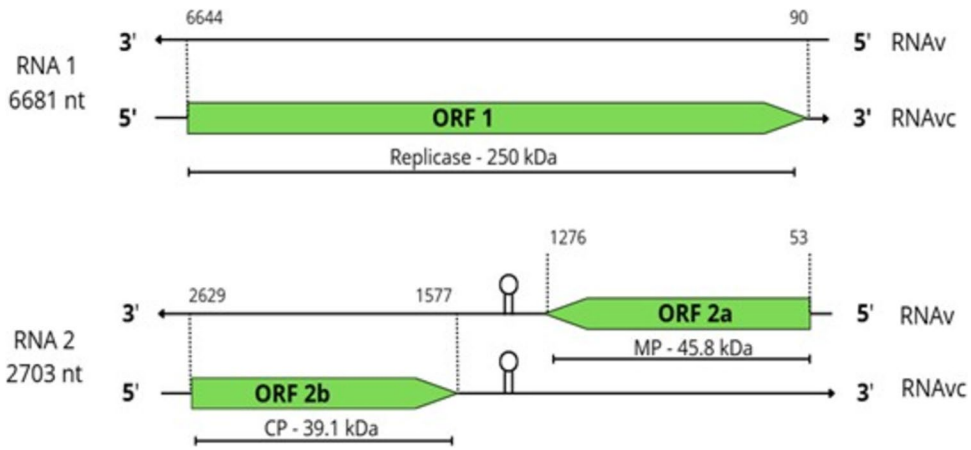

Fig. 1 Genomic organization of the citrus concave gum-associated virus (CCGaV) isolate characterized in this study. Open reading frames (ORFs) are shown as rectangles, and translated proteins are represented by solid lines, with their respective molecular weight (kDa). RdRp, RNA dependent-RNA polymerase (replicase); MP, movement protein; CP, coat protein

RNA2 contains two ORFs in opposite orientations separated by an AU-rich region that form a long hairpin structure (Navarro et al. 2018; Liu et al. 2021). In the isolate characterized here, this region spans positions 1,335 to 1,462. ORF2a spans positions 53 to 1,276, encoding a 407 aa protein of 45.8 kDa, which showed 100% amino acid identity with the movement protein of a Chinese isolate (GenBank UEC79360) found in an apple tree (unidentified cultivar). ORF2b of RNA2 spans positions 2,629 to 1,577, encoding a 350 aa, 39.1 kDa nucleocapsid protein (Fig. 1). The size and molecular weight of these proteins were similar between the apple and citrus isolates. The ORF2b sequence shared 99.71%–100% nucleotide identity with a Brazilian isolate from "Mishima" cultivar (GenBank QDK54403) and 99.43%–99.71% identity with a Czech isolate (GenBank UUR59962) from an apple tree (unidentified cultivar).

Viral populations can experience significant reductions in size and genetic diversity due to factors such as vector transmission, environmental conditions, and the availability of susceptible hosts (Ali et al. 2006). For instance, viruses exposed to genetically uniform hosts often exhibit local adaptation, as observed in graft transmitted viruses (Elena 2017). The high nucleotide identities (>99%) observed in this study, coupled with similar molecular characteristics among the different isolates, may be directly attributable to the movement of plant propagules, resulting in reduced host genetic diversity.

Pairwise nucleotide identity analysis revealed two distinct groups: one comprising citrus (from Italy) CCGaV isolates, and the other comprising apple CCGaV from Brazil, China, USA, Italy and Turkey (Supplementary Fig. 1). Phylogenetic analysis indicated that CCGaV-RNA1 is most closely related to Brazilian isolates from apple cultivars "Fuji", "Gala," and "Mishima" (Nickel et al. 2020; Nickel and Fajardo 2021), and more distantly related to CCGaV-MG764563.1 and CCGaV-NC035759.1, both from citrus trees in Italy (Fig. 2).

In addition to CCGaV, HTS also detected apple hammerhead viroid (AHVd). RT-PCR using specific primers confirmed AHVd in four of the 14 samples sequenced via HTS. The infected samples included the "Santa Maria" pear cultivar (isolate 1, from SC) and the apple rootstocks "G.210" (isolate 2, from SC), "G.213" (isolate 3, from RS), and "G.814" (isolate 4, from RS). For these samples, HTS generated 41 million raw reads (151 nt each). These reads were processed as described above for CCGaV. A contig was assembled and aligned, and exhibiting strong similarity to AHVd. BBmap (Bushnell 2014) was used to map the processed reads to the AHVd reference sequence (NC_028132). A total of 137,613 reads mapped to the complete AHVd sequence, which exhibited over 94% nucleotide identity with AHVd isolates worldwide. The RNA sequence detected in the pear tree was 439 nt in length, while the previously characterized isolate described by Zhang et al. (2014) was 434 nt. Both sequences are predicted to form self-cleaving hammerhead structures. The predicted structure (RNAfold; Hofacker et al. 1994) has a free energy of −183.99 kcal mol^{-1}. Structurally, the molecule can be divided into a stem-like region, encompassing the nucleotides that comprise the hammerhead structures, and a branched, loop-rich region with multiple loops and secondary helices (Fig. 3).

Fig. 2 Phylogenetic relationships of CCGaV isolate G.210 and other CCGaV isolates from different countries and hosts, based on RNA1 (**A**) and RNA2 (**B**) nucleotide sequences. Phylogenetic trees were constructed using the maximum likelihood method in MEGA 10 with 2000 bootstrap replicates. Numbers at nodes indicate bootstrap support values. Apple mosaic virus (ApMV) was used as the outgroup. The blue circle highlights CCGaV-G.210 from *Malus domestica* in SC

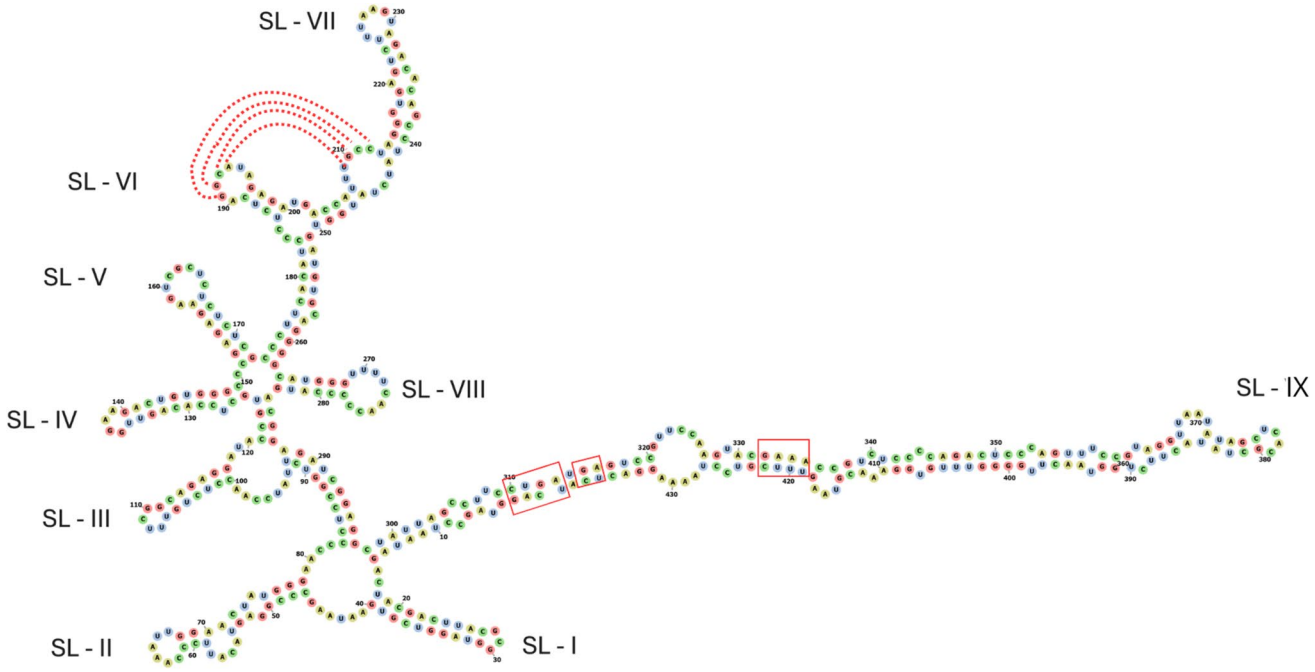

Fig. 3 Predicted secondary structure of the apple hammerhead viroid (AHVd) isolate. The figure displays the modeled secondary structure of the pear sample isolate, generated using Vienna RNAfold software. Predicted stem-loops (SL-I to SL-IX) are indicated. Nucleotides involved in the "kissing loop" interaction and conserved nucleotides are highlighted with red lines and rectangles, respectively

The branched region differs from those of previously characterized AHVd isolates (Zhang et al. 2014; Messmer et al. 2017; Chiumenti et al. 2019). However, the associated hammerhead structures are highly conserved and contain the nucleotides previously described by Zhang et al. (2014), except for a GU pair preceding the first multi-branched loop. In this position, AU nucleotides maintain base pairing, a characteristic also observed in a Brazilian AHVd isolate from apple (Nickel and Fajardo 2021). Nine stem-loops (SLs) were observed in the characterized isolate (Fig. 3), a typical feature of this viroid species (Zhang et al. 2014). SL-VI forms a "kissing loop" interaction with an internal loop through partial sequence dimerization within the loops (Gamache et al. 2017). This dimerization event, occurring between a stem-loop and an internal loop, has been previously observed, although dimerization between two stem loops is also possible (Chiumenti et al. 2019; Canales et al. 2021). Phylogenetic analysis clustered the isolates into two clades: one containing isolates from Europe, North America, Africa, and Asia; and the other containing Brazilian isolates from apple (AHVd - MK947213) and the pear isolate characterized in this study (Supplementary Fig. 2). Despite the lower phylogenetic relatedness between the two Brazilian isolates, likely due to their different hosts, their co-clustering within the same clade suggests a geographical link. Both isolates may share a common ancestor introduced to Brazil through vegetative material. Plant pathogen biogeography is complex (Scott et al. 2019). Landscape changes and the movement of host material, particularly infected plant material, are increasingly leading to the emergence of new host-pathogen associations (Wingfield et al. 2016; Paini et al. 2016). Plant protection authorities are tasked with preventing pathogen dispersal while maintaining trade between geographically separated markets with limited and inconsistent data on species biogeography (Bradley et al. 2012; Wingfield et al. 2016). Trade in biological material is largely data-driven, but current practices often do not sufficiently address the diversity of unknown pathogens in regions with limited data (Scott et al. 2019).

To determine relationships between AHVd isolates, pairwise alignments of the HTS-derived sequence were performed using the MUSCLE in SDT1.3 (Muhire et al. 2014; Roossinck et al. 2015). The AHVd isolate obtained in this study showed the highest identity (95%) with a Brazilian isolate, and high similarity (ranging from 86% to 95%) with isolates from the Czech Republic, Japan, Tunisia, the USA, Canada, and Spain (Supplementary Fig. 3). These results indicate a global distribution of AHVd, likely influenced by host plant trade and agricultural dissemination, important factors in the spread of plant viruses and viroids to new areas (Anderson et al. 2004; Elena 2017). The high degree of identity between isolates from geographically distant countries suggests a recent common origin or multiple introductions from a shared ancestor, as

observed by Kraberger et al. (2018) for faba bean necrotic yellows virus isolates in Spain and Tunisia.

Virus testing results for 53 apple and pear samples (14 previously mentioned and 39 additional samples) from five municipalities in RS and SC are detailed in Supplementary table 2. The extent of infection varied across the municipalities. Nevertheless, ASPV (64.15%) and ASGV (62.3%) were significantly predominant in all sampled locations, with a total of 42 positive samples (Supplementary table 2). While ASPV, ASGV, and ACLSV are latent viruses known to be widespread globally, ACLSV was detected in only four of the analyzed samples. The high prevalence of these viruses in the collected samples is not unexpected, given their established presence in various parts of the world, including Brazil (Nickel et al. 2001; Nickel and Fajardo 2021), as discussed by (Xiao et al. 2022).

First identified in Italy (Navarro et al. 2018) and originally linked to citrus plants, CCGaV has subsequently been found to be highly prevalent in apple trees in the United States and, more recently, in the Brazilian state of RS (Wright et al. 2020; Nickel and Fajardo 2021). In the present study, CCGaV was detected in 16 samples, 13 of which were from SC, marking the first report of the virus in this state and highlighting its continued spread within Brazil. Although independent introduction of vegetative material in the states of SC and RS cannot be excluded. AHVd, previously detected in apple trees in Brazil (Nickel et al. 2021), was identified in 10 samples: nine from apples and one from pears. This finding represents the first global record of AHVd in pear. It is important to note that of the 53 samples collected, only 10 were from pear trees (all from the municipality of São Joaquim, SC), and these 10 were infected with AHVd, ASPV, and ASGV.

Forty-five of the tested samples showed evidence of infection: 12 with single infections and 33 with co-infections (Supplementary Table 2). Within the co-infected group, 23 samples were simultaneously infected with both ASPV and ASGV, demonstrating the prevalence of this particular viral co-infection. Furthermore, 18 samples exhibited co-infection with three or more viruses and one viroid. Regarding the pear samples, two showed co-infection, three were positive for single infections, and five were negative for all analyzed viruses and viroids. Decline of apple trees is recurrent in the last decades, generally associated with multiple virus infections, and the symptoms include low vigor, lack of terminal growth, die back of central leaders, small fruits, yield reduction, and reduction or ausence of active roots (Nickel et al. 2020; Nickel and Fajardo 2021). Apple growers have reported symptoms of decline in some combinations of scion and rootstock in southern Brazil. This could be related to the mixed infections presented in this study, which in many cases include CCGaV, which has already been associated with symptoms of decline by other authors (Navarro et al. 2018; Nickel et al. 2020; Nickel and Fajardo 2021; Nickel et al. 2023).

Another important aspect of this study is the detection of viruses and viroids in scions and rootstocks from nurseries (Supplementary Table 2), which once again demonstrated the importance of using virus-free propagation material and recurrent indexing that should be applied to the plant material present in the nurseries. In this way, it can be ensured that high quality material is available to growers in terms of virus and viroids infections.

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Authors' contributions Conceptualization: VMS, GCM and FNS; Methodology: VMS, SCN, PADO, GCM, TVMF, DRB, and FNS; Formal analysis and investigation: VMS, SCN, and PADO; Writing - original draft preparation: VMS and FNS. Writing – review, and editing: GCM, TVMF, DRB, LR, AB, and FNS. Funding acquisition: GCM, LR, AB, and FNS; Resources: GCM, LR, and FNS; Supervision: FNS.

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Data availability All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics declaration Not applicable.

Conflict of interest The authors declare no conflict of interest.

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