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Challenges to elucidating how endornaviruses influence fungal hosts: Creating mycovirus-free isogenic fungal lines and testing them

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ABSTRACT

Determining roles of mycoviruses in fungal biology is complicated, especially when fungi are co-infected with multiple viruses. Genetically identical (isogenic) fungal lines that are infected by and not infected by viruses must be created and compared. Here, we study an isolate of *Ceratobasidium* sp., a fungus isolated from pelotons in roots of a wild terrestrial orchid. The fungal isolate was co-infected with three distinct endornaviruses, isolates of Ceratobasidium endonarvirus B (CbEVB), Ceratobasidium endonarvirus C (CbEVC) and Ceratobasidium endonarvirus D (CbEVD). An experiment to reveal natural distribution of the three mycoviruses within a fungal colony revealed no sectoring; they were all evenly distributed throughout the colony. Hyphal tipping and treatments with one of five antibiotics (kanamycin, streptomycin, cycloheximide, rifampicin and ampicillin) were applied in attempts to 'cure' fungal lines of one, two or three of the viruses present. Surprisingly, the three mycoviruses responded differentially to each curing approach. The isolate of CbEVC was eliminated upon treatment with cycloheximide, but not with kanamycin or streptomycin, whereas the isolate of CbEVD did not respond to cycloheximide. The isolate of CbEVB was eliminated upon all treatments. In some cases, a virus was undetectable by species-specific RT-PCR assay after treatment, but when the fungus was cultured for a period on non-selective medium, the virus was detected again. Effects of mycoviruses on growth characteristics of isogenic fungal lines on two nutrient media were studied. Co-infection by the three viruses reduced mycelial growth rate on both media. In contrast, some fungal lines infected with one or two mycoviruses grew more rapidly than virus-free lines.

1. Introduction

Mycoviruses are viruses that infect fungi. It has been more than 50 years since the first mycovirus was discovered in cultured *Agaricus bisporus* (Portobello mushroom) lines suffering from die-back disease (Hollings, 1962). The International Committee on Taxonomy of Viruses (ICTV) recognises 17 virus families from which at least one species infects a fungus (Kotta-Loizou and Coutts, 2017).

Unlike viruses that infect other eukaryotic groups, most mycoviruses are not transmitted to new hosts *via* a vector or by other extracellular routes, and consequently, these are usually not infectious agents in the classical sense. An exception is Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), that was transmitted extracellularly to *Sclerotinia sclerotiorum* (Yu et al., 2013). Most mycoviruses are transmitted to new hosts by hyphal fusion (anastomosis), and they spread over distance within asexual spores (Nuss, 2011).

How mycoviruses influence the biology of host fungi in natural conditions is largely unknown. Where studies have been done, mycoviruses are referred to as being either hypovirulent, latent, or hypervirulent. These terms are somewhat confusing because they do not refer to virulence of the virus(es) *per se*, but rather they refer to changes in pathogenicity of the fungal host. Thus, Cryphonectria parasitica hypovirus 1 (CHV1) is known as a hypovirulent mycovirus because its presence in the fungus *Cryphonectria parasitica* induces lower pathogenicity of the fungus when it infects plants of *Castanea dentata* (American chestnut) (Eusebio-Cope, Sun et al. 2015). Similarly, Nectria radicicola virus L1 (NrV-L1) is referred to as a hypervirulent mycovirus because its infection of *Nectria radicicola* induces greater pathogenicity of the fungus on plants of *Panax ginseng* (ginseng) (Ahn and Lee, 2001). Many mycoviruses do not obviously influence virulence of their hosts either way, and these are referred to as latent or cryptic mycoviruses (Pearson, Beever et al. 2009). Mycoviruses may influence the biology of their fungal hosts in ways other than pathogenicity of the fungus on its

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host. For example, co-infection by three mycoviruses increased growth rate of a *Monilinia fructicola* (the causal agent of brown rot on *Prunus* species) isolate *in vitro*, but did not apparently influence growth rate of lesions on plums infected by isogenic lines of *M. fructicola* (Tran, Li et al. 2019).

Determining how mycoviruses affect the biology of their primary host can be challenging. In some cases, fungi are co-infected with multiple viruses (Ghabrial, 1998; Ong, Li et al. 2016) and ascribing influences to individual viruses, or certain combinations of them, is problematic. In order to do so, isogenic (genetically identical) lines of the fungal host must be created that are both infected by and free of the virus(es). Comparisons are then made of various characteristics of the isogenic lines. Two approaches are possible to obtain isogenic fungal lines: i. introduction of mycovirus(es) to virus-free fungal lines, and ii. eliminate (cure) mycovirus(es) from infected fungal lines. In the first strategy, transmission of the virus from infected lines to non-infected lines could be obtained naturally by hyphal anastomosis. Artificially, either purified virus particles or plasmid-derived infectious clones of viruses are used to infect a virus-free fungal line. In cases where the mycovirus is unencapsidated, e.g. viruses in the family *Endornaviridae* (Fermin, Mazumdar-Leighton et al. 2018), purification of virus particles for transfer to virus-free lines may not be possible (Roossinck et al., 2015); we are not aware of reports of gel-purified mycoviral genomes being used to infect fungal hosts. Infectious clones are challenging to construct when viruses such as endornaviruses have large (12–24 kb) genomes. The 'curing' approach often centers around growing cultures from hyphal tips, protoplast isolation and spore isolation (Zhang, Liu et al. 2014; Herrero Asensio, Sánchez Márquez et al. 2013), and/or treatment of fungal cultures with chemical agents, often antibiotics such as cycloheximide, that act on ribosomal proteins to inhibit replication of viral genomes (Stöcklein and Piepersberg, 1980). Other methods involve freeze/thawing to disrupt virus particles (Redman, Sheehan et al. 2002).

In this research, we tested hyphal tipping and five antibiotic treatments, some not previously reported, to cure a *Ceratobasidium* sp. fungal isolate of one to three endornaviruses. We undertook this research as part of ongoing research into the roles that viruses play in natural biological systems.

2. Methods

2.1. Fungi and mycoviruses

Ceratobasidium sp. isolate C02 was previously extracted from a single peloton in root tissue of an indigenous wild *Pterostylis sanguinea* (red-banded greenhood orchid) plant in Western Australia in 2012 (Ong, Li et al. 2016). The isolate was maintained on oatmeal agar medium at 4 °C. The fungal isolate was co-infected with three endornaviruses: Ceratobasidium endornavirus B (CbEVB) which, at 23.6 Kb is the largest endornavirus genome described, Ceratobasidium endornarvirus C (CbEVC) which has a 21 Kb genome, and Ceratobasidium endornarvirus D (CbEVD) which has a 19.4 Kb genome (Ong, Li et al. 2016). These three viruses represent the type isolates of their species, and were granted GenBank accessions NC_031463, NC_031461 and NC_031449, respectively.

2.2. Mycovirus treatments

Hyphal tipping and exposure to antibiotics were approaches tested to eliminate mycoviruses from the fungal isolate. Each treatment was performed on nine clones of the fungal isolate, all of which were derived from a single fungal culture.

2.3. Hyphal tipping

Hyphae were subcultured on water agar medium, then incubated in the dark at 25 °C for 72 h. Under a binocular microscope, the growing tips (~1 mm) of single hyphae were excised and transferred to fresh water agar plates. Hyphae were allowed to grow in the conditions described above, and this process was repeated five times.

2.4. Antibiotics

Before beginning the curing experiment, the sensitivity of the fungus to each antibiotic was tested. Mycelium was cultured on water agar plates containing concentrations of 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1} and 200 mg L^{-1} of an antibiotic. The fungi were grown on nine replicate plates of each antibiotic concentration, and growth was measured after seven days. The concentration used was the one in which growth was impeded by 50% compared to that on antibiotic-free medium. The concentrations of antibiotic used for subsequent experiments were streptomycin 200 mg L^{-1}, kanamycin 50 mg L^{-1}, cycloheximide 50 mg L^{-1}, rifampicin 25 mg L^{-1}, and ampicillin 100 mg L^{-1} (Sigma Aldrich).

For curing experiments, a five-mm^2 agar cube of actively-growing mycelium was cultured on water agar in which one of the five antibiotics was present. Subculturing to fresh antibiotic plates was done at seven-day intervals, as described above. After five subcultures (total 35 days), the mycelium was tested for presence of each of the three viruses using virus-specific primers. They were also transferred to 'recovery plates' of water agar lacking antibiotics and incubated for seven days in the dark at 25 °C to recover then tested again for presence of each virus as above.

A five-mm^2 agar cube of mycelium taken from the fifth subculture of (1) hyphal tipping method cultures and (2) the antibiotic treated cultures, and (3) the recovery (antibiotic-free) cultures was transferred to 25 mL antibiotic-free potato dextrose broth (PDB) and incubated at 25 °C in the dark with shaking at 100 rpm until 80–100 mg of fungal biomass could be collected for RNA extraction.

2.5. Virus distribution

Distribution of the three endornaviruses within mycelial colonies was tested. One isogenic line contained all three viruses. Another isogenic line was free of all three viruses. The mycelium was allowed to grow to the edge of the plate, upon which each plate was divided into 52 sections. A 3-mm diameter plug of mycelium was removed from the centre of each section (Fig. 1). The mycelial plug was used to inoculate 25 mL of PDB, and shaken at 100 rpm at 25 °C in the dark until 80–100 mg of fungal biomass could be collected for RNA extraction. The presence of each of the three endornaviruses was tested in each of the 52 sections using virus-specific primers by RT-PCR.

2.6. RNA extraction

RNA was extracted from 80 to 100 mg fungal mycelia using a cellulose powder-based extraction procedure (Morris and Dodds, 1979) designed to enrich double-stranded (ds) RNA. Although endornaviruses have single-stranded (ss) RNA genomes, the replicative form is dsRNA (Fukuhara and Gibbs, 2012). The aqueous phase from a phenol-chloroform extraction was added to CF-11 cellulose powder (Whatman). RNA was eluted from the cellulose power in elution buffer (0.1 M NaCl, 50 mM Tris − HCl, pH 8 and 0.5 mM EDTA, pH 8), and then precipitated in cold absolute ethanol and stored at −80 °C.

2.7. cDNA synthesis

cDNA was synthesized in a 20 μL volume using approximately 500 ng heat-denatured RNA, 160 units of GoScript™ reverse transcriptase (Promega Corp.), 3 mM $MgCl_2$, 0.5 mM dNTPs and 0.5 mM of random primer (Promega Corp). Reactions were incubated at 25 °C for 5 min for primer annealing, then strand-extention at 42 °C for 60 min,

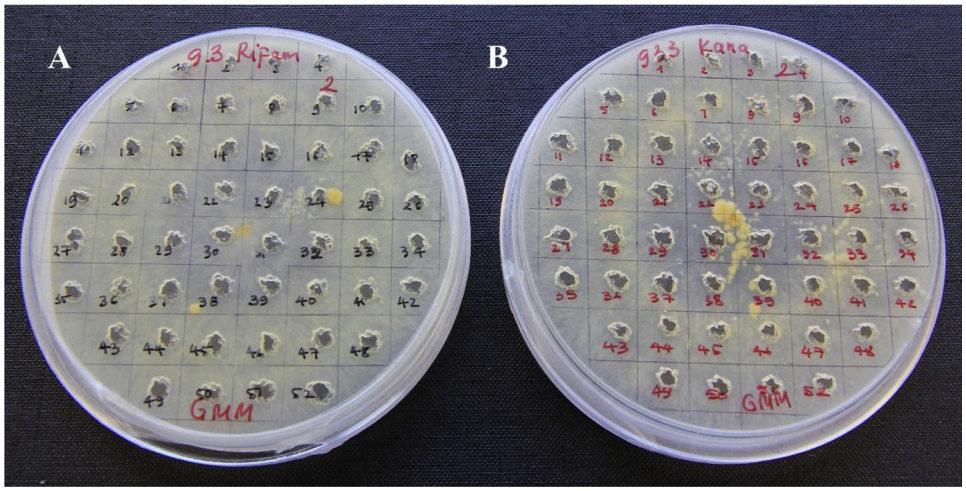

Fig. 1. Virus distribution experiment. (A) *Ceratobasidium* isolate co-infected with three endornaviruses, (B) isogenic isolate free of three endornaviruses. Plates were divided into 52 sections and a plug of mycelium taken from the centre of each for virus testing.

followed by incubation at 72 °C for 15 min to denature the enzyme.

2.8. Mycovirus detection

The presence of each mycovirus was established using specific primers (Table S1) (Ong, Li et al. 2016). Each primer pair was designed to amplify a region of the large open reading frame (ORF) coding for the RNA-dependent RNA polymerase (RdRp) gene of each virus. PCRs were set up in 20 µL reaction volumes comprising of GoTaq® Green Master Mix (Promega Corp.), 0.5 mM of each forward and reverse primer, and 2 µL of cDNA. PCRs were done with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 1 min at 55 °C, extension for 1 min at 60 °C, after which a final extension was carried out at 72 °C for 10 min. Amplicons were separated on 1% agarose gels.

2.9. Effect of mycovirus on fungal growth

Five 'recovered' isolates from the virus elimination experiment results were chosen. They contain different virus combinations: (1) CbEVB, CbEVC and CbEVD, (2) CbEVB and CbEVD, (3) CbEVC and CbEVD, (4) only CbEVC, and (5) no viruses. All five isolates were tested to ensure the presence of virus. Thirty replicate cultures of each virus combination derived from one mother culture (total 150 cultures) were cultured in glucose minimal agar (GMM) and the same number was cultured on soil extraction agar (SEA). The 300 plates were incubated at 25 °C in the dark for five days. Each fungal colony was measured across three diameters based on the final growth of the colony, and the means were calculated. A *t*-test was performed using the Tukey tool in R v3.4.1. at the 95% confidence level ($P = 0.05$).

3. Results

3.1. Responses of viruses to six curing treatments

Untreated fungal cultures were tested for the presence of CbEVB, CbEBC and CbEVD before starting virus elimination experiments. After five sequential rounds of each treatment, virus-specific assays revealed that all treatments eliminated at least one endonarvirus from at least one treatment replicate. However relative efficacies of treatments differed (Table 1).

After 35 days of treatment, hyphal tipping and antibiotic eliminated CbEVB in a high proportion of replicates. Treatments by cycloheximide, kanamycin and rifampicin resulted in 89–100% of lines that were virus-free after the 5th and 6th rounds of subculturing. The most effective treatment for CbEVC was cycloheximide (78% of replicates) followed by rifampicin (56% of replicates), while hyphal tipping, streptomycin and kanamycin treatments were totally unsuccessful at eliminating CbEVC. In contrast, cycloheximide was ineffective at eliminating CbEVD as assessed at day 35 of treatment, while hyphal tipping, and kanamycin were effective, eliminating CbEVD in 100% and 78% of replicate plates, respectively.

Table 1
Curing isogenic fungal lines of mycoviruses. Success rate (percentage of free-virus isolates) of curing a fungus of mycoviruses using six different treatments, including hyphal tipping and five antibiotics. Fungal lines were tested for on the 5th treatment subculture (T) and on the recovery (6th) subculture (R) using species-specific primers. Each treatment was performed on nine isolates.

	CbEVB[a]		CbEVC		CbEVD	
	T	R	T	R	T	R
Hyphal Tipping	89	100	0	0	100	100
Streptomycin 200 mg L^{-1}	89	89	0	0	33	44
Kanamycin 50 mg L^{-1}	100	89	0	0	78	67
Cycloheximide 50 mg L^{-1}	100	100	78	33	0	0
Rifampicin 25 mg L^{-1}	100	33	56	0	56	33
Ampicillin 100 mg L^{-1}	89	67	11	22	11	22

[a] CbEVB, Ceratobasidium endornavirus B; CbEVC, Ceratobasidium endornavirus C; CbEVD, Ceratobasidium endonarvirus D.

3.2. Reappearance of virus after apparent elimination

After being subjected to five rounds of antibiotic treatments, all replicate fungal cultures were grown on recovery medium lacking antibiotics. After recovery, cultures were tested again at 7 and 14 days for presence of each virus. Surprisingly, some of the cultures in which a virus had apparently been eliminated, as verified by negative RT-PCR results after the fifth subculture, were shown to harbour the virus. In case of rifampicin treatment, CbEVB could not be detected in any of the replicate cultures, but after recovery on rifampicin-free medium, only a third of the cultures were found to be free of CbEVB. After rifampicin treatment, over half of the cultures were apparently free of CbEVC, but after recovery treatment, CbEVC was detected in every replicate line. After cycloheximide treatment, CbEVC was detected in about a quarter of replicate plates, but after recovery treatment, CbEVC was detected in about two-thirds of cultures (Table 1).

Although virus eventually reappeared in some apparently virus-cured lines after recovery, in others it was never detected. After recovery, in two lines, the fungus was cured of all three viruses, while in

Table 2
List of viruses detected in *Ceratobasidium* sp. isogenic lines.

Virus(es) present	Number of isogenic *Ceratobasidium* sp. lines after 5th subculture on selection medium	Number of isogenic *Ceratobasidium* sp. lines after culture on recovery medium	Name of isogenic line used in subsequent experiments
None	3	2	Free1, Free2
CbEVB only	0	0	–
CbEVC only	21	20	C
CbEVD only	10	3	D[a]
CbEVB and CbEVC	1	2	BC[b]
CbEVC and CbEVD	17	18	CD
CbEVB and CbEVD	0	1	BD
CbEVB, CbEVC and CbEVD	2	8	BCD

[a] Three isolates containing only CbEVD, however CbEVC was present again after two weeks.
[b] Two isolates containing CbEVB and CbEVC lose CbEVB after two weeks.

others, one or two viruses were eliminated (Table 2). The recovered isolates were subjected to subsequent experiments in which distribution within a fungal colony and their effect on fungal host growth was tested. These isolates were considered to be isogenic and differed in virus presence only.

3.3. Viral distribution within a fungal colony

To test whether the apparent disappearance then reappearance of viruses was due to uneven distribution of viruses within fungal colonies, two colonies were sampled at 52 points across the whole colony (Fig. 1). Colony A harboured three endornaviruses and colony B was free of endornaviruses after treatment by kanamycin. Mycelium from each of the 104 segments was grown in liquid medium, RNA was extracted, cDNA synthesised using random primers, and RT-PCRs performed using specific primers for each endornavirus. All three viruses were detected in each segment from colony A and none from colony B. Thus, the three viruses were evenly distributed across the infected colony. This finding suggested that uneven virus distribution was not the reason why fungal cultures were apparently cured of virus(es), that later reappeared.

3.4. Viral effect on fungal host growth

Effects of virus infection on fungal growth rate were assessed on two media: glucose minimal medium (GMM) and soil extract agar (SEA).

At the 95% confidence level ($P = 0.05$), infection of all three viruses together inhibited radial growth (diameter) of fungal colonies on both media (Fig. 2). When comparing the effects of lines with triple-virus infection with some combinations of single- (CbEVC) and double-virus (CbEVB & CbEVD or CbEVC & CbEVD) infections, lines with all three viruses grew slower on SEA medium but faster on GMM. Effects of combinations of two viruses, CbEVB with CbEVD and CbEVC with CbEVD, displayed opposite radial growth rate characteristics on GMM and SEA media. Lines CbEVB with CbEVD, and lines CbEVC with CbEVD grew slower on GMM but faster in SEA medium. Lines with CbEVC alone grew significantly slower in GMM compared to virus-free lines, but this was not the case on SEA medium. In general, infection with one or two viruses slowed fungal colony growth on GMM, but their presence increased growth rate on SEA medium (Table 3, Fig. 2).

4. Discussion

The three endonarviruses that coinfected an isolate of *Ceratobasidium* sp. responded differently to hyphal tipping and five antibiotic treatments, but the reasons for this are unclear. CbEVB was eliminated more readily than the other two viruses. On the other hand, CbEVC was the most recalcitrant, and when it had apparently been eliminated on an antibiotic, it was subsequently detected when the antibiotic was removed. Our experience confirmed that mycovirus elimination is a challenging process, requiring ongoing monitoring of lines to confirm maintenance of their virus-free status.

The effects of endornavirus presence on growth of the fungal host was also complex. When infected with all three viruses, the fungal colony grew slower on both SEA and GMM media. Although a similar response occurred on GMM in dually- and singly-infected lines, on SEA medium for the virus combinations tested, presence of one or two mycoviruses stimulated mycelial growth.

4.1. Curing fungi of mycoviruses

Hyphal tipping was effective at eliminating these viruses. Mycoviruses replicate within fungal cells and move between cells during cell division. Distribution of mycoviruses within fungal mycelia is reported to be uneven (Ghabrial, 1980). Virus-like particles are more likely to be present in older hyphae than in actively-growing hyphae at the tip (Border, Buck et al. 1972; DeMarini et al., 1977), and the same is reported in plant cells (Toussaint et al., 1984). The reason for this is thought to be replication of viruses lags behind host growth, as shown when *Penicillium stoloniferum* was infected by Penicillium stoloniferum virus F (PsV-F) and Penicillium stoloniferum virus S (PsV-S) (Buck, 2018). Thus, growing tips may be free of viruses, and so culturing them is an established method of curing fungi of viruses. For example, mycoviruses were successfully eliminated from members of *Aspergillus* species (van Diepeningen et al., 2006), and *Sclerotinia sclerotiorum* strain DT8 was cured of Sclerotinia Sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) using hyphal tipping approaches (Yu, Li et al. 2010).

Here, hyphal tipping eliminated CbEVB and CbEVD, but not CbEVC, but the reasons for this is unclear. In *Rhizoctonia solani*, a relative of *Ceratobasisidum* (Mosquera-Espinosa, Bayman et al. 2013), there has been varied success in curing lines of viruses. *R. solani* lines were not cured of two endornaviruses by hyphal tipping, but other unidentified mycoviruses (dsRNA species) were eliminated from other lines. Ability to cure strains of mycoviruses may vary across different anastomosis groups (Table 4).

To our knowledge, kanamycin, streptomycin, and ampicillin have not previously been examined for their ability to cure fungi of viruses. Kanamycin and streptomycin have similar modes of action, that is inhibition of protein synthesis by binding to the 30S subunit of the ribosome (Overington, Al-Lazikani et al. 2006). Rifampicin is a derivative of rifamycin that prevents the binding of RNA polymerase to DNA and acts as an inhibitor of RNA viruses (Hartmann, Honikel et al. 1967; Campbell, Korzheva et al. 2001). Although the curing mechanism is not clear, a treatment of rifamycin with cAMP in a minimal medium was reported to cure a culture of the edible mushroom *Pleurotus ostreatus* from the RNA mycoviruses oyster mushroom spherical virus (OMSV) and oyster mushroom isometric virus (OMIV) (Kwon et al., 2012).

On the other hand, cycloheximide has been used widely to cure fungi of viruses. The ability of cycloheximide to affect RNA replication

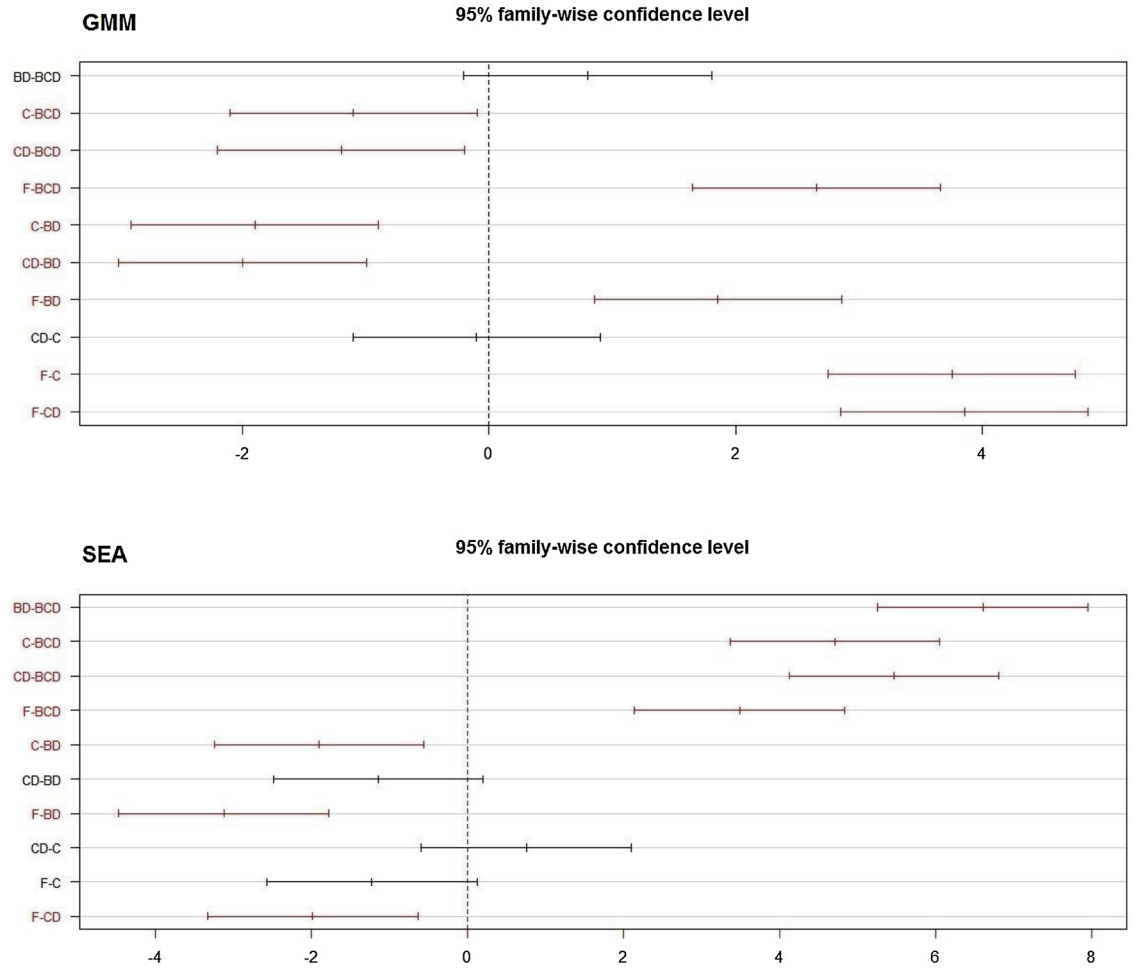

Fig. 2. Comparisons of growth between isogenic *Ceratobasidium* sp. lines either free of all three viruses (F), or infected with different combinations of CbEVB (B), CbEVC (C), and CbEVD (D) on glucose minimal agar medium (GMM) and soil extract agar medium (SEA). There were 30 replicate colonies for each virus combination. The difference in mean diameter of fungal colonies between two-virus combinations (X axis) is presented in millimetres (Y axis). A *t*-test was performed using the Tukey tool available in R v3.4.1. $P = 0.05$. Statistically-significant differences are highlighted in red. BCD = triple infection with CbEVB, CbEVC and CbEVD; BD = dual infection with CbEVB and CbEVD; CD = dual infection with CbEVC and CbEVD; C = single infection with CbEVC (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 3
Summary of pairwise comparisons between isogenic lines infected with different virus combinations on two different growth media at 95% confidence level ($P = 0.05$). The letters BCD refer to CbEVB, CbEVC and CbEVD, respectively; BD = infection with CbEVB and CbEVD; CD = infection of CbEVC and CbEVD; C = infection of CbEVC; Free = no viruses. The symbol > indicates faster growth rate, < = slower growth rate, no = there is no statistically-significant difference in growth rate. The differences in growth (means of diameter of fungal colonies after five days incubation) were measured in millimetres. GMM = glucose minimal agar medium. SEA = soil extract agar medium.

Comparison	GMM	SEA
BD with BCD	no	BD > BCD
C with BCD	C < BCD	C > BCD
CD with BCD	CD < BCD	CD > BCD
Free with BCD	Free > BCD	Free > BCD
C with BD	C < BD	C > BD
CD with BD	CD < BD	no
Free with BD	Free > BD	Free < BD
CD with C	no	no
Free with C	Free > C	no
Free with CD	Free > CD	Free < CD

is linked to its ability to alter clearance of translating ribosomes from the viral genome. Cycloheximide inhibits polypeptide chain elongation by “freezing” ribosomes on the viral RNA genome and prevents ‘clearance’ of ribosomes from them, thereby preventing both translation and replication of the viral genome from the negative strand RNA (Barton et al., 1999). Endornaviruses have ssRNA genomes with a dsRNA replicative stage (Fukuhara and Gibbs, 2012), and it is at this replicative stage that cycloheximide is active. As cell division proceeds, dsRNA is diluted, especially in actively-growing hyphal tips, and so cultures become free of viruses (Fink and Styles, 1972).

Some antibiotics are reported to induce branching activities in filamentous fungi (Baráthová, Betina et al. 1969). Hyphal branching is induced by cell division. Inducing branching activities through treatment with antibiotics should increase the number of hyphal tips, and thereby increase opportunities for generating virus-free cultures from hyphal tips.

The antibiotics used in this study had differential influences on eliminating the three endornaviruses, and the reasons for this are unclear. Effectiveness probably reflects both the mode of action of the antibiotic and aspects of biology of each endornavirus. Streptomycin and kanamycin act on a similar mechanism, which could explain why

Table 4
Outcomes of attempts to eliminate mycoviruses by hyphal tipping in different anastomosis groups (AG) of *Rhizoctonia solani*.

Strain and anastomosis group	Mycovirus	Outcome	Reference
Rhizoctonia solani GD-11 of AG-1 IA	Rhizoctonia solani endornavirus 2, RsEV2	Unsuccessful	(Zheng et al., 2014)
Rhizoctonia solani GD-2 of AG-1 IA	Rhizoctonia solani endornavirus 1, RsEV1	Unsuccessful	(Zheng, Shu et al. 2019)
Rhizoctonia solani Rhs 717 of AG 2	Unidentified. dsRNA segments	Successful	(Tavantzis and Bandy, 1988)
Rhizoctonia Solani AG 3	Unidentified. More than one dsRNA segment	Partially successful. Elimination of smaller dsRNA segments	(Robinson and Deacon, 2002)
Rhizoctonia Solani AG 4	Unidentified. More than one dsRNA segment	Partially successful	(Finkler, Koltin et al. 1985)

kanamycin and streptomycin had similar patterns of elimination of viruses. Both kanamycin and streptomycin eliminated CbEVD quite effectively, but seemed to have mild effects on CbEVC, indicating a viral component to effectiveness of the antibiotics.

4.2. Difficulties in elimination of certain viruses, and re-emergence of others

CbEVC was the most recalcitrant virus, by both hyphal tipping and antibiotic treatments, while CbEVB was relatively simple to eliminate. In other systems, similar outcomes were observed. In *Aspergillus fumigatus* lines infected with two mycoviruses, cycloheximide eliminated Aspergillus fumigatus chrysovirus (AfuCV), but not Aspergillus fumigatus partitivirus (AfuPV) (Bhatti, Jamal et al. 2011). In *Aspergillus* sect. *flavi,* cycloheximide was effective against ssRNA molecules, but the same treatment was ineffective against dsRNA molecules (Elias and Cotty, 1996). The reasons for this are unclear, but our findings with three ssRNA viruses suggest the effect is mediated by a viral component. It is unclear if the viral component is based on its genome structure, viral proteins, or an aspect of its biology within the cell (Khankhum, Valverde et al. 2015).

We considered whether differential distribution of the viruses within the colony might explain the differences in responses of CbEVB and CbEVC to treatments. Two virus-like particles that coinfected a *Penicillium cirtinum* isolate were unevenly distributed within the colony (Ghabrial, Sanderlin et al. 1979; Ghabrial, 1980). In our study, differential distribution does not appear to explain the apparent disappearance, and then reappearance of the viruses when the antibiotic was removed. The levels of virus present was apparently below the detection limit of the RT-PCR assays used. The reappearance of mycoviruses in fungal strains after apparent curing was reported in *R. solani* isolate Papa that was apparently cured of a 12 Kb dsRNA, which was later detected again (Robinson and Deacon, 2002). In another case, *R. solani*, dsRNA fragments were found in a previously reported dsRNA-free isolate (Kotlin, Finkler et al. 1987; Robinson and Deacon, 2002). In *Agaricus bisporus*, curing Agaricus bisporus endornavirus 1 (AbEV1) using hygromycin B resulted in apparent loss of the virus, but after a month in culture without the antibiotic, the virus was redetected (Maffettone, 2007). Similarly, *Pseudogymnoascus destructans* harbouring two virus-like RNA segments was treated with cycloheximide (25 mg L^{-1}) and ribavirin (300 μM). After three subcultures, the viruses were apparently eliminated, but they were detected again after transfer to an antibiotic-free medium (Thapa, Turner et al. 2016).

It was suggested that presence of antibiotics kept the level of the dsRNA below RT-PCR detection level, but no quantitative RT-PCR tests have been done to measure virus titres during and after treatments. Confirmation of the viral status in the 'cured' fungal lines is required for some time after treatment. In our study, fungal isolates were tested for one to two weeks after apparent elimination by antibiotic treatment, and we recommend that future researchers in the field do the same.

A less-likely explanation for apparent reappearance of dsRNA elements from fungi may be the presence of integrated DNA copies of the dsRNA elements (Robinson and Deacon, 2002). There is evidence of sequence identity between dsRNA segments and parts of the genome of *R. solani*, which supports this explanation (Tavantzis, 1994). Comparisons between sequences of mycovirus genomes and the fungal host genome would give more evidence to support this possible explanation.

4.3. Possible roles of mycoviruses

When infected with all three viruses, the fungal host grew slower on both SEA and GMM compared to virus-free isolates. This growth pattern could be conceptualised as the cost of virus replication on the host. However, this concept is probably simplistic. When fungal lines were infected with one or two viruses, they grew at a slower rate on GMM than did the virus-free line. However, on SEA the presence of one or two mycoviruses stimulated growth above virus-free line. A somewhat similar response was observed in triple virus-infected *Monilinia fructicola* isogenic lines that grew faster on PDA medium than virus-free lines (Tran, Li et al. 2019). SEA medium may more closely (than GMM) resemble the natural soil habitat the fungus is used to, and its response on SEA may more closely resemble what happens in nature, but more research is required to address this hypothesis. It is noted that linear growth of fungal colonies, as was measured here, may not correlate directly with fungal biomass, which is a function of other factors including density and branching frequency of mycelia.

Co-infections by two or more viruses may result in commensal interactions between viruses. For example, coinfection of a hypovirus and a mycoreovirus in an isolate of chestnut blight fungus, *Cryphonectria parasitica*, resulted in enhanced vertical transmission of the mycoreovirus, facilitated by suppression of the host RNA silencing-associated gene *dcl2* by components of the hypovirus (Aulia et al., 2019). Commensal interactions between endornaviruses have not been recorded.

In the natural environment, fungi must encounter numerous biotic and abiotic stresses, such as attack by pathogens and competition for resources by other soil inhabitants. Our study showed that presence of mycoviruses differentially affected fungal growth on two media. GMM is a defined medium commonly used to grow fungi (Ouedraogo, Lagendijk et al. 2013). It contains the basic micro- and macro-elements (Zn^{2+} 44.5 × 10^{-4} M, B^{3+} 9 x 10^{-4} M, Mn^{+2} 20 × 10^{-4} M, Fe^{2+} 15 × 10^{-4} M, Co^{2+} 5 × 10^{-4} M, Cu^{2+} 5 × 10^{-4} M, Mo^{3+} 65 × 10^{-4} M) with glucose as the carbon source. SEA is undefined with a complex mixture of nutrients and other compounds more closely resembling soil. To make SEA, soil was collected at the original fungus/orchid sample collection site, which is a sandy loam containing organic matter from *Eucalyptus* trees and other plants. Eucalyptus fresh leaf tissue contains phenols and tannins which typically make up 4–40% and 0–27%, respectively, of leaf dry weight (Macauley and Fox, 1980). The fact that presence of virus(es) stimulated colony growth on SEA is an indication that viruses may play a positive role in Ceratobasidium biology, but what these roles are and the mechanisms of action requires further research. Likewise, the roles, if any, that mycoviruses play in the complex symbiotic relationship between fungi and orchids is a topic for further research.

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Declaration of Competing Interest

The authors declare that they have no known competing financial

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113745.

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