

Mycoviruses: A New Dimension In Microbiology

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Introduction

Knowledge of mycoviruses is so new that most scientists are barely aware that they exist, yet they are widespread and affect many parameters. Their possible effect on the levels of toxins and metabolites produced by fungi enhance their significance in environmental health research. Evidence for the occurrence of a lysogenic virus in yeast was presented as early as 1936 (1), and the viruses of higher fungi were first suspected in 1950 when Sinden and Hauser (2) reported a degenerative disease of mushrooms. *Olpidium brassicae* was shown in 1958 to be the vector of the virus which causes the big vein disease of lettuce but the evidence did not suggest that the fungus supported multiplication of the virus (3, 4).

The first virus of a fast growing fungus was reported by Ellis and Kleinschmidt (5, 6). The discovery resulted from several years of intensive investigation of the active factor in *Penicillium stoloniferum* responsible for the stimulation of interferon in test animals. Lampson *et al.* (7), working with another antiviral substance from *P. funiculosum* also capable of inducing interferon (8), found this activity associated with a double-stranded RNA extracted from mycelia of this mold. Subsequently, the RNA extracted from purified *P. stoloniferum* mycovirus was shown to be double stranded in nature (9).

The finding of mycovirus in *P. stoloniferum* was confirmed and also elucidated in *P. funiculosum* (10).

At Boyce Thompson Institute, our interest in mycovirus lies primarily in the effect of the virus on the pathogenicity and toxin production of the host fungus. The early work with *P. stoloniferum* (5, 6) was a definite stimulation. Whereas, in the past detection of a new virus had usually followed the recognition of a disease caused by the virus, it was now apparent that viruses of fungi might be found by screening procedures which involved purely physical methods, i.e. partial purification and electron microscopy. A number of laboratories, including our own, began screening fungus isolates for the presence of virus and met with considerable success.

The purpose of this review is to present an overview of the problem rather than a detailed synopsis of all of the published works, and to discuss the possible implications of these to interferon induction, toxin production, genetics of fungi, and plant diseases. Reviews of the published work through early 1971 have been presented by Hollings and Stone (11) and Spire (12).

Mycoviruses are viruses isolated from fungi. Although the term mycophage has been used by some workers (5, 13), it seems inappropriate since lysis of infected cells is not a prominent characteristic and in no case has lysis been shown to be a direct effect of mycovirus infection. The term virus-like particles (VLPs) has been used in

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reference to particles with one or more of the physical characteristics of viruses. In the purest sense, only those VLPs which have been transmitted by cell free preparation or by heterokaryosis qualify as viruses, namely the mushroom viruses (11) and the viruses of *Penicillium stoloniferum* (14, 15), *Aspergillus niger* (15), and *Ustilago maydis* (16). For the purpose of this review, all VLPs isolated from fungi or observed in thin section which are uniform in shape and size will be referred to as mycoviruses. Random spherical particles or pleomorphic particles observed by electron microscopy are not

considered mycoviruses even though they might have considerable substructure.

The Occurrence of Mycoviruses

Mycoviruses have been shown to occur in all major groups of fungi. A total of 55 different mycoviruses has been observed in 56 different species and 44 genera (Table 1). Random sampling of fungus cultures has indicated that 10 to 15% of the species contain mycoviruses and there is good reason to believe that a virus can be found in any species with diligent searching.

Table 1. Fungi Which Have Been Found to Contain Mycoviruses

Fungus	Reported description	Citation
<u>Phycomycetes</u>		
<i>Aphelidium</i> sp.	Iridescent type (f)	20
<i>Choanephora</i> sp.	—	0 (a)
<i>Mucor</i> sp.	—	0 (a)
<i>Plasmodiophora brassicae</i>	—	56
<i>Rhizopus</i> sp.	—	0 (a)
<i>Syncephalastrum</i> sp.	—	0 (a)
<u>Ascomycetes</u>		
<i>Daldinia</i> sp.	—	0 (a)
<i>Diploclaron rosae</i>	Isometric 34, 32 nm	24
<i>Hypoxylon</i> sp.	—	0 (a)
<i>Neurospora crassa</i>	Isometric 60 nm	93
<i>Ophiobolus graminis</i>	Isometric 29 nm	52, 78, 81
<i>Peziza ostracoderma</i>	Rods 17 x 350 nm	19
<i>Saccharomyces carlsbergensis</i>	Phage type (e)	61
<u>Basidiomycetes</u>		
<i>Agaricus bisporus</i> (virus 1)	Isometric 25 nm	11, 28
<i>A. bisporus</i> (virus 2)	Isometric 29 nm	11, 28, 31
<i>A. bisporus</i> (virus 3)	Bacilliform 19 x 50 nm	11, 28, 31
<i>A. bisporus</i> (virus 4)	Isometric 35 nm	11, 28, 31
<i>A. bisporus</i> (virus 5)	Isometric 50 nm	11, 28
<i>A. bisporus</i>	Rods 17 x 350 nm	19
<i>Boletus</i> sp.	Isometric 50 nm	11
<i>Hypholoma</i> sp.	—	0 (a)

Table 1. Fungi Which Have Been Found to Contain Mycoviruses (Continued)

Fungus	Reported description	Citation
<i>Basidiomycetes (Continued)</i>		
<i>Laccaria amethystina</i>	Isometric 28 nm	94
<i>Laccaria laccata</i>	Isometric 28 nm	94
<i>Polyporus</i> sp.	—	0 (a)
<i>Tilletiopsis</i> sp.	Isometric 40 nm	0 (c)
<i>Ustilago maydis</i>	Isometric 41 nm	16
<i>Fungi Imperfecti</i>		
<i>Alternaria tenuis</i>	Isometric 30-40 nm	11
<i>Arthrobotrys</i>	—	0 (b)
<i>Aspergillus flavus</i>	Isometric 30 nm	75
<i>Aspergillus foetidus</i> (IMI 41871)	Isometric 40-42 nm	66
<i>A. foetidus</i> S (IMI 41871)	Isometric 40 nm	22
<i>A. foetidus</i> F (IMI 41871)	Isometric 40 nm	22
<i>Aspergillus glaucus</i>	Isometric 25 nm	11
<i>Aspergillus niger</i> (IMI 146891)	Isometric 40-42 nm	66
<i>Aspergillus</i> sp.	—	0 (a)
<i>Aspergillus</i> sp.	—	0 (b)
<i>Botrytis</i> sp.	—	0 (a)
<i>Candida</i> sp.	—	0 (a)
<i>Cephalosporium acremonium</i> ATCC 11550	—	95
<i>Cephalosporium</i> sp.	—	0 (a)
<i>Chrysosporium</i> sp.	—	0 (b)
<i>Fusarium moniliforme</i>	Isometric 40 nm	0 (c)
<i>Fusarium</i> sp.	—	0 (a)
<i>Gliocladium</i> sp.	—	0 (a)
<i>Gliomastic</i> sp.	—	0 (b)
<i>Helminthosporium maydis</i>	Isometric 40 nm	18
<i>H. victoriae</i>	Isometric 40 nm	0 (c)
<i>Kloeckera</i> sp.	—	0 (a)
<i>Mycogone perniciosa</i>	Isometric 42 nm	96
<i>Mycogone perniciosa</i>	Rods 18 x 120 nm	96
<i>Paecilomyces</i> sp.	—	0 (a)
<i>Penicillium brevi-compactum</i>	Isometric 40 nm	17
<i>P. brevi-compactum</i>	—	0 (c)
<i>P. chrysogenum</i> ATCC 9480, NRRL 1951, B25, X1612, Q176, WIS 48-701, WIS 51-20C, BRL 700	Isometric 35 nm	42
<i>P. chrysogenum</i> ATCC 9480	Isometric 40 nm	23

Table 1. Fungi Which Have Been Found to Contain Mycoviruses (Continued)

Fungus	Reported description	Citation
<i>Fungi Imperfecti (Continued)</i>		
<i>P. chrysogenum</i> NRRL 1951	Isometric 35 nm	45, 46
<i>P. citrinum</i>	Isometric 40-50 nm	13
<i>P. cyaneo-fulvum</i> CMI 58138	Isometric 32 nm	49
<i>P. funiculosum</i>	Isometric 25-30 nm	10
<i>P. funiculosum</i>	—	0 (c)
<i>P. funiculosum</i>	—	0 (c)
<i>P. multicolor</i>	Isometric 34, 32 nm	0 (d)
<i>P. notatum</i>	Isometric 25 nm	11
<i>P. stoloniferum</i> ATCC 14586	Isometric 25-30 nm	5, 10
<i>P. stoloniferum</i> F ATCC 14586	Isometric 32, 34 nm	21
<i>P. stoloniferum</i> S ATCC 14586	Isometric 32, 34 nm	21
<i>P. variable</i>	Isometric 40-50 nm	13
<i>Penicillium</i> sp.	—	0 (b)
<i>Penicillium</i> sp.	—	0 (a)
<i>Piricularia oryzae</i>	Isometric 32 nm	25
<i>Piricularia oryzae</i>	Isometric 36 nm	26
<i>Rhizoctonia solani</i>	—	0 (c)
<i>Sclerotium cepivorum</i>	Isometric 30 nm	52, 97, 98
<i>Scopulariopsis</i> sp.	—	0 (a)
<i>Spicaria</i> sp.	—	0 (b)
<i>Stemphylium botryosum</i>	—	11, 99
<i>Trichothecium</i> sp.	—	0 (a)
<i>Verticillium</i> sp.	—	0 (a)

0, Unpublished results of (a)Merfyn Richards, Beecham Research Laboratories, Betchworth, England; (b)Rodger Crosse, Glaxo Research Ltd., Stoke Poges, Bucks, England; (c)R.F. Bozarth and H.A. Wood; (d)D.E. Mackenzie and J.P. Adler, Cornell Medical School, New York, N.Y.; (e)phage type. Viruses are similar to the t-bacteriophage with heads 70 x 80 nm and tails 70 nm long; (f)this virus is 200 nm in diameter and similar to the iridescent viruses (100).

Some genera are especially rich in mycoviruses; thus we isolated mycoviruses from five of six isolates of *Penicillium* species screened in a random sampling of cultures. While only one of these has been studied in detail (17), the other four appear to be distinctly different from each other. In *Helminthosporium maydis* (18), six mycoviruses were found among 12 isolates sampled from cultures obtained from geographically isolated areas. On the other

hand, we have tested 10 isolates of *Verticillium* and have thus far not located an infected strain.

Most of the mycoviruses described are spherical although rods (19) and polyhedral types (20) have been found. All of them studied thus far contain double-stranded RNA (ds-RNA) but there is no reason to believe that mycoviruses with single-stranded RNA (ss-RNA) and DNA will not be found.

Some fungi are infected with more than

one mycovirus (21). In the case of the *Penicillium stoloniferum* virus complex two quite similar viruses infect the species. They can be distinguished serologically and by the properties of their RNA. A strain of *Aspergillus foetidus* (22) also appears to contain two mycoviruses.

Our experience indicates that most of the mycoviruses occur in concentrations of less than 0.01 µg/g dry weight; however, the viruses of *P. chrysogenum* (23) and *P. stoloniferum* (21) occur in concentrations of 1 mg/g dry weight or higher. Related viruses do not necessarily occur at the same concentration level, thus the *P. brevicompactum* virus (17) which is serologically related to *P. chrysogenum* (23) virus occurs in its host at about 1/100 the concentration of the latter. A similar situation exists with the *Diplocarpon rosae* (24) virus which is related to one of the viruses of the *P. stoloniferum* (21) virus complex.

It is already apparent that the classification of the mycoviruses will be a major task complicated by the fact that more than one virus can be found in a single species. Some confusion already exists. For instance, Banks *et al.* (10) found a virus in a strain of *P. funiculosum* from England which is probably different from the virus in an American strain of the fungus (7). There is no evidence that the mycoviruses of *Piricularia oryzae* isolated in France (25) and Japan (26) are related. The identification of mycoviruses by fungus species and culture number will become increasingly more important as new mycoviruses are reported.

Transmission

Mycoviruses may be disseminated in spores, multiply, and possess all the physical characteristics of viruses but few workers will accept their designation as true viruses unless evidence of transmissibility is presented. This should be accomplished with cell free preparations.

Gandy (27) obtained the first evidence for transmission of the watery stipe disease of

cultivated mushrooms by hyphal anastomosis of mycelial cultures. Mycelial fragments of diseased tissue placed on trays of healthy mushrooms resulted in transmission, whereas filtrates of this diseased tissue did not. The virus nature of this disease was shown by Hollings (28) and Hollings *et al.* (29) who isolated a complex of three types of mycoviruses from diseased mushrooms and produced the disease in healthy mushrooms by injecting cell free preparations into the first flush of sporocarps. Later flushes of sporocarps showed the disease symptoms. Rasmussen *et al.* (30) confirmed these transmission experiments. Dieleman-van Zaayen and Temmink (31) performed similar experiments, produced the disease in mushrooms, and reisolated the three types of particles. It was not determined if all three types of particles are required for infectivity.

Whereas transmission experiments using purified preparations were comparatively easy with the mushroom viruses since the sporocarps could be easily injected with a virus suspension, growth of the fungus in pure culture is slow and at present unsatisfactory for propagation of large quantities of virus. Therefore, progress on defining the properties of the mushroom virus has been quite slow. Quite the reverse is true with viruses of filamentous fungi. Their growth in culture is fast and some mycoviruses reach high concentrations (21, 23). Several have been studied rather thoroughly, yet their cells are so small that direct injection of purified virus preparations cannot be made and special techniques must be worked out to demonstrate their transmissibility. The undisputed pioneer in this field is Pol Lhoas of Imperial College, London.

Lhoas (32) mixed the green spored culture of *Penicillium stoloniferum* ATCC 14586, which was previously shown to contain a mycovirus complex (10, 33-35), with the white spored mutant of *P. stoloniferum* ATCC 10111, which was previously shown not to contain mycovirus (10), and obtained white and green conidia in the same culture of heterokaryotic mycelium. Colonies which developed from white single spores were

shown to contain the mycovirus by electron microscopy and by polyacrylamide gel electrophoresis. He obtained similar results using the same technique with a visible mutant of *P. stoloniferum* CMI 91966 and also with *Aspergillus niger*.

Analogous results were obtained by Day *et al* (36) and Day and Anagnostakis (37) working in cooperation with our laboratory (16). A mycovirus of *Ustilago maydis* was transmitted by heterokaryon transfer from P1 isolates which contained a virus and a killer substance to P2 isolates which contained no virus and were sensitive to the killer substance. These results are discussed more fully below.

In order to obtain transmission of viruses using cell free extracts, Lhoas (14) produced protoplasts from virus free strains of *Penicillium stoloniferum* ATCC 10111 and CMI 91966 by incubating mycelium with snail gut enzyme. He mixed partially purified preparations of the *P. stoloniferum* (ATCC 14586) virus complex with the protoplasts, incubated them, and then plated the protoplast suspensions on agar media. He obtained transmissions to 12 isolates of strain ATCC 10111 and 15 isolates of strain CMI 91966 out of 20 tested for each. These experiments have been confirmed by Ian Pallett of Beecham Research Laboratories, Betchworth, England (personal communication).

Lhoas (15) next transmitted the mycoviruses (*Aspergillus niger* CMI 146891 and *Penicillium stoloniferum* ATCC 14586) to yeast (*Saccharomyces cerevisiae*) using an elegant procedure which required incubating compatible haploid yeast cells under conditions for zygote formation in the presence of the purified virus preparation. One to five per cent of the zygote colonies stained with methylene blue when incubated on methylene blue medium indicating the death of some cells. Serial single cell isolations from these blue colonies yielded lines in which 95% of the colonies were blue. Colonies in which some cells stained blue were found to contain mycovirus whereas colonies with no stained cells were virus free. Thin section electron microscopy (38) showing a large

concentration of mycovirus suggests that both viruses multiply to a high titer in yeast cells. Lhoas (15) hypothesized that cell fusion was necessary for infection since five zygotes grown under similar conditions in the presence of the virus did not become infected. The low percentage of cells infected suggested that only a few virus particles entered each cell but yields sufficient to induce the death of the cells were eventually obtained.

Saccharomyces may constitute a "common host" where viruses of broad phylogenetic origin may be propagated. Kovács *et al.* (39) reported the successful infection of *S. cerevisiae* with polyoma virus. In contrast to the viruses of *Aspergillus* and *Penicillium*, this DNA virus seemed to require no special conditions for infection of *Saccharomyces*.

Still another example of possible transmission of a virus to a fungus was supplied by Brants (40, 41) who inoculated shake cultures of *Pythium sylvaticum* with tobacco mosaic virus and maintained infectivity for 2½ years.

Biophysical Properties of Mycoviruses

Kleinschmidt and Ellis (6) first reported the occurrence of mycovirus in *Penicillium stoloniferum* (ATCC 14586). It was later found that it contained more than one virus (34, 35).

Bozarth *et al.* (21) obtained highly purified preparations of this virus complex by a combination of differential centrifugation and sucrose density gradient centrifugation. Two electrophoretically distinct virus species designated PsV-f (fast) and PsV-s (slow) according to their mobility were purified by sucrose density gradient electrophoresis. Each reacted in a specific reaction line in serological tests with antiserum prepared to the complex (Fig. 1). Electron micrographs of both PsV-s and PsV-f showed isometric particles, some of which were penetrated by the uranylacetate used for negative staining (Fig. 2). Penetrated particles measured 32 nm and unpenetrated particles measured 34 nm in diameter.

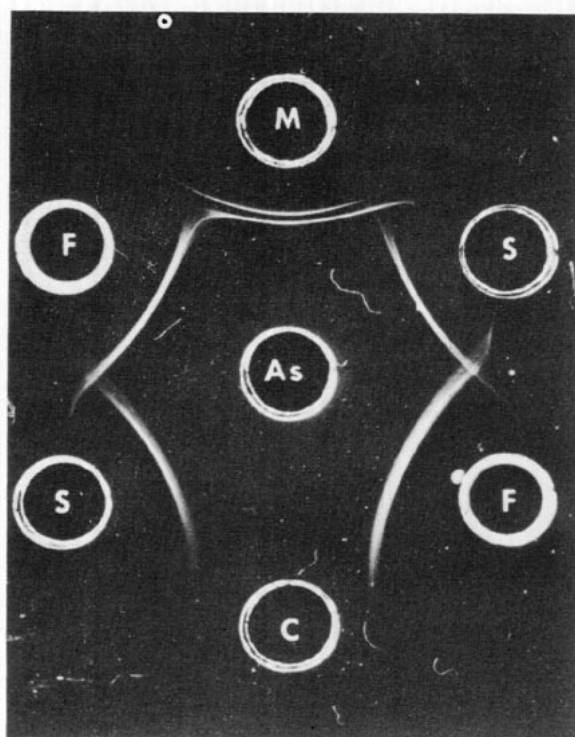


FIGURE 1. Ouchterlony double-diffusion serological reaction between antiserum (As) to viruses isolated from *Penicillium stoloniferum* ATCC 14586 and the viruses. The two viruses, PsV-s (S) and PsV-f (F), were purified by sucrose density gradient electrophoresis. The natural mixture of the two viruses is contained in well M. The control (C) is *P. brevi-compactum* mycovirus (17, 21).

The RNAs of all mycoviruses studied so far are double stranded and occur as more than one size class. PsV-s contained two molecular weight classes of ds-RNA (1.10 and 0.94×10^6 daltons) and the melting profile was typical of ds-RNA. PsV-f contained three classes of ds-RNA with molecular weights of 0.99 , 0.89 , and 0.23×10^6 daltons. In addition, PsV-f contained heterogeneous low molecular weight RNA which gave an RNA pattern typical of single-stranded RNA and was sensitive to digestion by ribonuclease. This is the only single-stranded RNA reported in mycoviruses to date but it constitutes a minor part of the total RNA of the virus.

Banks *et al.* (42) reported that *Penicillium chrysogenum* (ATCC 9420) and six strains

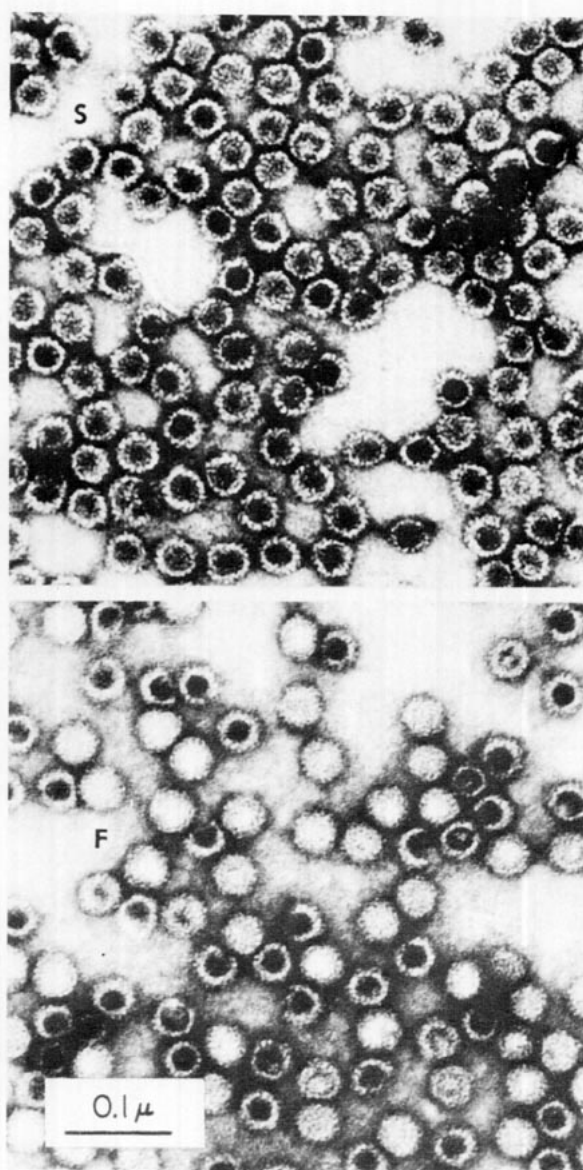


FIGURE 2. Isometric virus particles PsV-f (F) and PsV-s (S) isolated from *Penicillium stoloniferum* ATCC 14586, stained with 1% uranyl acetate (21).

derived from it contain an isometric mycovirus approximately 35 nm in diameter. They noted that all strains currently used by industry for the production of penicillin are also derived from this same strain and therefore probably contain the virus. It is the most studied of all mycoviruses thus far reported. Purification of the virus has been reported by Buck

et al. (43), Banks *et al.* (44), Wood and Bozarth (23), and Nash *et al.* (45).

The virus is isometric, 40 nm in diameter, and contained 11 to 15% RNA (23). Others have reported a particle diameter of 35 nm (42, 43, 45). A molecular weight of 13×10^6 daltons was computed from sedimentation and diffusion data (23).

Because of its importance as an inducer of interferon, the ds-RNA of this virus has been studied more thoroughly than the virus itself (46). Wood and Bozarth (23) extracted the RNA from highly purified virus preparations. It was double stranded, as indicated by its resistance to nuclease and hypochromicity profile (Fig. 3). It consisted of three classes with molecular weights of 2.18, 1.99, and 1.89×10^6 daltons, respectively (Fig. 4).

Three classes of ds-RNA are also suggested from the data of Buck *et al.* (43), Nash *et al.* (45), and Cox *et al.* (47, 48). Classes of ds-RNA reported by Cox *et al.* to sediment at 7.3 S (47, 48) have not been observed by other investigators (23, 43, 45).

We found (Wood and Bozarth, unpublished), as did others (46), that *P. chrysogenum* contains a significant quantity of unencapsulated ds-RNA. Lemke and Ness (46) extracted the ds-RNA directly, removed host DNA and ss-RNA by nucleases, and finally chromatographed the ds-RNA on DEAE cellulose. It was identical in molecular weight and ratio of the three RNA classes to that obtained from purified virus preparations.

By reacting the virus particles with sodium persulfate on a protein monolayer and transferring the partially degraded particles to an electron microscope grid, it was determined that each particle had one single strand of ds-RNA (23).

The acceptance of the idea of three classes of RNA in a virus preparation whose chemical and physical analysis suggests that there is only one RNA molecule per particle posed a problem. Nash *et al.* (45) suggested that the three peaks observed on polyacrylamide gel are best explained by conformation differences; however, the sedimentation heterogeneity and melting behavior indicated

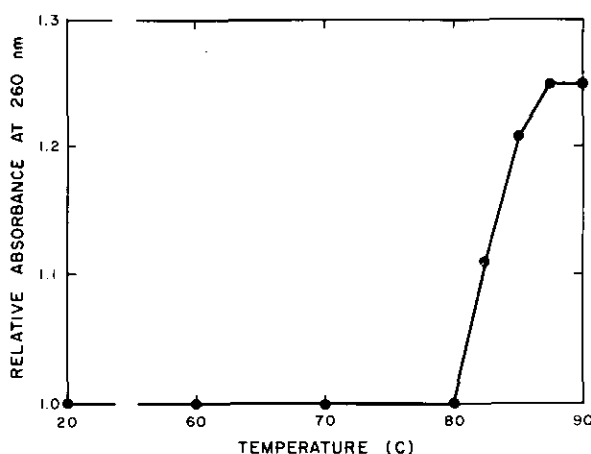


FIGURE 3. Hyperchromicity profile of *P. chrysogenum* ds-RNA.

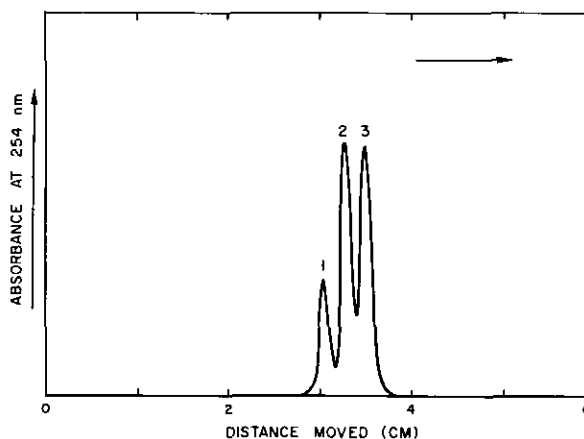


FIGURE 4. Electrophoretogram following electrophoresis of *P. chrysogenum* mycovirus ds-RNA indicates three classes of ds-RNA with molecular weights of 1.89, 1.99, and 2.18×10^6 daltons (23).

that three species do indeed exist (23, 43, 47, 48).

A likely explanation for the three species of ds-RNA found in the *P. chrysogenum* strain 9480 is that the virus is a mixture of virus strains. Since the virus can be maintained by vegetative propagation of the host, a stable relationship of these strains could easily be envisioned. Alternately, they may represent three segments of a split genome (23).

Antiserum to the *P. chrysogenum* has been produced by Buck *et al.* (43) and by Wood and Bozarth (23). The latter authors reported a serological cross reaction between the antiserum of the virus with the *P. brevicompactum* virus (17).

Biophysical properties of the mycoviruses of *P. cyaneo-fulvum* (49), *P. brevicompactum* (17), and *Aspergillus foetidus* (22) have also been recorded. The *P. brevicompactum* (17) mycovirus was found to be similar in all respects to the *P. chrysogenum* mycovirus (23) except that the ratio of the three RNA classes was different and a spur reaction was obtained in serological tests of antigen and antiserum. Thus they were considered to be related strains of the same virus (23). Ratti and Buck (22) recently reported that *Aspergillus foetidus* contains two electrophoretic mycovirus species which they designated fast and slow according to their mobility. A total of six ds-RNA classes was reported for the two viruses. Ratti and Buck (22) showed that each RNA class was associated with a specific density peak when the virus was centrifuged in CsCl density gradients.

With a few exceptions most of the other VLPs recorded in Table 1 are known only from electron microscopy evidence and their properties are yet to be described.

Ultrastructure of Mycovirus Infected Fungi

Although there are a number of reports of ultrastructure of mycovirus infected tissues, none has done more than demonstrate the presence of the virus in the intact cells. Probably the best studies to date are those of Dieleman-van Zaayen and Igesz (50) and Dieleman-van Zaayen (51). Three types of mycoviruses (spheres 25 and 34 nm, rods 19 x 50 nm) known from previous work (31) to be present in cultivated mushrooms, *Agaricus bisporus*, were observed in thin section. Particles were observed in packed arrays and near electron dense areas which were interpreted to be viroplasm. Many of the cells observed had mitochondria, Golgi bodies, endoplasmic reticulum, and nuclei,

indicating that they were viable metabolizing cells when fixed. The particles occurred frequently near a septum and in the region of dolipores which suggested the possibility of cell-to-cell movement. Similar results were obtained by Albouy and Lapierre (52).

Mycoviruses are most readily seen in old cells devoid of cytoplasm and most cellular constituents. Hooper *et al.* (53) demonstrated their presence in old cells of *P. brevicompactum* (17, 54) and *P. stoloniferum* (21, 33, 34). In *Penicillium chrysogenum* (23, 55) which normally yields large quantities of purified virus (23, 45), mycovirus was easily observed in thin sections of old cells (Fig. 5), but not in sections of younger cells with a normal complement of cell components (Bozarth, Wood, and Granados, unpublished). This difficulty may arise from the fact that the virus is only about twice the size of ribosomes, or it may be that in the growing fungus hyphae the cytoplasm is near the point of natural disintegration by the time the virus matures. Albouy and Lapierre (52) also observed 30 nm particles in old, disorganized cells of *Ophiobolus graminis* and in *Sclerotium cepivorum*. This may represent a kind of internal lysis wherein the cell is lysed but the cell wall remains intact and prevents the particles from escaping. Mycoviruses are typically found within an apparent membrane structure (Fig. 5). Others have also reported that the particles appear to be enclosed in a membrane (51-53). Mycoviruses are readily visible in spores (11, 51, 53) and in zoospores (56). From the concentration observed in spores of *P. stoloniferum* ATCC 14586 (Fig. 6) it is obvious why spores from infected mycelium have generally been found to transmit mycoviruses (11, 21, 23, 51, 57, 58).

Thin section studies have been used to confirm the presence of mycoviruses from *Aspergillus niger* and *Penicillium stoloniferum* in yeast cells artificially infected with these viruses (15, 38). Packed arrays were found in both cases confirming other results which indicated that the viruses were actually transmitted and were multiplying in the yeast cells (38).

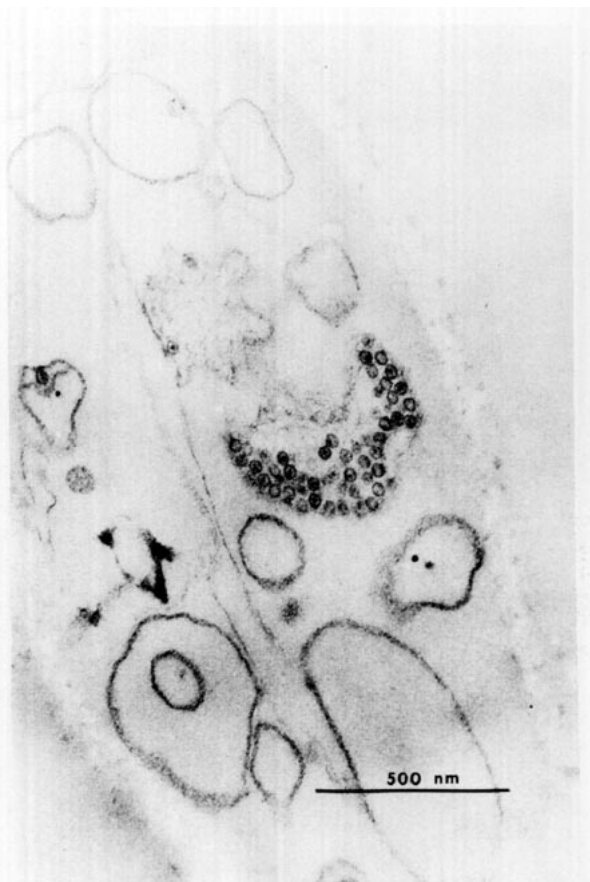


FIGURE 5. Thin section of *P. chrysogenum* ATCC 9480 containing mycovirus. (Electron micrograph by R. R. Granados.)

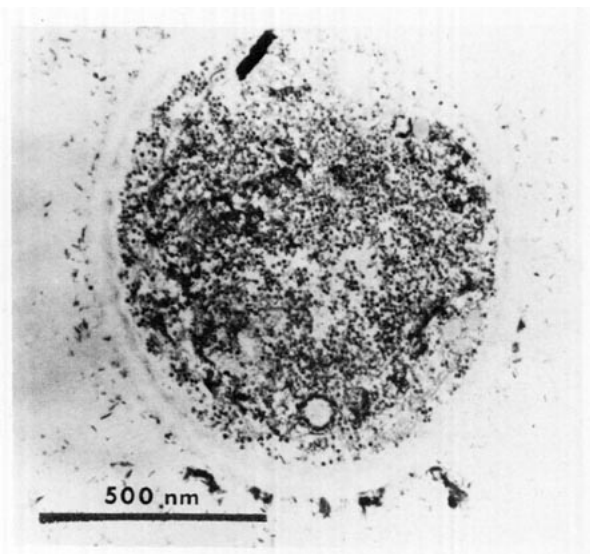


FIGURE 6. Thin section of *P. stoloniferum* ATCC 14586 spore containing mycoviruses. (Electron micrograph by G. Hooper.)

Other mycoviruses are known only as a result of ultrastructure studies. Aist and Williams (56) reported the occurrence of unidentified bodies which appear to be mycovirus in thin sections of *Plasmodiophora brassicae*. They occurred both singly and in crystalline arrays and showed considerable fine structure.

Schnepf *et al.* (20) observed mycovirus within cells of the phycomycete *Aphelidium* which were parasitizing *Scenedesmus*. The 200 nm isometric particles had a core of 150 nm separated from an outer shell by an electron transparent zone of about 13 nm. The authors considered them to be similar to the smaller *Tipula* iridescent virus and pointed out the similarity to the virus found in a gecko (59) and in a frog (60).

In yeast, *Saccharomyces carlsbergensis*, Volkoff and Walters (61) observed phage-like particles with heads of 80 x 90 nm and tails 70 nm long. They were first seen in buds of cells in cultures with an abnormally high frequency of buds. The particles were observed for a short time in the growth cycle and were not seen again. No plaques or lyses were observed macroscopically. Lindegren *et al.* (62) observed particles in thin section of yeast which were considered to be virus in nature.

One of the few rod viruses in fungi was observed in thin section of *Peziza ostracoderma*, an ascomycete frequently found growing in mushroom beds as a weed (19).

Significance of Mycoviruses

The rapid, almost logarithmic, increase in mycovirus research began five years ago with the discovery of a virus in *P. stoloniferum* (5). While it is still too early to appraise their significance fully, it is already apparent that mycoviruses have a profound effect on many aspects of biology. The current interest in mycoviruses is a direct outgrowth of interferon research.

Interferon induction. Interferon has come to be recognized as a primary defense mechanism against viral invasion and is the

earliest detectable body defense following infection by a virus (63). Recent reviews by Kleinschmidt (64) and by Baron (63) discuss the mechanism of induction and the biological significance of interferon. It was first described by Isaacs and Lindenmann (65) and their investigations suggested that viral RNA was an inducer. Two fungal products, statolon from *Penicillium stoloniferum* and helenine from *P. funiculosum*, were found to have the capacity to induce interferon in test animals. It was ultimately found that the active factor in both statolon and helenine was ds-RNA of viral origin (6, 7, 9, 10). Since then the ds-RNA of mycoviruses isolated from *P. chrysogenum* (23, 42, 43, 46, 55), *P. cyaneo-fulvum* (49), *Aspergillus foetidus* (66), and another strain of *P. funiculosum* (10) has been shown to induce interferon in test animals. This and other research suggest that most mycoviruses contain ds-RNA which will induce interferon. Single-stranded RNA (67), DNA (68), replicative form RNA (69), as well as other substances including pyran and tilorone (64) have been reported to induce interferon, but the most consistent results are obtained with ds-RNA -- either natural (43, 70), or synthetic (64, 71-73).

Interferon may be induced by either the intact mycovirus or its extracted ds-RNA (9, 43). Buck *et al.* (43) compared the interferon titer in test animals following injections of *P. chrysogenum* mycovirus and *P. chrysogenum* mycovirus ds-RNA. The titer was higher and achieved earlier when the ds-RNA was used. The interferon titer returned to the "normal" amount at about the same rate.

One problem in the use of the intact virus as the inducer is the high antigenicity of the viral protein. Another is the comparatively short duration of the interferon response following a single injection of inducer; however, protection against virus multiplication could be maintained for periods of 11 days by daily injection of a small quantity of ds-RNA (70). Sharpe *et al.* (70) reported that mice injected daily with 50 μ g of mycovirus ds-RNA from *P. stoloniferum* were pro-

tected against encephalomyocarditis virus.

Our preparation of *P. chrysogenum* mycovirus ds-RNA (23) was compared to reference preparations of poly I · poly C for its ability to induce resistance to vesicular stomatitis virus plaque formation in cultures of rabbit kidney cells and interferon production in human cells. It proved superior to the synthetic ds-RNA in both cases (Vilček, Wood, and Bozarth, unpublished).

Mycovirus ds-RNA of discrete molecular sizes can be obtained in highly purified form. The ds-RNA associated with the mycovirus of *P. chrysogenum* is a uniform product consisting of three molecular weight classes of approximately 2×10^6 daltons (23). The *P. stoloniferum* virus complex contains five molecular weight species (21) and the *A. foetidus* complex contains six molecular weight species (22). For experimentation on the effect of molecular size of the inducer RNA on interferon production, a molecular weight ranging from 0.24 to 2.76×10^6 daltons could be chosen. In this respect, it should have a significant advantage over poly I · poly C which is comparatively heterogeneous and varies in molecular size from batch to batch.

P. chrysogenum mycovirus can be obtained in yields of 1 mg per gram dry weight of tissue. There is evidence that an equal or greater quantity of ds-RNA occurs free in the tissue (46). It could be recovered in large quantities as a by-product of penicillin production.

Toxin production. A number of lines of research suggest that the level of toxins or other metabolites produced by fungi may be affected by the presence of mycoviruses. All of the strains of *P. chrysogenum* used in the commercial production of penicillin are infected with a mycovirus (42). Buck *et al.* (74) reported that the virus infected strain of *P. stoloniferum* contained 18 to 45 times more galactosamine than did five non-virus containing strains of the same species. Recently MacKenzie and Adler (75) reported a microvirus from a non-toxin-producing culture of *A. flavus*, whereas their toxin-producing culture did not contain microvirus.

The corn blight which swept through the American corn belt in recent years is caused by the fungus, *Helminthosporium maydis*. We tested six blight-producing strains and six mild strains and found that strains of *Helminthosporium* which produce severe disease in corn contain mycovirus. Those which do not produce severe disease do not contain mycovirus (18). A strain of *Fusarium moniliforme* which produces a severe vitamin B deficiency when fed to chicks was found to contain a mycovirus (Mislivec, Wood, and Bozarth, unpublished).

While the number of known toxin-producing fungi which contain mycoviruses is growing, there is as yet no firm evidence for a positive relationship between mycovirus and toxin production and at least one line of evidence suggests that a negative relationship (75) exists.

Cytoplasmic inheritance. There is an increasing body of evidence that many cytoplasmically inherited factors are the result of virus infection which is passed to the progeny. Day (76) has reviewed the known cases of cytoplasmic inheritance in fungi. A positive correlation between the presence of a mycovirus and the marker factor has been established in five cases (18, 25, 37, 77, 78).

Puhalla (77) described a cytoplasmically inherited killer factor in *Ustilago maydis*. Strains designated P1 contained the killer factor and produced a substance which was lethal to cells of the P2 type. P1 strains are also insensitive to the killer substance produced by other P1 strains. Hankin and Puhalla (79) isolated a proteinaceous substance from P1, a strain which was lethal to the P2 strain. The fact that the killer factor was cytoplasmically inherited suggested that a virus was present. A cooperative research effort between our laboratory and that of P. R. Day (Conn. Agr. Expt. Sta., New Haven, Conn., USA) revealed that P1 strains contained mycovirus, whereas P2 strain did not (16, 36). By growing P1 strains on minimal medium containing charcoal, production of the killer substance by P1 could be avoided. In this way, mating between P1 and P2 strains was carried out in Petri dishes (37) and

growth of dikaryotic hyphae developed at the junction of the two cultures. When the dikaryotic mycelium was transferred to a complete medium without charcoal, the two haploid types were isolated. Nearly all clones of single cell origin recovered from the P1 + P2 mating were of the P1 phenotype. One of these which had a P2 genotype was tested and found to contain mycovirus (16, 36). Thus the cytoplasmically inherited factor which makes a strain P1 was transmitted along with the mycovirus. Crosses of P1 + P2 may also generate a third cell type designated P3 (77) which has the insensitivity of P1 strains to the killer protein but does not produce it. P3 cells were also found to contain mycovirus.

There appears to be a positive correlation between a cytoplasmically inherited factor in P1 and P3 which confers resistance to the killer protein and the presence of the virus. The study of the virus has just begun and it is possible that there are actually two viruses with similar appearances. One virus genome would be responsible for the production of killer protein and one virus genome responsible for resistance to it. There is a precedent for such a conclusion (21).

Diseases of fungi caused by viruses. A very severe disease of cultivated mushrooms is widespread and has been shown to be caused by one or more of a complex of at least five mycoviruses (11). The most severe manifestations of the disease result in crop loss and degeneration of the mycelium in the compost. Evidence for the viral nature of the disease is discussed under transmission of mycoviruses (above) and a complete review of the problem and etiology of the disease has been presented (11). If infection with mycoviruses is found to adversely affect the pathogenicity of a fungus, then mycoviruses may offer a means of biological control of some fungus diseases. This would be especially useful in the control of fungi which affect the roots of plants.

Lapierre *et al.* (78) and Lemaire *et al.* (80) recently isolated a mycovirus from *Ophiobolus graminis*, a fungus which causes a severe root rot in wheat. Isolates which

contained the mycovirus grew and sporulated poorly and were only weakly pathogenic to wheat. In contrast normal isolates grew well on agar medium, sporulated profusely, and were severe pathogens which caused the disease of wheat called "take-all." The weak pathogenicity of the virus infected fungus suggested the possibility of biological control. Lemaire *et al.* (81) completed the first series of experiments which indicated that virus infected strains of *O. graminis* produced little or no disease in wheat. When virus infected strains were mixed with normal strains in greenhouse pots and field plots planted with wheat, the disease severity was greatly reduced compared to controls infected with the non-virus infected strain of *O. graminis*. This constitutes the first report of biological control of a plant pathogen with a mycovirus.

Jinks (82) described a condition of lysis and breakdown in *Aspergillus glaucus* which resulted in "vegetative death" in some strains of this species. Jinks inferred that the factor was cytoplasmic in origin and Hollings and Stone (11) later found 25 nm particles in strains showing the condition. Apparently the maintenance of cultures which show the condition was quite difficult and no further work was done on the problem.

Lindberg (83) described a transmissible disease of *Helminthosporium victoriae* which appeared to be virus induced. The disease was transmissible by hyphal anastomosis and by dipping plugs of "healthy" mycelium in macerates of diseased tissue. Transmission by cell free preparations was not possible but phenol extracts (supposedly containing viral RNA) from both diseased and normal mycelia induced the disease (84). He found that diseased isolates produced less toxin and caused less severe blight in inoculated oat plants (85). Diseased isolates grew slowly and produced few viable spores. Those spores which did germinate produced normal mycelium (86). Psarros and Lindberg (86) described two stages of the disease - acute and chronic. In the acute stage hyphal branches stood out sharply at right angles from the main hyphal strands and most cells

appeared devoid of organized contents. In the chronic stage multinucleate cells were common and the cultures appeared to be similar in appearance to normal cultures. Antisera produced against extracts of diseased isolates were reported to contain antibodies to antigens not present in diseased isolates (87).

All of the diseased isolates of *H. victoriae* studied by Lindberg (83) were obtained from sectors of normal isolates and each had the ability to convert its normal homolog to the diseased form following hyphal anastomosis. Similar diseased isolates were obtained from *H. victoriae* (83, 85), *H. maydis* (88), and *H. oryzae* (89). We screened two isolates of *H. victoriae* in our laboratory and one which showed disease symptoms, not unlike those described by Lindberg, was found to contain a mycovirus (Bozarth, Wood, and Goenaga, unpublished). M. Richards also found particles in *Helminthosporium* (personal communication).

It is now evident that viruses are quite common in *Helminthosporium*. We tested 12 strains of *H. maydis* for the presence of virus and found mycoviruses in six strains, all of which caused severe blight in corn. Mycoviruses were not found in six strains which produced only mild symptoms in corn (18). Our results indicate that there are several different types of mycoviruses in *Helminthosporium*.

It is still too early to generalize on the effect of mycoviruses on the virulence of *Helminthosporium*. Lindberg's results provide interesting grounds for speculation but it is not yet known whether his diseased isolates actually contain mycovirus. Our own data, which must be regarded as preliminary, indicate a correlation between virulent strains of *H. maydis* and the presence of mycoviruses. If further research continues to support this correlation, then virus infection in *H. maydis* will have the opposite effect from mycovirus infection in *Ophiobolus graminis* (81). There is no *a priori* reason why virus infection should be expected to increase or decrease disease severity. Each may occur in a given set of circumstances.

Lysis of fungal cells similar to that of bacteria resulting from phage infection has been rarely observed. Borré *et al.* (13) reported that cultures of *Penicillium citrinum* and *P. variable* which had areas of white fluffy mycelium had lytic plaques which were observable from the bottom of plates. Mycelium taken from the center of the plaques consisted of swollen hyphae, with little or no cytoplasm.

Other fungal abnormalities which suggest the presence of viruses have been found in *Podospora anserina* (90), *Pestalozzia* sp. (91), and *Endothia parasitica* which causes the well known chestnut blight (92). To date there is no positive correlation of mycoviruses with these abnormalities.

Summary

Since the first report of mycoviruses in fungi in 1962 and in filamentous fungi in 1967, 56 mycoviruses have been discovered. Those which have been studied in detail include the mycoviruses of *Penicillium stoloniferum*, *P. chrysogenum*, *P. brevicompactum*, *P. cyaneo-fulvum*, and *Aspergillus foetidus*. All of these are isometric particles in the size range of 30 to 40 nm, containing several classes of ds-RNA. In some mycoviruses it has been shown that each virion contains a single strand of ds-RNA. Some mycoviruses isolated from different species and genera have been shown to be serologically related and contain ds-RNA classes of the same molecular weight.

Transmission by means of hyphal anastomosis, heterokaryosis, and protoplasts has been demonstrated. Yeast, *Saccharomyces cerevisiae*, has been inoculated with cell-free preparations of mycoviruses from *Penicillium stoloniferum* and *Aspergillus niger* by applying cell-free extracts of the mycovirus during mating of haploid cells.

The ds-RNA of mycoviruses has been found to be a potent inducer of interferon and thus is important to the study of natural defense mechanisms against virus infection.

Correlations have been found between the presence of mycoviruses and cytoplasmically-inherited factors in fungi. A number of toxigenic fungi have been found to contain mycoviruses and there is enough preliminary evidence to warrant further investigation of the relationship between the presence of mycovirus and toxin production by these fungi.

Mycoviruses have been shown to be responsible for lethal diseases of higher fungi and some plant pathogenic fungi contain mycoviruses. Preliminary evidence indicates that a severe disease of wheat is rendered less severe if virus infected fungi are present in the soil along with the pathogenic fungi of the same species. Other preliminary evidences suggest that virus infected fungi may be more severe pathogens of higher plants than non-infected fungi of the same species.

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