Biocontrol via mycoviruses: a neglected option for bioprotection?

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1 Introduction

‘Mycoviruses’ is the term used for viruses infecting fungi and fungus-like organisms. Mycoviruses have been reported in Ascomycota, Basidiomycota, Chytridiomycota, Deuteromycota and Zygomycota (Ghabrial et al., 2015). Most reported mycoviruses consist of linear double-stranded RNA (dsRNA; ~70%), while a small number are composed of negative single-stranded RNA (−ssRNA) or positive single-stranded RNA (+ssRNA; ~30%) (Ghabrial et al, 2015; King et al, 2011). More rarely, mycoviruses with circular single-stranded DNA (ssDNA) have been found (Jiang et al., 2013; Li et al., 2020; Yu et al., 2010). Like animal and plant viruses, mycoviruses require the living cells of their host to replicate. The main differences between animal and plant viruses on the one hand, and mycoviruses on the other, lie in the fact that most mycoviruses lack an extracellular route for infection and are transmitted between cells through cell division, sporulation and occasionally cell fusion, as well as lacking movement proteins (Son et al., 2015).
Many of these mycoviruses are considered to be cryptic, and supposedly have little to no effect on their host. However, some notable exceptions have been described, in which the viruses cause deleterious effects on their host, and where the host is a plant pathogen, may cause hypovirulence, reducing the infection, growth and reproduction of the fungus. Virocontrol, the biocontrol of pathogens via hypovirulence-causing mycoviruses, is a small field, but one which is now receiving more attention. This chapter discusses the characteristics of mycoviruses and the conditions that need to be met to make a successful virocontrol agent. The chapter describes one of the success stories so far, that of the virocontrol of Cryphonectria parasitica, the chestnut blight pathogen. The chapter ends by reviewing future trends and information on sources of more information on mycoviruses and virocontrol.

2 Mycovirus types and origins

In over 50 years of mycovirus research, an enormous diversity of mycoviruses has been found. They vary in their make-up from DNA to RNA, single-stranded or double-stranded, consisting of a single fragment or multiple fragments, linear or circular, with folds or without folds at their ends, with or without a protein coat, consisting of one or multiple protein components, multiple fragments packed singly or together, and localised in the fungal cytoplasm or the mitochondrion. Mycoviruses are classified based on their genetic organisation, their form and resemblance to other (myco)viruses. Mycovirus names are generally derived from their fungal host names, with the first letters deriving from the fungal genus and species epithet, some letters relating to virus organisation followed by a number.

The type of nucleic acids and the number of fragments forming the mycovirus genome determine the virus families they belong to. The majority of mycoviruses consist of dsRNA and are grouped in the families of the Chryso-, Megabirna-, Quadri-, Partiti-, Reo-, and Totiviridae (Ghabrial et al., 2015; King et al, 2011, 2012; Wang et al., 2016). Some dsRNA mycovirus species have also been found to belong to new, as yet unnamed virus families (e.g. Liu et al., 2019). Linear positive-sense +ssRNA mycoviruses can belong to the Alphaflexi-, Ambigui-, Barna-, Beny-, Botourmia- Endorna-, Fusari-, Gammaflexi-, Hypo-, Narna-, Tombus-, Tymo-, Virga- and Yadokariviridae families. Some of the described reverse transcribed +ssRNA mycoviruses fall in the Meta- and Pseudoviridae families. The rarer negative-sense −ssRNA viruses belong to the Mycomononega- and Ophioviridae (Ghabrial et al., 2015; Gilbert et al., 2019; Marzano et al., 2016) families. Finally, the ssDNA mycoviruses fall in the Genomoviridae or unclassified groups (Ghabrial et al., Li et al., 2020). For characteristics of the different virus families, see Table 1. Members of quite a number of diverse mycovirus families have been found in the past 50+ years, but undoubtedly many more are to be discovered.
Mycoviruses are usually located in the cytoplasm of the fungal host, but may be associated with the mitochondria (e.g. Heaton and Leslie, 2004; Hong et al., 1998, 1999; Polashock and Hillman, 1994). Many mycoviruses have a protein coat, but naked mycoviruses also occur, without a protein coat (e.g. Qiu et al., 2010). Isometric forms identified by electron microscopy are the predominant coat morphology, but rigid or flexuous rods, club-shaped particles, enveloped bacilliform particles and herpes-like particles have also been described (e.g. Ghabrial et al., 2015; Kazama and Schornstein, 1973; Varga et al., 2003; van Diepeningen et al., 2008). Wickner (1996) described a virus capsid protein that both provides protection in the form of subcellular compartmentalisation for transcription and replication, and has a catalytic function in decapping host messenger RNA (mRNA) in favour of viral mRNAs. The isometric particles are generally based on a 120-subunit $T=1$ capsid with a dimer as the asymmetric unit, while insertions at the capsid’s outer surface are likely linked to its enzymatic activities (Luque et al., 2018). Some ssRNA viruses code for RNA-capping methyl transferases as a mechanism to protect their genome (e.g. Howitt et al., 2001; Roossinck et al., 2011). Others use a cap-snatching mechanism and borrow from the 5’-part of mRNA of their host as protection and as a primer to synthesise viral transcripts (e.g Fujimura and Esteban, 2011). Some mycoviruses have been described where the mycovirus is associated with or enveloped in colloidal cellular components (e.g. Kanhayuwa et al., 2015; Kozlakidis et al., 2009). Other mycoviruses protect their RNA by folding their 5′- and 3′-UTR regions in stem-loop structures for protection (e.g. Torres-Trenas and Pérez-Artés, 2020).

Different hypotheses exist about the origin of mycoviruses. The two main hypotheses are: (1) the ancient co-evolution hypothesis and (2) the plant virus hypothesis (Ghabrial, 1998). In the case of ancient co-evolution, Koonin et al. (1991) suggested the acquisition of an ancestral ssRNA virus from a plant based on sequence similarities, and during co-evolution of fungus and virus, a subsequent loss of protein coat and a shift towards the dominant dsRNA replication type as observed in a Cryphonectria parasitica hypovirus. Support for the co-evolution hypothesis can also be found in the large number of chromosomal genes needed for replication of the so-called killer viruses in Saccharomyces cerevisiae (Wickner, 1996), the use of host-coded enzymes for capsid processing (Huang et al., 1997) and use of the mitochondrial genetic translation code in mycoviruses located in the mitochondria (Park et al., 2006). Several phylogeographic studies (e.g. Arthur et al., 2007; Voth et al., 2006) also support co-evolution.

The trade-off theory explains why a reduced virulence of a pathogen is needed when the transmission of it is limited, since it would go extinct when a host is too affected to transmit the pathogen. This theory may explain why
### Table 1 Virus families of which members have been observed in fungi, and their characteristics

<table>
<thead>
<tr>
<th>Genome build-up</th>
<th>Virus family</th>
<th>Number of fragments $^1$</th>
<th>Phenotype $^1$</th>
<th>Hosts $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsRNA</td>
<td>Chrysoviridae</td>
<td>(3), 4, (5 or 7)</td>
<td>Isometric ~40 nm</td>
<td>Ascomycetes, Basidiomycetes, plants, (insects)</td>
</tr>
<tr>
<td></td>
<td>Megabirnaviridae</td>
<td>2</td>
<td>Isometric ~52 nm</td>
<td>Ascomycetes, Basidiomycetes</td>
</tr>
<tr>
<td></td>
<td>Quadriviridae</td>
<td>4</td>
<td>Isometric ~45 nm</td>
<td>Filamentous fungi</td>
</tr>
<tr>
<td></td>
<td>Partitiviridae</td>
<td>2</td>
<td>Isometric ~25-43 nm</td>
<td>Fungi, plants</td>
</tr>
<tr>
<td></td>
<td>Reoviridae</td>
<td>9,12</td>
<td>Isometric ~60-80 nm, 1-3 capsid layers, with or without spikes</td>
<td>Fungi, animals</td>
</tr>
<tr>
<td></td>
<td>Totiviridae</td>
<td>1</td>
<td>Isometric ~40 nm</td>
<td>Fungi, parasitic protozoa</td>
</tr>
<tr>
<td>+ssRNA</td>
<td>Alphaflexiviridae</td>
<td>1</td>
<td>Flexuous filaments, 12-13 nm × 470-800 nm</td>
<td>Fungi, plants</td>
</tr>
<tr>
<td></td>
<td>Ambiguiviridae</td>
<td>1</td>
<td>-</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Barnaviridae</td>
<td>1</td>
<td>Bacilliform 18-20 nm × 48-53 nm</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Benyviridae</td>
<td>≥ 2</td>
<td>Rod-shaped, 20 nm × 65-390 nm</td>
<td>Plants, fungi</td>
</tr>
<tr>
<td></td>
<td>Botourmiaviridae</td>
<td>1-3</td>
<td>Bacilliform, 18 nm × 30-63 nm</td>
<td>Plants, fungi</td>
</tr>
<tr>
<td></td>
<td>Endornavirida</td>
<td>1</td>
<td>Pleomorphic membrane vesicles</td>
<td>Fungi, plants, oomycetes</td>
</tr>
<tr>
<td></td>
<td>Fusariviridae</td>
<td>1</td>
<td>-</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Gammaflexiviridae</td>
<td>1</td>
<td>Flexuous filaments 13 nm × 720 nm</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Hypoviridae</td>
<td>1</td>
<td>Pleomorphic vesicles 50-80 nm</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Narnaviridae</td>
<td>1</td>
<td>Ribonucleoprotein complex with RNA-dependent RNA polymerase</td>
<td>Fungi, cytoplasm-mitochondrial</td>
</tr>
<tr>
<td></td>
<td>Tombusviridae</td>
<td>1</td>
<td>Isometric ~32-35 nm</td>
<td>Plants, fungi</td>
</tr>
<tr>
<td>Virus Family</td>
<td>Species Distribution</td>
<td>Morphology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------------------------------</td>
<td>-----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tymoviridae</em></td>
<td>Variable</td>
<td>Plants (animal vectors)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Virgaviridae</em></td>
<td>1-3 Rod-shaped ~20 x 300 nm</td>
<td>Plants, fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Yadokariviridae</em></td>
<td>1 Variable</td>
<td>Animals, fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−ssRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycomononegaviridae</em></td>
<td>1 Filaments ~ 15-50 nm × 1000 nm</td>
<td>Fungi, related to animal viruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ophioviridae</em></td>
<td>3-4 pleiomorphic</td>
<td>Plants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Genomoviridae</em></td>
<td>1, circular</td>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data on the virus families are available at [https://talk.ictvonline.org/ictv-reports/ictv_online_report](https://talk.ictvonline.org/ictv-reports/ictv_online_report).
so many presumably co-evolved mycoviruses are cryptic, with no apparent effects in their host and limited or no transmission. Göker and colleagues (2011) studied the codivergence of mycoviruses with their hosts. Their study was significantly influenced by the numbers of viruses sampled per family, but for well-sampled mycovirus families, gave significant support to co-evolution. However, not all mycoviruses are consistent with the first hypothesis, as they have deleterious effects on their host or do not follow the evolutionary pattern of their host (e.g. Carbone et al., 2004; Liu et al., 2003).

The plant virus hypothesis is supported by observations that some mycoviruses - both dsRNA and ssRNA mycoviruses - are more closely related to certain plant viruses than to other mycoviruses (e.g. Adams et al., 2004; Chu et al., 2002; Fauquet et al., 2005; Howitt et al., 2001, 2006; Linder-Basso et al., 2005; Martelli et al., 2007; Xie et al., 2006) or have a similar genome organisation and gene expression strategy as certain plant viruses (Kwon et al., 2007). Some fungi are known to be vectors of plant viruses, and carry those virions on the outside of the fungal structures, where a rare event may have led to internalisation (Varga et al., 2003). Besides having an evolutionary link, also the cological link between plant viruses and plant pathogenic fungi is clear (Roossinck, 2019).

Recent work has shown that transfer, replication and expression of plant viruses from plant to different (plant pathogenic) fungi and lichens is possible (Andika et al., 2017; Mascia and Gallitelli, 2016; Mascia et al., 2019; Petrzik et al., 2014). In reverse, transfer of fungus to plant has been observed where the mycoviruses of an endophytic fungus successfully replicated in plant cells (Nerva et al., 2017), suggesting that transfer of viruses between plant and fungus may occur in both directions.

A third alternative hypothesis would be the transfer of animal viruses to fungi. There is as yet less evidence for this. However, the Reoviridae contain mostly animal viruses and a few plant and fungal viruses, with only low to moderate levels of similarity between them (Hillman et al., 2004; Suzuki et al., 2004; Tanaka et al., 2012; Wei et al., 2003). More recently discovered fungal members of Yadokariviridae and the Mycomononegaviridae have animal pathogenic relatives. With the diversity of mycoviruses observed in the fungal kingdom, it seems likely that multiple hypotheses (ancient co-evolution, plant and animal transfer hypotheses) are needed to explain the diversity observed.

3 Mycovirus detection and incidence

The first hints of the existence of mycoviruses came from fungi showing abnormalities for which no visible pathogens could be detected. The work of Hollings (1962) can be seen as the first evidence of mycoviruses being the
cause of die-back diseases such as La France disease and virus X disease in the white button mushroom *Agaricus bisporus*. The phenotypic evidence of mycoviruses can also present itself as slower growth, irregular colony rims, reduced spore production and/or different colony colour (e.g. Sivanesan and Holliday, 1981; van Diepeningen et al., 2006).

Hollings (1962) is also the first to show that infection with multiple mycoviruses in one host can occur. In Holling’s electron microscopic photographs, both isometric particles and bacilliform rods were visible, while infectivity of the virus was demonstrated by injection of the tissue of the *Agaricus* sporocarp with a hypodermic needle. Electron microscopy used for the visualisation of *A. bisporus* mycoviruses is still the main technique, often with preceding (density gradient) centrifugation, to visualise mycoviruses (e.g. Sharma et al., 2011).

Many (cryptic) mycoviruses have been detected based on the presence of their (dsRNA) genomes. After viral nucleic acid isolation, determination of the (double-stranded) RNA nature of mycovirus genomes was first resolved chemically (Banks et al., 1968), and later enzymatically (e.g. Buck et al., 1973; Varga et al., 1994). The numbers and sizes of fragments were determined via agarose or acrylamide gel electrophoresis (e.g. Buck and Ratti, 1975). In many of the first (dsRNA) mycovirus detection protocols, a phenol-step to deproteinise viral fragments (Banks et al., 1968) in the nucleic acid extraction protocols proved essential for isolation. This compound was later replaced by sodium dodecyl sulfate and potassium acetate (SDS/KOAc) treatments, followed by CF-11 chromatography to separate dsRNA from other nucleic acids (e.g. DePaulo and Powell, 1995).

Direct cloning, amplification and sequencing of dsRNA genomes have advanced gradually (for an overview, see Potgieter et al., 2009), providing more insight into the diversity and functioning of mycoviruses. Many viruses are now detected *in silico* after analysis of (meta)transcriptome data (e.g. Gilbert et al., 2019; Marzano et al., 2016; Myers et al., 2020; Zoll et al., 2018).

Estimates of the numbers of mycovirus-infected strains in a population suggest that 30–80% of fungal species may be infected (Ghabrial and Suzuki, 2009), while infection rates per species vary between 0% and 100% (Table 2). Mycoviruses occur commonly both in Ascomycetes and Basidiomycetes as well as in earlier-diverging lineages (Myer et al., 2020). Multiple infections with two or more, often unrelated, mycoviruses in a host appear quite common, irrespective of the detection technique (e.g. Gilbert et al., 2019; Hao et al., 2018; Herrero and Zabalgogeazcoa, 2011; Hollings, 1962; Osaki et al., 2016; Ran et al., 2016). Defective dsRNA and/or satellite dsRNA can also be found (Ghabrial and Suzuki, 2008). Osaki and co-workers (2016) even reported a single strain of *Fusarium poae* that appeared to be infected with 16 different mycoviruses of the dsRNA and –ssRNA types.
## Table 2
Examples of estimated incidences of virus infections and debilitating/hypovirulence-causing infections in different fungal and oomycete genera and species

<table>
<thead>
<tr>
<th>Fungal genus/ species</th>
<th>% Mycovirus infected strains in population</th>
<th>% Debilitating/ hypovirulence phenotype(^1) in population</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria alternata (Japanese pear)</td>
<td>44</td>
<td>4</td>
<td>Hayashi et al., 1988; Fuke et al., 2011</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>0</td>
<td>0</td>
<td>Coenen et al., 1997</td>
</tr>
<tr>
<td>Aspergillus section flavus</td>
<td>8–11</td>
<td>0</td>
<td>Elias and Cotty, 1996; van Diepeningen et al., 2008</td>
</tr>
<tr>
<td>Aspergillus section nigeri</td>
<td>10</td>
<td>0.1</td>
<td>Ratti and Buck, 1972; van Diepeningen et al., 2006, 2008</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>72</td>
<td>≤1.5</td>
<td>Howitt et al., 1995; Hao et al., 2018; Wu et al., 2010</td>
</tr>
<tr>
<td>Chalara elegans</td>
<td>84</td>
<td>18.6</td>
<td>Bottacin et al., 1994</td>
</tr>
<tr>
<td>Cryphonectria parasitica</td>
<td>2–36</td>
<td>2–36</td>
<td>Adamčíková et al., 2012; Montenegro et al., 2008; Murolo et al., 2018; Park et al., 2008; Peever et al., 1998</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
<td>3.7</td>
<td>1.5</td>
<td>Aminian et al, 2011; Chu et al., 2002, 2004; Darissa et al., 2011</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>11</td>
<td>5</td>
<td>Klic and Griffin, 1998</td>
</tr>
<tr>
<td>Fusarium oxysporum f.sp dianthi</td>
<td>≥15</td>
<td>≥0.4–2</td>
<td>Torres-Trenas et al., 2019; Torres-Trenas and Pérez-Artés, 2020</td>
</tr>
<tr>
<td>Fusarium poae</td>
<td>100</td>
<td>0</td>
<td>Fekete et al., 1995</td>
</tr>
<tr>
<td>Fusarium proliferatum</td>
<td>4</td>
<td>0</td>
<td>Heaton and Leslie, 2004</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>2.9</td>
<td>0</td>
<td>Nogawa et al., 1993</td>
</tr>
<tr>
<td>Monilia fructicola</td>
<td>63</td>
<td>0</td>
<td>Tsai et al., 2004; Tran et al., 2019</td>
</tr>
<tr>
<td>Magnaporthe oryzae</td>
<td>19–71</td>
<td>≤6</td>
<td>Hunst et al., 1986; Moriyama et al., 2018; Okada et al., 2015</td>
</tr>
<tr>
<td>Phytophthora infestans</td>
<td>36</td>
<td>0</td>
<td>Tooley et al., 1989</td>
</tr>
<tr>
<td>Pythium irregular</td>
<td>85</td>
<td>0</td>
<td>Gillings et al., 1993</td>
</tr>
<tr>
<td>Rosellinia necatrix</td>
<td>14–20</td>
<td>0</td>
<td>Arjona-Lopez et al., 2018; Arakawa et al., 2002</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>20</td>
<td>≤20</td>
<td>Boland, 1992</td>
</tr>
<tr>
<td>Ustilago maydis</td>
<td>34–100</td>
<td>0</td>
<td>Voth et al., 2006</td>
</tr>
</tbody>
</table>

\(^1\) Debilitation is defined as when the comparison of isogenic virus-containing and virus-free strains show clear phenotypic differences in their growth. Percentually small effects due to use of host resources are not taken into account. Hypovirulence – when a plant pathogenic strain shows reduced pathogenicity in bioassay.
4 Mycovirus effects

The majority of mycoviruses are cryptic or latent, with no apparent effect on their host. In theory, every mycovirus that draws on its host’s resources should have at least a small negative effect on its host. The impact of this theft of resources may depend on the availability of resources from the fungal environment. In lab tests under nutrient-restricted conditions, the growth effects on a host by a mycovirus were more pronounced than under nutrient-rich conditions (van Diepeningen et al., 2006, 2008). This section summarises the different phenotypic effects caused by mycoviruses, providing an overview of what is known about the cellular mechanistic effects of the mycoviruses causing such phenotypic effects.

4.1 Debilitation and hypovirulence

A small number of mycoviruses (0–20%, depending on the host species) (Table 2) show clear debilitating effects on their host, with reduced growth rate, sporulation and/or virulence. In contrast, the reduction of pathogen virulence is called hypovirulence. Hypovirulence has been studied in some of the major plant pathogens like *Alternaria alternata*, *Cryphonectria parasitica* and *Magnaporthe oryzae*, and is linked to a reduced ability to infect, colonise, kill, and/or reproduce on susceptible host tissues (Table 3). In studies with green fluorescent marker strains from *Fusarium oxysporum* f.sp. *dianthi*, Torres-Trenas and co-workers (2019) showed that the initial colonisation of the carnation root was similar for virus-infected and virus-free strains, but that colonisation of internal tissues of the carnation root by mycovirus-infected strains was slower and less dense. They also showed that the infection more rarely became intracellular through the virus-infected strain, whereas virus-free strains readily infected the cells in the medulla.

Hypovirulence has been observed in all major groups of mycoviruses. This includes dsRNA mycoviruses like chrysoviruses (e.g. Moriyama et al., 2018), +ssRNA viruses like the hypoviruses (e.g. Koonin et al., 1991), a −ssRNA member of *Mycomononegaviridae* (Liu et al., 2014) and even in the ssDNA mycovirus of *Genomoviridae* (Yu et al., 2010). The strength of effects of a mycovirus on its host not only depends on the virus type but also on the virus titre in the mycelium (Aoki et al., 2009; Darissa et al., 2012; van Diepeningen et al., 2006).

Hypovirulence may be caused by the fitness effects of nutrient depletion due to mycovirus infection. Different virulence-related mechanisms of the fungus may be influenced directly or indirectly by the viral infection. Melanins, for example, are linked to microbial virulence and protection against oxidising agents. Moriyama and co-workers (2018) observed that chrysovirus infection in *M. oryzae* tends to lead to lighter-coloured colonies due to reduced melanin
<table>
<thead>
<tr>
<th>Plant pathogen</th>
<th>Host(s)</th>
<th>Hypovirulence mycovirus</th>
<th>Virus type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternaria alternata</em> f.sp. <em>mali</em></td>
<td>Apple</td>
<td>AaHV1</td>
<td>Hypoviridae, 1 dsRNA fragment</td>
<td>Li et al., 2019</td>
</tr>
<tr>
<td><em>Alternaria alternata</em></td>
<td>Japanese pear</td>
<td>AaCV1</td>
<td>Chrysoviridae, 5 dsRNA fragments</td>
<td>Okada et al., 2018</td>
</tr>
<tr>
<td><em>Botryosphaeria dothidea</em></td>
<td>Fruit and wood trees</td>
<td>BdRV1</td>
<td>Unknown, 5 dsRNAs</td>
<td>Zhai et al., 2016</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>&gt;200 dicotyledons and monocotyledons</td>
<td>Virus from <em>B. cinerea</em> CCg425 BcMV1</td>
<td>Mitovirus 1 dsRNA</td>
<td>Wu et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BcHV1</td>
<td>Hao et al., 2018</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BcFV1</td>
<td>Hao et al., 2018</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BcPV2</td>
<td>Kamaruzzaman et al., 2019</td>
</tr>
<tr>
<td><em>Cryphonectria parasitica</em></td>
<td>Major hosts in the genus Castanea/chestnut blight</td>
<td>CHV1-EP713</td>
<td>1 dsRNA</td>
<td>Shapira et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MYRV-1</td>
<td>Enebak et al., 1994; Suzuki et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NB631 dsRNA</td>
<td>Polashock et al., 1997</td>
</tr>
<tr>
<td><em>Fusarium graminearum</em></td>
<td>Cereals and other crops</td>
<td>FgV1-DK21</td>
<td>1 ssRNA</td>
<td>Cho et al., 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FgGMTV1</td>
<td>Genomoviridae, 3 circular ssDNAs</td>
<td>Li et al., 2020</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em> f.sp. <em>dianthi</em></td>
<td>Carnation</td>
<td>FodV1</td>
<td>Chrysoviridae, 4 dsRNA fragments</td>
<td>Lemus-Minor et al., 2015; Torres-Trenas et al., 2019</td>
</tr>
<tr>
<td><em>Magnaporthe oryzae</em></td>
<td>Rice blast</td>
<td>MoCV1-A</td>
<td>Chrysoviridae, 5 dsRNAs</td>
<td>Moriyama et al., 2018</td>
</tr>
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<td></td>
<td></td>
<td>MoCV1-B</td>
<td>Chrysoviridae, 4-5 dsRNAs</td>
<td>Moriyama et al., 2018</td>
</tr>
<tr>
<td><em>Ophiostoma novo-ulmi</em></td>
<td>Dutch Elm</td>
<td>3a</td>
<td>Narnaviridae, 1 dsRNA</td>
<td>Hong et al., 1998</td>
</tr>
<tr>
<td>Pathogen</td>
<td>Hosts</td>
<td>Viral Component</td>
<td>Type</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------</td>
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<td>--------------------</td>
</tr>
<tr>
<td><em>Sclerotinia homeocarpa</em></td>
<td>Turfgrasses (dollar spot)</td>
<td>L-dsRNA of Sh12B</td>
<td>Mitochondrial dsRNA</td>
<td>Deng et al., 2003</td>
</tr>
<tr>
<td><em>Sclerotina sderotiorum</em></td>
<td>Many herbaceous plants</td>
<td>SsHADV-1</td>
<td>Unknown, related to geminiviruses circular ssDNA</td>
<td>Yu et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SsHV1</td>
<td><em>Hypoviridae</em>, 3 dsRNAs</td>
<td>Xie et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SsNSRV-1</td>
<td>Unknown, (−) ssRNA mycovirus, 1 fragment</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em></td>
<td>Many hosts</td>
<td>M2 of Rhs 1A1</td>
<td>dsRNA fragment</td>
<td>Lakshman et al., 1998</td>
</tr>
<tr>
<td><em>Rosellinia necatrix</em></td>
<td>Over 400 woody and herbaceous plants</td>
<td>RnMBV1</td>
<td><em>Megabirnaviridae</em>, 2 dsRNAs</td>
<td>Kondo et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RnMPV1-W8 in combination with RnMBV2</td>
<td><em>Partitiviridae</em>, 2 dsRNAs</td>
<td>Kondo et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MYRV3-W370</td>
<td><em>Mycoreoviridae</em>, 12 dsRNAs</td>
<td>Kanematsu et al., 2004; Kanematsu et al., 2013</td>
</tr>
</tbody>
</table>

Biocontrol via mycoviruses: a neglected option for biocontrol?
production and due to hypovirulence. Mycotoxins in many fungi are also linked to increased host pathogenicity. Virus infection decreases mycotoxin production in many fungi, for instance in different *Aspergillus* species (Elias and Cotty, 1996; Kotta-Loizou and Couuts, 2017; Nerva et al., 2019) and in *Fusarium graminearum* (Aminian et al., 2011; Chu et al., 2002; Li et al., 2016).

### 4.2 Hypervirulence

Hypervirulence is the opposite of hypovirulence, and has also been observed in mycovirus-infected strains, where infected strains show an increased growth rate, sporulation and/or virulence on their host. In *Chalara elegans* (a.k.a *Thielaviopsis basicola*), two different dsRNA mycoviruses have been observed, one of which caused hypovirulence while the other caused hypervirulence (Bottacin et al., 1994). In *Nectria radicicola*, a viral dsRNA upregulates host virulence (Ahn and Lee, 2001). In *A. alternata*, pathogenic to Japanese pear, both hypovirulent and hypervirulent mycoviruses have been observed. The hypervirulent virus AaCV1 caused impaired growth of its host fungus, but enhanced pathogenicity against Japanese pear plants, related to an increase in AK-toxin (Okada et al., 2018). In the US-8 lineage of *Phytophthora infestans*, most strains harbour the PiRV-2 virus that enhances its host's ecological fitness through down-regulation of ammonium and amino acid uptake, thus achieving stimulated sporulation (Cai et al., 2019). However, virus infection may also stimulate fungal growth without causing increased virulence (Tran et al., 2019).

In the human pathogen *Aspergillus fumigatus*, the AfuPmV-1 virus increases host virulence. An analysis of the nucleotide composition and codon usage showed that, in comparison to non-hypervirulent viruses, the AfuPmV-1 has a higher GC content and a preferred codon usage ending on G or C, while codons ending with A and U were not observed (Je et al., 2019).

The trade-off hypothesis is one way to study the symbiotic relationship between viruses and their hosts. In the tripartite system of mycovirus–fungal pathogen–plant host, the trade-off hypothesis can explain situations where either microbial (hypovirulence) or macrobial host (hypervirulence) has the larger cost, and also situations where everyone wins (Márquez and Roossinck, 2012).

### 4.3 Killer mycoviruses and other beneficial traits

In several yeasts and filamentous fungi, killer-mycoviruses have been observed that kill off any uninfected strains in the proximity of the fungus. The satellite dsRNAs of these killer-mycoviruses code both for secreted protein toxins and immunity to the toxin. Such ‘killer-virus’-infected strains may have a direct selective advantage above killed or non-infected competitors, depending on
environmental conditions (e.g. Czárán and Hoekstra, 2003; Wickner, 1996; Wloch-Salamon et al., 2008). This is seen as an extreme form of beneficial interaction. In *Saccharomyces cerevisiae*, several different satellite dsRNAs, called M1, M2, M28 or Mlus each encode a protein toxin, killing by a different mechanism (Schmitt and Breinig, 2006). The plant pathogenic yeast *Ustilago maydis* possesses multiple mycovirus-encoded killer systems based on different cellular mechanisms (e.g. Brunn, 1986; Koltin, 1986; Park et al., 1996). Expression of the killer protein by transgenic maize renders it virtually immune to *U. maydis* (Allen et al., 2013). In the plant pathogenic fungus *Helminthosporium victoriae* (a.k.a *Cochliobolus victoriae*), a protein with antifungal properties has been isolated from a virus-infected strain (de Sa et al., 2010).

A different type of beneficial trait conferred by a mycovirus has been observed in *Curvularia protuberata*, an endophyte of panic grass, *Dichanthelium lanuginosum*. This fungus, its host and a mycovirus of *C. protuberata* have an interesting three-way symbiosis, where the dsRNA mycovirus (called *Curvularia* thermal tolerance virus (CThTV)) confers thermal tolerance to both the endophyte and host plant (Márquez et al., 2007).

In *Trichoderma harzianum*, the removal of *Trichoderma harzianum* mycovirus 1 (ThMV1) resulted in improved biocontrol of *Fusarium oxysporum* f.sp. *cucumerinum*. On the other hand, the same virus improved the growth of cucumber (Liu et al., 2019). Recent work in *Sclerotinia sclerotiorum* has identified a 2-kb mycovirus that converts this pathogen into beneficial endophytes for the *Brassica* species and increases yield (Zhang et al., 2020).

### 4.4 Intracellular mechanisms

Mycoviruses affect their hosts in different ways and via diverse mechanisms. With so many different mycoviruses, different cellular compartments where mycoviruses are located and different mechanisms within the fungal cell at different physiological stages, the intracellular processes affected by mycoviruses may vary over time.

RNA silencing can function as a virus defence mechanism in a diverse range of eukaryotes, and many viruses are capable of suppressing the silencing machinery targeting them. Hammond and co-workers (2008) have shown that *Aspergillus* viruses are both targets and suppressors of RNA silencing, and that *Aspergillus* virus 1816 in particular shows strong deleterious effects on host growth, with sporulation suppressing RNA silencing through a mechanism that alters the level of small interfering RNA. In *Fusarium graminearum*, viral fragments interact with different components of the RNA interference (RNAi) pathway (Yu et al., 2018). In *Rosellinia necatrix*, the mycoreovirus suppresses RNA silencing (Yaegashi et al., 2013). In *Cryphonectria hypovirus1* (CHV1), p29, a papain-like protease suppresses hairpin RNA-induced, virus-induced RNA...
silencing, by inhibiting the expression of the RNAi genes (Choi et al., 1991; Segers et al., 2006). In addition to their roles in silencing viral replication by degrading viral RNAs, RNAi components also promote viral RNA recombination in C. parasitica (Sun et al., 2009a; Zhang et al., 2008).

*C. parasitica* has at least four RNA-dependent RNA polymerases (RdRPs) that may be involved in transcriptional and posttranscriptional gene silencing and antiviral defence (Zhang et al., 2014). The Aspergillus fumigatus polymycovirus-1 (AfuPmV-1) seems to have adapted its own RdRPs codon usage to that of its fungal host, and increased its virulence (Je et al., 2019).

Genome-wide studies have shown the transcriptional regulation by viruses to be very complex, for instance, in *Aspergilli* (Ejmal et al., 2018), *F. graminearum* (Cho et al., 2012), *R. necatrix* (Shimizu et al., 2018) and *S. sclerotiorum* (Li et al., 2008). The study of Shimizu et al. (2018) showed that 545 genes in the host were upregulated due to virus infection, while 615 were downregulated. With such profound effects on the expression of genes, mycoviruses also affect the secretion of specific gene products (Kazmierczak et al., 2012). Moriyama et al. (2018) suggested that mycovirus infection, with all its effects on the fungal cell and metabolism, can be a driving force behind the development of physiological diversity of strains and the evolution of pathogenic races.

Transcriptional reprogramming or usage of specific host genes has also been observed in several mycovirus-host systems. This started with studies on single genes. In *F. graminearum*, the hex1 gene was found necessary for fungal asexual reproduction and pathogenesis as well as for efficient viral RNA accumulation (Son et al., 2013). In *C. parasitica*, the transcription factor gene pro1 proved responsive to hypovirus, necessary for female fertility, asexual spore development and the stable maintenance of virus infection (Sun et al., 2009b). In *C. parasitica*, the vir2 gene is also downregulated by the virus, resulting in reduced asexual sporulation and pycnidium production, as well as impaired sexual crossing ability (Zhang et al., 1993).

**5 Transmission of mycoviruses**

Transfer of (hypovirulence-causing) mycoviruses is important for effective biocontrol. One of the main differences between mycoviruses and viruses from plants and animals lies in the fact that mycoviruses in general have no extracellular phase. Mycoviruses depend on vertical transmission through asexual structures or sexual spores, and on horizontal transmission after hyphal contact for new infection. However, with so many different types of viruses and virus effects on their host, mycoviruses can differ in their modes and rates of transfer.
5.1 Asexual transmission

The cellular location of mycoviruses can vary. Some are found in the mitochondria, like the mitoviruses in *C. parasitica*, *F. proliferatum* and *Ophiostoma novo-ulmi* (e.g. Heaton and Leslie, 2004; Polashock and Hillman, 1994; Hong et al., 1998, 1999). Others are found in association with the trans-Golgi network (Jacob-Wilk et al., 2006), while for many, the exact intracellular location is not known. They may spread within the growing mycelium or between dividing yeast cells. However, mycovirus titres need not be homogenous throughout the mycelium, as variations in mycovirus-titres have been observed especially in mycovirus infections with negative effects (Aoki et al., 2009; Darissa et al., 2012; van Diepeningen et al., 2006). As a consequence of this inhomogeneous spread, (sequential) hyphal tip isolations have been a way to obtain cured or partially-cured isolates (e.g. Tran et al., 2019; van Diepeningen et al., 2006). However, the mycovirus spread throughout the growing fungal mycelium in general seems sufficient to infect the large majority of cells within the mycelium.

Whether the viral transport through the mycelium takes place by an active or a passive process is unknown. Plasma streaming in general may be a transport mechanism (Sasaki et al., 2006), but transport via the microtubules may also be possible. In the case of mitochondria-associated mycoviruses (‘mitoviruses’), transport with the organelle may be likely (Wu et al., 2010).

Many fungi are capable of producing asexual reproductive structures throughout their mycelium. The asexual reproduction varies from the production of conidiospores on special conidiophores or in asexual fruiting bodies like pycnidia, to a transformation of mycelial cells to survival structures like chlamydospores and sclerotia. The transfer rates to such asexual structures seem generally high (e.g. Buck, 1998; van Diepeningen et al., 1997, 1998), but clearly can vary by mycovirus within a species and even within a multiple-infected strain (Herrero and Zabalgogeazcoa, 2011).

5.2 Sexual transmission

The vertical transmission of mycoviruses through sexual spores seems usually much less effective than via asexual spores in the same species. This is the case, for example, in several *Aspergillus* species, where the majority of the sexual spores prove virus-free, while asexual spores of the same species prove infected (Coenen et al., 1997; Varga et al., 2001). Exclusion of dsRNA segments from sexual spores was further observed in, for example, the ascomycetes *Gaeumannomyces graminis* strains (McFadden et al., 1983), *Ophiostoma ulmi* (Brazier, 1983), *Epichloë festucae* (Romo Vaquero et al., 2007) and *R. necatrix* and in the basidiomycete *Helicobasidium mompa* (Ikeda et al., 2004). The exclusion of mycovirus from the sexual spore is in line with the selection arena
hypothesis – to exclude deleterious elements and mutations from the germ line (Bruggeman et al., 2004).

However, there are also species where the transmission to the sexual offspring is very efficient. This is the case, for example, in the basidiomycetes Ustilago maydis (Koltin and Day, 1976) and Heterobasidion annosum (Ihrmark et al., 2004) and ascomycetes like S. cerevisiae (Brewer and Fangman, 1980) and F. graminearum (Chu et al., 2004).

5.3 Heterokaryon incompatibility and horizontal transmission

Horizontal transmission is the transfer of one mycelium to another, as opposed to the vertical transmission to sexual and asexual offspring. This horizontal transmission via hyphal fusion or anastomosis seems under the control of the fungal self/non-self-recognition system regulated by vegetative (vic) or heterokaryon (het) incompatibility genes. This system is thought to have evolved to limit the spread of harmful organisms like mycoviruses (Caten, 1972). In many model organisms, this vegetative/heterokaryon incompatibility system is under the regulation of multiple allelic or non-allelic vic or het genes, and most species seem to involve different genes (Glass and Dementhon, 2006; Saupe, 2000).

In 1970, Lhoas described how mycoviruses could be transferred via heterokaryosis in Aspergillus niger (Lhoas, 1970). Since then, transmission between isolates carrying the same vic or het genes has been reported in many species, for example, in C. parasitica (Liu and Milgroom, 1996) and A. nidulans (Coenen et al., 1997). In contrast, incompatibility blocked virus transfer in Aspergillus and Cryphonectria (e.g. Coenen et al., 1997; Milgroom and Cortesi, 2004; van Diepeningen et al., 1997). However, transfer may depend on the strength of the incompatibility reaction in the fungal species as well as on the virus (Biella et al., 2002). Transmission of mycoviruses has been found to be effective between intersterility groups of H. annosum (Ihrmark et al., 2002), and by anastomosis between incompatible individuals of Beauveria bassiana (Dalzoto et al., 2006). Carbone and co-workers (2004) found that transmission may occur in one direction, while it may not be as efficient in the reciprocal pairing. The systemic disruption of multiple vic/het loci can be a way to engineer super mycovirus-donor strains, as has been shown in C. parasitica (Zhang and Nuss, 2008).

So far, one virus, the Sclerotinia sclerotiorum mycoreovirus 4 (SsMYRV4), has been found to suppress the host’s self/non-self-recognition system and thus enable its own intermycelial transfer (Wu et al., 2017). Environmental conditions may also influence intermycelial transfer. Zinc compounds have, for example, been found to attenuate heterokaryon incompatibility reactions in R. necatrix (Ikeda et al., 2013).
5.4 Protoplast fusion and other alternative infection routes

The rigid cell wall of fungi has been thought to serve as a structural barrier against the uptake of virus particles from extracellular sources. This is because protoplasts, competent cells or cells during the sexual reproduction stage of the yeast *S. cerevisiae* could be infected by purified virus particles (el-Sherbeini and Bostian, 1987). Removal of the fungal cell wall and subsequent protoplast fusion has been a way to transfer mycoviruses within a species, though vegetative/heterokaryon-incompatible combinations of strains could still result in limited or no virus transfer when cytoplasms are mixed (e.g. van Diepeningen et al., 1998; Wu et al., 2012). The transmission of mycoviruses to protoplast particles has been a way to expand the host range of (hypovirulent) mycoviruses to other (plant pathogenic) fungi (e.g. Kanematsu et al., 2010; Lee et al., 2011; van Diepeningen et al., 1998).

An alternative way to transfer hypovirulence within a population of *C. parasitica* has been the construction of infectious cDNA clones of hypoviruses and the subsequent transgenic lines (Chen and Nuss, 1999; Choi and Nuss, 1992). Such transgenic hypovirulent *C. parasitica* strains differ from natural hypovirulent strains in having the ability to transmit hypoviruses to ascospore progeny. However, vegetative/heterokaryon incompatibility still limits the colonisation and conversion of treated cankers (Root et al., 2005).

5.5 Extracellular transmission

The majority of mycoviruses have a strictly intracellular lifestyle, but more mycoviruses are being discovered that have an extracellular stage. The first mycovirus found with an extracellular phase was a mycoreovirus (Hillman et al., 2004; Fauquet et al., 2005). A mycovirus consisting of 4 double-stranded RNA fragments from the human pathogenic fungus *A. fumigatus*, the Aspergillus fumigatus tetramycovirus-1, proved infectious both as purified particles as well as in naked dsRNA form (Kanhayuwa et al., 2015).

The first DNA mycovirus discovered, *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1), was also found to have an extracellular phase (Yu et al., 2010, 2013). Isolated virus particles of SsHADV-1 proved to be able to transfer hypovirulence under field conditions, reduce disease severity and enhance rapeseed yield. SsHADV-1 could be transmitted to sister species *Sclerotinia minor* and *S. nivalis*, but not to other species (Yu et al., 2013).

There is relatively little evidence for the use of vectors for the transmission of mycoviruses. SsHADV-1 proved able to use a mycophagous insect – *Lycoriella ingenua* – as a vector for transmission. SsHADV-1-infected fungus could suppress the production of repellent volatile substances to attract adults to lay eggs on its colony. Larvae fed on virus-infected fungus became viruliferous adults that could transmit SsHADV-1 transovarially, while stimulating the female
adults to produce more eggs (Liu et al., 2016). *Cryphonectria parasitica* with mycovirus has been found in dejecta of the mite *Thyreophagus corticalis* fed with the fungus *in vitro*. The faecal pellets in turn could transmit the infection further (Bouneb et al., 2016).

In *Helicobasidium mompa*, mycophagous nematodes found in the basidiocarps of the fungus may play a role in virus-fungus interactions (Ikeda et al., 2005). In *C. parasitica*, the mycophagous nematode *Aphelenchoides hylurgi* and relatives can be vectors for hypovirulent strains (Griffin et al., 2009, 2012).

### 5.6 Phylogenetic studies do suggest interspecies transfer

Indirect evidence for interspecies transfer can be derived from (phylo)genetic studies. Such studies suggest that closely-related mycoviruses may occur in different host species, and that, despite interspecies barriers, interspecies transfer may occasionally be possible. Interkingdom transfer of viruses between plant and fungus and *vice versa* has been proven possible (Andika et al., 2017; Mascia and Gallitelli, 2016; Mascia et al., 2019; Nerva et al., 2017; Petrzik et al., 2014).

van Diepeningen et al. (2008) found that within species and between closely related species, mycovirus transfer via protoplast fusions seems to occur less than the transfer between more distantly related species when cell wall restrictions are removed. Mycoviruses transferred via protoplast fusion or transfection are often viable in related hosts (e.g. van Diepeningen et al., 2008; Yu et al., 2013), but transfer to other genera is also possible (e.g. van Diepeningen et al., 2000). Transferred mycoviruses even may keep their debilitating and hypovirulent effects in their new hosts (e.g. van Diepeningen et al., 1998).

In *H. mompa*, related mycovirus fragments were found in different vegetative/heterokaryon compatibility groups, suggestive of horizontal transfer (Ikeda et al., 2005). The same variants of *Cryphonectria* HypoVirus-1 (CHV1) have been detected in different species of the genus *Cryphonectria* (Liu et al., 2003). At least one virus, the Sclerotinia sclerotiorum mycoreovir us 4 (SsMYRV4), suppresses host non-self-recognition and facilitates horizontal transmission of itself and heterologous viruses in *S. sclerotiorum* (Wu et al., 2017). Mycoviruses may also have as yet unknown extracellular infective stages or use an animal vector for transfer (see Section 5.5). In their analysis of pathogenic, saprotrophic and mycorrhizal fungi inhabiting the same forest stand, Vainio et al. (2017) showed that horizontal transmission of mycoviruses between the different groups was possible. In the context of potential virocontrol applications, they did not find a major infection pressure towards the indigenous fungal community, but the ecological consequences of such putative interspecies virus transmission will require further investigation.
6 Essential traits for a virocontrol product

The principle of virocontrol is the biocontrol of fungal plant pathogens via hypovirulence-causing mycoviruses. It has mostly been studied in the Chestnut - C. parasitica-hypovirus model system. More host plant-fungal pathogen and mycovirus interactions are being studied such as fruit tree-white root rot fungus-mycoviruses (e.g. Chiba et al., 2010). From these studies of plant, pathogen and mycovirus interactions, and studies on mycoviruses in different fungal systems, the two most important essential traits needed for effective virocontrol can be identified:

- Hypovirulence; and
- Transferability.

These are discussed in the following sections.

6.1 Hypovirulence

Whereas most viruses have cryptic effects in their host, quite a number have deleterious effects that could cause hypovirulence. Occasionally, mycovirus infection may cause hypervirulence and stronger adverse effects of the pathogenic fungus on their host plant. The three-way interaction of plant, pathogen and mycovirus should be studied. Hypovirulence is not a trait limited to a particular mycovirus family, but may occur in all major types of mycoviruses. Many fungal species harbour mycoviruses themselves, but mycoviruses may be transferred to new host species either naturally or via protoplast fusions. Transfer between related species (or species with similar physiology and codon usage) may be more effective, and mycoviruses may then retain their effects on host physiology, including hypovirulence.

6.2 Transferability

The majority of mycoviruses do not have an extracellular route, and rely on intercellular transmission between the hyphae of different mycelia. The population structure of the pathogen in the field with respect to heterokaryon/vegetative incompatibility is important in the choice of a suitable and compatible vector strain or strains for introduction in the field. A pathogen with one clonal lineage will be easier to control than a pathogen with abundant incompatibility in the field that limits the effectiveness and transfer of the virocontrol agent. Selection or construction of an omni- or pluri-compatible strain could be advantageous, but may need genetic modification. For the rare mycoviruses that do not rely on intercellular transmission, but have an extracellular route, the fungal host strain is of importance for multiplication but presumably not for transmission.
6.3 Developing a commercial virocontrol product

To develop a successful commercial biological control product, Köhl et al. (2011, 2019) described and tested a useful series of steps for screening. Considering virocontrol, these steps could be adapted as shown below:

- Step 1: Assessment of targeted crops, diseases and markets;
- Step 2: Origin and isolation of candidate organisms;
- Step 3: Stepwise assessment of biomass production, safety and ecological characteristics of candidate antagonists in rapid-throughput screening systems;
- Step 4: Assessment of potential risks for use and patent positions of candidate antagonists;
- Step 5: Efficacy testing of candidate antagonistic mycovirus/fungal host combinations on infected plants or infected parts of plants;
- Step 6: Preliminary assessments of mass production;
- Step 7: Development and testing of a pilot formulation and estimation of registration costs;
- Step 8: Upscaling mass production and full-field testing; and
- Step 9: Integration into cropping systems.

These steps are discussed in more detail below.

**Step 1: Assessment of targeted crops, diseases and markets** for a virocontrol plant protection product. With regard to the plant, it is important to take into account crop characteristics and the affected and targeted parts of the plants. With regard to the pathogen, one must take into account the life cycle of the pathogen, heterokaryon incompatibility within the population, natural presence of a suitable hypovirulence-causing mycovirus and the potential for genetically modified organisms (e.g. when needing omni- or pluri-compatible strains). Market size, competing products and regulations should also be taken into account.

**Step 2: Origin and isolation of candidate organisms.** Candidate mycovirus/fungal combinations from the relevant niche and location should ideally be used. Standard growth media and growth conditions are preferable to reduce production costs. In view of the convention of biological diversity and the Nagoya Protocol, it is important to avoid restricted isolates.

**Step 3: Stepwise assessment of biomass production, safety and ecological characteristics of candidate antagonists in rapid-throughput screening systems.** A key criterion is purity of the colony. This is described as regular colony development and conclusive barcoding sequences. For
mycovirus-containing fungal strains, the conclusive barcoding sequences would be the more important criteria, as colony morphology may be irregular due to the mycovirus infection and its effects. The sporulation criterion of $10^5$ spores after 21 days on oat meal agar at 18°C may be more variable due to virus content. The criteria for safety (no germination and growth at 36°C; no mycotoxin production) and for field applicability (cold tolerance, high or low pH tolerance, draught tolerance and UV-B radiation) may be normally met. Compatibility with fungicides against the target pathogen and with pesticides against non-target pathogens as applied in the agricultural setting of the host plant is also important.

**Step 4: Assessment of potential risks for use and patent positions of candidate antagonists.** With regard to intellectual property rights (IPR) protection, a potential application already protected by patents or already published can affect the commercial value of the product. It is essential to carry out safety/risk assessments for human/animal pathogenicity, allergies and mycotoxins. Pathogenicity of the fungal pathogen to plants other than the target plant is an important issue. The availability of relevant registration data in dossiers or publications and toxicology profiles can make registration and marketing easier.

**Step 5: Efficacy testing of candidate antagonistic mycovirus/fungal host combinations on infected plants or infected parts of plants.** When assessing the efficacy of the mycovirus/host combinations, it is necessary to check for disease control *in planta* but also for possible growth effects on the host plant.

**Step 6: Preliminary assessments of mass production.** The mass production of the fungus and mycovirus will probably take place using either solid or liquid fermentation. Mycovirus infections may lower both mycelial growth rates as well as spore production. In general, mycovirus effects seem less severe under nutrient-rich conditions than under nutrient-restricted growth conditions (van Diepeningen et al., 2006), making it important to optimise mass production as well as minimise production costs.

**Step 7: Development and testing of a pilot formulation and estimation of registration costs.** Scaling up mass production, downstream processing, product formulation and shelf life are the next steps, all of which may be affected by the intracellular presence of the mycovirus. Human toxicity and environmental safety of the formulated product are important issues for the estimation of registration costs.

**Step 8: Upscaling mass production and full-field testing.** The best candidates for both the production process and the formulation must finally be selected. Candidates with expected low-risk profiles will probably have lower assessment costs for registration.
**Step 9: Integration into cropping systems.** In this last phase before registration, evaluation of the virocontrol within common plant protection schemes in target crops and other crops, and with existing or under-development non-chemical control should be evaluated. Furthermore, persistence in the environment should be evaluated.

Though quite a number of hypovirulence-inducing mycoviruses have been described, no biocontrol product based on mycovirus/fungal host combinations has been registered and/or is commercially available. Only the *Cryphonectria parasitica* virus I has been extensively used experimentally, but not commercially in the field, to reduce chestnut blight. This means that as there are no guidelines yet detailing whether a mycovirus-based product faces the same requirements as a fungal biocontrol product or whether additional requirements need to be met.

### 7 Success story: *Cryphonectria parasitica*

In 1904, chestnut blight caused by *C. parasitica* was first observed in American chestnut trees in New York City. Within forty years, the pathogen had killed around 3.5 billion trees in North America and destroyed much of the former oak-chestnut forest in Eastern North America, in the triangle between Ontario, Maine and Alabama, and impacted forest ecology, including insects and wildlife. In Europe, the chestnut blight was first discovered in 1938, where it was less destructive and killed fewer trees, partly because of higher blight resistance levels in the European chestnut, and partly due to the natural occurrence of hypovirulent strains (Anagnostakis, 1982). The origin of *C. parasitica* lies in Asia, and the presumably co-evolved resistance in chestnuts is observed there (Anagnostakis, 1992). The Chinese chestnut is generally considered to be highly resistant, but the pathogen can affect various other members of the genus *Castanea* in China (Qin et al., 2002). In Japan, the pathogen is sometimes destructive on the otherwise relatively blight-resistant Japanese chestnut (Uchida, 1977).

The pathogen causing chestnut blight, *Cryphonectria parasitica* (Murrill) M.E. Barr was first described as *Diaporthe parasitica* (Murrill, 1906), renamed *Endothia parasitica* by Anderson and Anderson (1912), until it was given its current name in 1978 (Barr, 1978). *C. parasitica* causes cankers on branches and stems of susceptible chestnut trees with conspicuous yellow to orange-brown stromata, containing fungal conidiomata (containing asexual spores), ascomata (containing sexual spores) as well as host cells. On resistant trees, the stromata are infrequent or inconspicuous. In natural populations, *C. parasitica* exhibits a mixed mating system and sexual offspring is formed both by outcrossing as well as selfing (Marra et al., 2004). *C. parasitica* also has a saprophytic phase on
dead wood with both perithecia and pycnidia being formed (Prospero et al., 2006). After introduction of *C. parasitica* in a new area, eradication efforts by cutting and burning the infected plants/trees have mostly failed (Rigling and Prospero, 2018).

Different dsRNA mycoviruses have been found in association with *C. parasitica*, some of which cause hypovirulence of their host, which results in less virulent, non-fatal infections of the chestnut tree. The infection rate of both non-virulent and hypovirulent mycoviruses in *C. parasitica* lies in the range of between 2% and 36% of the isolates being infected (e.g. Adamčíková et al., 2012; Montenegro et al., 2008; Murolo et al., 2018; Park et al., 2008; Peever et al., 1998). The first hypovirulence virus described was CHV-1 (Anagnostakis, 1982) which is also the type virus of the *Hypoviridae*, but since then, more hypovirulence-causing viruses of *Hypoviridae* as well as other mycovirus families have been found (e.g. Hillman et al., 2004). Different subtypes of CHV-1 based on sequence variation and RFLP have been described. Phylogenetic analysis of these subtypes and their spread suggests that the subtypes diverged several hundreds of years ago, and that different subtypes were introduced in Europe and elsewhere afterwards (Gobbin et al., 2003). The mycovirus-induced hypovirulence caused by CHV-1 in *C. parasitica* has been well studied both in the field, in the lab, and back in the field again in recurrent cycles, to enhance its potential as a biocontrol method (Nuss, 2000).

The effects of CHV-1 on its host vary from transcriptional repression of specific host genes (Kazmierczak et al., 1996; Sun et al., 2009b; Zhang et al., 1993) to influencing the secretion of proteins by its host (Kazmierczak et al., 2012). The virus-induced RNA-silencing process can be suppressed by a papain-like protease of CHV-1 (Choi et al., 1991; Segers et al., 2006). In addition to their roles in silencing viral replication by degrading viral RNAs, RNAi components also promote viral RNA recombination in *C. parasitica* (Sun et al., 2009a; Zhang and Nuss, 2008). *C. parasitica* has at least four RNA-dependent RNA polymerases (RdRPs) that may be involved in transcriptional and post transcriptional gene silencing and antiviral defence (Zhang et al., 2014).

Genetic variation exists in the interaction of different *C. parasitica* isolates and different CHV variants (Peever et al., 2000; Brusini et al., 2019). Some observed effects may be mitigated by environmental conditions, for instance, the typical ‘white’ phenotype of *C. parasitica* upon infection with a hypovirus and lower conidiation can be overcome with a high dose of light (Hillman et al., 1990).

CHV-1 does not have an extracellular life stage. Vertical transmission of mycovirus occurs to asexual offspring, where transfer rates can vary with host and mycovirus genotype (Brusini et al., 2019). The production of sexual offspring is largely inhibited by virus infection (Nuss, 2005). However, the construction of transgenic lines with cDNA clones of hypoviruses has been a way to construct hypovirulent lineages that have the ability to transmit the
hypovirulence to ascospore progeny (Chen and Nuss, 1999; Choi and Nuss, 1992). In the saprophytic phase on dead wood, perithecia yield only hypovirus-free sexual spores, while pycnidia contain 5–41% hypovirulence-infected conidia (Prospero et al., 2006).

Horizontal transmission between isolates carrying the same vic genes occurs readily (Liu and Milgroom, 1996), but incompatibility effectively blocks virus transfer (Milgroom and Cortesi, 2004), also from virulence encoded by the transgenic lines (Root et al., 2005). The systemic disruption of multiple vic loci can be a way to engineer super mycovirus-donor strains (Zhang and Nuss, 2008).

In North America, the transmission of virulence-attenuating mycoviruses is severely hampered by the at least 120 vegetative incompatibility groups present in the field, and therefore the effects of virocontrol are very limited. To restore some of the former oak-chestnut forests, breeding programmes exist where some of the remaining American chestnuts, mainly existing as understory sprouts from extant root systems, are crossed with the blight-resistant Asian chestnut species and back-crossed afterwards towards producing trees with the form, phenology and growth characteristics of the American chestnut (Anagnostakis, 2012).

In Europe, less vegetative incompatibility groups occur, and hence the more ready transfer of viruses from hypovirulent strains. In Europe, however, hypovirulence was already present, caused by different viral subtypes (Anagnostakis, 1982; Bissegger et al., 1997; Gobbin et al., 2003). The European chestnut also has a higher resistance level (Anagnostakis, 1982). Bissegger and colleagues (1997) in their study in two fields in Switzerland observed 21 Vegetative Compatibility Groups (VCGs). European vegetative compatibility groups proved to have equal infection rates with the hypovirulent virus (Bissegger et al., 1997). Young chestnut sprouts were especially killed in their first year, while over the following years, the growth of cankers decreased and hypovirulent strains were observed on all surviving sprouts, suggesting natural spread of the hypovirus infection and/or of hypovirulent strains (Bissegger et al., 1997).

When looking at spreading infections throughout Europe, one can see that in general, more recently colonised areas contain lower numbers of VCGs or only one mating type may be present (e.g. Montenegro et al., 2008; Robin and Heiniger, 2001; Robin et al, 2009). The spontaneous introduction and further spread of hypovirulence in these populations may now occur to mitigate the disease (e.g. Montenegro et al., 2008; Trapiello et al., 2017). On the other hand, as the success of biocontrol with the hypovirus CHV1 is negatively correlated to the number of VCGs present, an increase in VCG diversity may jeopardise biocontrol efforts. Movement of chestnut plants and wood should be restricted and mycelial mixtures of CHV1-infested C. parasitica for biocontrol should only contain the local VCG and mating types (Robin and Heiniger, 2001).
In Asia, the place of origin of *C. parastica*, the numbers of VCGs appear to be highest. In Japan, at least 71 groups could be recognised in 79 isolates, while in China, two populations of 28 and 11 isolates all consisted of unique VCGs, while a third population consisted of 15 VCGs in 25 isolates (Anagnostakis et al., 1998; Liu and Milgroom, 2007). However, due to the high resistance to chestnut blight in the different chestnut species in Asia, the disease causes only minor problems.

The application of hypovirulent *C. parasitica* in the field is quite laborious. Holes are drilled around cankers on sprouts and branches, and are then filled with hypoviral inoculum, with each canker treated separately. Heiniger and Rigling (2009) found that while treated cankers were cured, the hypovirus might slowly spread to untreated cankers. The choice of strains of the right locally present VCGs, mating type and the type of hypovirulent virus are important. Virus types that are too virulent/hypovirulent may limit host growth and spore production, thereby limiting their own spread. The spread of hypovirulent inoculum may be helped by certain arthropods, for example, mites (Bouneb et al., 2016) or mycophagous nematodes (Griffin et al., 2009, 2012).

No simple all-round product for the treatment of *C. parasitica* is available, as locally abundant VCGs have to be taken into account, as well as choice of the virus. However, *C. parasitica* is still the best-studied example of virocontrol. While *C. parasitica* eradicated many of the American chestnuts in America, the largely natural biocontrol of the pathogen in Europe by mycoviruses has proved quite successful.

8 Future trends

Mycoviruses have been reported in Ascomycetes, Basidiomycetes as well as in Deuteromycetes, and in groups formerly considered as fungi, like Chytridiomycota and Zygomycota (Ghabrial et al., 2015). As whole-genome sequencing and RNA-Seq are becoming ever more available and are integrated into routine descriptions of fungi, more viruses will be detected, even in low titres. Some of these viruses - both DNA, ss and dsRNA viruses - cause hypovirulence and debilitation in their host (Table 3). Virus and hypovirulence incidences vary per species, and sometimes per population (Table 2). For species with no known natural mycovirus infections, mycoviruses transfer via protoplast fusion has shown to be a successful way of interspecies transfer (e.g. Ejmal et al., 2018; Lee et al., 2011; van Diepeningen et al., 1998, 2000). The integration of cDNA and the construction of transgenic hypervirulent lines may also be an option to get hypovirulence out in the field (e.g. Choi and Nuss, 1992; Chen and Nuss, 1999). Hypovirulence or debilitation-causing viruses often retain this trait in a new host (e.g. Ejmal et al., 2018; Lee et al., 2011; van
Diepeningen et al., 2008). There is thus a range of potential virocontrol viruses available for the control of pathogenic fungi.

The limited vegetative transfer between different isolates of a species, due to the omnipresent heterokaryon or vegetative incompatibility genes, may be a more serious limitation to virocontrol than the availability of mycoviruses. So far, especially in natural populations with lower diversity in het/vic variation, virus transmission is established via donor strains compatible with the occurring pathogen(s). Engineering of super mycovirus donor strains disrupted in multiple het/vic loci may be an option to overcome the problem in more diverse populations (Zhang and Nuss, 2016). Alternatively, adding compounds like zinc to the virocontrol formulation to attenuate incompatibility reactions and enhance anastomosis rates between heterogenic strains, may increase virus transfer (Ikeda et al., 2013). How and where to apply virocontrol of fungi in the field is another question, and depends on the host-pathogen system and spread of hypovirulent fungal propagules via naturally present routes or via necessary additional vectors, be it human or animal.

There is also the question of whether a risk of virocontrol is that plant pathogens are used as donor strains. In principle, the plant pathogens used are hypovirulent and should not be a risk in themselves, in comparison to the virus-free pathogens. Chances of losing a mycovirus spontaneously are relatively small as shown by laboratory experiments on curing infected strains (e.g. van Diepeningen et al., 2006), while the presence of other isogenic and thus vegetative-compatible biocontrol isolates will lead to rapid re-infections and consequent hypovirulence. However, one should evaluate carefully if a hypovirulent strain is sufficiently a-virulent and that the introduction of high amounts of a mild pathogen does not cause problems, whether applied preventively or curatively.

There are currently no examples of non-pathogenic variants of pathogen species being used (only hypovirulent ones). However, with new insights into the molecular organisation of pathogenicity in many species and whole-genome sequencing techniques, one can use donor strains containing less virulence-related genes or make knock-out constructs without certain virulence or toxigenic genes. How registration regulations will deal with application of a hypovirulent pathogen or potentially of a non-pathogenic strain of this fungus as donor is not certain, as no products have been registered yet.

Present heterokaryon or vegetative incompatibility in and between species limits both needed and unwanted spontaneous transfer, and reduces the risk of accidental infection of neutral or beneficial fungal species. As most mycoviruses lack a viable extracellular phase, transfer via that route is also very limited. Some mycoviruses are related to plant viruses, most seem co-evolved with their fungal host, while a relation to animal viruses seems very limited (Göker et al., 2011). Recently, Andika et al. (2017) showed the natural occurrence of a cross-kingdom infection of pathogenic fungi with a plant virus. No research has been
done to see if the reverse also may occur. That does leave the risk observed by Moriyama and co-workers (2018) in *M. oryzae* and *A. alternata*, that at least some chrysoviruses may not only confer hypovirulence but may also be the driving force behind the development of physiological diversity in the hosts, including new pathogenic races.

Virocontrol via hypovirulent fungi is not only used in plant pathology, but is also gaining interest in human and animal mycology. A lack of applicable, effective antifungals is limiting treatment, while the number of vulnerable, often immunocompromised, patients is growing. Treatment of *A. fumigatus*, the main cause of Aspergillosis, with introduced (Refos et al., 2013) or intrinsic (Kanhayuwa et al., 2015; Takahasi-Nagaguchi et al., 2020; Zoll et al., 2018) mycoviruses has been explored. In the case of the AfuTmV-1, which seems to be an intermediate between a dsRNA and positive strand ssRNA virus in build-up, the virus is infectious both as purified entity and as naked DNA (Kanhayuwa et al., 2015). One study shows such a natural mycovirus may cause hypovirulence when tested in the *Galleria mellonella* model (Özkan and Coutts, 2015). On the other hand, recent studies on mycoviruses in *Malassezia* yeasts, common inhabitants of the human skin and occasionally causing infections, show that mycovirus infection may enhance skin colonisation and enhance interferon production (Applen Clancey et al., 2020; Park et al., 2020).

**9 Conclusion**

Virocontrol and the use of hypovirulent mycoviruses is a small field in biocontrol research. However, advances in genomic techniques have made the screening for mycoviruses and assessing (molecular) effects on their host easier, and a plethora of mycoviruses have been found in all divisions of fungi and related phyla. Infections with mycoviruses appear quite common in most fungal species, with infection rates varying between 0% and 100%, with corresponding effects on host pathogenicity. For those pathogens that do not contain hypovirulent mycoviruses themselves in nature, alternative ways of infection are available, using viruses from other (related) species and protoplast techniques.

Fungal (population) biology has taught us a lot about the reproduction modes of fungi and vegetative (in)compatibility, and how that may affect mycovirus transmissions. Vertical transmission rates of viruses to sexual and asexual offspring can vary per species and per mycovirus, as does horizontal transfer between isolates. For successful application in the field, virus donors need to be compatible with the target field isolates and demonstrate incompatibility and species boundaries to limit further spread.

As yet, no commercial virocontrol product exists for the control of plant pathogenic fungi. However, mycovirus research so far has shown that for
many of the major plant pathogens, potential candidates for virocontrol with hypovirulent mycoviruses exist.

10 Where to look for further information

10.1 Mycoviruses in general


10.2 Principles of virocontrol and biocontrol


10.3 C. parasitica mycoviruses and hypovirulence

• Liu, Y.-C. and Milgroom, M. G. (1996), Correlation between hypovirus transmission and the number of vegetative incompatibility (vic) genes
different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology* 86, 79–86.


### 10.4 *Rosellinia necatrix* mycoviruses and hypovirulence

The white root rot fungus *Rosellinia necatrix* is a soil-borne pathogen that causes serious economic losses worldwide in various crops, including fruit trees. After *C. parasitica*, it probably is the best studied plant-fungal pathogen–mycovirus system studied.


### 11 References


Biocontrol via mycoviruses: a neglected option for bioprotection?


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