microbial biotechnology

Grapevine virome and production of healthy plants by somatic embryogenesis

Floriana Nuzzo,[1,†] Amedeo Moine,[1,†] Luca Nerva,[1,2,†]
Chiara Pagliarani,[1] Irene Perrone,[1] Paolo Boccacci,[1]
Ivana Gribaudo,[1] Walter Chitarra[1,2] and
Giorgio Gambino[1] (iD)

[1]Institute for Sustainable Plant Protection, National
Research Council (IPSP-CNR), Torino, Strada delle
Cacce 73, Torino, 10135, Italy.
[2]Council for Agricultural Research and Economics –
Research Centre for Viticulture and Enology CREA-VE,
Via XXVIII Aprile 26, Conegliano, 31015, Italy.

Summary

Grapevine (*Vitis* spp.) is a widespread fruit tree hosting many viral entities that interact with the plant modifying its responses to the environment. The production of virus-free plants is becoming increasingly crucial for the use of grapevine as a model species in different studies. Using high-throughput RNA sequencing, the viromes of seven mother plants grown in a germplasm collection vineyard were sequenced. In addition to the viruses and viroids already detected in grapevine, we identified 13 putative new mycoviruses. The different spread among grapevine tissues collected in vineyard, greenhouse and *in vitro* conditions suggested a clear distinction between viruses/viroids and mycoviruses that can successfully be exploited for their identification. Mycoviruses were absent in *in vitro* cultures, while plant viruses and viroids were particularly accumulated in these plantlets. Somatic embryogenesis applied to the seven mother plants was effective in the elimination of the complete virome, including mycoviruses. However, different sanitization efficiencies for viroids and grapevine pinot gris virus were observed among genotypes. The absence of mycoviruses in *in vitro* plantlets, associated with the absence of all viral entities in somaclones, suggested that this regeneration technique is also effective to eradicate endophytic/epiphytic fungi, resulting in gnotobiotic or pseudo-gnotobiotic plants.

Introduction

Grapevine (*Vitis* spp.) is one of the most important and widespread fruit trees in the world. In the last 15 years, the availability of a reliable draft genome (Jaillon *et al.*, 2007), the sequencing of several genotypes (Liang *et al.*, 2019), a deep -omics characterization (Adam-Blondon *et al.*, 2016) associated with genetic transformation, and functional genomics studies (Dalla Costa *et al.*, 2017), elevated grapevine to the rank of model species for fruit trees. For this purpose, it is important to control the pathogens that can infect it and particularly viruses, which are always present in grapevine and can influence the behaviour of the plant. Grapevine can be infected by more than 80 viral entities (Martelli, 2017; Fuchs, 2020), and this number has steadily increased in recent years thanks to high-throughput sequencing (HTS) approaches that have been used as a viral detection method (Maliogka *et al.*, 2018; Bertazzon *et al.*, 2020a). These viral entities are easily transmitted by vegetative propagation, the multiplication method normally used for grapevine and by vectors, such as insects, mites and nematodes (Fuchs, 2020). Consequently, all grapevines cultivated in vineyards and in nurseries are affected by at least one, or more commonly many viruses and viroids, which inevitably influence the growth and environmental responses of the plant. The impact of virus diseases in grapevine can include reduction in plant vigour, yield and fruit quality leading to large economic losses in the whole grapevine agribusiness, including the wine industry (Rienth *et al.*, 2021). Several works showed evidence of the overall best performances of virus-free vines, in terms of yield, bunch size, juice sugar concentration, acidity, phenols and the chemical and sensory quality of wines (Guidoni *et al.*, 1997; Cretazzo *et al.*, 2010; Girardello *et al.*, 2019).

In addition to viruses/viroids infecting grapevine, in recent years, mycoviruses, or fungal viruses, have been largely studied because widespread in all taxonomic

groups of fungi (Nerva and Chitarra, 2021). Infection by mycoviruses can be latent, beneficial or can induce severe symptoms to fungi and indirectly to the plant host (Ghabrial and Suzuki, 2009; Xie and Jiang, 2014). The positive or negative effects that these virus-infected endophytic or epiphytic fungi can induce in host plants make mycoviruses a class of viruses to be carefully considered for gaining a comprehensive understanding of plant–environment interactions. Mycoviruses associated with culturable fungal pathogens, such as *Botrytis cinerea* (Ruiz-Padilla *et al.*, 2021), *Neofusicoccum parvum* (Marais *et al.*, 2021), grapevine trunk pathogens (Nerva *et al.*, 2019a) or non-culturable endophytes/epiphytes (Chiapello *et al.*, 2020a) have also been identified in grapevine by HTS. In the case of mixed samples, containing RNAs from plants and its associated fungi, the distinction among plant and fungal viruses can be difficult (Chiapello *et al.*, 2020b; Silva *et al.*, 2021). Therefore, for metagenomics analyses of plant samples, it would be useful to establish some biological parameters for a proper identification of these two virus categories.

Studies on grapevine have generally demonstrated a great influence of the genotype on the results obtained due to the high level of heterozygosity of the species (Figueroa-Balderas *et al.*, 2019). Indeed, the genotype represents a key factor to be considered before, during and after an experiment, while the virome or the microbiome of the plant has been erroneously rarely considered. The best way to standardize an experiment and use grapevine as a model species is to start with plants with a homogeneous virome or ideally free from viral entities (Gilardi *et al.*, 2020). The standard methods for obtaining virus-free grapevine plants include thermotherapy, chemotherapy, cryotherapy and meristem culture, which show different sanitation efficiencies based on the virus and plant genotype (Maliogka *et al.*, 2015). More in detail, the application of these methods is often ineffective in producing healthy plants, since viroids (Gambino *et al.*, 2011) and some widespread viruses, such as grapevine rupestris stem pitting-associated virus (GRSPaV), are very difficult to be eliminated (Gribaudo *et al.*, 2006; Turcsan *et al.*, 2020). Somatic embryogenesis, that is, non-zygotic embryo formation from somatic cells, is the most used method for grapevine regeneration following genetic transformation in functional genomic studies (Martinelli and Gribaudo, 2009). Moreover, over the years, somatic embryogenesis has been proved to be an effective strategy for the production of virus/viroid-free plants from different grapevine genotypes with percentages close to 100% (Goussard *et al.*, 1991; Gambino *et al.*, 2006). However, in recent years, more effective detection methods, such as HTS, have shown that some residual infections can persist even in regenerated somaclones (SCs; Turcsan *et al.*, 2020).

Based on the above, we characterized the complete virome of seven mother plants (MPs) of grapevine (*Vitis vinifera* and rootstocks), maintained in a germplasm collection, using the RNA sequencing method. We evaluated the presence of these viral entities in different organs (leaf and wood), in the vineyard, in pots after vegetative propagation, and in *in vitro* culture. Furthermore, for the first time, we assessed the effectiveness of somatic embryogenesis to produce grapevine plants that are free of mycoviruses, in addition to plant viruses and viroids. Most of the SCs were virus/viroid/mycovirus-free, thus suggesting the elimination of endophytic or epiphytic fungi that host the mycoviruses and the generation of gnotobiotic or pseudo-gnotobiotic plants with a reduced microbiome complexity than plants grown in the field.

Results and discussion

Characterization of viruses and viroids infecting grapevine mother plants

The viromes of seven MPs used for the induction of somatic embryogenesis were analysed by RNA-seq. The RNA was mixed in two libraries, with 'Sangiovese', 'Cabernet Sauvignon', '110 Richter', and *Vitis rupestris* in library #6 and 'Nebbiolo', 'Chardonnay', and 'Brachetto' in library #7. The identified viral entities and the number of associated reads are reported in Tables 1, S1 and S2. Overall, ten viruses and two viroids that were previously reported in grapevine were identified in the two RNA-seq libraries. The sequencing data were confirmed by RT-qPCR, further attesting the excellent reliability of the two techniques and the correct association between viruses/viroids and MP was carried out. The MPs were collected in the same germplasm collection vineyard but had different origins, therefore justifying the different viromes observed among the plants. 'Sangiovese' and 'Cabernet Sauvignon' were infected by most of the known plant viruses/viroids, while the rootstocks '110 Richter' and *V. rupestris* hosted less viral entities. GRSPaV and grapevine pinot gris virus (GPGV) were the only two viruses that infected all seven MPs. GRSPaV is a member of the genus *Foveavirus*, belonging to the Betaflexiviridae family, associated with a disorder named as 'Rugose Wood complex' (Martelli, 2017; Fuchs, 2020) and usually found in a latent state in *V. vinifera* cultivars. GRSPaV is one of the most prevalent viruses of grapevine (Meng and Gonsalves, 2003), and as expected, all MPs were infected by this virus. Conversely, the ubiquitous presence of GPGV was surprising. GPGV, a *Trichovirus*, belonging to the Betaflexiviridae family, is the presumptive causal agent of the 'Grapevine Leaf Mottling and Deformation' disease (Saldarelli *et al.*, 2015) and is probably spread by the

Table 1. Identification of viruses and viroids in the two RNA-seq libraries containing the pools of grapevine mother plants (MPs). In addition to the number of specific virus/viroid reads identified in each library, the correct association between virus/viroid and MP carried out by RT-qPCR is reported.

Virus/viroid	RNA-seq		RT-qPCR						
	Reads library #6	Reads library #7	SG	CAB	110R	RUP	NE	CH	BRA
Grapevine leafroll-associated virus 2	5905			+					
Grapevine leafroll-associated virus 3	47268		+	+					
Grapevine leafroll-associated virus 4	4522		+						
Grapevine rupestris stem pitting-associated virus	88481	284744	+	+	+	+	+	+	+
Grapevine pinot gris virus	92920	314614	+	+	+	+	+	+	+
Grapevine virus A	5667			+					
Grapevine virus D	1001		+						
Grapevine fanleaf virus	5546			+					
Grapevine virus T		13443						+	
Grapevine fleck virus	11584	48136	+	+			+		+
Grapevine yellow speckle viroid 1	14777	47719	+			+	+	+	+
Hop stunt viroid	28769	33172	+	+	+	+	+	+	+

+, positive sample; 110R, '110 Richter'; BRA, 'Brachetto'; CAB, 'Cabernet Sauvignon'; CH, 'Chardonnay'; NE, 'Nebbiolo'; RUP, *Vitis rupestris*; SG, 'Sangiovese'.

grapevine eriophyid mite *Colomerus vitis* (Malagnini *et al.*, 2016). It was first identified in vineyards in north-east Italy (Giampetruzzi *et al.*, 2012), but it is currently distributed in many viticultural areas of the world. This virus is still not considered common in northwest Italy, although our data suggest that it is also likely widespread in this geographical area and, notably, that is able to infect grapevine genotypes that have been rarely associated with it, such as 'Nebbiolo' and 'Brachetto' (Nerva *et al.*, 2019b). Furthermore, GPGV showed a rapid spread in the vineyard starting from a few infected plants; this means that, in a few years, it could easily invade new environments (Bertazzon *et al.*, 2020b).

The causal agent of 'Fleck disease', grapevine fleck virus (GFkV, genus *Maculavirus*, family Tymoviridae), is distributed worldwide and generally latent in European grape cultivars and in many rootstocks. In the present study, this virus was detected in four *V. vinifera* genotypes (Table 1). Viruses associated with most harmful and economically impacting grapevine diseases, such as *Ampelovirus* (grapevine leafroll-associated virus-2, −3 and −4), responsible for 'Grapevine leafroll disease', grapevine virus A (GVA, *Vitivirus*) associated with 'Kober stem grooving', another syndrome of the 'Rugose Wood complex', and grapevine fanleaf virus (GFLV, *Nepovirus*), responsible for 'Grapevine infectious degeneration' (Martelli, 2017), were found only in 'Sangiovese' and 'Cabernet Sauvignon', which were the most infected by viruses among the MPs (Table 1). Finally, two minor viruses spread in the northwest of Italy, grapevine virus T (GVT, *Foveavirus*) and grapevine virus D (GVD, *Vitivirus*), both associated with the 'Rugose Wood complex' (Jo *et al.*, 2017; Martelli, 2017), were identified only in 'Chardonnay' and 'Sangiovese' respectively.

In addition to viruses, all plants were infected by the hop stunt viroid (HSVd), and in five of the seven MPs we found the grapevine yellow speckle viroid 1 (GYSVd-1). Both viroids are very common in this viticultural area, as previously reported (Gambino *et al.*, 2014).

Identification of new putative mycoviruses infecting grapevine mother plants

In addition to plant viruses and viroids identified in the metatranscriptomic samples, other viral entities putatively associated with mycoviruses were identified. However, in the germplasm collection vineyard, several antifungal treatments with pyraclostrobin, metiram, copper and sulfur were applied during the season to limit the development of downy and powdery mildews and other fungi, and no symptoms of fungal/oomycete infections were observed. Therefore, the epiphytic fungal community and the mycoviruses associated with it had been influenced by these antifungal treatments. Seven of the putative mycoviruses detected (Table S1) had a very high sequence homology with mycoviruses that were previously identified, such as Sclerotinia sclerotiorum virga-like virus 1 (Jia *et al.*, 2021), or mycoviruses identified in grapevine, such as Gremmeniella abietina RNA virus MS1, Plasmopara viticola lesion-associated vivivirus 1 and Erysiphe necator-associated narnavirus 33 associated with grapevine downy and powdery mildew lesions (Chiapello *et al.*, 2020a) and Neofusicoccum parvum mitovirus 3 associated with grapevine trunk disease (Marais *et al.*, 2021). In the metatranscriptome, we also found three RNA segments corresponding to grapevine-associated jivivirus 1 (GaJV1), which was originally identified in *Plasmopara viticola*-infected *grapevine samples*

and that is considered a plant virus (Chiapello et al., 2020b). Nonetheless, it cannot be ruled out here that GaJV1 is instead associated with fungal endophytes (Silva *et al.*, 2021). Thirteen new viruses associated with the tissue of grapevine plants were also identified (Table S2): two of them belonged to the double-stranded RNA (dsRNA) clade and 11 to the positive single-stranded RNA (+ssRNA) clade. Interestingly, most of the viruses detected here belonged to a group of viruses that host mycoviruses, such as the *Botourmiaviridae* family. In addition, five incomplete sequences likely belonging to mycoviral entities were observed (Table S2).

RdRPs belonging to the dsRNA clade showed similarities with other RdRPs of partitiviruses (Fig. 1). The first sequence found the highest similarity to Plasmopara viticola lesion-associated partitivirus 8 (QHD64792.1). The phylogenetic analysis of the RdRP revealed that the new virus belonged to the *Gammapartitivirus* genus. Here, we proposed to name it Grapevine-associated partitivirus 3 (GaPV3) (two other viruses named Grapevine-associated partitivirus 1 and 2 were previously reported (NCBI accessions MW648510.1 and MW648511.1). A second RdRP encoding segment showed the highest similarity to Plasmopara viticola lesion-associated partitivirus 5 (QHD64809.1). Phylogenetic analysis of the RdRP revealed that the new virus belongs to the *Betapartitivirus* genus, and for this reason, we proposed to name it Grapevine-associated partitivirus 4 (GaPV4). Together with the two RdRPs, we also identified two sequences encoding putative capsid proteins (CP) of partitiviruses. The first one (TRINITY_DN102118_c0_g4_i2) was only a partial sequence showing similarity to CP of Talaromyces marneffei partitivirus 1 (KM235304.1). Looking at the mapping data (Table S2), it seemed that this CP fragment was associated with GaPV3. On the contrary, qPCR analysis was able to detect this CP fragment also in a sample where the GaPV3 RdRP was not present. The second contig (TRINITY_DN117791_c0_g1_i1) was a complete sequence coding for a 74.94 kDa protein with a very low homology with the CP of Ceratocystis polonica partitivirus (AY260757.1), and was detected, following read remapping and qPCR analysis, only when the RdRP of GaPV4 was present (Table 2 and Table S2).

The 11 viruses belonging to the +ssRNA clade were divided among the Lenarviricota phylum (6), the Tombusviridae family (1), the Martellivirales order (3) and one sequence outside form of the known viral groups (1). Among the six viruses of the Lenarviricota phylum, four of them were similar to Botourmiaviridae (Ayllón *et al.*, 2020) species. Specifically, the phylogenetic analysis suggested that two viruses belonged to the *Magoulivirus* genus, one to the *Scleroulivirus* genus and one to the *Penoulivirus* genus (Fig. 2). The four viruses were

named Grapevine-associated ourmia-like viruses 1, 2, 3 and 4 (GaOlV1, GaOlV2, GaOlV3 and GaOlV4). The other two viruses of this phylum showed phylogenetic placement in the *Mitovirus* genus and in a clade close to the *Narnavirus* genus. For this reason, we named them Grapevine-associated mitovirus 1 (GaMV1) and Grapevine-associated narnavirus 1 (GaNV1) respectively.

Among the RdRPs showing similarity to Martellivirales, we identified a sequence that presented a phylogenetic relationship with already known fungal viruses. The other two RdRPs showed hints of phylogenetic links with Martellivirales, however, as reported in Fig. 3, the phylogenetic analysis suggested that they likely belonged to a new and previously undescribed group. Due to the nature of the three viruses, we proposed the names Grapevine-associated RNA virus 13, 14 and 16 (GaRV13, GaRV14 and GaRV16). Similarly, two other sequences displayed similarities with the *Tombusviridae* family (Fig. 3). Specifically, the first one fell within a group of RdRPs ascribed to mycoviruses or to sequences previously identified by metagenomics approaches (Marzano and Domier, 2016; Nerva *et al.*, 2019a; Jo *et al.*, 2020). This sequence was named Grapevine-associated tombus-like virus 5 (GaTlV5). The second sequence referred to the *Tombusviridae* family, although the relationship was weaker. For this reason, we propose to call it with a more general name: Grapevine-associated RNA virus 15 (GaRV15).

In addition to the previously described viral sequences, we identified three contigs that likely belonged to viral entities but that were incomplete and could not be analysed for phylogeny due to the lack of conserved motifs (e.g. GDD for RdRPs). The first incomplete contig (TRINITY_DN106149_c0_g1_i1) encoded a protein with 88.51% identity to Actinidia yellowing virus 2 and was renamed Grapevine-associated RNA virus 17 (GaRV17). The second contig (TRINITY_DN33323_c0_g1_i1) encoded a protein with 62.99% identity to Plasmopara viticola lesion-associated mononegaambi virus 7 and was renamed Grapevine-associated RNA virus 18 (GaRV18). The third contig (TRINITY_DN83177_c0_g1_i2) encoded a portion of an RdRP probably ascribed to the *Botourmiaviridae* family (named Grapevine-associated ourmia-like virus 5). Different bioinformatics approaches were used to recover longer contigs or near complete genomes, but unfortunately, we were unable to further extend these sequences.

Putative mycoviruses were associated with specific MPs by RT-qPCR. A complete congruence between RNA-seq and RT-qPCR results was observed (Table 2), as described above for plant viruses, thus confirming that mixing multiple samples together prior to RNA sequencing is a less expensive and reliable strategy
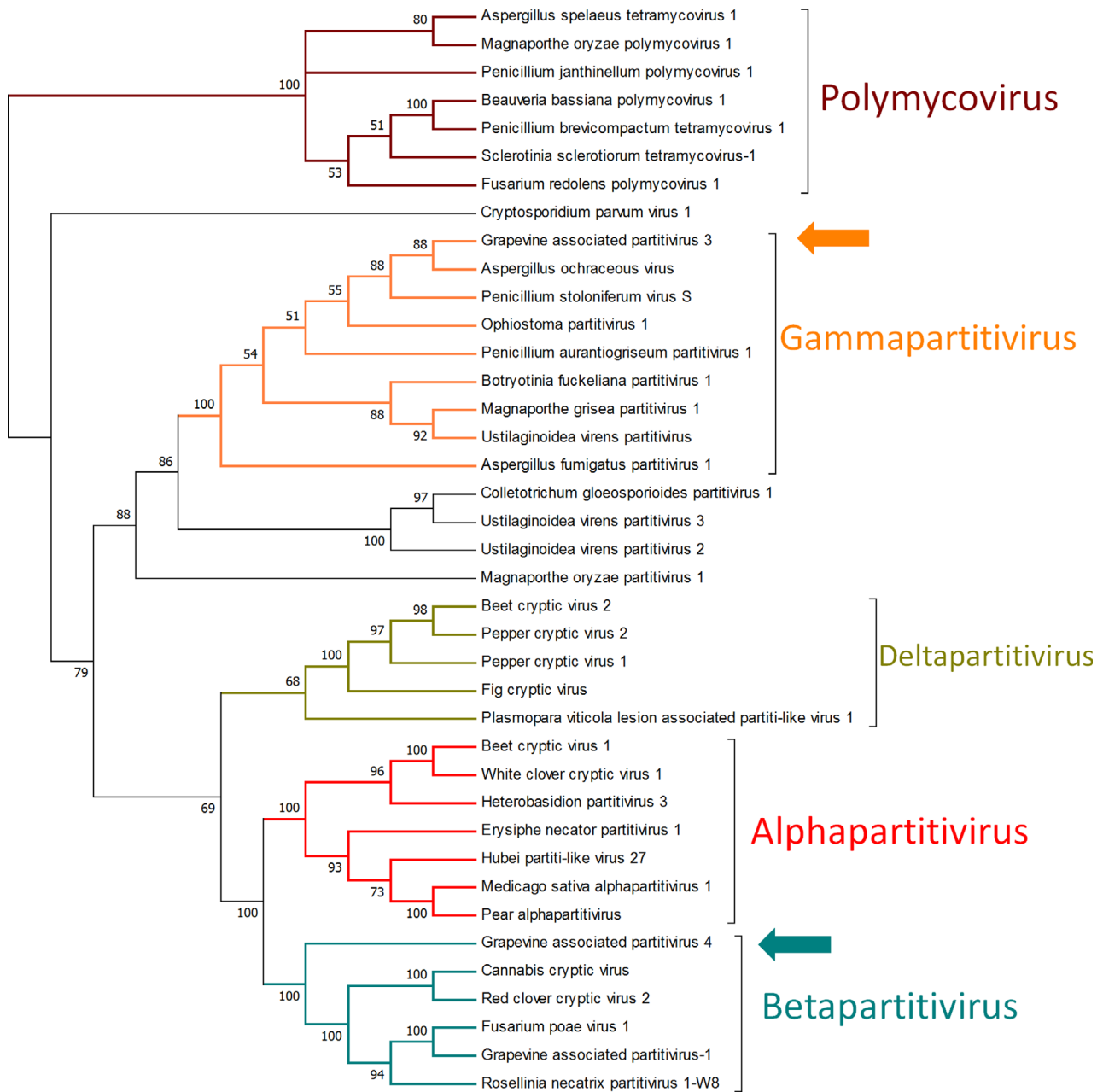
Fig. 1. Phylogenetic analysis of a selected number of RdRP sequences from dsRNA clade closely related to Grapevine-associated partitivirus 3 and 4. The maximum likelihood (ML) methodology was used to infer the best tree. Arrows indicate the viruses assembled in this work. The percentage of bootstrap values is indicated below each node.

allowing mycovirus detection. The distribution of these putative mycoviruses was very variable within the MPs: 'Brachetto' and 'Nebbiolo' contained the majority of mycoviruses (9 and 10 respectively), while 'Sangiovese' and 'Cabernet Sauvignon' the least (only two species). Although cultivated in the same vineyard, the MPs displayed a very variable mycovirome, even after 10 years of cultivation, as reported above for the plant viruses. The variability in the mycovirome suggested that the

population of endophytic and/or epiphytic fungi hosting these mycoviruses was likely highly variable among genotypes in the same vineyard, and that the time span here considered (10 years from planting) was not enough for the exchange of endo/epiphytes between plants, even though they were planted closely in the same vineyard (Kraus *et al.*, 2019). Furthermore, year, location, cultivar and pre-existing microbial communities could impact on the composition of the grapevine

Table 2. Identification of putative mycoviruses in the two RNA-seq libraries containing the pool of grapevine mother plants (MPs). In addition to the number of specific mycovirus reads identified in each library, the correct association between mycovirus and MP carried out by RT-qPCR was reported.

Mycovirus	RNA-seq		RT-qPCR						
	Reads library #6	Reads library #7	SG	CAB	110R	RUP	NE	CH	BRA
Gremmeniella abietina RNA virus MS1		96							+
Grapevine-associated jivivirus 1	119	1024		+	+	+	+	+	+
Sclerotinia sclerotiorum virga-like virus 1		924						+	+
Neofusicoccum parvum mitovirus 3	193			+					
Plasmopara viticola lesion-associated vivivirus 1		177					+		+
Erysiphe necator-associated narnavirus 33	861					+			
Erysiphe necator-associated ourmia-like virus 101	318				+				
Grapevine-associated narnavirus 1	233					+			
Grapevine-associated mitovirus 1		273					+	+	
Grapevine-associated tombus-like virus 5		1335							+
Grapevine-associated partitivirus 3	118		+						
Grapevine-associated partitivirus 4		10670							+
Grapevine-associated ourmia-like virus 1	288				+				
Grapevine-associated ourmia-like virus 2		277					+		+
Grapevine-associated ourmia-like virus 3	563					+			
Grapevine-associated ourmia-like virus 4		88					+		
Grapevine-associated ourmia-like virus 5	184					+			
Grapevine-associated RNA virus 13	189				+				
Grapevine-associated RNA virus 14		1300					+	+	+
Grapevine-associated RNA virus 15		1011					+		+
Grapevine-associated RNA virus 16	759			+					
Grapevine-associated RNA virus 17		62					+		
Grapevine-associated RNA virus 18		73					+		
Ceratocystis polonica partitivirus / Grapevine-associated partitivirus 4		1952							+
Talaromyces marneffei partitivirus-1/ Grapevine-associated partitivirus 3	181	106	+					+	

+, positive sample; 110R, '110 Richter'; BRA, 'Brachetto'; CAB, 'Cabernet Sauvignon'; CH, 'Chardonnay'; NE, 'Nebbiolo'; RUP, *Vitis rupestris*; SG, 'Sangiovese'.

mycobiota (Kernaghan *et al.*, 2017; Nerva *et al.*, 2019b; Cureau *et al.*, 2021).

Virome distribution is different in grapevine organs

The distribution of viruses and viroids in grapevine is notoriously variable during the vegetative season and in different organs (Vega *et al.*, 2011; Gambino *et al.*, 2012; Krebelj *et al.*, 2015; Chitarra *et al.*, 2018; Shabanian *et al.*, 2020), and they are easily transmissible by vegetative multiplication and *in vitro* cultures (Martelli, 2017). Conversely, the behaviour of mycoviruses closely linked to the fungal community is still poorly understood. Starting from the virome of seven MPs, obtained from a mix of different tissues (wood, leaf and *in vitro* plantlets), the presence of viruses/viroids and mycoviruses was verified in the wood and leaves collected from vineyards and in the wood and leaves collected from potted plants after vegetative multiplication by woody cuttings and *in vitro* cultures.

As expected, all viruses and viroids identified in the seven MPs (Table 1) were also detected by RT-qPCR in winter woods and in leaves collected in the vineyards in July, while putative mycoviruses were detected only in wood and not in leaves collected in the vineyard. The woody cuttings of all seven MPs were planted in pots and placed in greenhouse in March, and after 1 year, the analysis conducted on the wood and leaves collected in these conditions confirmed the results obtained from the samples obtained from the vineyard, that is, (i) the complete virome was again detected in the wood of potted plants; (ii) only viruses and viroids infected the leaves, confirming the absence of all putative mycoviruses in these samples. Finally, green buds of MPs were sterilized and placed in *in vitro* culture for the development of new plantlets, and only viruses and viroids were transmitted to the *in vitro* plantlets with an efficiency of 100%, whereas no putative mycoviruses were found.

These findings suggested some interesting considerations. The putative mycoviruses identified in this work exclusively derived from woody tissues, since no RT-qPCR amplification was observed in leaf samples. Most likely, most of the fungi hosting these mycoviruses were endophytes of the wood. Nonetheless, it cannot be excluded that some of them were epiphytes, as the
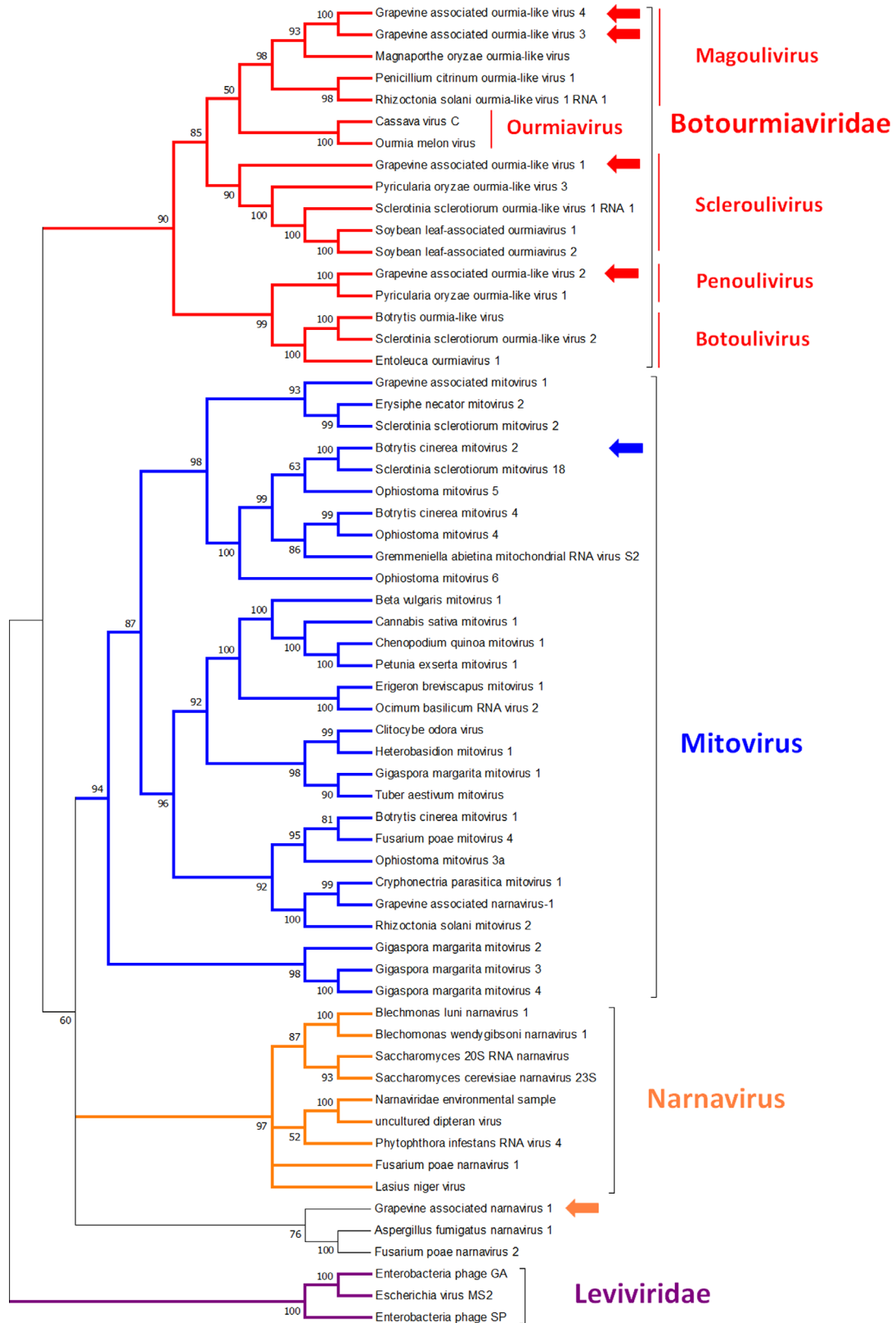
Fig. 2. Phylogenetic analysis of a selected number of RdRP sequences from +ssRNA clade closely related to *Lenarviricota* phylum. The maximum likelihood (ML) methodology was used to infer the best tree. Arrows indicate the viruses assembled in this work. Bootstrap values are indicated as percentage below each node.

Fig. 3. Phylogenetic analysis of a selected number of RdRP sequences from +ssRNA clade closely related *Tombusviridae* family and the *Martellivirales* order. The maximum likelihood (ML) methodology was used to infer the best tree. Arrows indicate the viruses assembled in this work. Bootstrap values are indicated as percentage below each node.
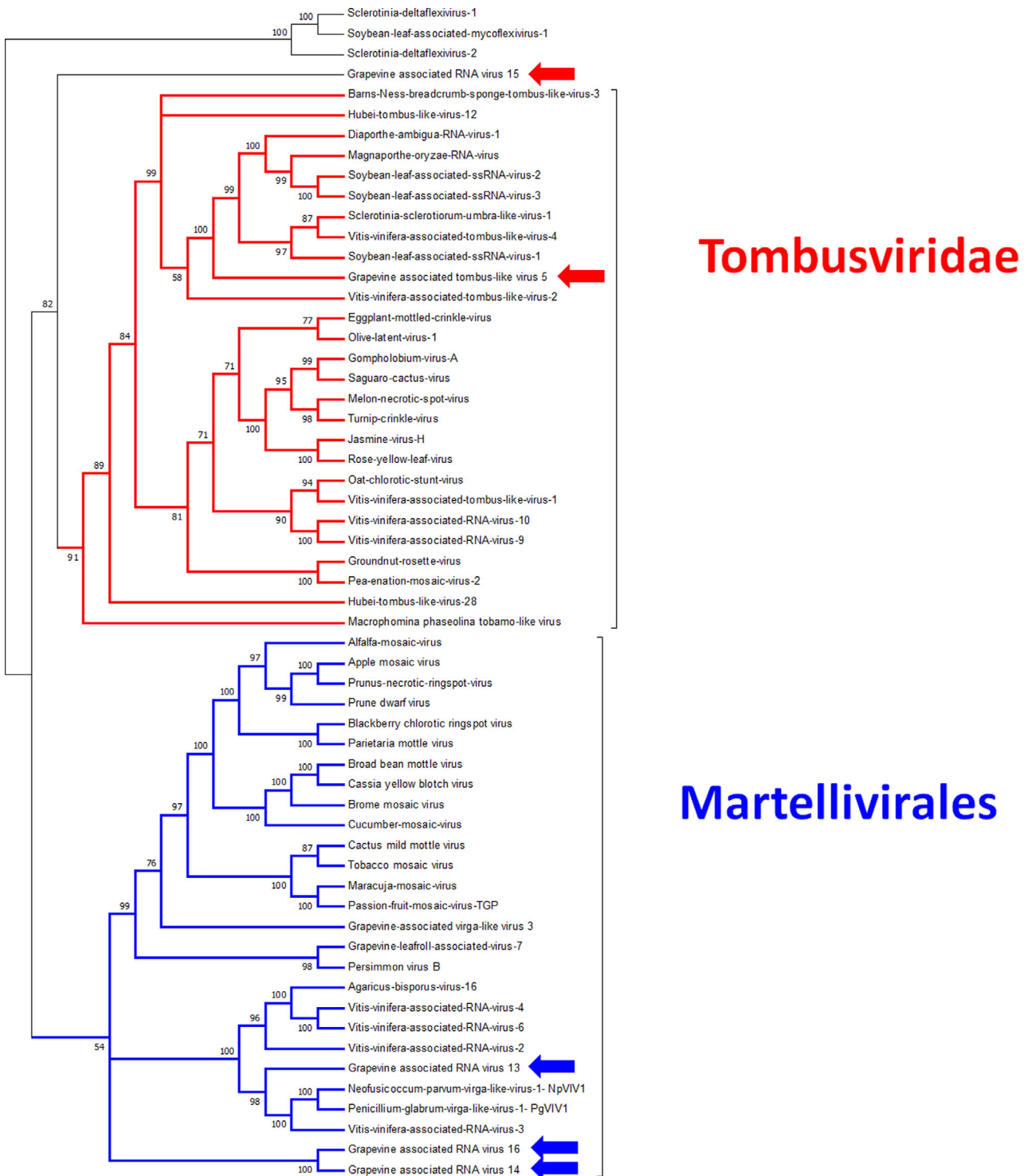
wood was not sterilized externally before or after material collection. The absence of mycoviruses in leaves and their presence in wood of the same plants implied a non-uniform distribution of these mycovirus-hosting endophytes within different organs of the plant. This hypothesis was supported by previous studies carried

out on grapevine and other species, in which the myco-biome was reported to be different among the various organs of the plant due to the slow colonization rate of endophytes and their tissue specificity (Hofstetter *et al.*, 2012; Qi *et al.*, 2012; Del Frari *et al.*, 2019; Fan *et al.*, 2020). However, some of those mycoviruses (Gremme-niella abietina RNA virus MS1, Plasmopara viticola lesion-associated vivivirus 1 and Erysiphe necator-associated narnavirus 33) were previously identified in leaves in presence of *P. viticola* infection (Chiapello *et al.*, 2020a). Under the conditions here adopted, both in the vineyard and in the greenhouse, several antifungal treatments were applied during the season, and no symptoms of fungal/oomycete infections were present during the sampling time. Therefore, we could also suggest that the viruses both newly described and previously reported, belong to the fungal endophytes inhabiting grape tissues (Nerva *et al.*, 2019a). This was more evident for those viruses that were detectable in wood samples, where the possibility of identifying actively growing *P. viticola* was quite limited. Furthermore, analysis of the RNA-seq data for *P. viticola* transcripts confirmed the absence of sequences belonging to this pathogen, strengthening the hypothesis of endophyte-associated viruses.

The putative mycoviruses identified in this work from grapevine wood after winter pruning were associated with fungi that seemed to have little mobility in other organs, but that were transmitted with extreme efficiency in the case of vegetative multiplication by hardwood cuttings. Mycoviruses can, therefore, be considered an ecological marker for fungal spread. For instance, the fungal community presents in the plants grown in nurseries integrated with the mycobiome of the vineyard could be easily evaluated by studying the spread of mycoviruses (Degola *et al.*, 2021).

Finally, there was a clear distinction between viruses/viroids and mycoviruses in *in vitro* plants. As already known, viruses/viroids are very easily transmitted and maintained in *in vitro* plants, whereas all mycoviruses identified in this work in the seven MPs were not. This suggested that, in *in vitro* conditions, the original plant mycobiome was profoundly altered. Moreover, the presence or absence of a virus in *in vitro* plants could facilitate the classification of a new viral entity in the case is unclear whether this virus is associated either with plants or fungi. For example, GaJV1 was originally detected on the surface of grapevine leaves and classified as a plant virus albeit with some doubts (Chiapello *et al.*, 2020b; Silva *et al.*, 2021). In our work, it infected six MPs, but it was never present in *in vitro* plantlets or in leaves from plants cultivated in vineyard and greenhouse, thus, pointing out that it was most likely a mycovirus.

Virus and viroid quantification

The uneven virus/viroid distribution in grapevine during the vegetative season and among different organs is well known; however, scarce information is available about the accumulation of viruses in *in vitro* cultured plants. Using RT-qPCR, we quantified all viruses and viroids identified in this work in woody tissues collected during winter pruning, in leaves sampled in July, and in *in vitro* plantlets. In all genotypes, the winter woody tissues displayed the lowest concentrations of viruses/viroids (Fig. 4 and S1). This result confirmed that, despite the widespread use of wood pruned during wintertime as tissue for viral diagnosis in grapevine, it is always advisable to use different organs, such as leaves in the vegetative period, which accumulate higher levels of viruses and viroids than other tissues (Gasparro *et al.*, 2019; Shabanian *et al.*, 2020; Soltani *et al.*, 2021).

Interestingly, the *in vitro* plantlets accumulated the highest levels of viruses and viroids in all analysed genotypes, representing an ideal tissue type for conducting effective diagnosis throughout the year and potentially for preserving the purity of viruses/viroids over time. In particular, HSVd showed very high levels of accumulation in *in vitro* plants, and in some genotypes ('Sangiovese', 'Nebbiolo' and 'Brachetto') the viroid was concentrated more than 20-fold compared to the levels detected in other plant organs *in vivo* (Fig. 4). Similarly, a higher accumulation was observed in almost all genotypes for the viroid GYSVd-1 and for two viruses, GRSPaV and GPGV. For all remaining viruses, a similar quantity of the virus was observed among leaves collected in the vineyard and leaves from *in vitro* plantlets. However, these virus concentrations were always higher in those samples than in the wood collected during winter pruning (Fig. S1).

Interestingly, looking at the viruses/viroids infecting several MPs, we observed a great variability in their concentration among genotypes, which was evident in all organs, including those from *in vitro* plantlets. For example, very low levels of GRSPaV were observed in the rootstocks '110 Richter' and *V. rupestris*. Similarly, a low level of GYSVd-1 was found in 'Chardonnay', and a low level of GLRaV-3 was observed in 'Cabernet Sauvignon'. However, a direct correlation between the quantities of viruses/viroids detected in wood and those present in leaves from the vineyard or *in vitro* plants was observed only for some genotype-virus combinations: GLRaV-3 in 'Cabernet Sauvignon', GFkV in 'Brachetto', GYSVd-1 in 'Chardonnay' and GRSPaV in '110 Richter' and *V. rupestris*. Essentially, this occurred in genotype-virus combinations showing very low pathogen levels in wood (Fig. 4, S1). In the other combinations, a similar direct
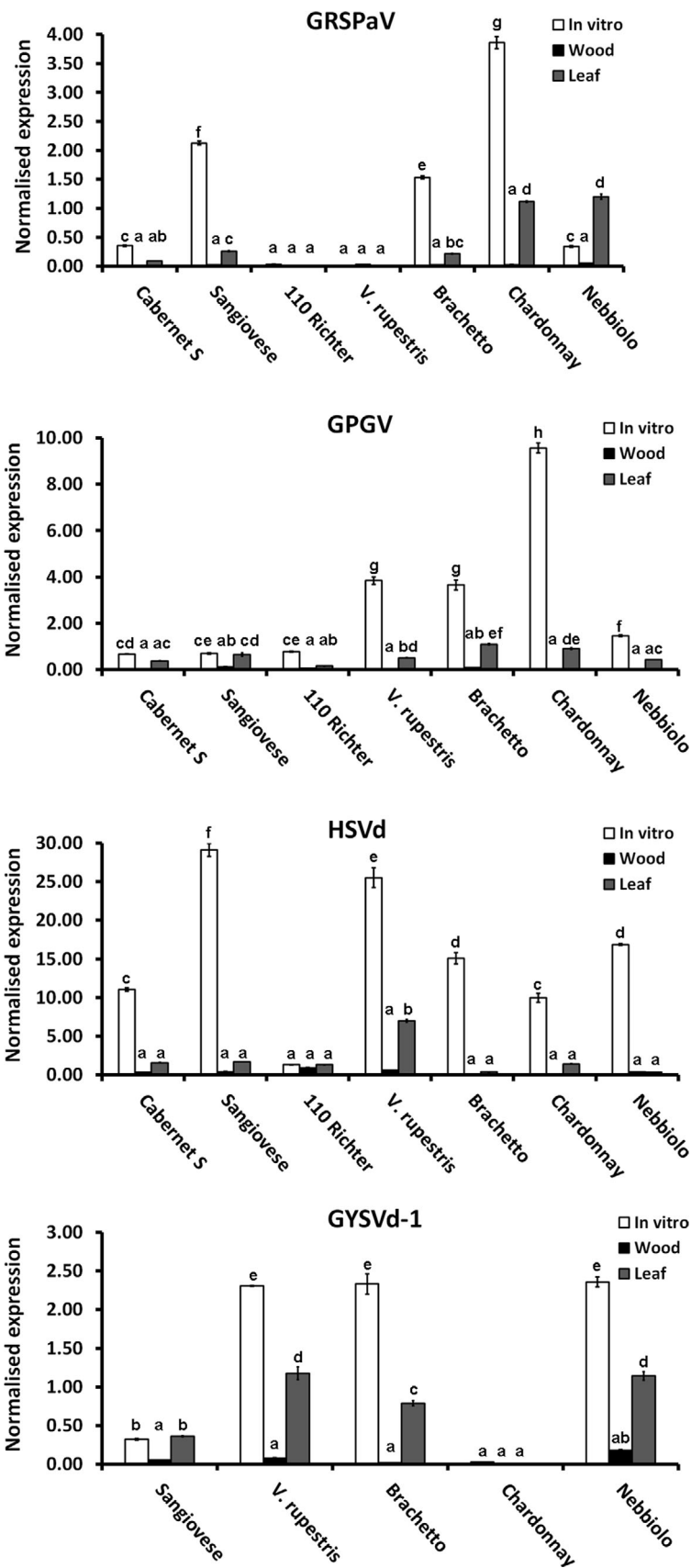
Fig. 4. Quantification of GRSPaV, GPGV, HSVd and GYSVd-1 RNA in wood and leaf collected in vineyard, and in *in vitro* plantlets of 'Sangiovese', 'Cabernet Sauvignon', '110 Richter', *Vitis rupestris*, 'Nebbiolo', 'Chardonnay' and 'Brachetto' as determined by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). RT-qPCR signals were normalized to *VvAct* and *VvUBI* transcripts. Data are presented as the mean ± standard deviation (SD) ($n = 3$). Lowercase letters denote significant differences attested by Tukey's honestly significant difference (HSD) test ($P < 0.05$).

correlation was not observed, supporting the notion that viral multiplication depends on different plant physiological and biological factors and not only on the starting inoculum, and further confirming the presence of specific interactions among viruses and genotypes.

Somatic embryogenesis is an effective tool to produce healthy plants in grapevine

Somatic embryogenesis was performed using immature flowers of the seven MPs, of which the complete virome and mycovirome were determined. As previously reported (Gribaudo *et al.*, 2017), the regeneration efficiency of SCs is strongly dependent on the genotype; in our work, we regenerated one plant from somatic embryogenesis in *V. rupestris* and up to 53 in 'Brachetto'. The viromes of the regenerated SCs were analysed by RNA-seq after depletion of ribosomal RNAs and mixed in five libraries, as reported in Tables S3 and 3. No reads associated with viral entities (virus/viroid/mycovirus) were detected in two libraries, #3 (containing SCs of 'Sangiovese' and 'Cabernet Sauvignon') and #5 ('Brachetto'), thus, suggesting that all plants were healthy from a viral point of view. In libraries #2 ('110 Richter' and *V. rupestris*) and #4 ('Chardonnay'), only reads of HSVd were identified, and in library #3 ('Nebbiolo'), only reads of GPGV and GYSVd-1 were found (Table 3). Putative mycoviruses were not identified in any of the five libraries, either suggesting that the invasion of the floral tissues by the fungal endophytes occurs with difficulty, as reported above for the leaves, or that the embryogenesis process is highly efficient in eliminating the endophytes that host these mycoviruses.

As performed previously for MPs, the association between viruses/viroids and SCs was determined by RT-qPCR, including in the analyses all plants regenerated

from SCs. All regenerated SCs of all genotypes were found to be free of nine of the ten viruses identified in MPs (Table 4). This finding confirmed previous works conducted using other genotypes, in which diagnostic systems less effective than HTS and RT-qPCR were adopted. In particular, GLRaV-2 (Goussard *et al.*, 1991), GLRaV-3 (Goussard *et al.*, 1991; Gambino *et al.*, 2006; Bouamama-Gzara *et al.*, 2017; San Pedro *et al.*, 2017; Malenica *et al.*, 2020), GRSPaV (Gambino *et al.*, 2006; Bouamama-Gzara *et al.*, 2017; Turcsan *et al.*, 2020), GFLV (Gambino *et al.*, 2009; San Pedro *et al.*, 2017; Malenica *et al.*, 2020), GFkV (San Pedro *et al.*, 2017; Malenica *et al.*, 2020; Turcsan *et al.*, 2020), GVA (Gambino *et al.*, 2006; Bouamama-Gzara *et al.*, 2017) and GVT (Turcsan *et al.*, 2020) were efficiently removed from SC. In addition, in the case of GLRaV-4 and GVD, our results represent the first report of sanitation obtained by SC in grapevine (Table 4). For GPGV, we identified only a single SC of 'Nebbiolo' that was still infected, making the sanitation ratio very close to 100% (99.1%), considering all 109 SCs analysed, and 90.9% considering only 'Nebbiolo'.

The sanitation rates for the two viroids HSVd and GYSVd-1 were lower than 100%, although higher success rates were observed in the past (Gambino *et al.*, 2011). We detected three plants still infected by GYSVd-1 in 'Nebbiolo', and eight plants in total infected by HSVd (Table 4). HSVd appeared to be much more difficult to eliminate in rootstocks (six SCs infected in '110 Richter' and one in *V. rupestris*) than in *V. vinifera*; only one SC of 'Chardonnay', out of the 93 SCs of *V. vinifera*, was infected. In the case of viroids, a strong effect of the genotype was evident on the success rate of sanitation. These data agree with a recent work conducted using small RNA-seq and RT-qPCR (Turcsan *et al.*, 2020), but differ from our previous work (Gambino *et al.*, 2011), in

Table 3. Identification of viruses and viroids in the five RNA-seq libraries containing the pools of somaclones (SCs). In addition to the number of specific virus/viroid reads identified in each library, the correct association between virus/viroid and SC carried out by RT-qPCR was reported.

Virus/viroid	RNA-seq reads					RT-qPCR						
	Library #1	Library #2	Library #3	Library #4	Library #5	SG	CAB	110R	RUP	NE	CH	BRA
Grapevine pinot gris virus	41581									+		
Grapevine yellow speckle viroid 1	10874									+		
Hop stunt viroid		19711		1702				+	+		+	

+, positive sample; 110R, '110 Richter'; BRA, 'Brachetto'; CAB, 'Cabernet Sauvignon'; CH, 'Chardonnay'; NE, 'Nebbiolo'; RUP, *Vitis rupestris*; SG, 'Sangiovese'.

Table 4. Incidence of virus and viroid infections in somaclones generated by somatic embryogenesis (SCs).

Virus/viroid	SG[a]	CAB[a]	110R[a]	RUP[a]	NE[a]	CH[a]	BRA[a]	Total[a]
Grapevine leafroll-associated virus 2		0/3						0/3
Grapevine leafroll-associated virus 3	0/4	0/3						0/7
Grapevine leafroll-associated virus 4	0/4							0/4
Grapevine rupestris stem pitting-associated virus	0/4	0/3	0/15	0/1	0/11	0/22	0/53	0/109
Grapevine pinot gris virus	0/4	0/3	0/15	0/1	**1/11**	0/22	0/53	1/109
Grapevine virus A		0/3						0/3
Grapevine virus D	0/4							0/4
Grapevine fanleaf virus		0/3						0/3
Grapevine virus T						0/22		0/22
Grapevine fleck virus	0/4	0/3			0/11		0/53	0/71
Grapevine yellow speckle viroid 1	0/4			0/1	**3/11**	0/22	0/53	3/91
Hop stunt viroid	0/4	0/3	**6/15**	1/1	0/11	**1/22**	0/53	8/109

110R, '110 Richter'; BRA, 'Brachetto'; CH, 'Chardonnay'; CAB, 'Cabernet Sauvignon'; NE, 'Nebbiolo'; RUP, *Vitis rupestris*; SG, 'Sangiovese'. In bold the number of SCs still infected after somatic embryogenesis.
a. Number of infected/number of tested SCs.

which SCs from rootstock were not analysed and the detection methods (RT-PCR) were less efficient. In addition to rootstocks, 'Nebbiolo' was the only *V. vinifera* genotype with several SCs still infected by viroids (GYSVd-1) or viruses (GPGV), despite only 11 plants were obtained from SCs. These outcomes were also confirmed in greenhouse plants of 'Nebbiolo' SCs 1 year after *in vivo* acclimation. Therefore, even within *V. vinifera*, there were differences among genotypes in terms of somatic embryogenesis sanitation efficiency.

The observed difference in the eradication efficiency of different viruses in SCs was most likely related to the ability of the viruses to invade plant tissues and calli; for instance, phloem-limited viruses, such as GLRaV-3 and GVA, had more difficulty in invading callus cells than GFLV (Gambino *et al.*, 2010). This could justify the persistence of GFLV in some SCs, as reported elsewhere (Gambino *et al.*, 2009; San Pedro *et al.*, 2017; Malenica *et al.*, 2020). Viroids move rapidly from cell to cell and in vascular tissues (Pallás and Gómez, 2017) and the infection of some SCs by HSVd and GYSVd-1 (Table 4) suggested that they easily spread in callus tissues and SCs. Based on our current knowledge, it is more difficult to justify the effect of the grapevine genotype on the somatic embryogenesis ability to eliminate viruses or viroids. This could be related to a different rate of growth and proliferation of callus cells among different genotypes, particularly among different grapevine species, or to some specific cellular interactions between some genotypes and some viruses/viroids that are not yet known. Such this subject is worth to be further explored in the future.

Conclusions

In this study, we determined the virome of seven grapevines used as MPs to produce healthy SCs by somatic embryogenesis. In addition to the viruses and viroids already reported as pathogens for grapevine, 20 viral entities (13 of which were new) classified as putative mycoviruses were identified in the analysed MPs. The different spread and viral concentrations among grapevine tissues in *in vivo* and *in vitro* conditions suggested a clear distinction between viruses/viroids and mycoviruses that is potentially exploitable for their identification. *In vitro* plantlets were excellent 'accumulators' of viruses and viroids, while mycoviruses were not present, suggesting that the endophytes that host mycoviruses are unable to invade these young tissues under sterile conditions.

Somatic embryogenesis is an effective technique for virus/viroid elimination in grapevine. Using the diagnostic techniques HTS and RT-qPCR, the sanitization efficiency was found to be less than 100% for viroids and GPGV; however, there were still significant differences between the genotypes, suggesting interesting and unknown genotype–virus interactions. The production of healthy plants without any viral entity (viruses, viroids or mycoviruses) from somatic embryogenesis could have both a practical value, being crucial for reducing the spread of these pathogens in the vineyard, and a scientific value, allowing researchers to understand the effects of a specific virus by using a healthy plant as control (Gilardi *et al.*, 2020). This aspect could be really important, especially because virus-free plants are not available from cultivated grapevines in vineyards and nurseries. In addition, the persistence of some viruses (GPGV) or viroids as a single pathogen infecting the SC also has a unique worth for studying the effects on plants of these virus/viroid without interactions with other viral entities. Finally, the absence of mycoviruses in *in vitro* plants and SCs suggests that the 'biological vacuum' generated by these regeneration techniques also involves fungi, resulting in gnotobiotic or

pseudo-gnotobiotic plants, thus, representing an extraordinary asset to understand the influence of the microbiome on plant growth and physiology.

Experimental procedures

Plant material

Seven MPs of grapevine, corresponding to five cultivars of *V. vinifera* ('Sangiovese', 'Cabernet Sauvignon', 'Chardonnay', 'Nebbiolo' and 'Brachetto g.l.' synonym of 'Bragat rosa', in the manuscript it is referred to simply as 'Brachetto') and two rootstocks (*Vitis berlandieri* x *Vitis rupestris* '110 Richter' and *V. rupestris*) maintained in a germplasm collection vineyard for 10 years (Grugliasco [TO], Northwest of Italy, GPS: 45° 03' 57.8" N, 7° 35' 29.5" E), were selected for somatic embryogenesis and virome analyses. In 2019, at winter pruning woody cuttings were collected from all seven MPs and used for: (i) RNA extraction and virus quantification, (ii) *in vivo* vegetative multiplication using a peat substrate (TS4, Turco Silvestro, Italy) in nine L pots placed in a greenhouse, and (iii) vegetative multiplication in *in vitro* conditions. Immature flowers were collected from each MP in spring (May 2019) and were used for somatic embryogenesis induction, while mature leaves were collected in July 2019 for viral quantification. Leaf and woody tissues from *in vivo* potted MPs were sampled in July 2020 and processed for viral diagnosis. In the germplasm collection vineyard and in greenhouse, commercial antifungal products were used according to the manufacturer's instructions. A standard control strategy used in conventional farms in the region, consisting of two treatments with pyraclostrobin and metiram (Cabrio® Top, BASF Agro, Cesano Maderno, Italy) and treatments with sulfur (Thiovit Jet, Syngenta Crop Protection, Basel, Switzerland) and copper hydroxide (Coprantol Hi Bio 2.0; Syngenta Crop Protection) was applied until the end of the season.

In vitro *culture and somatic embryogenesis*

Woody cuttings from MPs were forced to sprout in water at room temperature. Herbaceous shoots emerging from the buds were surface sterilized for 15 min with sodium hypochlorite (1.5% available chlorine) and rinsed several times with sterile distilled water before being cultured in a medium containing 4.4 μM benzyl aminopurine (BAP). Single plantlets were micropropagated by repeatedly subculturing apical cuttings on medium without plant growth regulators (PGRs), as previously described (Gribaudo *et al.*, 2006).

Somatic embryogenesis was induced in all seven MPs starting from immature anthers and ovaries according to Gambino *et al.* (2007). Somatic embryos were isolated

from the callus, and germination was induced with BAP, following a previously published protocol (Gambino *et al.*, 2021). The obtained SCs were micropropagated independently by subculturing apical cuttings on medium without PGRs and maintained in *in vitro* conditions. SCs of 'Nebbiolo' were acclimated to *in vivo* conditions using pots filled with a peat substrate (TS4, Turco Silvestro, Italy) and placed in a greenhouse and submitted to viral diagnosis which was performed after 1 year of culture.

RNA sequencing and virome analysis

The total RNA of the MPs was extracted using a CTAB-based protocol previously reported (Gambino *et al.*, 2008) from woody cuttings collected at winter pruning, from mature leaves collected in July from the vineyard, and from *in vitro* plantlets. For each MP, RNA samples from wood, leaves and *in vitro* plantlets were mixed in an equimolar ratio, and then the seven MPs were mixed in two pools containing three and four MPs (Table S3). Each MP included in each pool had the same amount of RNA to avoid bias. RNA extracted from SCs *in vitro* plantlets were mixed in five pools containing 7–13 SCs per pool using the same amount of RNA for each SC (Table S3).

Pooled RNA samples were subjected to ribosomal RNA depletion (Ribo-Zero™ Gold Kit; Epicentre, Madison, WI, USA), Illumina TrueSeq library preparation (TrueSeq total RNA sample kit; Illumina, San Diego, CA, USA), and sequenced by an external service (Macrogen, Seoul, Republic of Korea), which provided approximately 100 million reads (150 bases, pair ends) for each library. De novo assembly of sequencing data was performed on high-quality and cleaned reads that were selected using Trimmomatic (Bolger *et al.*, 2014). Trinity (version 2.3.2) was then used to assemble reads into contigs (Haas *et al.*, 2013), and the BLAST suite (version 2.6.0+) was adopted to search conserved viral proteins among the assembled contigs using blastx and a custom-built reference database of viral sequences (Nerva *et al.*, 2018; Bertazzon *et al.*, 2020a). To confirm reliable coverage, reads were aligned against the identified viral contigs using BWA 0.7.15-r1140 (Li and Durbin, 2010) and SAMtools 1.3.1 (Li *et al.*, 2009). Coding open reading frames (ORFs) were detected with ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi), blasted against the non-redundant NCBI protein database, and then the deduced molecular weight was calculated for each protein using the ExPASy online tool (https://web.expasy.org/compute_pi/). The CDD/SPARCLE tool from NCBI was used to search for conserved domains along contigs (Lu *et al.*, 2020).

The core conserved part of each identified viral RNA-dependent RNA polymerase (RdRP) was used for

multiple sequence alignments using MUSCLE (Edgar, 2004). The produced alignments were then used for phylogenetic inference using the maximum likelihood methodology in IQ-TREE (Trifinopoulos *et al.*, 2016). Statistical analysis for each clade was carried out through bootstrap analysis with 1000 replicates.

Quantitative real-time RT-PCR

To confirm that the viral contigs were not artefacts and to determine the association between specific virus/viroid/mycovirus identified *in silico* in pooled samples and specific MP or SC, RT-qPCR was carried out using specific primers (Table S4). In addition to SCs subjected to RNA-seq (Table S3), we analysed *in vitro* SCs of '110 Richter' (six SCs), 'Chardonnay' (10 SCs), and 'Brachetto' (40 SCs) only by RT-qPCR for the viral entities identified in the corresponding MPs to increase the number of SCs analysed. The viral status of 'Nebbiolo' SCs after 1 year of cultivation in greenhouse was confirmed by RT-qPCR.

Total RNA was treated with DNase (DNase I; Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions. qPCR reactions were performed in a CFX Connect Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA), using SYBR Green (SensiFAST™ SYBR® No-ROX Kit; Meridian Bioscience, Memphis, Tennessee, USA) with the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Amplified fragments were sequenced to confirm the viral sequence.

The relative quantification of the viruses/viroids in the wood and leaves of MPs, collected in the vineyard and from *in vitro* plantlets, was carried out using the same protocol described above and normalized with grapevine ubiquitin (*VvUBI*) and actin1 (*VvACT1*) as internal controls. Three biological replicates and three technical replicates were run for each RT-qPCR reaction. Viral accumulation data were subjected to one-way analysis of variance (ANOVA), followed by the Tukey's HSD post hoc test ($P \leq 0.05$). The SPSS statistical software package (SPSS Inc., Cary, NC, USA, v.23) was used to run statistical analyses.

Conflict of interests

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal participants.

Data Availability Statement

Raw sequences from the RNA-seq libraries were deposited at the NCBI Sequence Read Archive, BioProject PRJNA770860, BioSample SAMN22242529 and SAMN22242530, SRA SRR16311689 and SRR16311690.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Quantification of GFkV, GLRaV-3, GLRaV-2, GLRaV-4, GFLV, GVA, GVD and GVT RNA in wood and leaf collected in vineyard, and in *in vitro* plantlets of 'Sangiovese', 'Cabernet Sauvignon', 'Nebbiolo', 'Chardonnay' and 'Brachetto' as determined by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). RT-qPCR signals were normalized to *VvAct* and *VvUBI* transcripts. Data are presented as the mean ± standard deviation (SD) (n = 3). Lowercase letters denote significant differences attested by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

Table S1. List of contigs that found match in NCBI database against viruses already reported.

Table S2. List of contigs that represent new putative viral species identified in the present work.

Table S3. Composition of RNA pools subject to RNA-seq.

Table S4. List of the oligonucleotides used in this study.