The use of mycoviruses in the control of forest diseases

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Abstract

Fifteen families of mycoviruses have been described and 80% of these catalogued. However, their evolutionary relationship with fungi is not clear. The mycovirus genome can be formed by single or double-stranded RNA or single-stranded DNA. The effects of mycoviruses range from the induction of a cryptic state (asymptomatic) to promotion of hyper- or hypovirulence in the host. Horizontal transmission of mycoviruses is determined by the presence of different vegetative compatibility types and mating types. Biocontrol of chestnut blight (*Cryphonectria parasitica*) has been found to be a successful mycovirus-based treatment and is considered a model in forest disease management. Development of this type of biological control tool for use in other forest pathologies requires a sound knowledge of viral symptomatology and transmission. The present review focuses on the application of mycoviruses and the prospects for future use in the biological control of forest diseases as well as on advances in mycovirus-applied research in forestry, landscape and culture of woody plants.

Keywords: biological control, forest protection, hypovirulence, vc types, virocontrol, virus transmission.

1. Introduction

Viruses that infect fungi, i.e. mycoviruses, are frequent in the subkingdom Dikarya (phyla Ascomycota and Basidiomycota), phyla Blastocladiomycota and Neocallimastigomycota (formerly Chytridiomycota) and Glomeromycota (formerly Zygomycota) (Herrero, Dueñas, Quesada-Moraga, & Zabalgogeazcoa, 2012; Hibbett et al., 2007). Most fungal genera, ranging from microscopic yeasts to the more evolved edible mushrooms, have been described as hosts of mycoviruses (Hammond, Andrewski, Roossinck, & Keller, 2008; Lim et al., 2005; Magae, 2012; Ro et al., 2007; Schmitt & Breinig, 2006; Stielow, Klenk, Winter, & Menzel, 2011; Strauss, Lakshman, & Tavantzis, 2000). This also applied to filamentous fungi that cause plant diseases.

Despite the apparent abundance of mycoviruses in nature, research on these infective agents is relatively scarce. Some recent studies have attempted to uncover the biological mechanisms that drive viral infection, replication and transmission in fungi and the ecological and management implications. As a result, agroforestry researchers have discovered the potential use of these viruses in biocontrol, with special attention given to mycoviruses that confer hypovirulence (weakened state) in their pathogenic hosts.

In this article, we review studies concerning the use of mycoviruses to control devastating forest diseases. Our main goal is to provide background information about biocontrol based on fungal virus research as well as on the degree to which different protection strategies are being implemented.

2. General aspects of mycoviruses

2.1. Taxonomy, diversity and biology

More than 250 fungus-related viral sequences have been identified and sequenced according to National Center for Biotechnology Information (NCBI, 2014; Xie & Jiang, 2014), resulting in 22 genera divided among 15 families according to the list published by the International Committee on Taxonomy of Viruses (ICTV, 2014) (Figure 1). Nevertheless, 20% of mycoviruses have not yet been catalogued (Pearson, Beever, Boine, & Arthur, 2009; Van Regenmortel et al., 2010).

Mycoviruses usually replicate in the cytoplasm, although some (e.g. *Mitovirus* sp.) replicate in mitochondria of the host species (Göker, Scheuner, Klenk, Stielow, & Menzel, 2011; Milgroom & Hillman, 2011). Structurally, mycovirus genomes contain one or more open reading frames (ORFs) that encode proteins required for virus replication and sometimes for capsid synthesis. The molecular size of mycovirus genomes varies somewhat, e.g. Rosellinia necatrix quadrivirus 1 (RnQV1) segments range in size from 3.70-4.90 kbp with a single ORF (Chiba et al., 2009), while the maximum size of Chalara elegans RNA Virus 1 (CeRV1) has been reported to be 5.31 kbp in length and contain three ORFs (Park, James, & Punja, 2005). Other mycoviruses may be longer, e.g. Cryphonectria hypovirus 1 (CHV-1) is 12.70 kbp in length and has at least two ORFs (Allemann, Hoegger, Heiniger, & Rigling, 1999; Shapira, Choi, & Nuss, 1991). Overall, the size of genome ranges between the extremes of *Partitiviridae* viruses (1.4-2.4 kbp and a single ORF) and *Hypoviridae* viruses (~9-13 kb and two overlapping ORFs); in addition, some families such as *Alphaflexiviridae* may contain several more or less overlapping ORFs (e.g. Botrytis virus X: ~7.0 kb and five ORFs) (Ghabrial, Castón, Jiang, Nibert, & Suzuki, 2015). In some cases, small RNA molecules may also occur as satellite elements associated with the main genome particles (e.g. 0.9-1.4 kb elements associated with 3.7-5.0 kpb mycovirus genome in basydiomicetous yeast *Xanthophyllomyces dendrorhous*; anamorph: *Phaffia rhodozyma* (Flores, Alcaíno, Fernandez-Lobato, Cifuentes, & Baeza, 2015)).

Mycoviruses can be differentiated on the basis of molecular structure. Thus seven families possess double-stranded RNA (dsRNA) genomes, and six families have single-stranded RNA (ssRNA) genomes. The latter are further divided into two subcategories: five families have ss(+)RNA genomes and one family has a ss(-)RNA genome (Figure 1). The mycoviruses belonging to ss(+)RNA families possess viral RNA with the same base sequence as mRNA. The functions of the RNA are similar to mRNA during replication, serving as a template for protein synthesis such as RNA-dependent RNA polymerase (RdRp) or capsid. On the other hand, ss(-)RNA mycoviruses require participation of RNA replicase for their single strain genome to be transcribed into positive sense RNA. Only a few mycoviruses are formed by single circular molecules of DNA (ssDNA) (Ghabrial, Castón, Jiang, Nibert, & Suzuki, 2015; Pearson et al., 2009).

<<Insert Figure 1 around here>>

The evolutionary relationship between mycoviruses and their hosts remains unclear. Two main hypotheses have been proposed. Briefly, one hypothesis is based on ancient co-evolution of mycoviruses and fungi whereby the speciation of viruses is closely related to vertical transmission (see below), and the asymptomatic presence of mycoviruses may denote a long period of coexistence between viruses and fungi. This would explain the complex relationships between host species and mycoviruses, which range between severe disadvantage to the host (antagonism) and mutualism where the infected host obtains some benefit under certain conditions, as suggested in other viral associations

(Botella, Vainio, Hantula, Diez, & Jankovsky, 2015; Roossinck, 2015a, 2015b). The other hypothesis suggests the eventual transfer of viruses from plants to saprophytic or pathogenic fungi. In this case, viral transmission may take place during co-existence of fungal endophytes with plants, and small differences detected even within mycovirus families can be explained by a recent change of host (Chiba et al., 2011; Ghabrial, 1998; Liu et al., 2010; Pearson et al., 2009).

2.2. Transmission of mycovirus

The mechanism of viral transmission is another important aspect of viral biology. Mycoviruses can be transmitted in three ways: by horizontal, vertical or extracellular transfer. Horizontal transmission takes place when a mycovirus colonises a new host through hyphal contact and subsequent mycelia fusion (anastomosis) between individuals during heterokaryon formation (mediated by a self/non-self recognition system). Nevertheless, isolates of the same species are not always compatible, even in the same population. In this type of transfer, different vegetative compatability groups (vc types or VCGs) play a special role, sometimes restricting movement of the virus (Leslie, 1993). Heterokaryon formation is genetically controlled by a specific *het* or *vic* loci. Heteroallelism in the *het* locus is not possible, resulting in reduction in cell lysis or mycelial growth (Saupe, 2000). At the same time, the presence of different mating types (MAT´s) in fungal populations makes transmission more complex (Coppin, Debuchy, Arnaise, & Picard, 1997; Milgroom & Hillman, 2011).

In vertical transmission, mycoviruses commonly infect asexual spores. Nevertheless, prevalence rates may vary significantly between species, e.g. in *Heterobasidion annosum* only 3% of conidia are infected (Ihrmark, Johannesson, Stenström, & Stenlid, 2002) in contrast to 100% infection in *Cryphonectria parasitica* (Ding et al., 2007). Fungal viruses can also colonise sexual spores, infecting a new generation of the host: 8-13% dsRNA infected ascospores of *Magnaporthe grisea* (Chun & Lee, 2009) whereas 10-84% dsRNA infected basidiospores of *H. annosum* (Ihrmark, Stenström, & Stenlid, 2004). However, in a more recent study, lower vertical transmission of *Heterobasidion parviporum* to basidiospores (8.3%) was observed in a spruce forest (Vainio, Müller, Korhonen, Piri, & Hantula, 2014). The authors of the latter study suggested that continuous spore load in stumps may be related to the high rate of infected basidiospores, in contrast to low rates of infection in standing trees, as previously reported. It is now considered that the predominant route of viral transmission is via asexual spores, and vertical transmission has not been reported to occur in many fungal species (Carbone, Liu, Hillman, & Milgroom, 2004; Milgroom & Hillman, 2011).

Extracellular transmission, in which purified viral particles of Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) infected extracellularly virus-free protoplasts, intact hyphae and hyphal fragments of white mould fungus (*Sclerotinia sclerotiorum*) either *in vitro* (PDA culture) or *in vivo* (leaves of infected plants has recently been described (Yu et al., 2013). These authors also mentioned that purified viral DNA did not infect mycelia or fungal protoplasts, suggesting that whole viral particles are needed for extracellular infection.

On a larger scale, transmission of mycoviruses between species has also been reported (Lee, Yu, Son, Lee, & Kim, 2011; Liu, Linder-Basso, Hillman, Kaneko, & Milgroom, 2003; Vainio et al., 2011a), opening up new research lines focusing on the genetic, evolutionary and ecological factors involved in transmission.

2.3. Hypovirulence process

The effects of mycoviruses infection can range from cryptic symptoms (asymptomatic) to the promotion of hypervirulence, through variations of colonial morphology and inducement of color changes (Ghabrial & Suzuki, 2009). In fact, the same mycovirus can have different effects on their host depending on ecological conditions (Hyder et al., 2013). One phenomenon caused by mycoviruses, especially interesting for agroforestry science, is hypovirulence. Only a few mycoviruses reduce spore production, causing slow mycelial growth or less aggressive invasion in pathogenic hosts, making viruses effective in biocontrol (Milgroom & Hillman, 2011; Nuss, 2005) or virocontrol (Chiba, Kondo, Kanematsu, & Suzuki, 2010). In this sense, hypovirulence have been proved according to Koch´s postulates using infectious cDNA of *C. parasitica* (Chen & Nuss, 1999) and *S. sclerotiorum* (Marzano et al., 2015), hyphae infection of *Sclerotinia* spp. using viral particles (Yu et al., 2013) and protoplast infection using dsRNA (Chiba, Lin, Kondo, Kanematsu, & Suzuki, 2013; Hillman, Supyani, Kondo, & Suzuki, 2004; Lee et al., 2011). Hence, knowledge about mycovirus-mediated hypovirulence is improving biocontrol strategies in many cases of agroforestry health (see next section).

Both hyper- and hypovirulence are strongly related to the presence of specific viruses, even in co-infection. Four dsRNA mycoviruses have been detected in *Nectria radicicola* (anamorph: *Cylindrocarpon destructans*) (Ahn and Lee, 2001). Removal of one virus, L1 (6.0 kbp), caused a reduction in virulence of the fungus, while later reinfection through anastomosis recovered the virulence of the isolate. Detailed laboratory studies complemented with pathogenicity field assays are essential for developing virocontrol techniques.

One challenge in plant pathology and the use of mycoviruses is the antiviral response of fungi or RNA silencing. When viruses infect healthy cells, dicer-type nucleases initiate a response that produces viral RNA processed segments (sRNAs). The RNA-induced silencing complex then identifies homologous sequences on mRNA and subsequently degrades sRNAs (Dang, Yang, Xue, & Liu, 2011; Hammond et al., 2008; Nuss, 2011; Schumann, Ayliffe, Kazan, & Wang, 2010; Tauati, Pearson, Choquer, Foster, & Bailey, 2014; Yaegashi, Yoshikawa, Ito, & Kanematsu, 2013). In a study attempting to clarify this evolutionary relationship, Segers, Zhang, Deng, Sun, and Nuss (2007) found symptomatic differences between hypovirulence-mycovirus infected *C. parasitica* isolates. The use of *C. parasitica* strains in which RNA silencing genes were disrupted enabled identification of genes coding for particular dicer and argonaute-like proteins as required elements in antiviral response (Sun, Choi, & Nuss, 2009; Zhang & Nuss, 2008).

3. Mycoviruses in forest diseases: case studies

3.1. Cryphonectria parasitica

C. parasitica is the causal agent of chestnut blight, a severe disease that causes widespread damage in North America, where it infects American chestnut (*Castanea crenata*), in Europe, where it infects the European chestnut (*Castanea sativa*) and in Asia, where it colonises Asian species of chestnut (*C. crenata* and *Castanea mollissima*). The disease is characterised by damage to cambial tissues and the appearance of cankers. These cankers tend to girdle the stem, killing the trees (Milgroom & Cortesi, 2004).

Many ss(+)RNA mycoviruses have been identified in *C. parasitica*, four of them belonging to the genus *Hypovirus*. Cryphonectria hypoviruses 1-4 (CHV-1, CHV-2, CHV-3 and CHV-4) have been reported in different parts of the northern hemisphere (Hillman, Halpern, & Brown, 1994; Shapira et al., 1991; Smart et al., 1999). In relation to dissemination, transmission in conidia has been reported as highly variable, ranging from 0% to 100%. Transmission through ascospores has not been observed in nature (Ding, Liu, Xu, & Wang, 2007). However, the presence of mycovirus in ascospores of field-released transgenic strains of fungi ranged between 30% and 50% depending on culture conditions (Anagnostakis, Chen, Geletka, & Nuss, 1998).

The best known example of a mycovirus that causes hypovirulence is CHV-1. When CHV-1 infects *C. parasitica* it causes weakness, reducing mycelial growth and sporulation. Infected fungi are only capable of forming superficial (healing) cankers on stems, and the trees can therefore survive the disease. Other symptoms of the presence of CHV-1 in isolates include changes in colony morphology and colour (Peever, Liu, Cortesi, & Milgroom, 2000; Rigling, Heiniger, & Hohl, 1989). CHV-1 originally occurred in Europe (Italy and France) and Asia (Japan, China and Korea) but was later introduced into the USA (Allemann et al., 1999; Liu, Double, MacDonald, & Milgroom, 2002). Five genetically characerised subtypes of CHV-1 have been identified: F1 and F2 (from France), I (Italy), D (Germany) and E (Spain) (Allemann et al., 1999; Gobbin, Hoegger, Heiniger, & Rigling, 2003; Zamora, Martín, San Martín, Martínez-Álvarez, & Diez, 2014). CHV-1 is now considered an important biocontrol tool in European.

CHV-2 and CHV-3 are both common in North America. However, although CHV-2 occurs in native *C. parasitica* in Asia (Hillman, Tian, Bedker, & Brown, 1992; Peever et al., 1998), CHV-3 is only present in the USA (Michigan) (Peever, Liu, & Milgroom, 1997). Both CHV-2 and CHV-3 have proved useful in biocontrol as they induce hypovirulence in American forests and plantations. The mycovirus most commonly associated with chestnut blight in American forests (CHV-4) is traditionally considered to induce a cryptic state and is therefore not useful for biocontrol purposes (Enebak, MacDonald, & Hillman, 1994; Linder-Basso, Dynek, & Hillman, 2005).

Mycoreovirus 1 (MyRV-1) (*Reoviridae*) has been identified in hypovirulent strains of chestnut blight fungus (Suzuki, Supyani, Maruyama, and Hillman, 2004). Viral transmission of this *Mycoreovirus* sp. to sexual spores has been reported (Deng, Allen, Hillman and Nuss, 2007), and reovirus-infected isolates have been shown to produce mature perithecia and viable ascospores, which in turn host MyRV-1. Other mycoviruses belonging to the *Reoviridae* and *Narnaviridae* families - respectively Mycoreovirus 2 (MyRV2) and Cryphonectria mitovirus 1 (CpMV1) - have also been identified (Hillman & Suzuki, 2004). This fungus can host many *Reoviridae*, *Partitiviridae*, *Totiviridae* and *Megabirnaviridae* mycoviruses that usually infect other fungi (Eusebio-Cope et al., 2015).

3.2. Ophiostoma novo-ulmi

Dutch elm disease (DED), caused by *Ophiostoma ulmi* and *O. novo-ulmi*, was the most devastating disease affecting elms (*Ulmus* spp.) in Europe during the 20th Century (some 30 million elms were killed in the UK) (Brasier, 2001; Potter, Harwood, Knight, & Tomlinson, 2011). These fungi cause death of the tree by vessel cavitation due to fungal growth in the xylem. Two pandemics have occurred. In the first, which began in the 1910s, *O. ulmi* spread through Europe causing severe damage to adult trees and later spread to North America. In the 1950s, two subspecies of *O. novo-ulmi* (Euro-Asian race: *O. novo-ulmi* subsp. *novo-ulmi;* and the North American race: *O. novo-ulmi* subsp. *americana*) caused high mortality in European and American forests. In both cases, bark beetles (Coleoptera, Scolytinae) played an important role as vectors of the disease (Brasier & Kirk, 2010; Brasier, 1976, 1991; Santini & Faccoli, 2014).

In relation to the presence of mycoviruses, the d-factor has been identified as a cytoplasmically transmitted agent. It is characerised as a dsRNA virus, causing a reduction in fungal growth in wounds made by feeding bark beetles and in amoeboid colony morphology as well as lower vigour and growth rates and low conidial viability (Brasier, 1986; Sutherland, Brasier, & Lodge, 1997). Thirteen dsRNA mycoviruses with similar symptoms to the d-factor were later identified as being responsible for infection of a specific isolate called Ld (Cole et al., 1998; Doherty et al., 2006; Hong, Dover, Cole, Brasier, & Buck, 1999; Hong, Cole, Brasier, & Buck, 1998a,b). The complete genomes of *O. novo-ulmi* mitoviruses (OnuMV1a, OnuMV1b, OnuMV3a, OnuMV3b, OnuMV4-Ld, OnuMV5-Ld and OnuMV6-Ld) have been sequenced and RdRp sequences for OnuMV1a, OnuMV1b and OnuMV3b have also been established (Hintz, Carneiro, Kassatenko, Varga, and James, 2013).

In addition, other *Ophiostoma* species have been demonstrated to harbour mycoviruses. *Ophiostoma minus* (causal agent of blue stain in pine wood), and the saprophyte *Ophiostoma quercus* hosts viruses belonging to the *Totiviridae* and *Partitiviridae* families (respectively Ophiostoma minus totivirus (OmV) and Ophiostoma quercus partitivirus 2 (OPV2) (Doherty et al., 2007). A distant relationship between OPV2 and Ophiostoma partitivirus 1 (OPV1) was suggested (Doherty et al., 2007). OPV1, which was previously detected in the pathogenic fungus *Ophiostoma himal-ulmi* (Crawford et al., 2006), is not currently used in biocontrol.

3.3. Heterobasidion annosum

H. annosum s.l. is one of the most destructive fungi in the northern hemisphere. It is the causative agent of root disease in many coniferous species (*Abies* spp., *Calocedrus decurrens*, *Juniperus* spp., *Larix* spp., *Picea* spp., *Pinus* spp., *Pseudotsuga menziesii*, *Sequoiadendron giganteum, Thuja plicata* and *Tsuga heterophylla*) as well as in some broadleaf species (*Betula*, *Fagus* and *Populus* species) (Garbelotto & Gonthier, 2013; Gonthier & Thor, 2013). This fungal infection causes the death of trees (especially on pines and junipers), severe root and butt rot, general decay and decreased diameter growth in boreal forest and plantations, making it a major threat to timber production and the forest industry. Infection can occur in two ways: primary infection is caused by airborne basidiospores, while secondary infection takes place through colonisation of mycelia after contact with roots or grafting between infected and healthy trees (Asiegbu, Adomas, & Stenlid, 2005; Thor, Ståhl, & Stenlid, 2005; Tokuda, Ota, Hattori, Shoda-Kagaya, & Sotome, 2011; Woodward, Stenlid, Karjalainen, & Hüttermann, 1998).

Additionally, dsRNA mycoviruses in P and S types of *H. annosum* (Heterobasidion partitivirus P (HaV-P) and Heterobasidion annosum virus (HaV)) have been partially sequenced (Ihrmark, Zheng, Strenstöm, & Stenlid, 2001). The authors included these mycoviruses in *Partitiviridae* and reported that *H. annosum* s.l. harbours dsRNA viruses at a frequency of approximately 15% in Europe and western Asia. A new putative member of *Partitiviridae*, Heterobasidion partitivirus 3 (HetPV3), was subsequently detected in Chinese strains of *Heterobasidion ecrustosum* (Vainio, Korhonen, Tuomivirta, and Hantula*,* 2010). In a later study, a new dsRNA virus belonging to *Partitiviridae* and designated Heterobasidion partitivirus 2 (HetPV2) clearly formed a subcluster with HaV-P due to their genomic similarities (Vainio et al., 2011b). In addition, three new putative viruses, also included in *Partitiviridae*, were catalogued and subsequently named Heterobasidion partitivirus 1 (HetPV1), HetPV4 and HetPV5 (Vainio et al., 2011a). These authors proposed a close genetic relationship between HetPV1 and HaV, while the two other viruses were found to be more similar to mycoviruses associated with Heterobasidion parviporum partitivirus Fr110B and other disease-associated viruses.

Another three partitiviruses have been identified more recently: Heterobasidion partitivirus 6, 7 (HetPV6 and HetPV7 respectively) (Vainio et al., 2012, 2013c) and Heterobasidion partitivirus 8, strain 1 from *Heterobasidion irregulare* (HetPV8-ir1) (Vainio et al., 2013a). All are taxonomically distant from all other *H. annosum* s.l. viruses. HetPV6 resembles Fusarium graminearum virus 4 (FgV4), with around 40% of protein level sequence similarities, while HetPV8-ir1 shares only 32% of RdRp similarities with HaV-P and 33% RdRp similarities with HetPV2 (Vainio et al., 2010, 2013a, 2013b). A recent study showed that four different viral species may be present in the same plot affected by *H. parviporum* (Vainio et al., 2014). Three of these were provisionally assigned to HetPV6 and two possible congeneric strains of *Betapartitivirus* sp., named HetPV2-pa1 and HetPV7-pa1, were also identified.

3.4. Gremmeniella abietina

Many coniferous tree species (mainly *Picea*, *Pinus*, *Abies* and *Larix* species) in Northern and Central Europe, North America and Japan host the fungus *Gremmeniella abietina* (anamorph: *Brunchorstia pinea*), leading to the appearance of stem cankers and shoot dieback and causing severe damage in woods and plantations when weather conditions are favourable. Three races of this fungus (European, North American and Asian) have been catalogued. The European race is subdivided into three biotypes (A, B and alpine) (Botella et al., 2010; Donaubauer, 1972; Hamelin, Lecours, Hansson, Hellgren, & Laflamme, 1996; Kaitera & Jalkanen, 1992; Romeralo, Botella, Santamaria, & Diez, 2012; Santamaria, Alves-Santos, & Diez, 2005; Senn, 1999), although the taxonomy is currently under revision (Romeralo pers. com.).

Three families of dsRNA mycoviruses have been detected in this forest pathogen: Gremmeniella abietina mitocondrial RNA virus S1 (GaMRV-S1, *Narnaviridae*) (Tuomivirta & Hantula, 2003a); Gremmeniella abietina RNA virus L1 (GaRV-L1, *Totiviridae*); and Gremmeniella abietina RNA virus MS1 (GaRV-MS1, *Partitiviridae*) (Tuomivirta & Hantula, 2003b), with a high frequency of occurrence; e.g. the mycoviruses have been detected in 89% of Spanish isolates (Botella, Tuomivirta, Hantula, and Diez, 2012) and in 50% of Turkish isolates (Aday et al., 2012). In addition, three mycoviruses were found together infecting the same isolates of *G. abietina* var. *abietina* type A (Tuomivirta and Hantula, 2005). Later, Botella, Tuomivirta, Vervuurt, Diez, and Hantula (2012) reported the absence of mitoviruses in biotype B from Turkey, biotype A from North America and European Alpine biotype. On the contrary, biotype A from Finland and Spain hosted mycoviruses. Specifically, Spanish populations hosted two mycoviruses (GMV1 and GMV2) in high proportion (74% of isolates hosted dsRNA). These authors discussed the possible factors determining presence and transmission of mitoviruses between fungal races and highlighted the role of asexual reproduction in virus widespread. In fact, the higher proportion of mitovirus presence was detected in Spain where only asexual reproduction has been reported. Regarding the high presence and the low genetic variability detected in GMV2 in Spanish isolates, the researchers suggested a possible recent host switch and a subsequent adaptation to these new conditions. The findings of recent RdRp sequencing studies support the idea of a low degree of genetic variation in *G. abietina* mitoviruses in the European population (Botella, Tuomivirta, Hantula, Diez, & Jankovsky, 2014).

3.5. Fusarium circinatum

Pine pitch canker is a virulent disease caused by *Fusarium circinatum* (teleomorph: *Gibberella circinata*) in many pine species and in Douglas fir (*Pseudotsuga menziesii*) worldwide. Infections have also been observed to cause significant damage in *Abies alba*, *S. giganteum, Larix decidua* and *Picea abies* (Martínez-Álvarez, Pando, and Diez, 2014a). The pathogen was first detected in the southeastern USA and Mexico (where it is probably endemic) an then in Haiti, South Africa, Chile, France, Korea, Spain, Italy, Japan, Portugal, Uruguay and Brazil (Aegerter, Gordon, Storer, & Wood, 2003; Enebak & Stanosz, 2003; Gordon, Kirkpatrick, Aegerter, Wood, & Storer, 2006; Martínez-Álvarez, Alves-Santos, & Diez, 2012; Pfenning, Costa, Melo, Costa, & Aires, 2014). This fungus causes dieback in trees due to the formation of bleeding and resinous cankers on trunk and branches. Moreover, *F. circinatum* frequently causes death and damping-off of seedlings through both pre- and post-emergence infection, making such infections a significant threat to nurseries and afforestations (Aegerter et al., 2003; Hammerbacher, Ganley, Steenkamp, Gordon, & Coutinho, 2008).

Three putative *Mitovirus* spp. (*Narnaviridae*) were recently identified in *F. circinatum* isolates from *Pinus radiata* in northern Spain and named Fusarium circinatum mitovirus

1, 2-1 and 2-2 (FcMV1, FcMV2-1 and FcMV2-2) (Martínez-Álvarez, Vainio, Botella, Hantula, and Diez, 2014b). The genetic structure of the mycoviruses hosted by *F. circinatum* isolates from Spain and South Africa has also been studied (Vainio, Martínez-Álvarez, Bezos, Hantula, and Diez, 2015). Only Spanish isolates were found to host mycoviruses, which showed very similar sequence variants (>95% similarity). Indeed, a high rate of asexual spore transmission of mycoviruses (ranging between 70% and 100%) has been preliminary observed (Bezos, Martínez-Álvarez, Romeralo, and Diez, 2015), indicating the potential use of the mycoviruses as biocontrol agents.

***3.6. Botryosphaeria* spp.**

Botryosphaeria spp. commonly occur as endophytic fungi in healthy hosts, but may become virulent when their host is subjected to environmental stress or physical damage (Burgess, Sakalidis, & Hardy, 2006; Smith, Crous, Wingfield, Coutinho, & Wingfield, 2001; Smith, Wingfield, Crous, & Coutinho, 1996). Despite its taxonomic complexity, *Botryosphaeria dothidea* (anamorph: *Fusicoccum aesculi*) is cited as the causal agent of stem and branch cankers on apple trees (*Malus domestica*), ring spot on pear trees (*Pyrus communis*) and dieback and stem cankers on eucalyptus trees (*Eucalyptus* spp.) among many other woody species (Brown-Rytlewski & McManus, 2000; Slippers & Wingfield, 2007). *Eucalyptus* sp. is one of the most common trees planted in commercial and clonal forestry at an international level. Eucalyptus dieback and cankers are of special interest in forest science because of the reduced growth, offspring failure and adult tree death caused by the pathogen (Pérez, Wingfield, Slippers, Altier, & Blanchette, 2010). The gummy exudation produced in cankers also makes the wood less valuable, causing significant economic losses in the forest industry (Rodas, Slippers, Gryzenhout, & Wingfield, 2009).

Two dsRNA mycoviruses were recently detected in non virulent isolates of *B. dothidea* infecting *Pyrus pyrifolia* (Wang et al., 2014). These researchers reported Botryosphaeria dothidea chrysovirus 1 (BdCV1) as a new member of *Chrysoviridae* and also identified Botryosphaeria dothidea partitivirus 1 (BdPV1). Although BdPV1 was included in *Partitiviridae*, the capsid proteins of the mycovirus do not show significant similarity to any other capsid proteins. Analysis of the RdRp sequence also suggests the inclusion of this mycovirus in a new *Partitiviridae* clade (with 39% RdRp similarity to the most closely related *Chrysovirus* sp.).

3.7. Hymenoscyphus fraxineus

Ash dieback is an invasive disease caused by the fungus *Hymenoscyphus fraxineus* (synonym: *Hymenoscyphus pseudoalbidus*; anamorph: *Chalara fraxinea*). The fungus infects *Fraxinus* spp. with notable incidence in common ash (*Fraxinus excelsior*) and narrow-leafed ash (*Fraxinus angustifolia*). This pathogen has been spreading in Europe since the 1990s and causes severe damage in forests (pure or mixed stands), nurseries and urban green areas (Hietala, Timmermann, Børja, & Solheim, 2013; Kowalski, 2006; Timmermann, Børja, Hietala, Kirisits, & Solheim, 2011). It has also been cited in East Asia and Japan infecting *Fraxinus mandshurica* and *Fraxinus chinensis* (Gross, Holdenrieder, Pautasso, Queloz, & Sieber, 2014). The fungus infects ash trees of all ages, causing rapid crown dieback in adult trees, cankers and bark lesions on stem and twigs, and also leaf wilt. The disease frequently causes the death of young trees a few years after infection. However, it may become a chronic disease in older trees, reducing the tree's defences against other pathogens and pests or environmental factors (Gross et al., 2014; Kowalski & Holdenrieder, 2009; Timmermann et al., 2011).

A new ssRNA mycovirus that infects this pathogenic fungus was recently discovered (Schoebel, Zoller, and Rigling, 2014). The authors proposed inclusion of the virus in the genus *Mitovirus* (*Narnaviridae*) and named it Hymenoscyphus fraxineus mitovirus 1 (HfMV1). They noted the possibility of rapid genetic divergence based on their findings of large differences in the strains isolated in Switzerland, Poland, Germany, Lithuania and Japan. They hypothesised that the similarities between Swiss and Japanese strains may denote a European pathogen introduction across infected host material from Asia. Moreover, the prevalence of this mycovirus was high (90% in Swiss isolates according to Schoebel et al., (2014)), supporting the most accepted hypothesis of predominance of vertical transmission via ascospores.

3.8. Other fungal pathogens in woody plants

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) causes grey mould disease in more than 200 crops species over the world, including farmland crops, ornamental species and fruit crops such as grapes (*Vitis vinifera*), pear trees, raspberries and blackberries (*Rubus* spp.) (Rodríguez-García, Medina, Alonso, & Ayllón, 2014; Williamson, Tudzynski, Tudzynski, & van Kan, 2007). The presence of different genera of mycovirus in this fungus has been widely reported (Castro, Kramer, Valdivia, Ortiz, & Castillo, 2003; Potgieter, Castillo, Castro, Cottet, & Morales, 2013; Rodríguez-García et al., 2014; Wu et al., 2007; Zhang, De Wu, Li, Jiang, & Huang, 2010). These studies highlight the wide diversity of viruses that this fungus is able to host and which provide a wide range of opportunities for research in the field of fungal virology. Another three mycoviruses that infect *Botrytis* sp. have recently been sequenced: Botrytis virus F (BVF, *Gammaflexiviridae*), Botrytis virus X (BVX, *Alphaflexiviridae*) and Botrytis porri RNA virus 1 (BpRV1, dsRNA virus) (Xie & Jiang, 2014).

Verticillium dahliae and *Verticillium albo-atrum* are both causal agents of *Verticillium* wilt disease. They have been cited in a broad range of hosts and more than 200 species, including bushes and trees (Schall & Davis, 2009; Smith, 1965). Specifically, *V. dahliae* can infect economically important woody crops such as gooseberry *(Ribes grossularia*), apricot (*Prunus armeniana*), olive (*Olea europea*), quince (*Cydonia oblonga*) and roses (*Rosa* spp.), as well as other species of ecological interest such as maple *(Acer palmatum*), sycamore (*Acer pseudoplatanus*), raspberry, honeysuckle (*Lonicera* sp.) and broom (*Cytisus scoparius*). *V. albo-atrum* causes damage to the tree of heaven (*Ailanthus altissima*), striped maple (*Acer pennsylvanicum*), yellow poplar (*Liriodendron tulipifera*) and other landscape species (Morehart, Donohue, & Melchior, 1980; Schall & Davis, 2009; Smith, 1965). Some studies have demonstrated the presence of mycoviruses in these pathogenic fungi. For example, a *Chrysovirus* sp. named Verticillium dahliae chrysovirus 1 (VdCV1) was identified by Cao et al. (2011). A novel member of the family *Partitiviridae* was identified in *V. albo-atrum*: Verticillium albo-atrum partitivirus 1 (VaaPV1) (Cañizares, Pérez-Artés, and García-Pedrajas, 2014), although no details were provided about the pathogenic effect of the mycovirus in its fungal host.

Some opportunistic fungal pathogens of *Pinus* spp., such as *Diplodia pinea* (synonym: *Sphaeropsis sapinea*) and *Diplodia scrobiculata* (Smith et al., 1996), also host mycoviruses. Two dsRNA mycoviruses have been identified in *D. pinea*: Sphaeropsis sapinea RNA virus 1 and 2 (SsRV1, SsRV2 respectively; *Totiviridae*) (Preisig, Wingfield, & Wingfield, 1998); and one in *D. scrobiculata*: Diplodia scrobiculata RNA virus 1 (DsRV1; related to *Chrysoviridae*) (De Wet, Bihon, Preisig, Wingfield, & Wingfield, 2011; De Wet, Preisig, Wingfield, & Wingfield, 2008).

Another pathogenic fungi of interest in agroforestry is the causal agent of root rot disease, *Rosellinia necatrix* (anamorph: *Dematophora necatrix*). The interest is due to the pathogenicity of the fungus in several woody species e.g. apple, olive, grape and poplar (*Populus* spp.) (Pérez-Jiménez, 2006). Many families of mycoviruses are known to infect this fungus, e.g. *Chrysoviridae, Quadriviridae, Partitiviridae, Reoviridae* and *Totiviridae* (Xie and Jiang, 2014). Two dsRNA mycoviruses have also been associated with hypovirulence: Rosellinia necatrix megabirnavirus 1 (RnMBV1), included in a new family of mycoviruses (*Megabirnaviridae*) and Rosellinia necatrix partitivirus 2 (RnPV2) (Xie and Jiang, 2014).

4. Future perspectives for use of mycoviruses in biocontrol

As already mentioned, many forest, horticultural and ornamental species harbour mycoviruses to a greater or lesser extent (Table 1). Although many of these have not yet been found to be of use for biocontrol purposes, many of them provide new opportunities for research in forestry science. Despite the promising outlook, the use of mycoviruses in biological control is limited by the need for detailed analysis of (a) the symptoms associated with mycovirus-caused hypovirulence, (b) transmission mechanisms and biological and ecological conditions, (c) treatment effectiveness in the field and (d) subsequent persistence in the host population.

4.1. Identification of factors leading to hypovirulence: research in progress and lessons learned

The best known example of a disease managed by mycoviruses is chestnut blight. In Europe, CHV-1 has been used to induce hypovirulence (Robin & Heiniger, 2001) with goods results in field inoculation trials (Juhásová, Adamcíková, & Robin, 2005; Robin, Anziani, & Cortesi, 2000; Zamora et al., 2014). CHV-1 and CHV-3 have been used with less success in the USA than in Europe, with natural hypovirulence being reported in Michigan (Milgroom & Cortesi, 2004). For other pandemics such as DED, mycoviruses infecting *O. novo-ulmi* appear promising for biocontrol, because of the symptoms that they cause in host isolates, such as slow mycelial growth, abnormal or amoeboid colony formation, reduction in asexual spore production, low cytochrome oxidase level and formation of mitochondrial DNA plasmids (Hong et al., 1999).

In relation to the application of biocontrol in diseased forests in boreal areas, no clear relationship between viral presence and fungus growth rate was observed in *H. annosum* s.l. (Vainio et al., 2010). However, significant variations in growth and changes in the effects of virus were observed in relation to the culture conditions. The effect of HetPV6 infection in relation to multiple variables (geographical, culture conditions and host) has been investigated in four *Heterobasidion* species (Vainio et al., 2012). No significant differences in growth were found in *H. parviporum* (*in vivo* and *in vitro*) or *H. annosum* (*in vivo*). However, significantly increased mycelial growth was observed in infected *H. annosum* cultures (laboratory assays condition: 6º C and 15ºC culture on MOS agar plates). Consequently, these results do not support a possible use of HetPV6 in virocontrol, although HetPV6 is very frequent in fungal populations and apparently does not interfere in subsequent viral infection (Vainio et al. 2013b).

Mycoviruses may eventually be used as tools in the management of invasive diseases, for example in ash dieback. Although Hymenoscyphus fraxineus mitovirus 1 does not show harmful effects in its host, future perspectives for its application in biocontrol are promising because of the phylogenetic position of this mitovirus relative to others that are known to cause hypovirulence (Schoebel et al., 2014). In fact, HfMV1 is closely related to *Cryphonectria cubensis*, *S. sclerotiorum* and *Helicobasidium mompa* mitoviruses.

Several *Totiviridae*, *Chrysoviridae* and *Partiviridae* mycoviruses have been identified in *Fusarium graminearum* (Lee, Son, & Kim, 2011; Yu et al. 2011). More specifically, a mycovirus described in *F. graminearum* infecting maize in Korea (named Fusarium graminearum virus 1-DK2; FgV1-DK2) is capable of reducing mycelial growth and sporulation, decreasing mycotoxin production and increasing pigmentation (Chu et al., 2002). In a later study addressing this topic, a mixed infection of two dsRNA viruses was reported, with no changes in mycelial morphology but with a high rate of transmission in conidia and ascospores (30-100%) (Chu et al., 2004). A recent study identified a new mycovirus associated with hypovirulence in *Fusarium virguliforme* and closely related to

F. graminearum mycoviruses (Marvelli et al., 2014). Moreover, two new putative mycoviruses belonging to the *Mitovirus* genus have been described in *Fusarium coeruleum* isolates, in addition to one new *Alphapartitivirus* sp. in *Fusarium solani* f. sp. *pisi* (Osaki et al., 2015). Mycoviruses infecting in *F. coeruleum* are closely related to FcMV1, which opens up a new line of phylogenetic research. Together these results encourage the continued study of hypovirulence induced by mycoviruses in *Fusarium* spp. (with special focus on *F. circinatum*) whose use in biocontrol may prove to be a profitable consequence of in-depth studies of this species.

Grey mold, caused by *B. cinerea*, is being investigated by various research groups around the world because of the global importance of this disease. The rare formation of multicellular penetration structures (infection cushions) and decreased mycelial growth are probably caused by hypovirulence induced by mycoviruses (especially Botrytis cinerea mitovirus 1 (BcMV1), main mycovirus implied in hypovirulence process) as suggested by Rodríguez-García et al. (2014); Wang et al. (2014) and Zhang et al. (2010). These advances are very encouraging in agroforestry technology and are leading the way to the development of new treatments in the control of tree diseases, at least for incipient infections, thus possibly reducing economic and ecological damage.

4.2. Mycoviruses transmission and biological conditions

The existence of vegetative incompatibility is a major limitation in virocontrol, due to the instability of hyphal fusion between fungi that have not the same vc type. In the case of *C. parasitica*, fungal viruses can be transferred thought anastomosis among different vc types (0.13-0.50 transmission rates between CHV-1 strains differentiated by one or two vegetative incompatibly genes), although slowly and in less proportion (3-4%) (Cortesi, McCulloch, Song, Lin, & Milgroom, 2001; Liu & Milgroom, 1996; Peters, Holweg, Rigling, & Metzler, 2012). This limitation in biocontrol may be reduced with more knowledge about vc types at the population level. Papazova-Anakieva, Sotirovski, Cortesi, and Milgroom (2008) studied CHV-1 transmission between vc types in Macedonia, where only five vc types were detected and high rates of transmission between isolates with predominance in one direction were found. So that, *vic* genes for this species has been characterised (Choi et al., 2012; Zhang, Spiering, Dawe, & Nuss, 2014) enabling multilocus PCR assays development in order to analyse incompatibility genes profiles in field populations of fungus (Short et al., 2015).

Zamora, Martín, Rigling, and Diez (2012) studied vc types and mating types involved in this disease in the region of Castilla y León (Spain) and 11 vc types were identified. Two of these accounted for 88% of *C. parasitica* in the sampled population. Five of the remaining vc types were scarce (<10 isolates/vc type). In relation to the mating types present in *C. parasitica*, two mating types were found: MAT-1 was the most frequent and MAT-2 was only present in two of the provinces studied. It was concluded that the low diversity of vc types may explain the low incidence of MAT-2, supporting the idea that the fungus mainly undergoes asexual reproduction. However, the presence of two mating types in the same area could increase vc type diversity in an scenario where sexual reproduction eventually dominates. Elaborating a complex database of vc types among different CHV subtypes involves a large sampling effort, especially in areas with a high diversity of subtypes (>130 vc types in China: Wang, Shao, and Lu, 1991), but could greatly improve biocontrol against chestnut blight disease. Similarly, the main problem in relation to the use of RnMBV1 (causal agent of hypovirulence process on *R. necatrix* under laboratory conditions) for biocontrol purposes is the presence of a diverse fungus population (with numerous vc types) leading to the prevalence of sexual spores over anastomosis (Chiba et al., 2009). The possibility of observing variations in the hypovirulence phenomenon caused by environmental conditions and genetic intervention was also suggested (Chiba et al., 2009). The long term transmission of virus between incompatible isolates of *R. necatrix* was studied in apple trees (Yaegashi et al., 2013). After 2-3 years, both strains of fungus originally inoculated (one virus-free and other infected by dsRNA element called N10) and their hybrids were detected in trees. Moreover, isolates of both lineages (initially infected and non-infected) contained mycovirus, despite the vegetative incompatibility. The number of viral particles increased during the study period and six new mycovirus sequences were identified. The authors suggested the possible role of mycoparasitic fungi and mycophagous invertebrates as vectors involved in virus transmission thus enabling the vc types restrictions to be overcome.

More detailed knowledge of the virus transmission process and vc types is needed in the case of *O. novo-ulmi,* especially in regions where vc types are limited, e.g. Canada (Hintz et al., 2013). Such conditions may be favourable for carrying out field assays. In the case of *F. circinatum,* the low vc type diversity detected in many locations such as Spain (Iturritxa et al., 2011; Pérez-Sierra et al., 2007) and other regions where recent introduction of the pathogen is plausible may be suitable for implementing biocontrol treatments. For example, the three previously mentioned mitoviruses (FcMV1, FcMV2-1 and FcMV2-2.) have been identified in Spanish isolates of *F. circinatum* belonging to the both local mating types, and it has been suggested that the occurrence of these mitoviruses is not restricted by the mating type compatibility (Vainio, Martínez-Álvarez, Bezos, Hantula, and Diez, 2015). Therefore, if any of the three recently identified mycoviruses (Martínez-Álvarez et al., 2014b) were found to cause hypovirulence, inoculation treatments could be implemented as in the European chestnut blight technique.

Only three different vc types of *V. dahliae* have been identified in ornamental woody plants in Illinois (USA) (Chen, 1994). The lower diversification in the population was suggested to be related to the eventual establishment of the fungus in nurseries with subsequent dispersion. The presence of virus in less aggressive fungal isolates and high affinity in vc types suggests that the use of VdCV1 or VaaPV1 for biocontrol purposes is feasible. Indeed, VdCV1 has been isolated in non-defoliating strains of fungus (Cao et al., 2011). Nevertheless, these mycoviruses have not been shown to induce hypovirulence. Similarly, in a study of vc types involved in ash dieback in the UK, strong vegetative incompatibility was found between isolates from the same population (Brasier and Webber, 2013). The authors concluded that the low degree of compatibility may be caused by the genotype heterogeneity as a result of the well-known dominance of sexual reproduction in the species (Gross, Zaffarano, Duo, & Grünig, 2012; Gross et al., 2014). The mycoviruses that infect this pathogen are known to be genetically diverse (estimated nucleotide reposition rate 0.16) and able to infect sexual spores (Schoebel et al., 2014). The low compatibly between isolates may preclude their use in biocontrol. However, rapid changes in the mycovirus genome and the infrequent role of ascospores as virus vectors imply new opportunities in virocontrol research for this invasive disease

Regarding inter-specific transmission of mycoviruses, the high level of genetic similarity between HetPV1 strains (98% in polymerase sequence) isolated from different species of *Heterobasidion* (*Heterobasidion australe* and *H. parviporum*) infecting the same host suggests that mycovirus transmission is frequent in this fungal complex in nature (Vainio, Hakanpää, et al., 2011). This is also supported by the findings of laboratory studies with *Heterobasidion* spp., which demonstrated inter and intraspecific transmission via anastomosis (Ihrmark et al., 2002; Vainio et al., 2010). Furthermore, the possibility of protoplasmic transmission of mycoviruses in *Fusarium boothii* was analysed (Lee, Yu, Son, Lee, and Kim, 2011). These authors used the protoplast fusion method to inoculate FgV1-DK21 into *F. graminearum*, *Fusarium asiaticum*, *Fusarium oxysporum* f. sp. *lycopersici* and *C. parasitica*. They showed that this method could be used for inter- and intraspecific virus transmission and reported changes in colony morphology caused by mycovirus presence, even in fungi with no known hypovirulence related to FgV1-DK21. The survival rate of tomato plants (*Solanum* sp.) infected with mycovirus-treated *Fusarium* spp. was higher (71.7%) than in virus-free isolates (23.3%). In *C. parasitica*, FgV1-DK21 was effectively transmitted via *F. boothii* protoplast, and the virulence was lower than in virus-free and CHV-1 infected isolates. These results have clear implications for the development of management strategies in the medium term, opening the way for a new area of research involving the use of fungal complex in virocontrol at the community level.

The RNA silencing process was investigated in Rosellinia necatrix partitivirus 2 (RnPV2) infecting a non-natural host (*C. parasitica* isolates) (Chiba, Lin, Kondo, Kanematsu, and Suzuki, 2013). A wild type fungus and another mutant strain with defective protein processing sRNAs (dicer-like 2) were used. The wild-type *C. parasitica* showed milder symptoms after infection than the defective RNA silencing mutant (called Δdcl-2 mutant), suggesting that the antiviral response mechanism detected nonspecific *Partitivirus* sp. as a target. Furthermore, infections involving a defective interfering dsRNA1 (DI-dsRNA1) strain were less effective. By contrast, the natural host (*R. necatrix*) remained asymptomatic after the same treatments. In conclusion, this study suggests the potential for using mycoviruses provided by other fungal species in virocontrol and highlights the need for more detailed knowledge about the RNA silencing process. In a study of transfection of *Partitivirus* sp. (RnPV1) and the *Mycoreovirus* sp. (MyRV3) from *R. necatrix* donor isolates to *Diaporthe* sp., *C. parasitica* and *Valsa* *ceratosperma* protoplasts, successful horizontal transmission into these fungi was reported (Kanematsu, Sasaki, Onoue, Oikawa, and Ito, 2010). Infection by MyRV3 caused hypovirulence symptoms in all these new hosts. This result suggests a new line in virocontrol techniques.

Sclerotinia sclerotiorum partitivirus 1 (SsPV1), a mycovirus isolated from hypovirulent strains of white mould (*S. sclerotiorum*), has been found to be able to infect *B. cinerea* and also to be transferred via anastomosis among vc types and even overcome incompatibility barriers (Xiao et al., 2014). With regard to the high specificity of this mycovirus in host selection, biosafety in field use is guaranteed (Yu et al., 2013). These noteworthy findings demonstrate the possibility of improving the biological control techniques by using different mycoviruses, even in different pathogenic fungi. This opens up new research lines involving forest pathology biocontrol.

4.3. Future challenges in mycovirus-based biocontrol

Hypovirulence caused by co-infection is an interesting topic in biocontrol. Hypovirulence has been associated with simultaneous infection between MYRV-1 and CHV-1 in *C. parasitica* isolates (Sun, Nuss, and Suzuki, 2006). The co-infection produced similar colony changes as single CHV-1 infection, while conidia production and mycelial growth decreased when both viruses were present. Furthermore accumulation of dsRNA and vertical transmission of MyRV1 increased with co-infection, with no negative effects on CHV-1 genome RNA accumulation. In a more recent study, infection of *B. dothidea* isolates with BdPV1 mycovirus alone did not reduce growth, although the idea of a possible synergistic hypovirulence effect caused by simultaneous infection by BdCV1 and BdPV1 was suggested (Wang et al., 2014). Indeed, co-infection caused by distantly related viruses was recently found to be more stable in isolates of

Heterobasidion sp. (Vainio et al., 2014). This has important consequences for the distribution of viruses and the co-existence of different viral strains in the same host population. A very recent study showed greater effects of RNA silencing in Rosellinia necatrix victorivirus 1 (RnVV1) hosted by *C. parasitica* than in other mycoviruses naturally hosted by this fungus (CHV1 and MyRV1), suggesting an antagonistic relationship between mycoviruses co-infecting the same isolates (Chiba and Suzuki, 2015). CHV1 and MyRV1 interfered in replication and lateral transmission of RnVV1 and were involved in RNA silencing activation; however, these mycoviruses showed higher resistance of antiviral defence effects and were mainly RnVV1 suppressed, even when the host dicer or Argonaute genes were disrupted. Further studies focusing on the co-infection process are needed. If the combined effects of mycoviruses in its hosts are clarified, new advances in the preventive inoculation of virus complex may be possible.

In depth study of the interactions between mycoviral infections and environmental features is also required. In laboratory assays of *G. abietina* cultures under multiple different growth conditions, mycelial growth was highest in mycovirus free isolates (Romeralo, Botella, Santamaria, and Diez, 2012). However, it was not clear whether this phenomenon was mediated by mycoviruses or only by individual virulence of the strain. New studies focusing on this aspect are required for the development of virocontrol methods.

Research on the persistence of mycoviruses after the use of biocontrol strategies is scarce. In one of the few studies of this aspect, American chestnut plots were evaluated 12 years after biocontrol implementation against chestnut blight (Liu et al., 2002). CHV-1 was not detected in any isolate, and biocontrol failure was proposed as a possible reason for this absence. The persistence of CHV-2 and CHV-3 was limited. By contrast, although CHV-4 was common in the study area, attributing its origin to the introduction during biocontrol treatment was regarded as doubtful. Another study reported the disappearance of CHV-1 in European treated plots 24 years after biocontrol application (Robin, Lanz, Soutrenon, & Rigling, 2010). The authors pointed out that the low diversity of vc is not necessarily related to low persistence, because similar results have been reported in other chestnut forests in Europe, and they concluded that differences in CHV-1 subtype fitness may be the most important factor in the persistence of mycoviruses in field. More research is required to establish the long-term effects of the use of mycoviruses in the field.

<<Insert Table 1 around here>>

5. Conclusions

1. Mycoviruses represent a relatively unknown group in virology and plant pathology sciences. However, the taxonomy of mycoviruses based on genetic sequences and biological characteristics (including antiviral response by hosts) is being improved greatly.

2. Chestnut blight caused by *C. parasitica* is the best known and most successful mycovirus-based biocontrol method in forest pathology. Moreover, it is the only case in which a mycovirus-based biocontrol technique has been satisfactorily implemented. This disease serves as a study model in forestry protection, with particular relevance in the development of new preventive and therapeutic measures centred on several tree species.

3. Mycovirus research focused on diseases caused by the *O. novo-ulmi*, *H. annosum* complex, *G. abietina*, *F. circinatum, B. dothidea, H. fraxineus* and *R. necatrix* is currently being developed in the forest context. Further studies involving *D. pinea*, *D. scrobiculata*, *V. dahliae* and *V. albo-atrum* pathologies are also needed.

4. Mycovirus-mediated hypovirulence is a current challenge in biocontrol research because of its potential role in the prevention and/or management of plant diseases. It could become an important tool for maintaining the health of woody species, complementing or totally replacing chemical treatments.

5. Inoculation of fungi with mycoviruses may become a new management tool for forest protection, as used in the treatment of chestnut blight disease.

6. The main targets of study in mycovirus-based biological control are: (i) the mycoviruses that induce hypovirulence in their hosts, (ii) the conditions that affect hypovirulence and the virus silencing process, (iii) the transmission ecology and its biological limitations, (iv) the taxonomical and phylogenetic relationships between mycoviruses and (v) the viability of field biocontrol measures.

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Tables and figures

Table 1. Summary of mycoviruses of agroforestry interest.

Fungus	Main Hosts	Mycoviruses	Family	References
C. parasitica	*Castanea* spp.	CHV-1; CHV-2; CHV-3; CHV-4; MyRV-1; MyRV2; CpMV1	*Hypoviridae;* *Reoviridae* *Narnaviridae*	Hillman et al. (1994); Hillman and Suzuki (2004); Linder-Basso et al. (2005); Shapira et al. (1991); Smart et al. (1999); Suzuki et al. (2004)
O. novo-ulmi	*Ulmus* spp.	OnuMV1a; OnuMV1b; OnuMV1c; OnuMV2; OnuMV3a; OnuMV3b; OnuMV4-Ld; OnuMV5-Ld; OnuMV6-Ld; OnuMV7-Ld; DsRNA01_ORF; DsRNA02_ORF	*Narnaviridae*	Hong et al. (1998a,b, 1999); Doherty et al. (2006); Hintz et al. (2013)
H. annosum complex	Various	HaV; HaV-P; HetPV1; HetPV2; HetPV3; HetPV4; HetPV5;	*Partitiviridae*	Ihrmark et al. (2001) Vainio et al. (2010, 2011a,b, 2012, 2013c, 2014)

		HetPV6; HetPV7; HetPV8; HetPV2-pa1; HetPV7-pa1		
G. abietina	*Pinus* spp., *Picea* spp., *Abies* spp., *Larix* spp.	GaMRV-S1; GaRV-L1; GaRV-MS1	*Narnaviridae;* *Totiviridae;* *Partitiviridae;*	Tuomivirta and Hantula (2003a,b)
F. circinatum	*Pinus* spp.; *Pseudotsuga menziesii*	FcMV1; FcMV2-1; FcMV2-2	*Narnaviridae*	Martínez-Álvarez et al. (2014b)
B. dothidea	*Pyrus* spp., *Malus* spp., *Eucalyptus* spp.	BdCV1; BdPV1	*Chrysoviridae;* *Partitiviridae*	Wang et al. (2014)
H. fraxineus	*Fraxinus* spp.	HfMV1	*Narnaviridae*	Schoebel et al. (2014)
B. cinerea	Various	BcMV1	*Narnaviridae*	Wu et al. (2010)
V. dahliae	Various	VdCV1	*Chrysoviridae*	Cao et al. (2011)
V. albo-atrum	Various	VaaPV1	*Partitiviridae*	Cañizares et al. (2014)
D. pinea	*Pinus* spp.	SsRV1; SsRV2	*Totiviridae*	Preisig et al. (1998)
D. scrobiculata	*Pinus* spp.	DsRV1	*Chrysoviridae-related*	De Wet et al. (2011)
R. necatrix	Various	RnMBV1; RnPV2	*Megabirnaviridae;* *Partitiviridae*	Chiba et al. (2009, 2013)

Figure 1. General taxonomy of mycoviruses according to ICTV classification criteria, Virus Taxonomy 2014 Release.*Classification under consideration; **Family proposed by Ghabrial et al. (2015).