

1 **The use of mycoviruses in the control of forest diseases**

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27 **Abstract**

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

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

43 **1. Introduction**

44 Viruses that infect fungi, i.e. mycoviruses, are frequent in the subkingdom Dikarya
45 (phyla Ascomycota and Basidiomycota), phyla Blastocladiomycota and
46 Neocallimastigomycota (formerly Chytridiomycota) and Glomeromycota (formerly
47 Zygomycota) (Herrero, Dueñas, Quesada-Moraga, & Zabalgogezcoa, 2012; Hibbett et
48 al., 2007). Most fungal genera, ranging from microscopic yeasts to the more evolved
49 edible mushrooms, have been described as hosts of mycoviruses (Hammond, Andrewski,
50 Roossinck, & Keller, 2008; Lim et al., 2005; Magae, 2012; Ro et al., 2007; Schmitt &
51 Breinig, 2006; Stielow, Klenk, Winter, & Menzel, 2011; Strauss, Lakshman, & Tavantzis,
52 2000). This also applied to filamentous fungi that cause plant diseases.

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

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

63 2. General aspects of mycoviruses

64 2.1. Taxonomy, diversity and biology

65 More than 250 fungus-related viral sequences have been identified and sequenced
66 according to National Center for Biotechnology Information (NCBI, 2014; Xie & Jiang,
67 2014), resulting in 22 genera divided among 15 families according to the list published
68 by the International Committee on Taxonomy of Viruses (ICTV, 2014) (Figure 1).
69 Nevertheless, 20% of mycoviruses have not yet been catalogued (Pearson, Beever, Boine,
70 & Arthur, 2009; Van Regenmortel et al., 2010).

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

87 satellite elements associated with the main genome particles (e.g. 0.9-1.4 kb elements
88 associated with 3.7-5.0 kpb mycovirus genome in basydiomicetous yeast
89 *Xanthophyllomyces dendrorhous*; anamorph: *Phaffia rhodozyma* (Flores, Alcaíno,
90 Fernandez-Lobato, Cifuentes, & Baeza, 2015)).

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

102 <<Insert Figure 1 around here>>

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

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

117 **2.2. Transmission of mycovirus**

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

131 In vertical transmission, mycoviruses commonly infect asexual spores. Nevertheless,
132 prevalence rates may vary significantly between species, e.g. in *Heterobasidion annosum*
133 only 3% of conidia are infected (Ihrmark, Johannesson, Stenström, & Stenlid, 2002) in
134 contrast to 100% infection in *Cryphonectria parasitica* (Ding et al., 2007). Fungal viruses

135 can also colonise sexual spores, infecting a new generation of the host: 8-13% dsRNA
136 infected ascospores of *Magnaporthe grisea* (Chun & Lee, 2009) whereas 10-84% dsRNA
137 infected basidiospores of *H. annosum* (Ihrmark, Stenström, & Stenlid, 2004). However,
138 in a more recent study, lower vertical transmission of *Heterobasidion parviporum* to
139 basidiospores (8.3%) was observed in a spruce forest (Vainio, Müller, Korhonen, Piri, &
140 Hantula, 2014). The authors of the latter study suggested that continuous spore load in
141 stumps may be related to the high rate of infected basidiospores, in contrast to low rates
142 of infection in standing trees, as previously reported. It is now considered that the
143 predominant route of viral transmission is via asexual spores, and vertical transmission
144 has not been reported to occur in many fungal species (Carbone, Liu, Hillman, &
145 Milgroom, 2004; Milgroom & Hillman, 2011).

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

153 On a larger scale, transmission of mycoviruses between species has also been reported
154 (Lee, Yu, Son, Lee, & Kim, 2011; Liu, Linder-Basso, Hillman, Kaneko, & Milgroom,
155 2003; Vainio et al., 2011a), opening up new research lines focusing on the genetic,
156 evolutionary and ecological factors involved in transmission.

157 **2.3. Hypovirulence process**

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

174 Both hyper- and hypovirulence are strongly related to the presence of specific viruses,
175 even in co-infection. Four dsRNA mycoviruses have been detected in *Nectria radicola*
176 (anamorph: *Cylindrocarpon destructans*) (Ahn and Lee, 2001). Removal of one virus, L1
177 (6.0 kbp), caused a reduction in virulence of the fungus, while later reinfection through
178 anastomosis recovered the virulence of the isolate. Detailed laboratory studies
179 complemented with pathogenicity field assays are essential for developing virocontrol
180 techniques.

181 One challenge in plant pathology and the use of mycoviruses is the antiviral response
182 of fungi or RNA silencing. When viruses infect healthy cells, dicer-type nucleases initiate

183 a response that produces viral RNA processed segments (sRNAs). The RNA-induced
184 silencing complex then identifies homologous sequences on mRNA and subsequently
185 degrades sRNAs (Dang, Yang, Xue, & Liu, 2011; Hammond et al., 2008; Nuss, 2011;
186 Schumann, Ayliffe, Kazan, & Wang, 2010; Tauati, Pearson, Choquer, Foster, & Bailey,
187 2014; Yaegashi, Yoshikawa, Ito, & Kanematsu, 2013). In a study attempting to clarify
188 this evolutionary relationship, Segers, Zhang, Deng, Sun, and Nuss (2007) found
189 symptomatic differences between hypovirulence-mycovirus infected *C. parasitica*
190 isolates. The use of *C. parasitica* strains in which RNA silencing genes were disrupted
191 enabled identification of genes coding for particular dicer and argonaute-like proteins as
192 required elements in antiviral response (Sun, Choi, & Nuss, 2009; Zhang & Nuss, 2008).

193 3. Mycoviruses in forest diseases: case studies

194 3.1. *Cryphonectria parasitica*

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

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

210 The best known example of a mycovirus that causes hypovirulence is CHV-1. When
211 CHV-1 infects *C. parasitica* it causes weakness, reducing mycelial growth and
212 sporulation. Infected fungi are only capable of forming superficial (healing) cankers on
213 stems, and the trees can therefore survive the disease. Other symptoms of the presence of
214 CHV-1 in isolates include changes in colony morphology and colour (Peever, Liu,
215 Cortesi, & Milgroom, 2000; Rigling, Heiniger, & Hohl, 1989). CHV-1 originally
216 occurred in Europe (Italy and France) and Asia (Japan, China and Korea) but was later

217 introduced into the USA (Allemann et al., 1999; Liu, Double, MacDonald, & Milgroom,
218 2002). Five genetically characterised subtypes of CHV-1 have been identified: F1 and F2
219 (from France), I (Italy), D (Germany) and E (Spain) (Allemann et al., 1999; Gobbin,
220 Hoegger, Heiniger, & Rigling, 2003; Zamora, Martín, San Martín, Martínez-Álvarez, &
221 Diez, 2014). CHV-1 is now considered an important biocontrol tool in European.

222 CHV-2 and CHV-3 are both common in North America. However, although CHV-2
223 occurs in native *C. parasitica* in Asia (Hillman, Tian, Bedker, & Brown, 1992; Peever
224 et al., 1998), CHV-3 is only present in the USA (Michigan) (Peever, Liu, & Milgroom,
225 1997). Both CHV-2 and CHV-3 have proved useful in biocontrol as they induce
226 hypovirulence in American forests and plantations. The mycovirus most commonly
227 associated with chestnut blight in American forests (CHV-4) is traditionally considered
228 to induce a cryptic state and is therefore not useful for biocontrol purposes (Enebak,
229 MacDonald, & Hillman, 1994; Linder-Basso, Dynek, & Hillman, 2005).

230 Mycoreovirus 1 (MyRV-1) (*Reoviridae*) has been identified in hypovirulent strains of
231 chestnut blight fungus (Suzuki, Supyani, Maruyama, and Hillman, 2004). Viral
232 transmission of this *Mycoreovirus* sp. to sexual spores has been reported (Deng, Allen,
233 Hillman and Nuss, 2007), and reovirus-infected isolates have been shown to produce
234 mature perithecia and viable ascospores, which in turn host MyRV-1. Other mycoviruses
235 belonging to the *Reoviridae* and *Narnaviridae* families - respectively Mycoreovirus 2
236 (MyRV2) and *Cryphonectria mitovirus 1* (CpMV1) - have also been identified (Hillman
237 & Suzuki, 2004). This fungus can host many *Reoviridae*, *Partitiviridae*, *Totiviridae* and
238 *Megabirnaviridae* mycoviruses that usually infect other fungi (Eusebio-Cope et al.,
239 2015).

240 **3.2. *Ophiostoma novo-ulmi***

241 Dutch elm disease (DED), caused by *Ophiostoma ulmi* and *O. novo-ulmi*, was the most
242 devastating disease affecting elms (*Ulmus* spp.) in Europe during the 20th Century (some
243 30 million elms were killed in the UK) (Brasier, 2001; Potter, Harwood, Knight, &
244 Tomlinson, 2011). These fungi cause death of the tree by vessel cavitation due to fungal
245 growth in the xylem. Two pandemics have occurred. In the first, which began in the
246 1910s, *O. ulmi* spread through Europe causing severe damage to adult trees and later
247 spread to North America. In the 1950s, two subspecies of *O. novo-ulmi* (Euro-Asian race:
248 *O. novo-ulmi* subsp. *novo-ulmi*; and the North American race: *O. novo-ulmi* subsp.
249 *americana*) caused high mortality in European and American forests. In both cases, bark
250 beetles (Coleoptera, Scolytinae) played an important role as vectors of the disease
251 (Brasier & Kirk, 2010; Brasier, 1976, 1991; Santini & Faccoli, 2014).

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

264 In addition, other *Ophiostoma* species have been demonstrated to harbour
265 mycoviruses. *Ophiostoma minus* (causal agent of blue stain in pine wood), and the

266 saprophyte *Ophiostoma quercus* hosts viruses belonging to the *Totiviridae* and
267 *Partitiviridae* families (respectively *Ophiostoma minus totivirus* (OmV) and *Ophiostoma*
268 *quercus partitivirus 2* (OPV2) (Doherty et al., 2007). A distant relationship between
269 OPV2 and *Ophiostoma partitivirus 1* (OPV1) was suggested (Doherty et al., 2007).
270 OPV1, which was previously detected in the pathogenic fungus *Ophiostoma himal-ulmi*
271 (Crawford et al., 2006), is not currently used in biocontrol.

272 **3.3. *Heterobasidion annosum***

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

286 Additionally, dsRNA mycoviruses in P and S types of *H. annosum* (*Heterobasidion*
287 *partitivirus P* (HaV-P) and *Heterobasidion annosum virus* (HaV)) have been partially
288 sequenced (Ihrmark, Zheng, Strenstöm, & Stenlid, 2001). The authors included these
289 mycoviruses in *Partitiviridae* and reported that *H. annosum* s.l. harbours dsRNA viruses

290 at a frequency of approximately 15% in Europe and western Asia. A new putative member
291 of *Partitiviridae*, Heterobasidion partitivirus 3 (HetPV3), was subsequently detected in
292 Chinese strains of *Heterobasidion ecrustosum* (Vainio, Korhonen, Tuomivirta, and
293 Hantula, 2010). In a later study, a new dsRNA virus belonging to *Partitiviridae* and
294 designated Heterobasidion partitivirus 2 (HetPV2) clearly formed a subcluster with HaV-
295 P due to their genomic similarities (Vainio et al., 2011b). In addition, three new putative
296 viruses, also included in *Partitiviridae*, were catalogued and subsequently named
297 Heterobasidion partitivirus 1 (HetPV1), HetPV4 and HetPV5 (Vainio et al., 2011a).
298 These authors proposed a close genetic relationship between HetPV1 and HaV, while the
299 two other viruses were found to be more similar to mycoviruses associated with
300 *Heterobasidion parviporum* partitivirus Fr110B and other disease-associated viruses.

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

312 **3.4. *Gremmeniella abietina***

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

323 Three families of dsRNA mycoviruses have been detected in this forest pathogen:
324 *Gremmeniella abietina* mitochondrial RNA virus S1 (GaMRV-S1, *Narnaviridae*)
325 (Tuomivirta & Hantula, 2003a); *Gremmeniella abietina* RNA virus L1 (GaRV-L1,
326 *Totiviridae*); and *Gremmeniella abietina* RNA virus MS1 (GaRV-MS1, *Partitiviridae*)
327 (Tuomivirta & Hantula, 2003b), with a high frequency of occurrence; e.g. the
328 mycoviruses have been detected in 89% of Spanish isolates (Botella, Tuomivirta,
329 Hantula, and Diez, 2012) and in 50% of Turkish isolates (Aday et al., 2012). In addition,
330 three mycoviruses were found together infecting the same isolates of *G. abietina* var.
331 *abietina* type A (Tuomivirta and Hantula, 2005). Later, Botella, Tuomivirta, Vervuurt,
332 Diez, and Hantula (2012) reported the absence of mitoviruses in biotype B from Turkey,
333 biotype A from North America and European Alpine biotype. On the contrary, biotype A
334 from Finland and Spain hosted mycoviruses. Specifically, Spanish populations hosted
335 two mycoviruses (GMV1 and GMV2) in high proportion (74% of isolates hosted
336 dsRNA). These authors discussed the possible factors determining presence and
337 transmission of mitoviruses between fungal races and highlighted the role of asexual

338 reproduction in virus widespread. In fact, the higher proportion of mitovirus presence was
339 detected in Spain where only asexual reproduction has been reported. Regarding the high
340 presence and the low genetic variability detected in GMV2 in Spanish isolates, the
341 researchers suggested a possible recent host switch and a subsequent adaptation to these
342 new conditions. The findings of recent RdRp sequencing studies support the idea of a low
343 degree of genetic variation in *G. abietina* mitoviruses in the European population
344 (Botella, Tuomivirta, Hantula, Diez, & Jankovsky, 2014).

345 **3.5. *Fusarium circinatum***

346 Pine pitch canker is a virulent disease caused by *Fusarium circinatum* (teleomorph:
347 *Gibberella circinata*) in many pine species and in Douglas fir (*Pseudotsuga menziesii*)
348 worldwide. Infections have also been observed to cause significant damage in *Abies alba*,
349 *S. giganteum*, *Larix decidua* and *Picea abies* (Martínez-Álvarez, Pando, and Diez,
350 2014a). The pathogen was first detected in the southeastern USA and Mexico (where it is
351 probably endemic) and then in Haiti, South Africa, Chile, France, Korea, Spain, Italy,
352 Japan, Portugal, Uruguay and Brazil (Aegerter, Gordon, Storer, & Wood, 2003; Enebak
353 & Stanosz, 2003; Gordon, Kirkpatrick, Aegerter, Wood, & Storer, 2006; Martínez-
354 Álvarez, Alves-Santos, & Diez, 2012; Pfenning, Costa, Melo, Costa, & Aires, 2014). This
355 fungus causes dieback in trees due to the formation of bleeding and resinous cankers on
356 trunk and branches. Moreover, *F. circinatum* frequently causes death and damping-off of
357 seedlings through both pre- and post-emergence infection, making such infections a
358 significant threat to nurseries and afforestations (Aegerter et al., 2003; Hammerbacher,
359 Ganley, Steenkamp, Gordon, & Coutinho, 2008).

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

362 1, 2-1 and 2-2 (FcMV1, FcMV2-1 and FcMV2-2) (Martínez-Álvarez, Vainio, Botella,
363 Hantula, and Diez, 2014b). The genetic structure of the mycoviruses hosted by *F.*
364 *circinatum* isolates from Spain and South Africa has also been studied (Vainio, Martínez-
365 Álvarez, Bezos, Hantula, and Diez, 2015). Only Spanish isolates were found to host
366 mycoviruses, which showed very similar sequence variants (>95% similarity). Indeed, a
367 high rate of asexual spore transmission of mycoviruses (ranging between 70% and 100%)
368 has been preliminary observed (Bezos, Martínez-Álvarez, Romeralo, and Diez, 2015),
369 indicating the potential use of the mycoviruses as biocontrol agents.

370 **3.6. *Botryosphaeria* spp.**

371 *Botryosphaeria* spp. commonly occur as endophytic fungi in healthy hosts, but may
372 become virulent when their host is subjected to environmental stress or physical damage
373 (Burgess, Sakalidis, & Hardy, 2006; Smith, Crous, Wingfield, Coutinho, & Wingfield,
374 2001; Smith, Wingfield, Crous, & Coutinho, 1996). Despite its taxonomic complexity,
375 *Botryosphaeria dothidea* (anamorph: *Fusicoccum aesculi*) is cited as the causal agent of
376 stem and branch cankers on apple trees (*Malus domestica*), ring spot on pear trees (*Pyrus*
377 *communis*) and dieback and stem cankers on eucalyptus trees (*Eucalyptus* spp.) among
378 many other woody species (Brown-Rytlewski & McManus, 2000; Slippers & Wingfield,
379 2007). *Eucalyptus* sp. is one of the most common trees planted in commercial and clonal
380 forestry at an international level. Eucalyptus dieback and cankers are of special interest
381 in forest science because of the reduced growth, offspring failure and adult tree death
382 caused by the pathogen (Pérez, Wingfield, Slippers, Altier, & Blanchette, 2010). The
383 gummy exudation produced in cankers also makes the wood less valuable, causing
384 significant economic losses in the forest industry (Rodas, Slippers, Gryzenhout, &
385 Wingfield, 2009).

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

394 **3.7. *Hymenoscyphus fraxineus***

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

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

419 **3.8. Other fungal pathogens in woody plants**

420 *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) causes grey mould disease in
421 more than 200 crops species over the world, including farmland crops, ornamental species
422 and fruit crops such as grapes (*Vitis vinifera*), pear trees, raspberries and blackberries
423 (*Rubus* spp.) (Rodríguez-García, Medina, Alonso, & Ayllón, 2014; Williamson,
424 Tudzynski, Tudzynski, & van Kan, 2007). The presence of different genera of mycovirus
425 in this fungus has been widely reported (Castro, Kramer, Valdivia, Ortiz, & Castillo,
426 2003; Potgieter, Castillo, Castro, Cottet, & Morales, 2013; Rodríguez-García et al., 2014;
427 Wu et al., 2007; Zhang, De Wu, Li, Jiang, & Huang, 2010). These studies highlight the
428 wide diversity of viruses that this fungus is able to host and which provide a wide range
429 of opportunities for research in the field of fungal virology. Another three mycoviruses
430 that infect *Botrytis* sp. have recently been sequenced: *Botrytis virus F* (BVF,
431 *Gammaflexiviridae*), *Botrytis virus X* (BVX, *Alphaflexiviridae*) and *Botrytis porri* RNA
432 virus 1 (BpRV1, dsRNA virus) (Xie & Jiang, 2014).

433 *Verticillium dahliae* and *Verticillium albo-atrum* are both causal agents of *Verticillium*
434 wilt disease. They have been cited in a broad range of hosts and more than 200 species,
435 including bushes and trees (Schall & Davis, 2009; Smith, 1965). Specifically, *V. dahliae*
436 can infect economically important woody crops such as gooseberry (*Ribes grossularia*),
437 apricot (*Prunus armeniana*), olive (*Olea europea*), quince (*Cydonia oblonga*) and roses
438 (*Rosa* spp.), as well as other species of ecological interest such as maple (*Acer palmatum*),
439 sycamore (*Acer pseudoplatanus*), raspberry, honeysuckle (*Lonicera* sp.) and broom
440 (*Cytisus scoparius*). *V. albo-atrum* causes damage to the tree of heaven (*Ailanthus*
441 *altissima*), striped maple (*Acer pennsylvanicum*), yellow poplar (*Liriodendron tulipifera*)
442 and other landscape species (Morehart, Donohue, & Melchior, 1980; Schall & Davis,
443 2009; Smith, 1965). Some studies have demonstrated the presence of mycoviruses in
444 these pathogenic fungi. For example, a *Chrysovirus* sp. named *Verticillium dahliae*
445 chrysovirus 1 (VdCV1) was identified by Cao et al. (2011). A novel member of the family
446 *Partitiviridae* was identified in *V. albo-atrum*: *Verticillium albo-atrum partitivirus 1*
447 (VaaPV1) (Cañizares, Pérez-Artés, and García-Pedrajas, 2014), although no details were
448 provided about the pathogenic effect of the mycovirus in its fungal host.

449 Some opportunistic fungal pathogens of *Pinus* spp., such as *Diplodia pinea* (synonym:
450 *Sphaeropsis sapinea*) and *Diplodia scrobiculata* (Smith et al., 1996), also host
451 mycoviruses. Two dsRNA mycoviruses have been identified in *D. pinea*: *Sphaeropsis*
452 *sapinea* RNA virus 1 and 2 (SsRV1, SsRV2 respectively; *Totiviridae*) (Preisig, Wingfield,
453 & Wingfield, 1998); and one in *D. scrobiculata*: *Diplodia scrobiculata* RNA virus 1
454 (DsRV1; related to *Chrysoviridae*) (De Wet, Bihon, Preisig, Wingfield, & Wingfield,
455 2011; De Wet, Preisig, Wingfield, & Wingfield, 2008).

456 Another pathogenic fungi of interest in agroforestry is the causal agent of root rot
457 disease, *Rosellinia necatrix* (anamorph: *Dematophora necatrix*). The interest is due to the

458 pathogenicity of the fungus in several woody species e.g. apple, olive, grape and poplar
459 (*Populus* spp.) (Pérez-Jiménez, 2006). Many families of mycoviruses are known to infect
460 this fungus, e.g. *Chrysoviridae*, *Quadriviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae*
461 (Xie and Jiang, 2014). Two dsRNA mycoviruses have also been associated with
462 hypovirulence: *Rosellinia necatrix* megabirnavirus 1 (RnMBV1), included in a new
463 family of mycoviruses (*Megabirnaviridae*) and *Rosellinia necatrix* partitivirus 2 (RnPV2)
464 (Xie and Jiang, 2014).

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465 **4. Future perspectives for use of mycoviruses in biocontrol**

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

474 ***4.1. Identification of factors leading to hypovirulence: research in progress and lessons*** 475 ***learned***

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

486 In relation to the application of biocontrol in diseased forests in boreal areas, no clear
487 relationship between viral presence and fungus growth rate was observed in *H. annosum*
488 s.l. (Vainio et al., 2010). However, significant variations in growth and changes in the

489 effects of virus were observed in relation to the culture conditions. The effect of HetPV6
490 infection in relation to multiple variables (geographical, culture conditions and host) has
491 been investigated in four *Heterobasidion* species (Vainio et al., 2012). No significant
492 differences in growth were found in *H. parviporum* (*in vivo* and *in vitro*) or *H. annosum*
493 (*in vivo*). However, significantly increased mycelial growth was observed in infected *H.*
494 *annosum* cultures (laboratory assays condition: 6° C and 15°C culture on MOS agar
495 plates). Consequently, these results do not support a possible use of HetPV6 in
496 virocontrol, although HetPV6 is very frequent in fungal populations and apparently does
497 not interfere in subsequent viral infection (Vainio et al. 2013b).

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

505 Several *Totiviridae*, *Chrysoviridae* and *Partiviridae* mycoviruses have been identified
506 in *Fusarium graminearum* (Lee, Son, & Kim, 2011; Yu et al. 2011). More specifically, a
507 mycovirus described in *F. graminearum* infecting maize in Korea (named *Fusarium*
508 *graminearum* virus 1-DK2; FgV1-DK2) is capable of reducing mycelial growth and
509 sporulation, decreasing mycotoxin production and increasing pigmentation (Chu et al.,
510 2002). In a later study addressing this topic, a mixed infection of two dsRNA viruses was
511 reported, with no changes in mycelial morphology but with a high rate of transmission in
512 conidia and ascospores (30-100%) (Chu et al., 2004). A recent study identified a new
513 mycovirus associated with hypovirulence in *Fusarium virguliforme* and closely related to

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

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

531 **4.2. Mycoviruses transmission and biological conditions**

532 The existence of vegetative incompatibility is a major limitation in virocontrol, due to
533 the instability of hyphal fusion between fungi that have not the same vc type. In the case
534 of *C. parasitica*, fungal viruses can be transferred through anastomosis among different
535 vc types (0.13-0.50 transmission rates between CHV-1 strains differentiated by one or
536 two vegetative incompatibility genes), although slowly and in less proportion (3-4%)
537 (Cortesi, McCulloch, Song, Lin, & Milgroom, 2001; Liu & Milgroom, 1996; Peters,

538 Holweg, Rigling, & Metzler, 2012). This limitation in biocontrol may be reduced with
539 more knowledge about vc types at the population level. Papazova-Anakieva, Sotirovski,
540 Cortesi, and Milgroom (2008) studied CHV-1 transmission between vc types in
541 Macedonia, where only five vc types were detected and high rates of transmission
542 between isolates with predominance in one direction were found. So that, *vic* genes for
543 this species has been characterised (Choi et al., 2012; Zhang, Spiering, Dawe, & Nuss,
544 2014) enabling multilocus PCR assays development in order to analyse incompatibility
545 genes profiles in field populations of fungus (Short et al., 2015).

546 Zamora, Martín, Rigling, and Diez (2012) studied vc types and mating types involved
547 in this disease in the region of Castilla y León (Spain) and 11 vc types were identified.
548 Two of these accounted for 88% of *C. parasitica* in the sampled population. Five of the
549 remaining vc types were scarce (<10 isolates/vc type). In relation to the mating types
550 present in *C. parasitica*, two mating types were found: MAT-1 was the most frequent and
551 MAT-2 was only present in two of the provinces studied. It was concluded that the low
552 diversity of vc types may explain the low incidence of MAT-2, supporting the idea that
553 the fungus mainly undergoes asexual reproduction. However, the presence of two mating
554 types in the same area could increase vc type diversity in an scenario where sexual
555 reproduction eventually dominates. Elaborating a complex database of vc types among
556 different CHV subtypes involves a large sampling effort, especially in areas with a high
557 diversity of subtypes (>130 vc types in China: Wang, Shao, and Lu, 1991), but could
558 greatly improve biocontrol against chestnut blight disease. Similarly, the main problem
559 in relation to the use of RnMBV1 (causal agent of hypovirulence process on *R. necatrix*
560 under laboratory conditions) for biocontrol purposes is the presence of a diverse fungus
561 population (with numerous vc types) leading to the prevalence of sexual spores over
562 anastomosis (Chiba et al., 2009). The possibility of observing variations in the

563 hypovirulence phenomenon caused by environmental conditions and genetic intervention
564 was also suggested (Chiba et al., 2009). The long term transmission of virus between
565 incompatible isolates of *R. necatrix* was studied in apple trees (Yaegashi et al., 2013).
566 After 2-3 years, both strains of fungus originally inoculated (one virus-free and other
567 infected by dsRNA element called N10) and their hybrids were detected in trees.
568 Moreover, isolates of both lineages (initially infected and non-infected) contained
569 mycovirus, despite the vegetative incompatibility. The number of viral particles increased
570 during the study period and six new mycovirus sequences were identified. The authors
571 suggested the possible role of mycoparasitic fungi and mycophagous invertebrates as
572 vectors involved in virus transmission thus enabling the vc types restrictions to be
573 overcome.

574 More detailed knowledge of the virus transmission process and vc types is needed in
575 the case of *O. novo-ulmi*, especially in regions where vc types are limited, e.g. Canada
576 (Hintz et al., 2013). Such conditions may be favourable for carrying out field assays. In
577 the case of *F. circinatum*, the low vc type diversity detected in many locations such as
578 Spain (Iturrutxa et al., 2011; Pérez-Sierra et al., 2007) and other regions where recent
579 introduction of the pathogen is plausible may be suitable for implementing biocontrol
580 treatments. For example, the three previously mentioned mitoviruses (FcMV1, FcMV2-
581 1 and FcMV2-2.) have been identified in Spanish isolates of *F. circinatum* belonging to
582 the both local mating types, and it has been suggested that the occurrence of these
583 mitoviruses is not restricted by the mating type compatibility (Vainio, Martínez-Álvarez,
584 Bezos, Hantula, and Diez, 2015). Therefore, if any of the three recently identified
585 mycoviruses (Martínez-Álvarez et al., 2014b) were found to cause hypovirulence,
586 inoculation treatments could be implemented as in the European chestnut blight
587 technique.

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

605 Regarding inter-specific transmission of mycoviruses, the high level of genetic
606 similarity between HetPV1 strains (98% in polymerase sequence) isolated from different
607 species of *Heterobasidion* (*Heterobasidion australe* and *H. parviporum*) infecting the
608 same host suggests that mycovirus transmission is frequent in this fungal complex in
609 nature (Vainio, Hakanpää, et al., 2011). This is also supported by the findings of
610 laboratory studies with *Heterobasidion* spp., which demonstrated inter and intraspecific
611 transmission via anastomosis (Ihrmark et al., 2002; Vainio et al., 2010). Furthermore, the
612 possibility of protoplasmic transmission of mycoviruses in *Fusarium boothii* was

613 analysed (Lee, Yu, Son, Lee, and Kim, 2011). These authors used the protoplast fusion
614 method to inoculate FgV1-DK21 into *F. graminearum*, *Fusarium asiaticum*, *Fusarium*
615 *oxysporum* f. sp. *lycopersici* and *C. parasitica*. They showed that this method could be
616 used for inter- and intraspecific virus transmission and reported changes in colony
617 morphology caused by mycovirus presence, even in fungi with no known hypovirulence
618 related to FgV1-DK21. The survival rate of tomato plants (*Solanum* sp.) infected with
619 mycovirus-treated *Fusarium* spp. was higher (71.7%) than in virus-free isolates (23.3%).
620 In *C. parasitica*, FgV1-DK21 was effectively transmitted via *F. boothii* protoplast, and
621 the virulence was lower than in virus-free and CHV-1 infected isolates. These results have
622 clear implications for the development of management strategies in the medium term,
623 opening the way for a new area of research involving the use of fungal complex in
624 virocontrol at the community level.

625 The RNA silencing process was investigated in Rosellinia necatrix partitivirus 2
626 (RnPV2) infecting a non-natural host (*C. parasitica* isolates) (Chiba, Lin, Kondo,
627 Kanematsu, and Suzuki, 2013). A wild type fungus and another mutant strain with
628 defective protein processing sRNAs (dicer-like 2) were used. The wild-type *C. parasitica*
629 showed milder symptoms after infection than the defective RNA silencing mutant (called
630 Δ dcl-2 mutant), suggesting that the antiviral response mechanism detected nonspecific
631 *Partitivirus* sp. as a target. Furthermore, infections involving a defective interfering
632 dsRNA1 (DI-dsRNA1) strain were less effective. By contrast, the natural host (*R.*
633 *necatrix*) remained asymptomatic after the same treatments. In conclusion, this study
634 suggests the potential for using mycoviruses provided by other fungal species in
635 virocontrol and highlights the need for more detailed knowledge about the RNA silencing
636 process. In a study of transfection of *Partitivirus* sp. (RnPV1) and the *Mycoreovirus* sp.
637 (MyRV3) from *R. necatrix* donor isolates to *Diaporthe* sp., *C. parasitica* and *Valsa*

638 *ceratosperma* protoplasts, successful horizontal transmission into these fungi was
639 reported (Kanematsu, Sasaki, Onoue, Oikawa, and Ito, 2010). Infection by MyRV3
640 caused hypovirulence symptoms in all these new hosts. This result suggests a new line in
641 virocontrol techniques.

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

650 **4.3. Future challenges in mycovirus-based biocontrol**

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

662 *Heterobasidion* sp. (Vainio et al., 2014). This has important consequences for the
663 distribution of viruses and the co-existence of different viral strains in the same host
664 population. A very recent study showed greater effects of RNA silencing in *Rosellinia*
665 *necatrix* victorivirus 1 (RnVV1) hosted by *C. parasitica* than in other mycoviruses
666 naturally hosted by this fungus (CHV1 and MyRV1), suggesting an antagonistic
667 relationship between mycoviruses co-infecting the same isolates (Chiba and Suzuki,
668 2015). CHV1 and MyRV1 interfered in replication and lateral transmission of RnVV1
669 and were involved in RNA silencing activation; however, these mycoviruses showed
670 higher resistance of antiviral defence effects and were mainly RnVV1 suppressed, even
671 when the host dicer or Argonaute genes were disrupted. Further studies focusing on the
672 co-infection process are needed. If the combined effects of mycoviruses in its hosts are
673 clarified, new advances in the preventive inoculation of virus complex may be possible.

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

681 Research on the persistence of mycoviruses after the use of biocontrol strategies is
682 scarce. In one of the few studies of this aspect, American chestnut plots were evaluated
683 12 years after biocontrol implementation against chestnut blight (Liu et al., 2002). CHV-
684 1 was not detected in any isolate, and biocontrol failure was proposed as a possible reason
685 for this absence. The persistence of CHV-2 and CHV-3 was limited. By contrast, although
686 CHV-4 was common in the study area, attributing its origin to the introduction during

687 biocontrol treatment was regarded as doubtful. Another study reported the disappearance
688 of CHV-1 in European treated plots 24 years after biocontrol application (Robin, Lanz,
689 Soutrenon, & Rigling, 2010). The authors pointed out that the low diversity of vc is not
690 necessarily related to low persistence, because similar results have been reported in other
691 chestnut forests in Europe, and they concluded that differences in CHV-1 subtype fitness
692 may be the most important factor in the persistence of mycoviruses in field. More research
693 is required to establish the long-term effects of the use of mycoviruses in the field.

694 <<Insert Table 1 around here>>

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695 5. Conclusions

- 696 1. Mycoviruses represent a relatively unknown group in virology and plant
697 pathology sciences. However, the taxonomy of mycoviruses based on genetic
698 sequences and biological characteristics (including antiviral response by hosts)
699 is being improved greatly.
- 700 2. Chestnut blight caused by *C. parasitica* is the best known and most successful
701 mycovirus-based biocontrol method in forest pathology. Moreover, it is the only
702 case in which a mycovirus-based biocontrol technique has been satisfactorily
703 implemented. This disease serves as a study model in forestry protection, with
704 particular relevance in the development of new preventive and therapeutic
705 measures centred on several tree species.
- 706 3. Mycovirus research focused on diseases caused by the *O. novo-ulmi*, *H.*
707 *annosum* complex, *G. abietina*, *F. circinatum*, *B. dothidea*, *H. fraxineus* and *R.*
708 *necatrix* is currently being developed in the forest context. Further studies
709 involving *D. pinea*, *D. scrobiculata*, *V. dahliae* and *V. albo-atrum* pathologies
710 are also needed.
- 711 4. Mycovirus-mediated hypovirulence is a current challenge in biocontrol research
712 because of its potential role in the prevention and/or management of plant
713 diseases. It could become an important tool for maintaining the health of woody
714 species, complementing or totally replacing chemical treatments.
- 715 5. Inoculation of fungi with mycoviruses may become a new management tool for
716 forest protection, as used in the treatment of chestnut blight disease.

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

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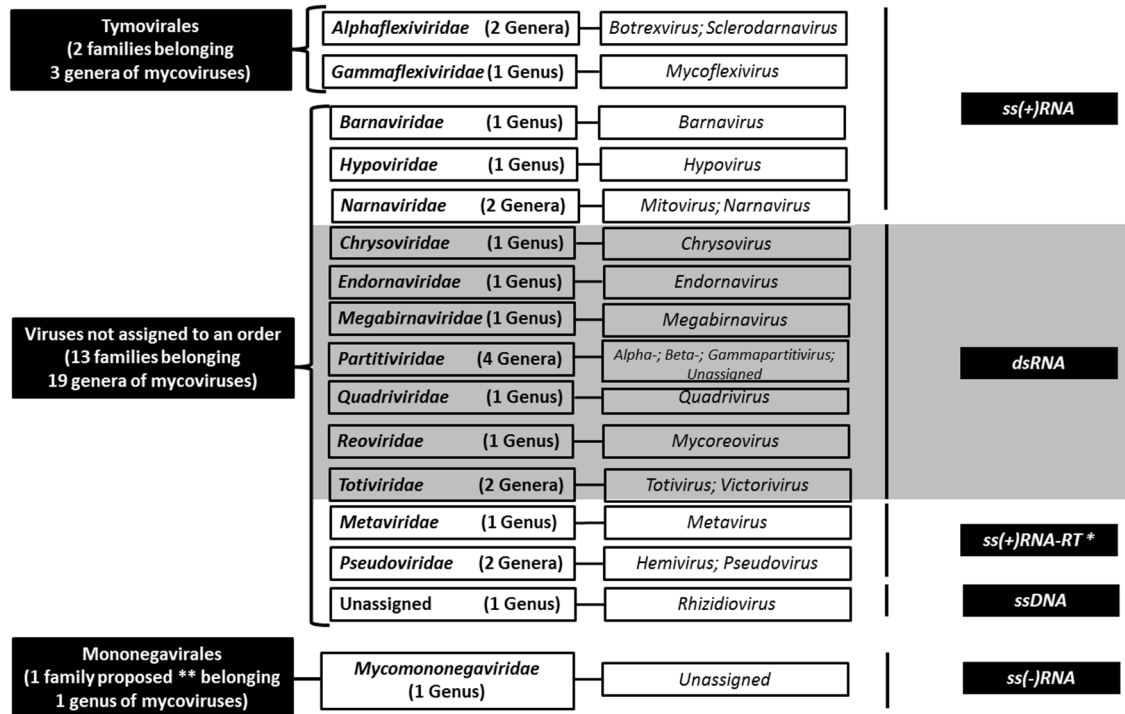
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1364 Table 1. Summary of mycoviruses of agroforestry interest.

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

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

1366 Figure 1. General taxonomy of mycoviruses according to ICTV classification criteria,
 1367 Virus Taxonomy 2014 Release.*Classification under consideration; **Family proposed
 1368 by Ghabrial et al. (2015).



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